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Functionalized Cyclopropenes As Bioorthogonal Chemical Reporters

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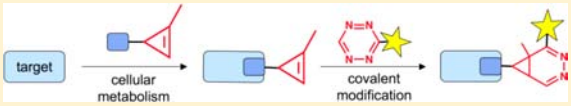
Supporting Information

ABSTRACT: Chemical reporters are unique functional groups that can be used to label biomolecules in living systems. Only a handful of broadly applicable reporters have been identified to date, owing to the rigorous demands placed on these functional groups in biological settings. We describe here a new chemical reporter—cyclopropene—that can be used to target biomolecules *in vitro* and in live cells. A variety of substituted cyclopropene scaffolds were synthesized and found to be stable in aqueous solution and in the presence of biological nucleophiles. Furthermore, some of the cyclopropene units were metabolically introduced into cell surface glycans and subsequently detected with covalent probes. The small size and selective reactivity of cyclopropenes will facilitate efforts to tag diverse collections of biomolecules *in vivo*.



INTRODUCTION

The bioorthogonal chemical reporter strategy is among the most popular methods to tag biomolecules in live cells and whole animals.[1] This technique relies on the metabolic introduction of a unique functional group (i.e., a chemical reporter) into a biomolecule of interest (Figure 1A). The reporter is detected in a second step using highly selective (i.e., bioorthogonal) chemistries.[2] Depending on the type of covalent labeling agent employed, this two-step approach can be used to visualize biomolecules in cellular environments or enrich them for further analyses.

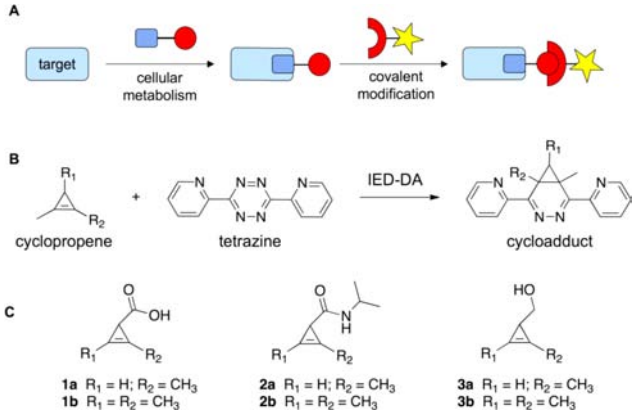


Figure 1. Chemical reporters and bioorthogonal chemistries. (A) Bioorthogonal chemical reporter strategy. A biomolecule of interest (light-blue rectangle) can be targeted with a chemical reporter group (red circle) appended to a metabolic precursor (dark-blue rectangle). Subsequent covalent reaction enables the target biomolecule to be visualized or retrieved. (B) Cyclopropenes undergo cycloaddition reactions with tetrazine scaffolds. (C) Panel of cyclopropene analogues examined in this study.

While powerful, the bioorthogonal chemical reporter strategy has been limited to only a handful of broadly functional reporter groups. This select class includes ketones, terminal alkynes, and organic azides.[3−6] Azides, in particular, have been widely utilized in live cells and animals owing to their remarkable biocompatibility and unique reactivity.[2,7,8] Azides can be readily affixed to metabolic precursors that target glycans, lipids, and numerous other biomolecules.[9−11] Once installed, these motifs can be selectively reacted with soft nucleophiles (via Staudinger ligation) or activated alkynes (via copper-free "click" chemistry) without detriment to the cell or organism.[12−16] Identifying new chemical reporters remains an important, yet challenging, goal as most functional groups do not meet the stringent criteria required for use in living systems. The scaffolds must remain inert to endogenous biological functionality yet react robustly with complementary probes in complex environments. Chemical reporters must also traverse biosynthetic pathways and, thus, be minimally perturbing to the cell's metabolic machinery.

In recent years, strained alkenes and alkynes have been identified that meet several of the criteria for broadly applicable chemical reporters.[8] These scaffolds, including *trans*-cyclooctene (TCO), norbornene (NB), and bicyclononyne (BCN), are abiotic and relatively stable in cellular environs.[17−20] Furthermore, they react rapidly with electron-poor tetrazines via inverse-electron-demand Diels−Alder (IED-DA) reactions. The remarkable speed of these reactions is well suited for sensitive imaging applications, and a variety of TCO- and NB-conjugated nanoparticles and antibodies have been utilized for this purpose.[21−23] More recently, Chin and others have demonstrated that amino acids outfitted with BCN, TCO, or NB can be incorporated into cellular proteins utilizing engineered strains of bacteria; the functionalized proteins can be subsequently targeted with visual probes via IED-DA ligations.[21,24,25] While useful, strained alkenes and alkynes have been slow to transition as reporter groups for other metabolic

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pathways. This is due, in part, to their large size and incompatibility with many endogenous biosynthetic pathways.

We aimed to examine a smaller strained olefin—cyclopropene—for use as a bioorthogonal chemical reporter. Cyclopropenes are not present in most eukaryotes, and are likely compatible with a variety of metabolic pathways owing to their small size. In fact, the steric demand of a cyclopropene unit is on par with diazirine, a widely used functional group in cellular labeling and photocross-linking studies.[26,27] Cyclopropenes also possess a large amount of strain energy that can drive IED-DA reactions and other cycloadditions under relatively mild conditions (Figure 1B).[28,29] These types of transformations are particularly attractive for use in biological settings and have been the subject of recent work by Devaraj and co-workers.[29] In this article, we describe the development and utilization of cyclopropenes as chemical reporters in living systems.

RESULTS AND DISCUSSION

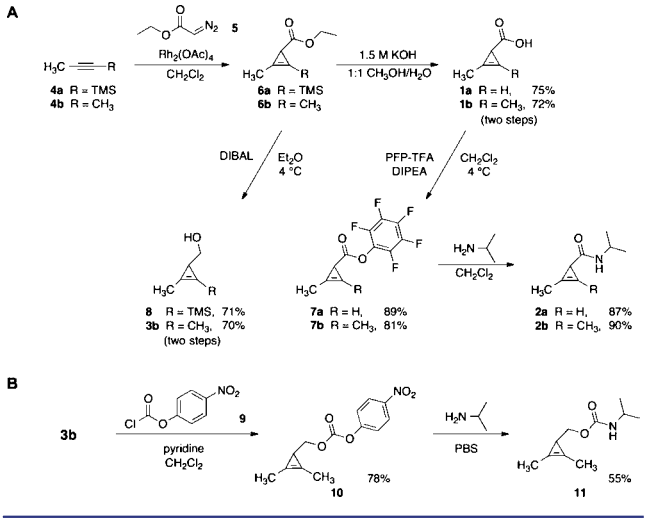
Design and Synthesis of Biocompatible Cyclopropenes. While cyclopropenes possess many favorable attributes for cell-based studies, they are not without limitation. Cyclopropene itself is prone to polymerization at room temperature and susceptible to attack by thiols and other biological nucleophiles.[30,31] However, several lines of evidence suggest that modifications to the cyclopropene core can markedly improve scaffold stability. For example, substituted cyclopropenes are found in both plant and marine natural products, indicating that C-1- and C-2-modified olefins possess some degree of metabolic stability.[32−34] Methyl-substituted cyclopropenes are also produced on the ton-scale in the agricultural industry and used in produce transport.[35] Additionally, carbonyls and other electron-withdrawing groups positioned at C-3 are known to stabilize cyclopropenes by imparting partial aromatic character to the ring.[36−38]

We reasoned that a combination of steric and electronic modifications to the cyclopropene core would provide a chemical reporter suitable for metabolic labeling without compromising cycloaddition reactivity. To test this hypothesis, we designed a panel of cyclopropenes with vinyl methyl substituents and various C-3 appendages (Figure 1C). The C-3 groups differed in their electron-withdrawing character and, in some cases, provided handles for eventual attachment to metabolic precursors. We were particularly attracted to the amide- and carbamate-functionalized scaffolds (**2** and **11**, respectively) as these linkages mimic those found in numerous bioconjugates.

Cyclopropenes can be readily accessed from alkynes, but the synthesis of such low-molecular weight compounds presents unique challenges. Many cyclopropenes are volatile and, as mentioned earlier, prone to polymerization upon concentration. To mitigate against these effects, we utilized disubstituted alkynes in the early stages of our syntheses. TMS-protected propyne and 2-butyne were first subjected to rhodium-catalyzed cyclopropenation with ethyl diazoacetate (**5**) to provide esters **6a,b** (Scheme 1A). Subsequent hydrolysis of the isolated esters afforded the free acids **1a,b** in good yield.

With **1** and **6** in hand, we were poised to access the remaining C-3-modified scaffolds. The amide-functionalized cyclopropenes **2a,b** were prepared by treating **1a,b** with pentafluorophenyl trifluoroacetate (PFP-TFA), followed by isopropylamine. The hydroxy-substituted cyclopropenes **3b** and **8** were generated via DIBAL-mediated reduction of **6**.

Scheme 1. Synthesis of Functionalized Cyclopropenes



Unfortunately, attempts to deprotect **8** to afford the monosubstituted cyclopropene **3a** were unsuccessful. NMR analyses suggested that **3a**—with a single methyl substituent and no electron-withdrawing group—polymerizes rapidly upon concentration (data not shown). Last, the carbamate scaffold **11** was isolated in two steps from **3b** (Scheme 1B). In contrast to **3a**, cyclopropenes **1**, **2**, **3b**, and **11** exhibited remarkable stability in aqueous buffer and in the presence of biologically relevant thiols. Scaffolds **2a** and **11**, in particular, were found to be stable for extended periods of time in solution and in the presence of cysteine (Figures S1, S2 in SI).

Analysis of Cyclopropene−Tetrazine Reactivity. To examine whether the substituted cyclopropenes were still amenable to facile cycloaddition, we subjected **1**, **2**, **3b**, and **11** to the model dipyridyl-tetrazine reagent **12**. Cycloadduct formation was observed in all cases when excess cyclopropene was used (Figure 2A, Figure S3, SI), although the products formed between **1** and **12** degraded rapidly in solution. The reactions also exhibited distinct fuchsia-to-yellow color changes that were used to calculate second-order rate constants for the transformations (Table 1, Figures S4 and S5 in SI). As expected, faster reactions were observed in more polar solvents and with less sterically congested cyclopropenes.[29] Additionally, cyclopropenes with reduced electron-withdrawing character at C-3 were found to react more expediently with **12**, in agreement with previous studies.[36] Scaffolds **2a** and **11** also exhibited comparable reactivity with a functionalized tetrazine probe.

While the cyclopropene reactions are markedly slower than other tetrazine-based ligations (~4−5 orders of magnitude slower than some TCO reactions),[39] they are still suitable for use in biological systems. In fact, the cycloaddition rates measured for **2a** and **11** are on par with two bioorthogonal reactions widely used in live cells and animals: the Staudinger ligation ($k = 0.25 \times 10^{-2}$ M^{-1} s^{-1} in 5% H_2O/CH_3CN) and the strain-promoted azide−alkyne cycloaddition with a difluorinated cyclooctyne ($k = 7.6 \times 10^{-2}$ M^{-1} s^{-1} in CH_3CN).[14,40] These reactions remain popular despite their relatively slow rates, as the need for small, nonperturbing chemical reporters (e.g., azides) can often trump the need for rapid reactivity in living systems.

Our analyses of the cycloaddition reactions also revealed important mechanistic details. Cyclopropene−tetrazine liga-

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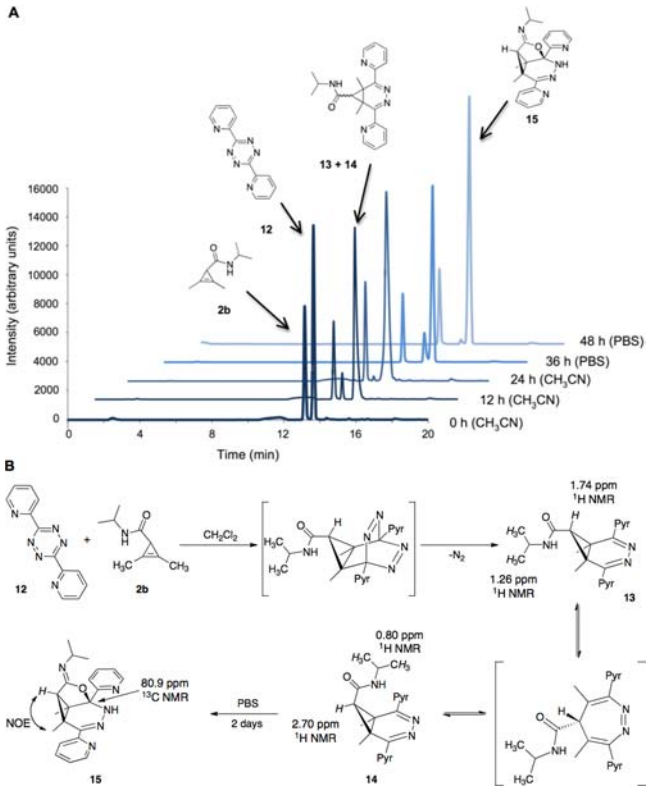


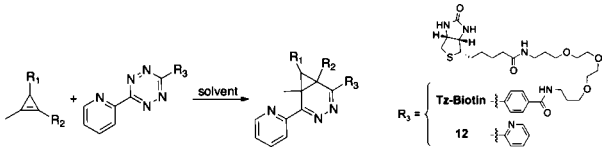
Figure 2. Cyclopropenes react with tetrazines to form covalent adducts. (A) HPLC analysis of the cycloaddition between **2b** and **12**. The reaction was initiated in organic solvent prior to the addition of aqueous buffer. (B) Cyclopropene–tetrazine ligation proceeds via an initial Diels–Alder reaction, followed by N_2 elimination. Subsequent ring-opening and closing provides a mixture of diastereomers (**13** and **14**). Intramolecular cyclization ultimately affords the tricyclic adduct **15**. Diagnostic NMR chemical shifts are noted.

tions proceed via an initial Diels–Alder cycloaddition, followed by N_2 expulsion. Facial selectively in the initial cycloadduct is dictated by steric considerations, with the anti-addition product likely predominating for most cyclopropenes (Figure 2 and Figures S6–S12 in SI).[41] For disubstituted cyclopropenes, though, the strain associated with multiple suprafacial substituents can drive further electrocyclic ring-opening.[42,43] Subsequent ring closure can ultimately alter the position of the C-3 substituent (placing it over the tetrazine ring, as in **14**). Indeed, when dimethyl cyclopropene **2b** was treated with **12**, resonances for both **13** and **14** were observed in the ^{1}H NMR spectrum. We also noticed that the yellow color of this solution faded over time in aqueous buffer. NMR and HPLC analyses revealed that the initial cycloadduct undergoes further intramolecular attack at the imine carbon to yield **15** (Figure 2 and Figures S6–S8 in SI). This internal cyclization was also observed when cyclopropene **3b** was treated with tetrazine **12**, although intramolecular attack proceeded at a faster rate (Figures S9, S10 in SI). By contrast, when **11** (lacking a suitable C-3 nucleophile) was treated with **12**, no intramolecular cyclization was observed following ring-opening (Figures S11, S12 in SI). While some cyclopropene–tetrazine adducts are prone to further rearrangement, it is important to note that the starting materials remain covalently linked.

Protein Modification via Cyclopropene–Tetrazine Ligation. In addition to undergoing rapid and selective ligation reactions, chemical reporters must function in complex environments. To evaluate the cyclopropene scaffolds in a biologically relevant setting, we appended the reporters to a model protein (lysozyme or BSA). Standard NHS-ester- and carbonate-coupling reactions were used to attach cyclopropene scaffolds **10** and **16**, respectively, to the protein surface (Figure 3A and Scheme S1 in SI). The modified protein samples were then reacted with a rhodamine-functionalized tetrazine scaffold (**Tz-Rho**, Scheme S2 in SI) and analyzed via mass spectrometry (Figure S13 in SI) or in gel fluorescence imaging (Figures 3A, Figure S14 in SI). As depicted in parts B–D of Figure 3 and in Figure S14 in SI, the ligations were both time- and dose-dependent, and no reaction was observed in the absence of either **Tz-Rho** or cyclopropene.

We also examined whether the cyclopropene–tetrazine ligation is compatible with azides and strained alkynes. The orthogonality of such reagents would enable cyclopropenes to be used in tandem with established bioorthogonal chemistries for dual labeling experiments. Cyclopropenes are known to react with organic azides and other 1,3-dipoles, but such reactions typically require strong heating.[44] Indeed, when cyclopropenes **2a** or **11** were subjected to azidoethanol or phenyl azide in organic solvent, no reaction was observed under ambient conditions over 24 h (data not shown). Additionally,

Table 1. Cycloaddition Rates Observed between Cyclopropene and Tetrazine Scaffolds



entry	cyclopropene	R_1	R_2	tetrazine	solvent	k (10^{-2} M^{-1} s^{-1})
1	**2a**	–C(O)NH–*i*-Pr	–H	**12**	CH_3CN	3.0 ± 0.2
2	**2a**	–C(O)NH–*i*-Pr	–H	**12**	CH_3CN/PBS	5.1 ± 0.7
3	**2a**	–C(O)NH–*i*-Pr	–H	**Tz-biotin**	CH_3CN/PBS	1.4 ± 0.2
4	**2b**	–C(O)NH–*i*-Pr	$-CH_3$	**12**	CH_3CN	ND[a]
5	**3b**	$-CH_2OH$	$-CH_3$	**12**	CH_3CN	1.9 ± 0.3
6	**3b**	$-CH_2OH$	$-CH_3$	**12**	CH_3CN/PBS	15 ± 2
7	**11**	$-CH_2OC(O)NH$–*i*-Pr	$-CH_3$	**12**	CH_3CN	0.28 ± 0.03
8	**11**	$-CH_2OC(O)NH$–*i*-Pr	$-CH_3$	**12**	CH_3CN/PBS	3.5 ± 0.5
9	**11**	$-CH_2OC(O)NH$–*i*-Pr	$-CH_3$	**Tz-biotin**	CH_3CN/PBS	3.9 ± 0.5

[a]Due to slow kinetics, rate was determined by ^{1}H NMR in CD_3OD (k = 0.037 ± 0.006 × 10^{-2} M^{-1} s^{-1}).

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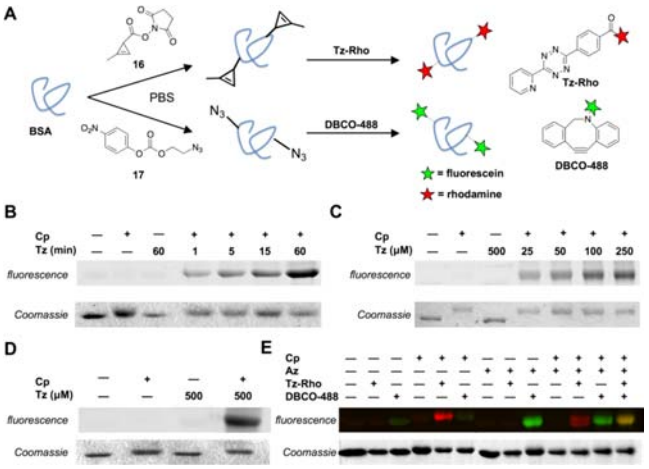

Figure 3. Methylcyclopropenes can be selectively modified on protein surfaces. (A) Cyclopropenes (Cp) and azides (Az) were appended to BSA (12.5 mg/mL in PBS) via NHS ester coupling or carbonate activation (8.4 mM labeling reagent). The labeled proteins (2 mg/mL) were subsequently reacted with either a tetrazine–rhodamine (**Tz-Rho**) conjugate or a cyclooctyne–fluorescein conjugate (**DBCO-488**). (B) Gel analysis of cyclopropene-modified BSA incubated with 100 μM **Tz-Rho** for 0–60 min or no reagent (–). (C) Gel analysis of Cp-modified BSA labeled with **Tz-Rho** (0–250 μM) for 1 h. (D) Gel analysis of Cp-modified BSA (+) or BSA only (–) treated with **Tz-Rho** (500 μM) or no reagent (–) at 37 °C for 1 h. (E) Gel analysis of BSA functionalized with Cp, Az, or both chemical reporters (lanes 10–13) and reacted for 1 h with either 100 μM **Tz-Rho**, **DBCO-488**, both reagents simultaneously, or no reagent. For B–E, protein loading was assessed with Coomassie stain (lower panels).

when cyclopropene (Cp)- and azide (Az)-modified BSA conjugates were mixed together (providing Cp/Az-BSA), both functional groups could be selectively targeted with covalent probes (either **Tz-Rho** or a dibenzocyclooctyne–fluorophore conjugate, **DBCO-488**),[45] suggesting that Cp and Az can coexist to a certain extent (lanes 11–13, Figure 3E). Reduced fluorescence intensities were observed when either **DBCO-488** or **Tz-Rho** was incubated with Cp/Az-BSA (compared to BSA samples modified with either Az or Cp alone), but this was likely due to fewer reporter groups present in the sample itself—Cp-BSA and Az-BSA were combined 1:1 to generate the mixed sample (Cp/Az-BSA), halving the number of available reporter groups (Figure 3E).

Cyclopropene reactivity with cyclooctynes and other strained molecules has not been extensively investigated. However, when cyclopropene-modified BSA (Cp-BSA) was treated with **DBCO-488**, no signal above background was observed under the labeling conditions employed (lane 6, Figure 3E). The faint fluorescence signal can be attributed to nonspecific **DBCO-488** reactivity (lane 3, Figure 3E). By contrast, the compatibility of tetrazine scaffolds with both azides and strained alkynes has been examined in more detail.[22,24,46] In a recent study, Hilderbrand and co-workers observed reactivity between a monosubstituted tetrazine and a DBCO conjugate ($k = 6 \times 10^{-2}$ M^{-1} s^{-1}). However, no reaction was observed when a disubstituted, deactivated tetrazine was employed.[22] The authors further demonstrated that the kinetically slower tetrazine could be used in tandem with DBCO to label TCOs and azides in cells. In our studies, **Tz-Rho** was expected to react with DBCO (albeit minimally) based on cycloaddition rates measured for similar tetrazines and TCO.[39] However, when **Tz-Rho** was incubated with **DBCO-488**, no reaction was observed over 12 h in PBS (Figure S15 in SI). Additionally, coadministration of **Tz-Rho** and **DBCO-488** to Cp/Az-BSA did not significantly diminish covalent protein labeling (Figure 3E, Figure S16 in SI). While a detailed analysis of tetrazine-DBCO reactivity has not been performed, our results suggest that the two reagents can be used concurrently to target cyclopropenes and azides under certain conditions.

Metabolic Incorporation of Cyclopropenes onto Live Cell Surfaces. Beyond biomolecule modification *in vitro*, chemical reporters must be able to traverse metabolic pathways *in vivo*. This requires that the scaffolds are stable in living systems and small enough to be tolerated by biosynthetic enzymes.[1] To investigate whether cyclopropenes would be useful for cellular labeling studies, we constructed a methylcyclopropene–sialic acid conjugate (**9-Cp-NeuAc**, Scheme S1 in SI). Modified sialic acids of this sort are known to be metabolized by cells and incorporated into cell surface glycans.[47–50] Jurkat cells were incubated with various concentrations of **9-Cp-NeuAc** for 24–48 h. The presence of cell surface cyclopropenes was subsequently probed by reaction with a tetrazine–biotin conjugate (**Tz-Biotin**, Scheme S2 in SI) and avidin staining (Figure 4A). The fluorescence of each cell

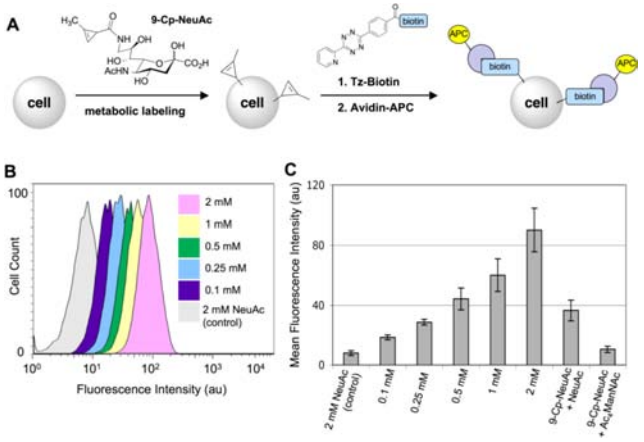

Figure 4. Cyclopropenes can be metabolically incorporated onto live cell surfaces. (A) Jurkat cells were incubated with **9-Cp-NeuAc** (0–2 mM), a control sugar (NeuAc, 2 mM) or both **9-Cp-NeuAc** and NeuAc (or Ac_4ManNAc) for 24 h. After washing, the cells were reacted with **Tz-Biotin** (100 μM) for 1 h at 37 °C. Subsequent staining with APC–avidin and flow cytometry analysis provided the plots in (B). (C) Mean fluorescence intensities (in arbitrary units, au) for the histograms in (B). Error bars represent the standard deviation of the mean for three experiments.

population was measured using flow cytometry. As shown in Figure 4B, a dose-dependent increase in signal was observed when cells were incubated with increasing concentrations of **9-Cp-NeuAc**, indicating successful metabolic incorporation of the chemical reporter. The incorporation efficiency of **9-Cp-NeuAc** was lower than that of a similarly functionalized azido sugar (**9-Az-NeuAc**, Scheme S1 in SI) but on par with other unnatural sialic acids used in metabolic engineering studies (Figure S17 in SI).[47–50] Importantly, the fluorescence signal also diminished when **9-Cp-NeuAc**-treated cells were cultured in the presence of unlabeled sugars (sialic acid, NeuAc, or peracetylated *N*-acetylmannosamine, Ac_4ManNAc) targeting the same metabolic pathway.[47]

We further investigated whether cyclopropene- and azide-modified sugars could be utilized concurrently for live cell

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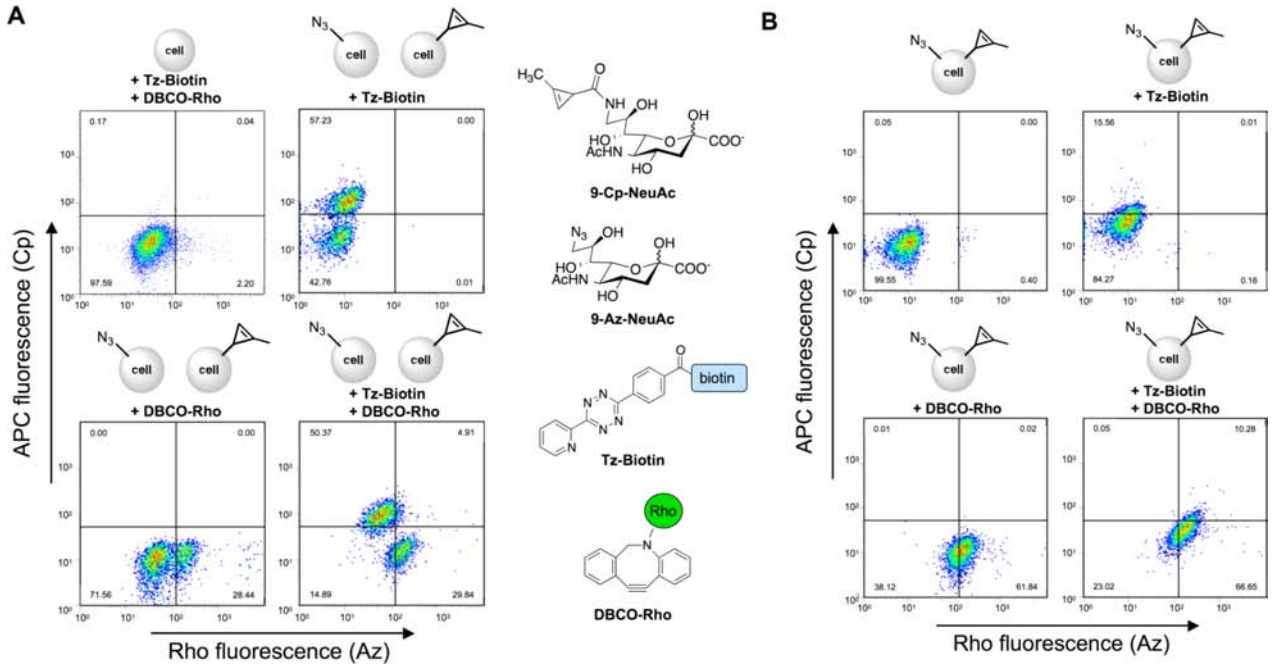

Figure 5. Methylcyclopropenes and organic azides can be utilized in tandem for cellular metabolic labeling. (A) Flow cytometry analysis of Jurkat cells treated with **9-Cp-NeuAc** (1 mM), **9-Az-NeuAc** (1 mM), or no sugar for 24 h. After washing, a portion of the **9-Cp-** and **9-Az-NeuAc** cells were mixed. Cell samples were then washed and subsequently reacted with **Tz-biotin** (100 μM), **DBCO-Rho** (100 μM), or both reagents for 1 h at 37 °C. Following staining with APC–avidin, cellular fluorescence was measured. Plots are shown with Rho (azide) and APC (cyclopropene) levels on the *x*- and *y*-axes, respectively. (B) Flow cytometry analysis of Jurkat cells incubated with **9-Cp-NeuAc** (1 mM) and **9-Az-NeuAc** (1 mM) simultaneously. After 24 h, the cells were washed, reacted, and analyzed as in (A). For (A) and (B), the same patterns of labeling were apparent in replicate experiments.

labeling. In one setup, Jurkat cells were incubated with **9-Cp-NeuAc**, **9-Az-NeuAc**, or no sugar. After 24 h, portions of the sugar-treated cells were combined. In a second setup, Jurkat cells were cultured with both sugars simultaneously. All samples were subsequently reacted with either **Tz-Biotin**, a water-soluble cyclooctyne-fluorophore conjugate (**DBCO-Rho**), or both reagents. Cells treated with **Tz-Biotin** were also stained with APC–avidin. The fluorescence of the resulting cell populations was analyzed via two-color flow cytometry, and the corresponding plots are depicted in Figure 5. For cells cultured separately with the unnatural sugars prior to mixing and covalent reaction, flow analysis revealed two distinct cell populations—one with robust APC fluorescence (corresponding to the **9-Cp-NeuAc**-treated cells) and one with robust rhodamine fluorescence (corresponding to the **9-Az-NeuAc**-treated cells) (Figure 5A). For cells cultured with the cyclopropenyl and azido sugars simultaneously, treatment with both covalent probes and flow analysis revealed a single population of cells labeled with both fluorophores (Figure 5B). The overall fluorescence signal attributed to the cyclopropene modification was reduced in this case, although likely due to the lower incorporation efficiency of **9-Cp-NeuAc** compared to that of **9-Az-NeuAc**. Nonspecific reactivity with **DBCO-Rho** was also observed in the cell-labeling studies, but importantly, no cross-reactivity was observed when **9-Az-NeuAc**-treated cells were labeled with **Tz-Biotin** or when **9-Cp-NeuAc**-treated cells were labeled with **DBCO-Rho** (Figure S18 in SI). Collectively, these results suggest that cyclopropene- and azide-based chemical reporters can be utilized together in live cells and will be useful for multiplexed metabolic engineering strategies.

CONCLUSIONS

In summary, functionalized cyclopropenes have been developed for use as chemical reporters in living systems. These scaffolds react with tetrazines to form covalent adducts in high yield and with rates suitable for biological labeling applications. Our data also indicate that cyclopropenes are stable in biological environs and can be used to derivatize proteins and other biomolecules. Moreover, these functional groups can be metabolically introduced into cellular glycans, suggesting they are small enough to traverse biosynthetic pathways in live cells. Methylcyclopropenes can also be used in tandem with organic azides, and we anticipate that combinations of these and other chemical reporters will be widely used for targeting multiple classes of biomolecules.[51]

This work also sets the stage for continued expansion of the bioorthogonal chemistry toolkit. We envision developing a collection of cyclopropene scaffolds suitable for use as both chemical reporters and secondary labeling agents. Toward this end, we are generating cyclopropenes that react more rapidly with tetrazine probes, along with identifying scaffolds with alternative modes of reactivity. These reagents will bolster efforts to monitor multicomponent biomolecular processes in living systems.

ASSOCIATED CONTENT

Supporting Information

Experimental details, full spectroscopic data for all new compounds, and additional images. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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