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Improved cyclopropene reporters for probing protein glycosylation†

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Cyclopropenes have emerged as a new class of bioorthogonal chemical reporters. These strained rings can be metabolically introduced into target biomolecules and covalently modified *via* mild cycloaddition chemistries. While versatile, existing cyclopropene scaffolds are inefficient reporters of protein glycosylation, owing to their branched structures and sluggish rates of reactivity. Here we describe a set of cyclopropenes for the robust detection of glycans on cell surfaces and isolated proteins. These scaffolds comprise carbamate linkages that are compatible with cellular biosynthetic pathways and exhibit rapid cycloaddition rates. Furthermore, these probes can be used in tandem with other classic bioorthogonal motifs—including azides and alkynes—to examine multiple biomolecules in tandem.

The chemical reporter strategy is a popular method to tag biomolecules with probes in live cells and animals.[1,2] This strategy relies on the metabolic introduction of unique functional groups (*i.e.*, chemical reporters) into target biomolecules.[3] The reporters can be selectively modified in a second step using highly specific (*i.e.*, bioorthogonal) chemistries. This two-step approach has been widely employed to visualize and profile cellular biopolymers, including glycoconjugates.[3–8] For example, sialylated glycans have been targeted with various *N*-acetyl mannosamine (ManNAc) and sialic acid precursors.[9–12] Similarly, mucin-type *O*-linked glycans and *O*-GlcNAc-modified proteins have been targeted with *N*-acetyl galactosamine (GalNAc) and *N*-acetyl glucosamine (GlcNAc) analogs, respectively.[5,13] In most cases, the sugars were equipped with azide or alkyne reporter groups and ultimately detected *via* Staudinger ligation,[14,15] copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC), or strain-promoted cycloaddition.[16]

In recent years, cyclopropenes have gained traction as broadly useful chemical reporters for biomolecule visualization and retrieval.[17–21] Cyclopropenes are small in size and likely compatible with a variety of endogenous biosynthetic pathways. These motifs can also be readily ligated with tetrazine probes *via* inverse electron-demand Diels–Alder (IED-DA) reactions or nitrile imines *via* 1,3-dipolar cycloaddition. Importantly, cyclopropenes can be used concurrently with organic azides and alkynes—the most established chemical reporters to date.[17,19,22,23] Thus, cyclopropenes are well suited for multi-component imaging studies.

We and others have recently utilized cyclopropene-modified sugars (including the ManNAc analog, **Ac$_4$ManNCyc**, Fig. 1A) to target sialic acid residues on live cell surfaces.[17,19] In these previous studies, cells were first incubated with **Ac$_4$ManNCyc**, and then treated with various tetrazine probes. Cyclopropene-specific signal was observed in all cases, but the intensities were quite low, likely due to poor metabolic conversion of the unnatural sugar, inefficient tetrazine ligation, or both of these issues. Indeed, the *N*-acyl unit in **Ac$_4$ManNCyc** is branched at the beta carbon; beta-substituted *N*-acyl chains are not well tolerated in the sialic acid biosynthetic pathway.[24,25] Additionally, cyclopropenes with amides or other electron-withdrawing groups at C-3 (see Fig. 1A) are sluggish IED-DA reactants.[17,21]

Here we report three cyclopropene-modified sugars that enable more facile tagging of mammalian cell glycoconjugates in a variety of assays. These monosaccharides comprise carbamate linkages between the requisite cyclopropene and sugar core (Fig. 1A). The *N*-cyclopropenyl carbamate derivative **Ac$_4$ManCCp** was designed to intercept the sialic acid biosynthetic pathway and target sialylated glycoconjugates.[26] The analogous GalNAc and GlcNAc analogs (**Ac$_4$GalCCp** and **Ac$_4$GlcCCp**) were designed to target mucin-type *O*-linked structures and *O*-GlcNAcylated proteins, respectively. Carbamates are relatively stable moieties, making them attractive for use in cells and live organisms. Indeed, Pratt and coworkers recently synthesized a set of *N*-propargyloxycarbamate sugars that can be readily detected *via* CuAAC for proteomics applications.[27]

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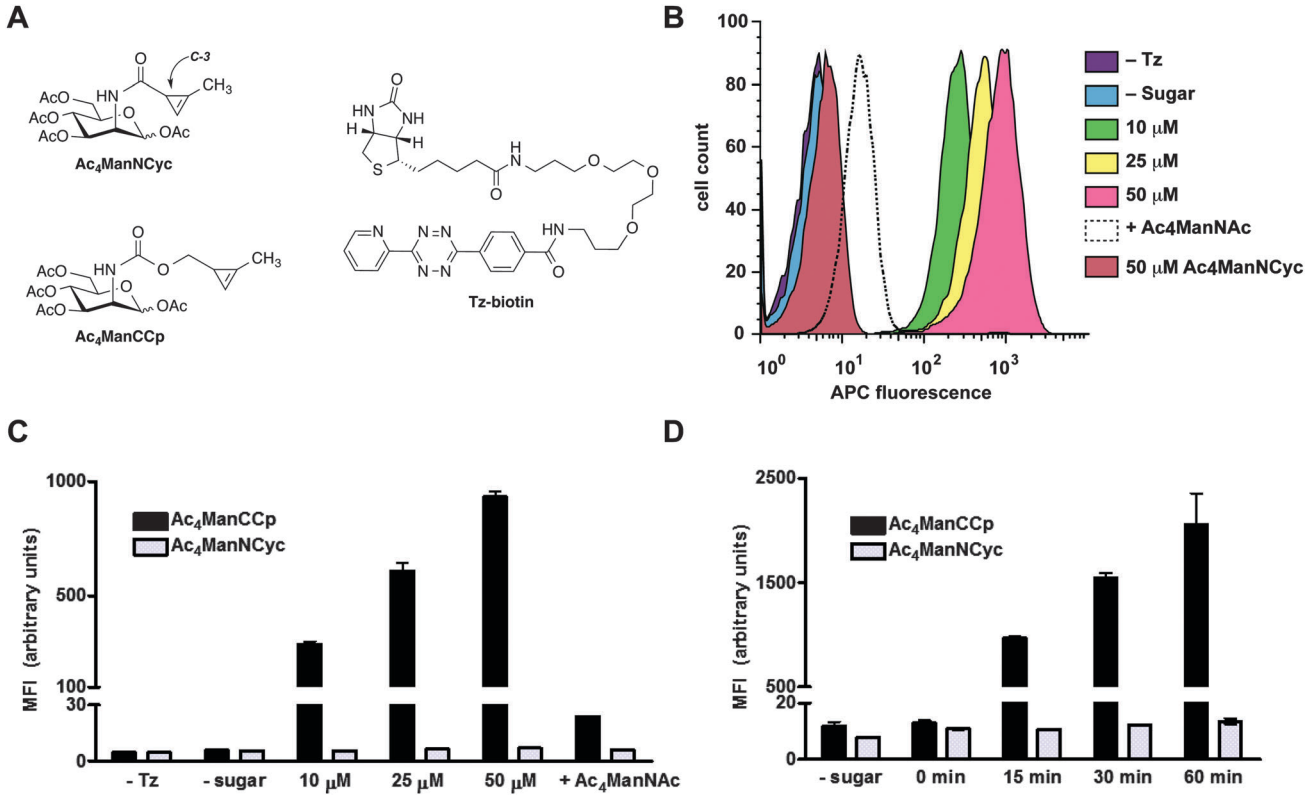
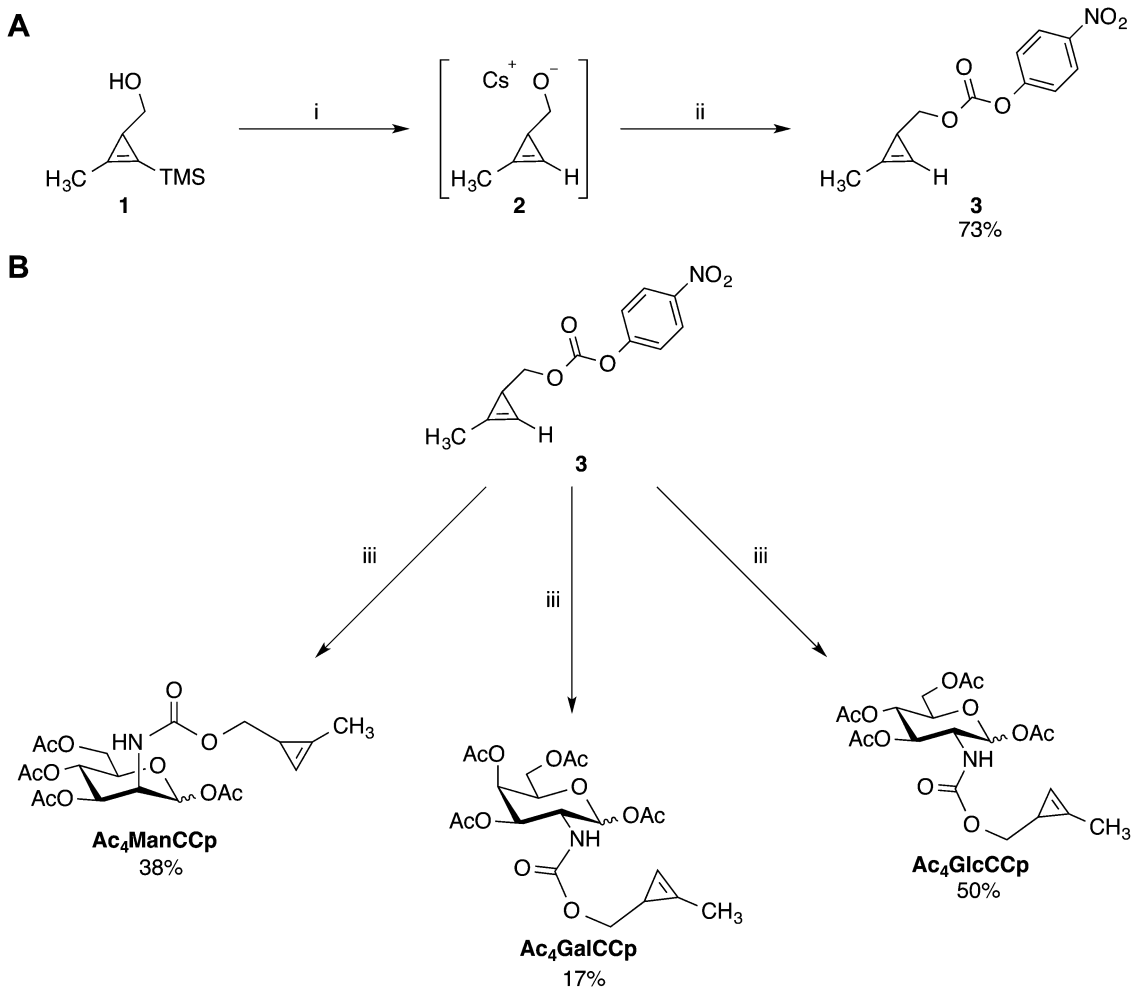


Fig. 1 Cyclopropene-modified ManNAc derivatives can be metabolically incorporated onto cell surfaces and covalently detected with tetrazine probes. (A) Structures of the ManNAc analogs (**Ac_4ManNCyc** and **Ac_4ManCCp**) and tetrazine reagent (**Tz-biotin**) used in this study. (B) **Ac_4ManCCp** is robustly incorporated onto live cell surfaces. Jurkat cells were incubated with **Ac_4ManCCp** (0–50 μM), **Ac_4ManNCyc** (0–50 μM) **Ac_4ManCCp** (50 μM) plus Ac_4ManNAc (10 μM, +Ac_4ManNAc) or no sugar (-sugar). Samples were then treated with **Tz-biotin** (10 μM) for 30 min at 37 °C. One **Ac_4ManCCp**-treated sample (10 μM) was not labeled with **Tz-biotin** (-Tz). All cells were then stained with APC-avidin and analyzed by flow cytometry. Representative histograms are shown. (C) and (D) **Ac_4ManCCp** enables more robust cell surface labeling than **Ac_4ManNCyc**. (C) The mean fluorescence intensities (MFI, in arbitrary units) for the histograms in (B) are plotted. MFI values for cells treated with **Ac_4ManNCyc** (10–50 μM) are also shown. (D) **Ac_4ManCCp** can be rapidly detected with **Tz-biotin**. Jurkat cells were incubated with **Ac_4ManCCp** (25 μM) or **Ac_4ManNCyc** (25 μM), then treated with **Tz-biotin** (10 μM) for 0–60 min at 37 °C. The cells were stained with APC-avidin and analyzed by flow cytometry. The mean fluorescence intensities of the cell populations are plotted. In (C) and (D), error bars represent the standard deviation of the mean for three labeling reactions.

For the cyclopropene probes, the carbamate linkage also alleviates steric congestion at the beta-position, improving the likelihood that cellular enzymes will efficiently process the sugars. Moreover, we and others have shown that cyclopropenes outfitted with carbamates (*versus* amides) at C-3 react ~100 times faster with electron-poor tetrazines.[17,18,21,26]

We prepared the desired probes (**Ac_4ManCCp**, **Ac_4GalCCp**, **Ac_4GlcCCp**, Scheme 1) *via* direct conjugation of the corresponding amino sugars with an activated cyclopropene unit (**3**). Carbonate **3** was prepared by treating alcohol **1** with anhydrous cesium fluoride (to remove the silyl group), followed by nitrophenyl chloroformate (Scheme 1A). These transformations were performed sequentially as intermediate **2** was not stable upon concentration. Direct activation of **1** also resulted in product decomposition. Ultimately, carbonate **3** was used to acylate the hydrochloride salts of mannosamine, galactosamine, and glucosamine (Scheme 1B). The resulting carbamate sugars were then globally acetylated to provide the desired probes **Ac_4ManCCp**, **Ac_4GlcCCp**, and **Ac_4GalCCp**. Acylation of sugar hydroxyl groups has been previously shown to facilitate probe uptake into mammalian cells.[28]

Once in hand, the modified sugars were used to metabolically target glycoconjugates in live cells. Jurkat T cells were first incubated with **Ac_4ManCCp** (0–50 μM) for 24 h, then reacted with a tetrazine-biotin probe (**Tz-biotin**, 10 μM, 30 min at 37 °C). Cell surface cycloadducts were detected upon staining with a fluorescent streptavidin conjugate and flow cytometry analysis. As shown in Fig. 1B and C, **Ac_4ManCCp**-dependent fluorescence was observed, indicating successful metabolism and cell surface incorporation of the unnatural sugar. Notably, **Ac_4ManCCp** provided enhanced cellular fluorescence compared to the *N*-acyl variant **Ac_4ManNCyc** at all reagent concentrations and labeling times investigated, with nearly a 130-fold improvement in signal at the maximal doses and times. This was likely due to the improved incorporation efficiency of the carbamate probe, along with its faster rate of reaction. Similar trends were observed in other cell lines cultured with **Ac_4ManCCp** (Fig. S1, ESI†).[26] The fluorescence signal from **Ac_4ManCCp**-treated cells was also diminished in the presence of Ac_4ManNAc, the native substrate, suggesting that the carbamate probe enters the sialic acid biosynthetic pathway. Furthermore, Western blot analysis of proteins



Scheme 1 (A) Synthesis of carbonate **3** *via* sequential deprotection and activation of **1**. (B) Synthesis of carbamate-linked cyclopropene sugars. (i) CsF (1.05 equiv.), 18-crown-6 (1.10 equiv.), THF, rt, 3 h; (ii) 4-nitrochloroformate (2 equiv.), pyridine (6 equiv.), CH_2Cl_2, rt, overnight; (iii) mannosamine, galactosamine, or glucosamine hydrochloride (0.25 equiv.), *N*,*N*-diisopropylethylamine (4 equiv.), DMF, rt, 4–12 h, followed by Ac_2O, pyridine.

harvested from **Ac_4ManCCp**-treated cells (and reacted with **Tz-biotin**) revealed a similar banding pattern—or "fingerprint"—compared to proteins isolated from cells treated with a previously validated reporter of sialylation, the azido-ManNAc analog Ac_4ManNAz (Fig. 2 and Fig. S3, ESI†).[29] In this experiment, Ac_4ManNAz-labeled glycoproteins were detected *via* CuAAC with an alkyne probe.

Ac_4GalCCp and **Ac_4GlcCCp**, the putative metabolic reporters for GalNAc and GlcNAc, respectively, were similarly evaluated in cultured cells. Jurkat or HEK293 cells were incubated with the unnatural sugars (0–50 μM) for 24 h prior to tetrazine ligation and flow cytometry analysis. Cell surface cyclopropenes were detected in all cases (Fig. S2, ESI†). The glycoprotein targets of these sugars were also analyzed. Soluble protein isolates from **Ac_4GalCCp**- or **Ac_4GlcCCp**-treated Jurkat cells were reacted with **Tz-biotin**, then separated by gel electrophoresis and analyzed by Western blot. As shown in Fig. 2, both **Ac_4GalCCp** and **Ac_4GlcCCp** produced "fingerprints" similar to their azido counterparts.[30–32] It should be noted that some *N*-acyl analogs of GalNAc, GlcNAc, and ManNAc have been observed to target multiple classes of biomolecules due to *N*-deacetylation and/or enzymatic scrambling.[30–32] The extent to which the carbamate sugars are interconverted remains to be determined, and further biochemical studies will ultimately elucidate their metabolic fates. Based on our work to date, though, the cyclopropene sugars appear to function similarly to the analogous azido probes (Fig. 2 and Fig. S4, ESI†).

Azide–alkyne cycloadditions and cyclopropene-tetrazine ligations can be used simultaneously to visualize distinct biomolecules in live cells. However, in most examples to date, the cyclopropene-tagging reactions required either extensive labeling times (>1 h with 100 μM tetrazine) or large probe concentrations (>100 μM **Ac_4ManNCyc** or >100 μM tetrazine). Such conditions resulted in cellular toxicity and higher levels of background labeling. With the carbamate-functionalized sugars, lower concentrations of reagents and shorter reaction times can be employed, facilitating glycan visualization in live cells. Indeed, when 4T1 cells were treated with **Ac_4ManCCp** or **Ac_4GalCCp** (25 μM), the targeted glycoconjugates could be readily detected with functionalized tetrazines in just 15 min (Fig. S5 and S7, ESI†). By contrast,

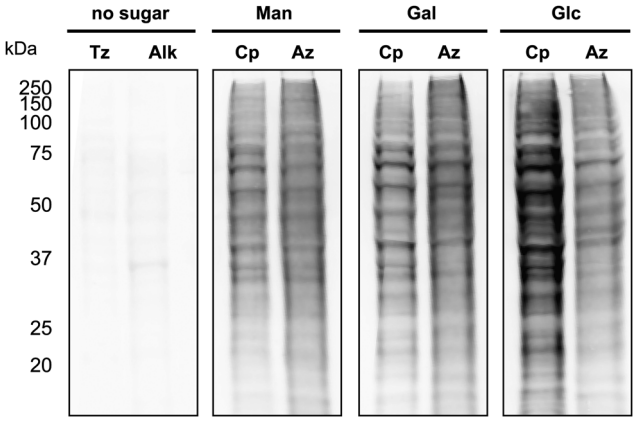


Fig. 2 Carbamate-linked cyclopropene sugars label cellular glycoproteins. Jurkat cells were incubated with cyclopropene (Cp) or azido (Az) analogs of ManNAc (Man), GalNAc (Gal), or GlcNAc (Glc) (75 μM) for 36 h, then lysed. Soluble protein isolates were treated with either 100 μM **Tz-biotin** to tag Cp-modified proteins or an alkyne-modified biotin (structure shown in Fig. S3, ESI,† 100 μM) to tag Az-modified proteins *via* CuAAC. All samples were separated by gel electrophoresis and analyzed *via* Western blot. Equivalent protein loading was confirmed using Ponceau S stain (Fig. S3, ESI†).

no detectable fluorescence was observed in **Ac_4ManNCyc**-treated cells even after extended tetrazine labeling times (1 h, Fig. S6, ESI†).

We used the optimized carbamate cyclopropenes in tandem with azido reporters to target unique subsets of cellular glycans (Fig. 3 and Fig. S8, ESI†). In brief, cells were treated with either **Ac_4ManCCp** (to target sialylated structures), the azido GalNAc analog (**Ac_4GalNAz**), both unnatural sugars, or no sugar. All cell samples were reacted concurrently with **Tz-biotin** (to tag cell surface cyclopropenes) and a strained alkyne (**DBCO-FLAG**, Scheme S1, ESI†) to tag cell surface azides. Selective labeling of each unnatural sugar was observed with no cross-reactivity. Unique sites of biomolecule co-localization were also observed near cellular junctions. Further insights into these and other multi-component processes will be aided by the cyclopropene chemical reporters described in this work.

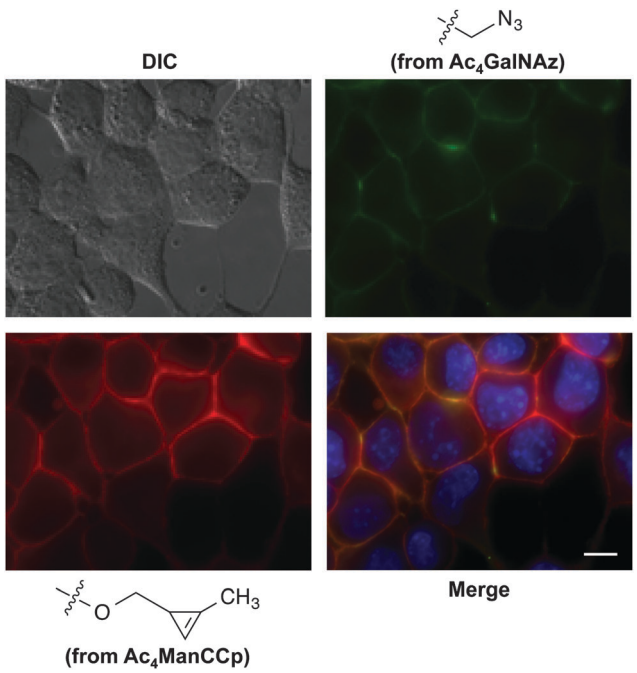


Fig. 3 Distinct metabolic targets can be simultaneously imaged using cyclopropene and azido reporters. 4T1 cells were cultured in the presence of both **Ac_4ManCCp** (25 μM) and **Ac_4GalNAz** (25 μM) for 24 h, followed by concurrent treatment with **Tz-biotin** (25 μM, 1 h at 37 °C) and **DBCO-FLAG** (100 μM, 1 h at 37 °C) to covalently tag cell surface cyclopropenes and azides, respectively. Cells were then stained with streptavidin-AF594 and FITC-α-FLAG and imaged *via* confocal microscopy. Representative images are shown. Red: AF594, green: FITC, blue: DAPI. Scale bar: 10 μm.

Conclusions

Cyclopropenes are versatile chemical reporters for biomolecule tagging in live cells. Despite their remarkable cellular compatibilities and unique chemistries, they have been inefficient reports of glycosylation to date. We developed a set of carbamate-functionalized cyclopropenes with improved utility for glycan imaging and profiling. These tools were readily processed in cultured cells and rapidly ligated with tetrazine probes. The carbamate-cyclopropene sugars can also be used in tandem with other chemical reporters including azides and alkynes, and we utilized combinations of these tools to tag two different subsets of glycans in live cells. Future multi-component imaging studies of glycans and related biomolecules will benefit from the versatility of the cyclopropene probes presented here.

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