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# Polar Dibenzocyclooctynes for Selective Labeling of Extracellular Glycoconjugates of Living Cells

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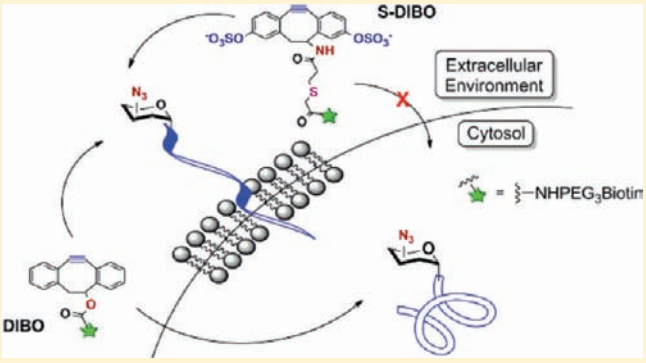
Supporting Information

**ABSTRACT:** Although strain-promoted alkyne−azide cycloadditions (SPAAC) have found wide utility in biological and material sciences, the low polarity and limited water solubility of commonly used cyclooctynes represent a serious shortcoming. To address this problem, an efficient synthetic route has been developed for highly polar sulfated dibenzocyclooctynylamides (S-DIBO) by a Friedel−Crafts alkylation of 1,2-bis(3-methoxyphenyl)ethylamides with trichlorocyclopropenium cation followed by a controlled hydrolysis of the resulting dichlorocyclopropenes to give bis(3-methoxyphenyl)-cyclooctacyclopropenones, which were subjected to methoxy group removal of the phenols, *O*-sulfation, and photochemical unmasking of the cyclopropenone moiety. Accurate rate measurements of the reaction of benzyl azide with various dibenzylcyclooctyne derivatives demonstrated that aromatic substitution and the presence of the amide function had only a marginal impact on the rate constants. Biotinylated S-DIBO **8** was successfully used for labeling azido-containing glycoconjugates of living cells. Furthermore, it was found that the substitution pattern of the dibenzylcyclooctynes influences subcellular location, and in particular it has been shown that DIBO derivative **4** can enter cells, thereby labeling intra- and extracellular azido-modified glycoconjugates, whereas S-DIBO **8** cannot pass the cell membrane and therefore is ideally suited for selective labeling of cell surface molecules. The ability to selectively label cell surface molecules will yield unique opportunities for glycomic analysis and the study of glycoprotein trafficking.




## INTRODUCTION

Metal-free cycloadditions of cyclooctynes with azides to give stable 1,2,3-triazoles have found wide utility in labeling glycans, proteins, and lipids of living cells, glycoprotein enrichment for proteomics, and tissue reengineering.[1] These reactions, which have been coined "strain-promoted alkyne-azide cycloadditions (SPAAC)", have also made entry in material sciences and have, for example, been employed for the assembly and surface modification of dendrimers,[2] derivatization of polymeric nanostructures,[3] and patterning of surfaces.[4,5] The first generation of cyclooctynes (compound **1**, Figure 1) exhibited relatively slow rates of reaction;[6] however, it has been found that significant increases in the rate of strain-promoted cycloaddition can be accomplished by appending electron-withdrawing groups to the propargylic position of cyclooctyne. For example, difluorinated cyclooctyne (DIFO, **2**) reacts with azides approximately 60 times faster than similar cycloadditions with an unsubstituted cyclooctyne.[7] We have found that derivatives of 4-dibenzocyclooctynol (DIBO, **3**, **4**) react fast with azido-containing biomolecules and can be employed for visualizing metabolically labeled glycans of living cells.[8] Attractive features of DIBO include easy access to the compound by a simple synthetic approach, nontoxicity, and the possibility of straightforward attachment of a variety of probes.

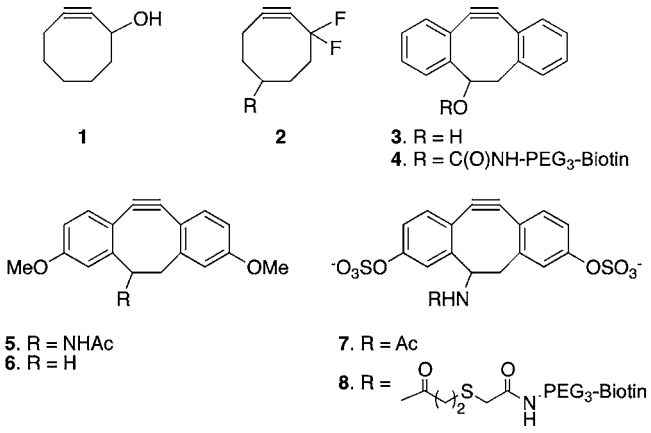



**Figure 1.** Reagents for labeling of azido-containing biomolecules.

Furthermore, dibenzocyclooctynes can be generated photochemically from corresponding cyclopropenones by short irradiation with UV light, thereby providing opportunities for the spatial and

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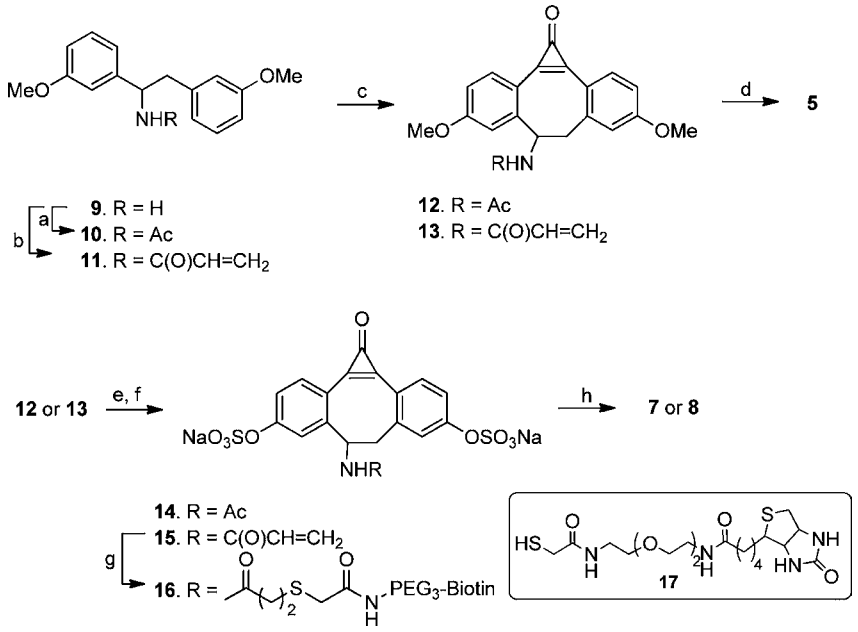
**Scheme 1**[a]




[a]Reagents and conditions: (a) $Ac_2O$, $Et_2i$-PrN, 0 °C to room temperature; (b) $CH_2$=CHC(O)Cl, $Et_2i$-PrN, 0 °C to room temperature; (c) $AlCl_3$, tetrachlorocyclopropene, $CH_2Cl_2$, −20 °C to room temperature, 5 $H_2O$; (d) *hν*, 350 nm, MeOH, $H_2O$; (e) $BBr_3$, $CH_2Cl_2$, −78 °C to room temperature; (f) $SO_3$·Py, DMF, then $Na^+$ resin; (g) **17**, Tris buffer, pH = 8, TCEP; (h) *hν*, 350 nm, $H_2O$.

temporal controlled labeling of the target substrates.[9] It has also been shown that by employing nitrones and nitrile oxides as 1,3-dipoles,[10,11] the rate of cycloaddition can be further enhanced, and this technology has, for example, made it possible to selectively tag proteins at the *N*-terminus or perform sequential modifications of complex compounds.[11] Several analogues of DIBO have also been reported that exhibit even higher rates of cycloaddition with azides.[5,12]

Despite the many attractive features of the second-generation cyclooctynes, their hydrophobicity and resulting limited water solubility represent a serious shortcoming. In particular, it can cause sequestration by membranes or nonspecific binding to serum proteins, thereby reducing bioavailability.[13] To address these difficulties, we report here the chemical synthesis of highly polar sulfated dibenzocyclooctynylamide (S-DIBO) derivative **8**, which was successfully used for labeling azido-containing glycoconjugates of living cells. Sulfate esters of phenols have excellent stabilities under moderately acidic and basic conditions[14] and are found in nature as post-translational modifications of tyrosine.[15] Therefore, it was expected that compounds such as **8** would possess appropriate properties for cell-based studies. The properties of **8** have been compared to those of parent derivative **4**, and it was found, for the first time, that their substitution pattern determines membrane permeability and in particular DIBO (**4**) can enter cells, thereby labeling intra- and extracellular azido-modified glycoconjugates, whereas S-DIBO (**8**) cannot pass the cell membrane and therefore is ideally suited for selective labeling of cell surface molecules.

## RESULTS AND DISCUSSION

**Chemical Synthesis.** We envisaged[9] that Friedel−Crafts alkylation of **10** and **11** with trichlorocyclopropenium cation would give, after a controlled hydrolysis, cyclopropenones **12** and **13**, respectively, which upon removal of the methoxy groups and subsequent *O*-sulfation and photochemically unmasking of the cyclopropenone moiety, would give easy entry into sulfated dibenzocyclooctynylamides (Scheme 1). Furthermore, it was expected that various tags could be introduced by a thio-Michael addition to the acrylamide moiety of the intermediate cyclopropenone **13** to give compounds such as biotin-containing **8**.[16] The attraction of a thio-Michael addition is that it possesses benefits similar to those of the parent thiol−ene click reaction,[17] such as mild reaction conditions, high functional group tolerance, and the formation of a biologically stable carbon−sulfur bond, but does not involve the need of generating radicals. Derivatives **5**, **6**, and **7** were prepared to determine the effect of aromatic substitution and the amide moiety on the kinetic parameters of cycloaddition.

Primary amine **9** (Scheme 1) could easily be prepared in high yield by condensation of 3-methoxybenzylmagnesium chloride (see the Supporting Information) with 3-methoxybenzonitrile followed by reduction of the resulting imine with sodium borohydride.[18] Acylation of the amine of **9** with acetic anhydride or acryloyl chloride in the presence of DIPEA in $CH_2Cl_2$ gave compounds **10** and **11**, respectively. Friedel−Crafts alkylation of **10** and **11** with tetrachlorocyclopropene in the presence of $AlCl_3$ (3−4 equiv)[9,19] yielded intermediate aromatic dichlorocyclopropenes, which were hydrolyzed in situ, to give the key cyclopropenones **12** and **13** in moderate yields of approximately 60%. The methyl ethers of **12** and **13** were cleaved with boron tribromide[20] and subsequent *O*-sulfation of the resulting phenoxy groups with $SO_3$·Py in DMF, followed by filtration through a $Na^+$ exchange resin gave cyclopropenones **14** and **15** as stable sodium salts. The acrylamide moiety of **15** could easily be modified by biotin by a thio-Michael addition[16] of **17** in Tris·HCl buffer (pH = 8), containing tris(2-carboxyethyl)phosphine hydrochloride (TCEP) to give **16**. Finally, photochemical decarbonylation of the cyclopropenone moieties of **12**, **14**, and **16** at 350 nm gave the target compounds **5**, **7**, and **8**, respectively. Compound **6** was prepared by a similar strategy starting from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1-methoxy-3-[2-(3-methoxyphenyl)ethyl]benzene **24** (see the Supporting Information).

It was observed that cyclooctynes **7** and **8** have an excellent shelf life in PBS buffer over a period of 24 h and remained intact after treatment with nucleophiles such as thiols and amines. In particular, no decomposition of **8** was observed by HPLC when incubated in the presence of a 10 mM glutathione (see the Supporting Information for details).

**Reaction Kinetics.** Accurate rate measurements of the cycloaddition of **3**, **5**, **6**, and **7** with benzyl azide were conducted by UV spectroscopy following the decay of the characteristic absorbance of the acetylene of the cyclooctynes at 312 nm. The rates were measured in methanol at 25 ± 0.1 °C. The kinetics of the cycloadditions were studied under pseudo first-order conditions by maintaining a fixed concentration of dibenzocyclooctyne while the concentration of benzyl azide was varied. Consumption of starting material followed a first-order equation, and the pseudo first-order rate constants were obtained by least-squares fitting of the data to a single exponential equation. The observed rate constants were linearly dependent on the concentration of dipoles (see the Supporting Information), and second-order cycloaddition rate constants calculated from the concentration dependencies of observed rates are listed in Table 1. As can be seen, the new

**Table 1. Second-Order Rate Constants for the Cycloaddition of Various Dibenzocyclooctynes with Benzyl Azide in Methanol at 25 ± 0.1 °C**

| compound | second-order rate constants ($M^{-1}$ $s^{-1}$) |
|---|---|
| **3** | 0.059 ± 0.006 |
| **5** | 0.128 ± 0.003 |
| **6** | 0.073 ± 0.003 |
| **7** | 0.116 ± 0.002 |

derivatives reacted slightly faster than DIBO **3**. Furthermore, the acetamido function of **5** makes the reaction 2 times faster than unfunctionalized **6**, and possible small differences in conformational properties may account for the difference in reaction rates. The methoxy- and sulfate-modified **5** and **7** exhibited similar kinetic parameters, highlighting that substitution of the aromatic ring does not impact the rate of cycloaddition.

**Cellular Labeling Studies.** Having established an efficient synthetic approach for the target sulfated DIBO derivative **8**, attention was focused on labeling living cells modified with azido moieties. Azides can be incorporated into biomolecules using a variety of strategies[21] such as post synthetic modification, in vitro enzymatic transfer, the use of covalent inhibitors, and metabolic labeling by feeding cells a biosynthetic precursor modified with an azido function. We opted for metabolic labeling with peracetylated *N*-α-azidoacetylmannosamine ($Ac_4$ManNAz), which is an appropriate substrate for the cell's glycosylation machinery, resulting in the incorporation of azido-containing sialic acids in glycoconjugates.[22] Elegant studies by Bertozzi and co-workers have demonstrated that glycoconjugates of various model organisms can be metabolically labeled with azido-containing sugars, and such an approach has been employed to demonstrate tissue-specific expression of glycoconjugates.[23]

Thus, Jurkat cells were cultured in the presence of $Ac_4$ManNAz (25 μM) for 3 days to metabolically introduce *N*-azidoacetyl-sialic acid (SiaNAz) moieties into glycoproteins.[22] As a negative control, cells were employed that were grown in the presence of peracetylated *N*-acetylmannosamine ($Ac_4$ManNAc). The cells were exposed to 30 μM of **4** and **8** for various time periods and, after washing, stained with avidin-FITC for 15 min at 4 °C. The efficiency of the two-step cell surface labeling was determined by measuring the fluorescence intensity of the cell lysates. As can be seen in Figure 2A, the two

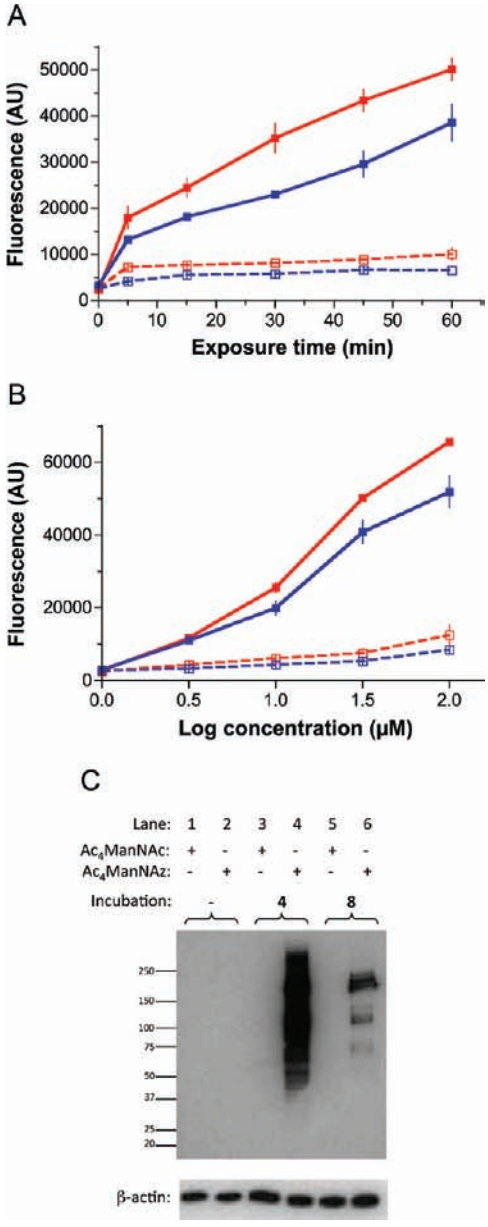


**Figure 2.** Cell surface labeling with compounds **4** and **8**. Jurkat cells grown for 3 days in the presence of $Ac_4$ManNAc (25 μM; – – –) or $Ac_4$ManNAz (25 μM; –) were incubated at room temperature with compounds **4** (red) and **8** (blue) at 30 μM for 0–60 min (A), at 0–100 μM for 1 h (B), or at 30 μM for 1 h (C). Next, either cells were incubated with avidin-FITC for 15 min at 4 °C, after which cell lysates were assessed for fluorescence intensity (A,B), or cell lysates were resolved by SDS-PAGE, and the blot was probed with an antibiotin antibody conjugated to HRP (C). Total protein loading was confirmed by Coomassie staining (Figure S1). AU indicates arbitrary fluorescence units.

compounds gave similar labeling intensities, which is in agreement with the kinetic data summarized in Table 1.

The concentration dependency of the cell surface labeling was studied by incubating the cells with various concentrations


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of **4** and **8** followed by staining with avidin-FITC (Figure 2B). As expected, cells displaying azido moieties showed a dose-dependent increase in fluorescence intensity. Reliable fluorescent labeling was achieved at a concentration of 3 $\mu$M of cyclooctyne; however, optimal results were obtained at concentrations ranging from 30 to 100 $\mu$M. Importantly, apolar DIBO derivative **4** is not soluble at high concentrations in aqueous solutions and therefore required DMF as a cosolvent (stock solution of 45 mM in DMF). On the other hand, polar S-DIBO **8** is readily soluble in water even at high concentrations (stock solution of 45 mM in water).

To examine whether DIBO (**4**) and S-DIBO (**8**) can cause unwanted side reactions with side-chain functional groups of proteins, the cell lysates were analyzed by Western blotting using an antibiotin antibody conjugated to HRP for detection (Figure 2C). Gratifyingly, the control cells gave negligible staining, demonstrating an absence of unwanted chemical reactions. A surprising observation was, however, that S-DIBO **8** exhibited a less robust and different pattern of staining as compared to parent DIBO **4**.

We contemplated that differences in cell permeability of the compounds might provide a rationale for the discrepancy of the cell labeling studies (Figure 2A,B) and Western blot analysis (Figure 2C). In this respect, the cell labeling experiments only detect surface glycoproteins because the avidin-FITC detection is performed on live cells at 4 °C, and under these conditions it cannot enter the cell and only bind to biotin-modified cell surface glycoproteins. On the other hand, detection by Western blotting is performed after cell lysis and therefore will identify all biotin-modified glycoproteins. Thus, the above-described results may be rationalized by the fact that DIBO **4** can enter the cell, resulting in labeling intracellular glycoproteins modified by azide, whereas S-DIBO **8** cannot pass the cell membrane and will only react with azido-containing glycoconjugates present in the extracellular environment.

**Chemical Modification of DIBO Results in Selective Permeability in Cultured Cells.** To address whether the DIBO derivatives exhibit differences in cell permeability, human fibroblasts were cultured in the presence of $Ac_4$ManNAz (25 $\mu$M) for 2 days followed by administration of compounds **4** and **8** (30 $\mu$M), and after an incubation time of 1 h at ambient temperature, the cells were fixed (formaldehyde in PBS), permeabilized (Triton X-100 in PBS), and then treated with streptavidin-Alexa fluor 568 to visualize azido-containing glycoconjugates. Human fibroblasts were chosen because their flat morphology and large size facilitate the visualization of intracellular organelles such as the Golgi apparatus. As shown in Figure 3A, intracellular staining using **4** was readily detected. This staining was primarily due to labeling of Golgi-localized sialoglycoproteins, as determined by the high degree of colocalization with the medial Golgi protein, giantin. Nuclear SiaNAz staining was also observed with **4** as judged by the colocalization with the ToPro stain. In contrast, very little or no intracellular staining (nuclear or Golgi) was observed with S-DIBO **8**, demonstrating that this compound is much less cell-permeable as compared to the parent compound **4**. The lack of Golgi staining was also confirmed by separating the individual confocal stacks of the maximum intensity projections shown in Figure 3A (Figure S2). Extracellular staining, consistent with the fibrillar network synthesized by fibroblasts, could also be detected in cells incubated with both compounds.

Azido-modified fibroblasts were also stained with Alexa Fluor 568-conjugated streptavidin prior to fixation and permealization to yield only staining of extracellular glycoproteins. As shown in Figure 3B, staining of cell surface glycoproteins, including those located within the fibrillar network, was observed, but no intracellular molecules could be seen. Together, these findings

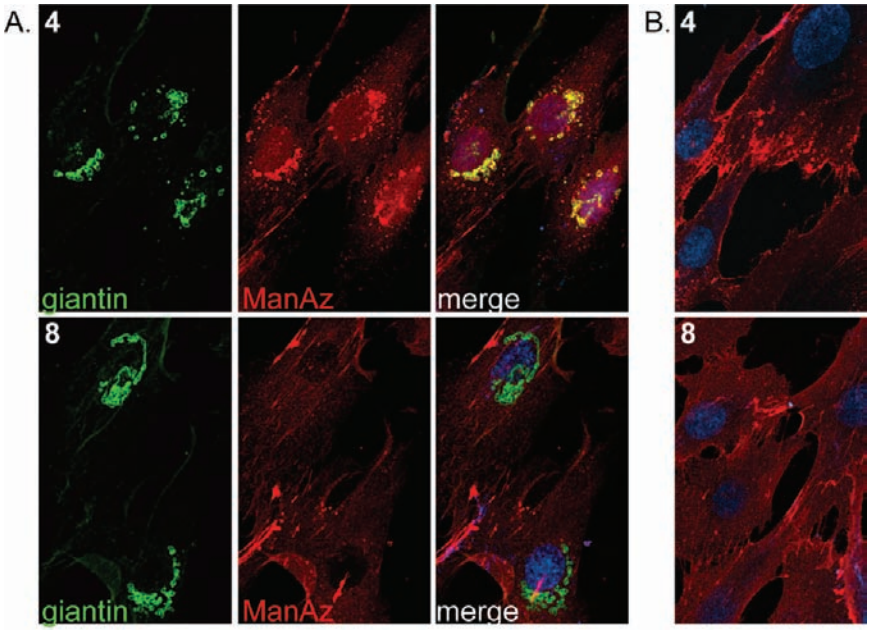


**Figure 3.** DIBO derivatives exhibit selective permeability in cultured human fibroblasts. Human skin fibroblasts grown for 2 days in the presence of $Ac_4$ManNAz (25 $\mu$M) were incubated for 1 h with **4** and **8** (30 $\mu$M) at room temperature. Following fixation, cells were permeabilized and incubated with a polyclonal antibody to the Golgi marker giantin. After incubation with appropriate conjugated fluorophores and a ToPro stain (blue) to mark the nucleus, cells were imaged. Only **4** was capable of labeling intracellular Golgi-localized proteins, indicating that this compound is cell permeable (A). Similar to (A), cells were labeled with $Ac_4$ManNAz and incubated with **4** and **8**, but staining with Alexa Fluor 568-conjugated streptavidin was performed prior to fixation and permeabilization to prevent internalization of labeled molecules. Under these conditions, DIBO **4** and its derivative **8** yield only surface staining of sialylated molecules (B). In both panels, maximum intensity projections of 6−8 individual confocal stacks are shown.


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demonstrate that DIBO derivatives can exhibit variable cell permeability that is evident under specific staining conditions.

To test whether the S-DIBO derivative **8** is capable of crossing the cell membrane following cycloaddition to SiaNAz-labeled glycoproteins at the cell surface, labeled fibroblasts were incubated with either **4** or **8** for 4 h at 37 °C. This extended labeling time was used to allow for a greater fraction of cell surface molecules to be labeled and internalized. As shown in Figure 4, staining of

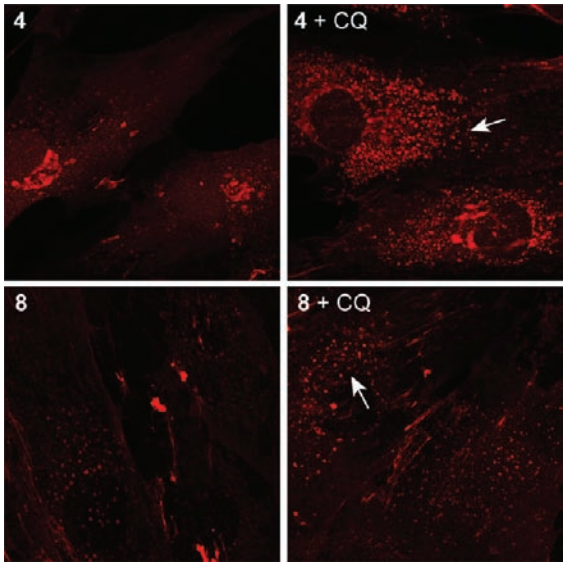


**Figure 4.** Intracellular staining of sialylated molecules can be detected using a nonpermeable DIBO derivative following chloroquine (CQ) treatment. Human skin fibroblasts grown for 2 days in the presence of $Ac_4$ManNAz (25 $\mu$M) were incubated for 4 h with **4** and **8** (30 $\mu$M) in the absence or presence of chloroquine (50 $\mu$M). Following fixation, cells were incubated with Alexa Fluor 568-conjugated streptavidin and imaged by confocal microscopy. Staining of intracellular vesicles can be readily detected using both compounds in the presence of chloroquine. Arrows denote the accumulation of SiaNAz-positive intracellular vesicles.

Golgi-localized sialoglycoproteins was again observed for **4**, but not for **8**.

Because the lack of intracellular staining in the case of **8** may be caused by the rapid degradation of internalized molecules within the lysosome, labeled cells were treated with the compounds in the presence of chloroquine to disrupt lysosomal pH and prevent efficient catabolism within this compartment.[24] Under these conditions, labeled molecules, localized within intracellular vesicles resembling late endosomes/lysosomes, are clearly noted for both **4** and **8**. The increased intensity of this intracellular staining when compound **4** is used is likely due to the ability of this molecule to cross the plasma membrane and react with sialylated molecules already present inside the cell. These results demonstrate that, although certain DIBO derivatives have very poor cell permeability, they can be internalized following reaction with cell surface sialylated molecules.

## CONCLUSIONS

Strain-promoted cycloadditions of azides with cyclooctyne derivatives provide unique opportunities for labeling biomolecules such as glycoconjugates. The currently employed reagents are, however, apolar, resulting in limited bioavailability. In our quest to develop highly polar cyclooctynes, we found that sulfated dibenzylcyclooctynamides can be prepared by an expedient synthetic route, react fast with azides, have excellent stabilities under moderately acidic and basic conditions, and can be employed for labeling azido-modified glycoconjugates of living cells. A surprising finding was that biotinylated dibenzylcyclooctynol **4** can enter cells, thereby labeling intra- and extracellular azido-modified glycoconjugates, whereas sulfated dibenzylcyclooctynamine **8** cannot pass the cell membrane. Therefore, such sulfated DIBO derivatives are ideally suited for selective labeling of cell surface molecules. The ability to selectively label cell surface glycoconjugates will offer unique opportunities for glycomics and glycoproteomics studies. For example, SPAAC can be employed for the isolation of glycoproteins. However, samples may be contaminated by biosynthetic intermediates, complicating the determination of biological relevant cell surface glycoconjugates. Labeling azido-modified glycoconjugates with reagents such as S-DIBO (**8**) that cannot pass the cell membrane will address this problem. Furthermore, selective labeling of cell surface glycoconjugates will provide new opportunities for monitoring retrograde trafficking. Upon endocytosis from the cell surface, most glycoproteins are either recycled through endosomes or targeted to lysosomes for degradation. Several human diseases are caused by defects in proteins involved in retrograde transport through the endosomal network or in enzymes responsible for the lysosomal catabolism of glycosylated molecules. We expect that selective tagging of cell surface glycoconjugates will make it possible to monitor trafficking and turnover of glycoproteins in healthy and diseased cells. For example, the studies reported here have already shown that accumulation of glycoconjugates in vesicular structures can easily be detected following chloroquine-induced disruption of intracellular transport and normal lysosomal function. Finally, the new polar dibenzylcyclooctynes have the obvious advantage that they are water-soluble and do not need an organic cosolvent for administration. We predict they will also have improved bioavailability and intend to address this in future studies.

## EXPERIMENTAL SECTION

***N*-(1,2-Bis(3-methoxyphenyl)ethyl)acetamide 10.** Acetic anhydride (1.1 mL, 11.6 mmol) was added dropwise to a solution of 1,2-bis(3-methoxyphenyl)ethanamine **9** (2.0 g, 7.8 mmol) and *N*-ethyldiisopropylamine (DIPEA) (4.0 mL, 23.3 mmol) in $CH_2Cl_2$ (20 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 18 h. The reaction mixture was diluted with $CH_2Cl_2$ (100 mL) and washed with 1 N HCl aq (2 × 50 mL), $NaHCO_3$ aq (50 mL), and brine (50 mL). The organic layer was then dried ($MgSO_4$), filtered, and the filtrate concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel using a gradient of 50−80% ethyl acetate to give *N*-(1,2-bis(3-methoxyphenyl)ethyl)-acetamide **10** as a light yellow oil, which solidified upon standing (1.57 g, 67%): IR (KBr) $\nu$ 3334 (s, NH), 2991 (w), 2936 (m), 2834 (w), 1646 (s), 1598 (s), 1535 (m), 1492 (m), 1463 (m), 1431 (w), 1374 (w), 1257 (m), 1041 (m), 857 (m), 791 (m), 729 (m), 700 (m) $cm^{-1}$; $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$ 1.85 (s, 3H, $CH_3$), 3.00 (d, $J$ = 7.4, Hz, 2H, $CH_2$CHNH), 3.66 (s, 3H, $OCH_3$), 3.68 (s, 3H, $OCH_3$), 5.22 (q, $J$ = 7.5 Hz, 1H, CHNH), 6.61−6.84 (m, 6H, 6 × $CH_{Ar}$), 6.93 (d, $J$ = 8.2 Hz, 1H, NH), 7.08−7.19 (m, 2H, 2 × $CH_{Ar}$); $^{13}C$ NMR (75.5 MHz, $CDCl_3$) $\delta$ 22.83 ($CH_3$), 42.39 ($CH_2$CHNH), 54.29 ($CH_2$CHNH), 54.76 ($CH_3O$), 54.84 ($CH_3O$), 111.80 ($CH_{Ar}$), 112.19 ($CH_{Ar}$), 112.46 ($CH_{Ar}$), 114.55 ($CH_{Ar}$), 118.70 ($CH_{Ar}$), 121.38 ($CH_{Ar}$), 128.95 ($CH_{Ar}$), 129.21 ($CH_{Ar}$), 139.05 ($C_{Ar}$), 143.46 ($C_{Ar}$), 159.20 (C−OMe), 159.38 (C−OMe), 164.40 (C═O); HRMS (MALDI) 322.0683 ($C_{18}H_{21}NNaO_3$ ($M + Na^+$) requires 322.1414).

***N*-(1,2-Bis(3-methoxyphenyl)ethyl)acrylamide 11.** Acryloyl chloride (0.78 mL, 9.6 mmol) was added dropwise to a solution of 1,2-bis(3-methoxyphenyl)ethanamine **9** (2.00 g, 7.80 mmol) and *N*-ethyldiisopropylamine (DIPEA) (5.56 mL, 32 mmol) in $CH_2Cl_2$


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(10 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 18 h. The reaction mixture was then diluted with $CH_2Cl_2$ (100 mL) and washed with 1 N HCl aq (2 × 50 mL), $NaHCO_3$ aq (50 mL), and brine (50 mL). The organic layer was dried ($MgSO_4$), filtered, and the filtrate concentrated under reduced pressure, and the residue was purified by flash chromatography on silica gel using a mixture of ethyl acetate and hexanes (1:1) to give acrylamide **11** as a light yellow oil, which solidified upon standing (2.21 g, 94%): $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 3.08 (dd, $J$ = 7.0, 1.8 Hz, 2H, $CH_2$CHNH), 3.69 (s, 3H, $OCH_3$), 3.72 (m, 3H, $OCH_3$), 5.30 (q, $J$ = 7.3 Hz, 1H, CHNH), 5.57 (dd, $J$ = 10.0, 1.6 Hz, 1H, CHHCHCO), 6.02−6.30 (m, 3H, CHHCHCONH), 6.59−6.84 (m, 6H, 6 × $CH_{Ar}$), 7.10−7.26 (m, 2H, 2 × $CH_{Ar}$); $^{13}$C NMR (75.5 MHz, $CDCl_3$) $\delta$ 42.37 ($CH_2$CHNH), 54.33 ($CH_2$CHNH), 55.00 ($OCH_3$), 55.09 ($OCH_3$), 112.24 ($CH_{Ar}$), 112.58 ($CH_{Ar}$), 112.61 ($CH_{Ar}$), 114.64 ($CH_{Ar}$), 118.85 ($CH_{Ar}$), 121.57 ($CH_{Ar}$), 126.54 ($CH_2$CHCONH), 129.21 ($CH_{Ar}$), 129.49 ($CH_{Ar}$), 130.70 ($CH_2$CHCONH), 138.70 ($C_{Ar}$), 142.97 ($C_{Ar}$), 159.43 (C−OMe), 159.60 (C−OMe), 164.78 (C═O); HRMS (MALDI) 334.0360 ($C_{19}H_{21}NNaO_3$ (M + $Na^+$) requires 334.1414).

**Friedel−Crafts Alkylation-Cyclization General Procedure.** Tetrachlorocyclopropene (0.19 mL, 1.90 mmol) was added to a suspension of aluminum trichloride (910 mg, 6.88 mmol) in dry $CH_2Cl_2$ (15 mL). The resulting mixture was stirred for 10 min at room temperature and then cooled to −20 °C. A cold (−20 °C) solution of amide **10** (510 mg, 1.72 mmol) or acrylamide **11** (543 mg, 1.72 mmol) in $CH_2Cl_2$ (10 mL) was then added dropwise. The reaction mixture was stirred for 2 h at −20 °C, then warmed to room temperature and stirred for one more hour. The reaction was then quenched by careful addition of water (5 mL). The resulting mixture was vigorously stirred for 30 min, and the organic phase was extracted with $CH_2Cl_2$ (3 × 20 mL) and ethyl acetate (3 × 10 mL). The organic fractions were combined, dried ($MgSO_4$), filtered, and the filtrate concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel using a gradient of 50−70% acetone in hexanes to give pure cyclopropenone **12** and **13**, respectively.

***N*-(4,9-Dimethoxy-1-oxo-6,7-dihydro-1*H*-dibenzo[*a*,*e*]-cyclopropa[*c*]cycloocten-6-yl)acetamide 12.** White solid (290 mg, 48%): IR (KBr) $\nu$ 3304 (s, NH), 3062 (w), 2941 (w), 2837 (w), 1846 (s, C═O cyclopropenone), 1644 (m), 1609 (s), 1562 (m), 1431 (m), 1348 (m), 1251 (m), 1137 (w), 1024 (w), 826 (w) cm$^{-1}$; $^1$H NMR (300 MHz, $d_6$-DMSO) $\delta$ 1.99 (s, 3H, $CH_3$), 2.88 (dd, $J$ = 14.5, 1.5 Hz, 1H, CHHCHNH), 3.17 (dd, $J$ = 14.8, 4.6 Hz, 1H, CHHCHNH), 3.87 (s, 6H, 2 × $CH_3O$), 4.73 (brs, 1H, CHNH), 7.02−7.08 (m, 4H, 4 × $CH_{Ar}$), 7.84−7.87 (m, 2H, 2 × $CH_{Ar}$), 8.79 (d, $J$ = 8.1 Hz, 1H, NH); $^{13}$C NMR (75.5 MHz, $d_6$-DMSO) $\delta$ 22.60 ($CH_3$), 44.51 ($CH_2$CHNH), 54.86 ($CH_2$CHNH), 55.67 (2 × $CH_3O$), 111.13 ($CH_{Ar}$), 111.72 ($CH_{Ar}$), 112.55 ($CH_{Ar}$), 114.52 ($C_{Ar}$), 115.73 ($CH_{Ar}$), 116.09 ($C_{Ar}$), 134.97 ($CH_{Ar}$), 135.41 ($CH_{Ar}$), 141.69 (C═C), 142.47 (C═C), 145.88 ($C_{Ar}$), 148.83 ($C_{Ar}$), 151.58 (C═O), 162.13 (C−OMe), 162.41 (C−OMe), 169.01 (C═O); HRMS (MALDI) 321.0652 ($C_{20}H_{19}NO_3$ ($M^+$ − CO) requires 321.1365).

***N*-(4,9-Dimethoxy-1-oxo-6,7-dihydro-1*H*-dibenzo[*a*,*e*]-cyclopropa[*c*]cycloocten-6-yl)acrylamide 13.** White solid (344 mg, 55%): IR (KBr) $\nu$ 3280 (m, NH), 3080 (w), 2927 (m), 2851 (w), 1848 (s, C═O cyclopropenone), 1728 (w), 1658 (m), 1608 (s), 1561 (m), 1500 (w), 1429 (w), 1407 (w), 1350 (m), 1309 (m), 1289 (m), 1252 (s), 1135 (m), 1074 (w), 1054 (w), 1020 (w), 957 (w), 837 (w), 832 (w) cm$^{-1}$; $^1$H NMR (300 MHz, $d_6$-DMSO) $\delta$ 2.94 (dd, $J$ = 14.6, 1.2 Hz, 1H, CHHCHNH), 3.23 (dd, $J$ = 14.8, 4.5 Hz, 1H, CHHCHNH), 3.85 (s, 3H, $OCH_3$), 3.86 (s, 3H, $OCH_3$), 4.85 (brs, 1H, CHNH), 5.71 (dd, $J$ = 10.2, 1.8 Hz, 1H, CHHCHCO), 6.11 (dd, $J$ = 17.1, 1.8 Hz, 1H, CHHCHCO), 6.44 (dd, $J$ = 17.1, 10.2 Hz, 1H, $CH_2$CHCO), 6.97−7.09 (m, 4H, 4 × $CH_{Ar}$), 7.85−7.89 (m, 2H, 2 × $CH_{Ar}$), 9.05 (d, $J$ = 8.2 Hz, 1H, NH); $^{13}$C NMR (75.5 MHz, $d_6$-DMSO) $\delta$ 44.46 ($CH_2$CHNH), 54.89 ($CH_2$CHNH), 55.65 ($OCH_3$), 55.67 ($OCH_3$), 111.14 ($CH_{Ar}$), 111.71 ($CH_{Ar}$), 112.62 ($CH_{Ar}$), 114.53 ($C_{Ar}$), 115.71 ($CH_{Ar}$), 116.08 ($C_{Ar}$), 126.36 ($CH_2$CHCONH), 131.08 ($CH_2$CHCONH), 135.01 ($CH_{Ar}$), 135.50 ($CH_{Ar}$), 141.63 (C═C), 142.53 (C═C), 145.73 ($C_{Ar}$), 148.39 ($C_{Ar}$), 151.56 (C═O), 162.16 (C−OMe), 162.39 (C−OMe), 164.28 (C═O); HRMS (MALDI) 333.0661 ($C_{21}H_{19}NO_3$ ($M^+$ − CO) requires 333.1365).

**General Procedure for the Deprotection of Phenols.** A solution of boron tribromide in $CH_2Cl_2$ (1.0 M, 2.86 mL, 2.86 mmol) was added dropwise to a suspension of amide-cyclopropenone **12** (100 mg, 0.29 mmol), or acrylamide-cyclopropenone **13** (105 mg, 0.29 mmol) in $CH_2Cl_2$ (10 mL) at −78 °C. The reaction was allowed to warm to room temperature and was stirred for 18 h. The reaction was carefully quenched by slow addition of water (5 mL), and the resulting mixture was extracted with $CH_2Cl_2$ (3 × 20 mL) and ethyl acetate (3 × 20 mL). The combined organic fractions were dried ($MgSO_4$), filtered, and the filtrate concentrated in vacuo. The resulting residue was purified by flash column chromatography on silica gel using a gradient of 5−10% methanol in $CH_2Cl_2$ to give diphenol **18** and **19**, respectively.

***N*-(4,9-Dihydroxy-1-oxo-6,7-dihydro-1*H*-dibenzo[*a*,*e*]-cyclopropa[*c*]cycloocten-6-yl)acetamide 18.** Orange solid (68 mg, 74%): IR (KBr) $\nu$ 3291 (s, br, OH), 1854 (s, C═O cyclopropenone), 1642 (m), 1600 (s), 1569 (s), 1448 (m), 1357 (m), 1298 (m), 1264 (w), 1232 (w), 1209 (w), 1137 (w), 870 (w), 827 (w) cm$^{-1}$; $^1$H NMR (300 MHz, $d_6$-DMSO) $\delta$ 1.99 (s, 3H, $CH_3$), 2.80 (d, $J$ = 13.9 Hz, 1H, CHHCHNH), 3.16−3.22 (m, 1H, CHHCHNH), 4.65 (brs, 1H, CHNH), 6.82−6.91 (m, 4H, 4 × $CH_{Ar}$), 7.69−7.72 (m, 2H, 2 × $CH_{Ar}$), 8.92 (d, $J$ = 7.8 Hz, 1H, NH); $^{13}$C NMR (75.5 MHz, $d_6$-DMSO) $\delta$ 22.65 ($CH_3$), 44.25 ($CH_2$CHNH), 55.29 ($CH_2$CHNH), 111.70 ($CH_{Ar}$), 113.11 ($C_{Ar}$), 114.09 ($CH_{Ar}$), 114.43 ($CH_{Ar}$), 114.80 ($C_{Ar}$), 116.72 ($CH_{Ar}$), 134.97 ($CH_{Ar}$), 135.37 ($CH_{Ar}$), 140.89 (C═C), 141.46 (C═C), 146.17 ($C_{Ar}$), 149.28 ($C_{Ar}$), 151.73 (C═O), 161.06 (C−OH), 161.30 (C−OH), 169.15 (C═O); HRMS (MALDI) 316.0553 ($C_{18}H_{15}NO_3$ (M + $Na^+$ − CO) requires 316.0944).

***N*-(4,9-Dihydroxy-1-oxo-6,7-dihydro-1*H*-dibenzo[*a*,*e*]-cyclopropa[*c*]cycloocten-6-yl)acrylamide 19.** Orange solid (58 mg, 60%): IR (KBr) $\nu$ 3153 (s, br, OH), 1849 (s, C═O cyclopropenone), 1661 (s), 1610 (m), 1447 (w), 1355 (w), 1302 (w), 1250 (w), 1133 (w), 980 (w), 874 (w), 826 (m), 754 (w), 665 (w) cm$^{-1}$; $^1$H NMR (300 MHz, $d_6$-DMSO) $\delta$ 2.88 (d, $J$ = 14.5, 1H, CHHCHNH), 3.14 (dd, $J$ = 14.2, 3.8 Hz, 1H, CHHCHNH), 4.76 (brs, 1H, CHNH), 5.71 (dd, $J$ = 10.2, 1.9 Hz, 1H, CHHCHCO), 6.11 (dd, $J$ = 17.1, 1.9 Hz, 1H, CHHCHCO), 6.43 (dd, $J$ = 17.1, 10.2 Hz, 1H, $CH_2$CHCO), 6.80−6.88 (m, 4H, 4 × $CH_{Ar}$), 7.68−7.77 (m, 2H, 2 × $CH_{Ar}$), 9.03 (d, $J$ = 8.0 Hz, 1H, NH), 10.56 (brs, 2H, 2 × OH); $^{13}$C NMR (75.5 MHz, $d_6$-DMSO) $\delta$ 44.43 ($CH_2$CHNH), 55.14 ($CH_2$CHNH), 111.48 ($CH_{Ar}$), 113.03 ($C_{Ar}$), 114.13 ($CH_{Ar}$), 114.42 ($CH_{Ar}$), 114.69 ($C_{Ar}$), 116.61 ($CH_{Ar}$), 126.33 ($CH_2$CHCONH), 131.10 ($CH_2$CHCONH), 134.93 ($CH_{Ar}$), 135.39 ($CH_{Ar}$), 140.82 (C═C), 141.45 (C═C), 145.86 ($C_{Ar}$), 148.76 ($C_{Ar}$), 151.51 (C═O), 161.09 (C−OH), 161.26 (C−OH), 164.21 (C═O); HRMS (MALDI) 306.0514 ($C_{19}H_{16}NO_3$ (MH$^+$ − CO) requires 306.1130).

**Sulfation General Procedure.** Sulfur trioxide pyridine complex (290 mg, 1.870 mmol) was added to a solution of amide-cyclopropenone **18** (30 mg, 0.093 mmol) or acrylamide-cyclopropenone **19** (31 mg, 0.093 mmol) in DMF (5 mL), and the resulting mixture was stirred for 2 h. The reaction was quenched by addition of pyridine in methanol (5 mL, 1:1 v:v). The solvent was then evaporated under reduced pressure at room temperature (please note that the pyridinium salt of sulfated product is unstable). The residue was then purified by C-18 column chromatography using a gradient of 0−15% $CH_3CN$ in $H_2O$ to give a pyridinium salt of **14** and **15**, respectively, which was then passed through a $Na^+$ ion-exchange resin (see for resin details the Supporting Information) and lyophilized to give compounds **14** and **15**, respectively.

***N*-(4,9-Sodium Disulfate-1-oxo-6,7-dihydro-1*H*-dibenzo-[*a*,*e*]cyclopropa[*c*]cycloocten-6-yl)acetamide 14.** White powder (28 mg, 57%): IR (KBr) $\nu$ 3456 (s, br), 1854 (s, C═O cyclopropenone), 1607 (s), 1565 (m), 1420 (w), 1360 (m), 1245 (s), 1051 (s), 957 (m), 894 (w), 786 (m) cm$^{-1}$; $^1$H NMR (300 MHz, $D_2O$) $\delta$ 2.17 (s, 3H, $CH_3$), 2.95 (d, $J$ = 13.6 Hz, 1H, CHHCHNH), 3.27 (dd, $J$ = 15.4, 4.5 Hz, 1H, CHHCHNH), 4.87 (brs, 1H, CHNH), 7.42−7.45 (m, 4H, 4 × $CH_{Ar}$), 8.04−8.08 (m, 2H, 2 × $CH_{Ar}$); $^{13}$C NMR (75.5 MHz, $D_2O$) $\delta$ 21.95 ($CH_3$), 44.13 ($CH_2$CHNH), 55.73 ($CH_2$CHNH), 116.94 ($CH_{Ar}$), 118.30 ($C_{Ar}$), 119.95 ($C_{Ar}$), 120.34


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(2 × $CH_{Ar}$), 122.33 ($CH_{Ar}$), 135.86 ($CH_{Ar}$), 136.34 ($CH_{Ar}$), 142.62 (C═C), 143.70 (C═C), 146.37 ($C_{Ar}$), 148.32 ($C_{Ar}$), 154.88 (C−$OSO_3Na$), 155.33 (C−$OSO_3Na$), 155.73 (C═O), 174.09 (C═O); HRMS (MALDI) 519.9465 ($C_{18}H_{13}NNa_3O_9S_2$(M + $Na^+$ − CO) requires 519.9719).

***N*-(4,9-Sodium Disulfate-1-oxo-6,7-dihydro-1*H*-dibenzo-[*a*,*e*]cyclopropa[*c*]cycloocten-6-yl)acrylamide 15.** White powder (46 mg, 90%): $^1$H NMR (300 MHz, $D_2O$) $\delta$ 2.84 (d, $J$ = 15.2, 1H, C*H*HCHNH), 3.27 (dd, $J$ = 15.4, 4.5 Hz, 1H, CH*H*CHNH), 4.91 (brs, 1H, C*H*NH), 5.86 (d, $J$ = 10.5 Hz, 1H, CH*H*CHCO), 6.23 (d, $J$ = 17.2 Hz, 1H, C*H*HCHCO), 6.50 (dd, $J$ = 17.1, 10.5 Hz, 1H, $CH_2CH$CO), 7.35−7.43 (m, 4H, 4 × $CH_{Ar}$), 7.96−8.02 (m, 2H, 2 × $CH_{Ar}$); $^{13}$C NMR (75.5 MHz, $D_2O$) $\delta$ 44.19 ($CH_2$CHNH), 55.64 ($CH_2$CHNH), 116.93 ($CH_{Ar}$), 118.14 ($C_{Ar}$), 119.77 ($C_{Ar}$), 120.34 (2 × $CH_{Ar}$), 122.34 ($CH_{Ar}$), 128.53 ($CH_2$CHCONH), 129.54 ($CH_2$CHCONH), 135.86 ($CH_{Ar}$), 136.33 ($CH_{Ar}$), 142.49 (C═C), 143.58 (C═C), 146.16 ($C_{Ar}$), 148.03 ($C_{Ar}$), 154.91 (C−$OSO_3Na$), 155.34 (C−$OSO_3Na$), 155.52 (C═O), 168.35 (C═O); HRMS (MALDI) 531.8558 ($C_{19}H_{13}NNa_3O_9S_2$ (M$Na^+$ − CO) requires 531.9719).

**Disulfated-Cyclopropenone-biotin Derivative 16.** A solution of cyclopropenone **15** (10 mg, 0.02 mmol) and thiol **17** (27 mg, 0.06 mmol) in degassed Tris·HCl buffer (2 mL, pH = 8, 0.1 M) containing tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (5 mM) was stirred at room temperature for 18 h. The mixture was loaded on a C-18 column and purified using a gradient of 0−15% $CH_3CN$ in $H_2O$. The fractions containing the desired product were combined, passed through a $Na^+$ ion-exchange resin, and lyophilized to give pure cyclopropenone **16** as a white powder (12.6 mg, 64%): $^1$H NMR (300 MHz, $D_2O$) $\delta$ 1.18−1.74 (m, 6H, 3 × $CH_2$), 2.12 (dt, $J$ = 7.4, 2.2 Hz, 2H, $CH_2CO$), 2.65−2.97 (m, 6H, $CH_2CO$, 2 × $SCH_2$), 3.03−3.18 (m, 2H, C*H*HCHNH, SCH), 3.27−3.70 (m, 15H, $CH_2S$, 2 × $CH_2NH$, CH*H*CHNH, 4 × $CH_2O$), 4.20−4.34 (dt, $J$ = 8.1, 4.5 Hz, 1H, C*H*NH), 4.47−4.52 (m, 1H, C*H*NH), 4.92 (brs, 1H, NHC*H*), 7.45−7.58 (m, 4H, 4 × $CH_{Ar}$), 8.09−8.13 (m, 2H, 2 × $CH_{Ar}$); $^{13}$C NMR (75.5 MHz, $D_2O$) $\delta$ 25.31 ($CH_2$), 27.77 ($SCH_2$), 28.10 ($CH_2$), 28.15 ($CH_2$), 35.21 ($SCH_2CO$), 35.48 ($CH_2CO$), 35.60 ($CH_2CO$), 39.09 ($CH_2NH$), 39.49 ($CH_2NH$), 39.88 ($SCH_2$), 44.43 ($CH_2$), 55.45 ($CH_2$CHNH), 55.82 (SCH), 60.34 (CHNH), 62.13 (CHNH), 68.90 ($CH_2O$), 69.01 ($CH_2O$), 69.65 (2 × $CH_2O$), 117.24 ($CH_{Ar}$), 118.15 ($C_{Ar}$), 119.72 ($C_{Ar}$), 120.28 ($CH_{Ar}$), 120.33 ($CH_{Ar}$), 122.24 ($CH_{Ar}$), 135.90 ($CH_{Ar}$), 136.42 ($CH_{Ar}$), 142.57 (C═C), 143.59 (C═C), 146.33 ($C_{Ar}$), 148.27 ($C_{Ar}$), 155.15 (C-$OSO_3Na$), 155.49 (C═O), 155.53 (C-$OSO_3Na$), 165.29 (C═O), 172.68 (C═O), 173.85 (C═O), 176.76 (C═O); HRMS (MALDI) 980.1951 ($C_{37}H_{45}N_5Na_3O_{14}S_4$ (M + $Na^+$ − CO) requires 980.1533).

***N*-(3,8-Dimethoxy-11,12-didehydro-5,6-dihydrodibenzo-[*a*,*e*]cyclooctyn-5-yl)acetamide 5.** A solution of cyclopropenone **12** (200 mg, 0.57 mmol) in MeOH:$CH_2Cl_2$ (20 mL, 1:1, v:v) was irradiated at 350 nm for 4 h. The solvent was evaporated under reduced pressure, and the residue was purified by flash column chromatography on silica gel using a gradient of 20−40% acetone in hexanes to give **5** as white solid (150 mg, 82%): IR (KBr) $\nu$ 3286 (s, NH), 2938 (w), 2835 (w), 1649 (s), 1613 (w), 1564 (m), 1489 (w), 1427 (w), 1371 (w), 1290 (m), 1035 (m), 818 (w) $cm^{-1}$; $^1$H NMR (300 MHz, $d_6$-DMSO) $\delta$ 1.98 (s, 3H, $CH_3$), 2.56 (dd, $J$ = 12.3, 2.6 Hz, 1H, C*H*HCHNH), 2.99 (dd, $J$ = 14.4, 3.5 Hz, 1H, CH*H*CHNH), 3.79 (s, 6H, 2 × $CH_3$), 4.51−4.54 (m, 1H, C*H*NH), 6.87−6.97 (m, 4H, 4 × $CH_{Ar}$), 7.27−7.31 (m, 2H, 2 × $CH_{Ar}$), 8.70 (d, $J$ = 8.6 Hz, 1H, NH); $^{13}$C NMR (75.5 MHz, $d_6$-DMSO) $\delta$ 22.68 ($CH_3$), 44.99 ($CH_2$CHNH), 53.72 ($CH_2$CHNH), 55.30 ($CH_3O$), 55.33 ($CH_3O$), 110.38 (C≡C), 111.05 ($CH_{Ar}$), 111.45 (C≡C), 112.01 ($CH_{Ar}$), 112.10 ($CH_{Ar}$), 113.71 ($C_{Ar}$), 115.40 ($C_{Ar}$), 116.23 ($CH_{Ar}$), 126.65 ($CH_{Ar}$), 126.98 ($CH_{Ar}$), 152.99 ($C_{Ar}$), 155.74 ($C_{Ar}$), 158.99 (C−OMe), 159.29 (C−OMe), 168.63 (C═O); HRMS (MALDI) 344.0544 ($C_{20}H_{19}NNaO_3$ (M + $Na^+$) requires 344.1257).

**General Procedure of Unmasking Cyclopropenones of Sulfated Cyclooctynes.** A solution of cyclopropenone **14** (30 mg, 0.060 mmol) or cyclopropenone **16** (12 mg, 0.012 mmol) in $H_2O$ (5 mL) was irradiated at 350 nm for 4 h. The solvent was evaporated, and the residue was purified by C-18 column chromatography using a gradient of 0−15% $CH_3CN$ in $H_2O$. Fractions containing the desired product were combined, passed through a $Na^+$ ion-exchange resin, and lyophilized to give compounds cyclooctyne **7** and **8**, respectively.

***N*-(3,8-Sodium Disulfate-11,12-didehydro-5,6-dihydrodibenzo-[*a*,*e*]cyclooctyn-5-yl)acetamide 7.** White powder (27.0 mg, 95%): IR (KBr) $\nu$ 3451 (s, br), 2923 (w), 1638 (s), 1565 (m), 1482 (m), 1431 (w), 1375 (w), 1244 (s), 1144 (w), 1052 (s), 950 (m), 890 (w), 845 (w), 780 (w) $cm^{-1}$; $^1$H NMR (500 MHz, $D_2O$) $\delta$ 2.15 (s, 3H, $CH_3$), 2.76 (d, $J$ = 14.7 Hz, 1H, C*H*HCHNH), 3.11 (dd, $J$ = 14.9, 3.2 Hz, 1H, CH*H*CHNH), 4.58 (brs, 1H, C*H*NH), 7.28−7.46 (m, 6H, 6 × $CH_{Ar}$); $^{13}$C NMR (75.5 MHz, $D_2O$) $\delta$ 22.01 ($CH_3$), 44.25 ($CH_2$CHNH), 54.86 ($CH_2$CHNH), 110.93 (C≡C), 112.49 (C≡C), 117.93 ($CH_{Ar}$), 119.54 ($C_{Ar}$), 120.38 ($CH_{Ar}$), 120.39 ($CH_{Ar}$), 121.24 ($C_{Ar}$), 122.89 ($CH_{Ar}$), 127.46 ($CH_{Ar}$), 127.98 ($CH_{Ar}$), 151.06 ($C_{Ar}$), 151.57 ($C_{Ar}$), 153.09 (C−$OSO_3Na$), 154.41 (C−$OSO_3Na$), 173.87 (C═O); HRMS (MALDI) 519.9779 ($C_{18}H_{13}NNa_3O_9S_2$ (M + $Na^+$) requires 519.9719).

**Disulfated-Cyclooctyne-biotin Derivative 8.** White powder (10.0 mg, 86%): IR (KBr) $\nu$ 3376 (s, br), 3073 (w), 2927 (m), 1694 (s), 1650 (s), 1561 (m), 1479 (w), 1253 (s), 1048 (s), 952 (w), 840 (w), 775 (w) $cm^{-1}$; $^1$H NMR (300 MHz, $D_2O$) $\delta$ 1.10−1.57 (m, 6H, 3 × $CH_2$), 2.12 (t, $J$ = 6.7 Hz, 2H, $CH_2CO$), 2.73−2.78 (m, 4H, C*H*HCHNH, $CH_2CO$, SC*H*H), 2.89−2.91 (m, 3H, $CH_2S$, SCH*H*), 3.28−3.62 (m, 16H, CHS, $CH_2S$, 2 × $CH_2NH$, CH*H*CHNH, 4 × $CH_2O$), 4.26 (dt, $J$ = 8.2, 4.2 Hz, 1H, C*H*NH), 4.45−4.50 (m, 1H, C*H*NH), 4.63 (brs, 1H, NHC*H*), 7.20−7.35 (m, 2H, 2 × $CH_{Ar}$), 7.40−7.50 (m, 4H, 4 × $CH_{Ar}$); $^{13}$C NMR (75.5 MHz, $D_2O$) $\delta$ 25.26 ($CH_2$), 27.74 ($SCH_2$), 28.06 ($CH_2$), 28.26 ($CH_2$), 35.29 ($CH_2CO$), 35.61 (2 × $CH_2CO$), 39.08 ($CH_2NH$), 39.49 ($CH_2NH$), 39.88 ($SCH_2$), 44.46 ($CH_2$), 54.91 ($CH_2$CHNH), 55.43 (SCH), 60.35 (CHNH), 62.13 (CHNH), 68.87 ($CH_2O$), 68.97 ($CH_2O$), 69.61 ($CH_2O$), 69.64 ($CH_2O$), 111.07 (C≡C), 112.50 (C≡C), 118.26 ($CH_{Ar}$), 119.38 ($C_{Ar}$), 120.39 (2 × $CH_{Ar}$), 121.09 ($C_{Ar}$), 122.86 ($CH_{Ar}$), 127.51 ($CH_{Ar}$), 128.02 ($CH_{Ar}$), 151.25 ($C_{Ar}$), 151.72 ($C_{Ar}$), 153.04 (C−$OSO_3Na$), 154.41 (C−$OSO_3Na$), 165.37 (C═O), 172.75 (C═O), 173.63 (C═O), 176.86 (C═O); HRMS (MALDI) 980.2696 ($C_{37}H_{45}N_5Na_3O_{14}S_4$ (M + $Na^+$) requires 980.1533).

**Reagents for Biological Experiments.** Synthetic compound **4** was reconstituted in DMF and **8** was reconstituted in double distilled water. Storage was at −80 °C. Final concentrations of DMF never exceeded 0.56% to avoid toxic effects.

**Cell Surface Azide Labeling and Detection by Fluorescence Intensity.** Human Jurkat cells (Clone E6-1; ATCC) were cultured in RPMI 1640 medium (ATCC) with L-glutamine (2 mM), adjusted to contain sodium bicarbonate (1.5 g/L), glucose (4.5 g/L), HEPES (10 mM), and sodium pyruvate (1.0 mM), and supplemented with penicillin (100 u/mL)/streptomycin (100 $\mu$g/mL; Mediatech) and fetal bovine serum (FBS, 10%; Hyclone). Cells were maintained in a humid 5% $CO_2$ atmosphere at 37 °C. Jurkat cells were grown in the presence of peracetylated *N*-azidoacetylmannosamine ($Ac_4$ManNAz; 25 $\mu$M final concentration) for 3 days, leading to the metabolic incorporation of the corresponding *N*-azidoacetyl sialic acid (SiaNAz) into their cell surface glycoproteins. Jurkat cells bearing azides and control cells grown in the presence of peracetylated *N*-acetylmannosamine ($Ac_4$ManNAc) were incubated with the biotinylated compound **4** and with the hydrophilic analogue **8** (0−100 $\mu$M) in labeling buffer (PBS, pH 7.4 containing 1% FBS) for 0−60 min at room temperature. The cells were washed three times with labeling buffer and then incubated with avidin conjugated to fluorescein isothiocyanate (FITC; 0.5 $\mu$g/mL; Molecular Probes) for 15 min at 4 °C. Following three washes and cell lysis, cell lysates were analyzed for fluorescence intensity (485 ex/520 em) using a microplate reader (BMG Labtech). Data points were collected in triplicate and are representative of three separate experiments. Cell viability was assessed at different points in the procedure with exclusion of trypan blue.

**Western Blot Analysis.** Jurkat cells were harvested by centrifugation (5 min at 500*g*) and resuspended as 5 × $10^6$ cells/mL. The cell suspensions (200 $\mu$L per sample) were incubated with biotin-conjugated compounds **4** and **8** (30 $\mu$M) or without compound as control for 1 h. The cells were washed (4 × 10 min) with cold DPBS, pH 7.4 containing FBS (1%), and lysed in passive lysis buffer. The cell


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lysates were clarified by centrifugation at 22 000*g* for 15 min, and the total protein content of the clear supernatants was assessed using the bicinchonic acid assay (BCA; Pierce Biotechnology). Cell lysate samples (20 $\mu$g of protein) in SDS-PAGE sample buffer containing 2-mercaptoethanol were boiled for 5 min, resolved on a 4−20% Tris-HCl gel (Bio-Rad), and transferred to a nitrocellulose membrane. Next, the membrane was blocked in blocking buffer (nonfat dry milk (5%; Bio-Rad) in PBST (PBS containing Tween-20 (0.1%) and Triton X-100 (0.1%)) overnight at 4 °C. The blocked membrane was incubated for 1 h at room temperature with an antibiotin antibody conjugated to horseradish peroxidase (HRP) (1:100 000; Jackson ImmunoResearch Lab, Inc.) in blocking buffer and washed with PBST (4 × 10 min). Final detection of HRP activity was performed using ECL Plus chemiluminescent substrate (Amersham), exposure to film (Kodak), and development using a digital X-ray imaging machine (Kodak). Coomassie staining with GelCode Blue Stain Reagent (Pierce, U.S.) was used to confirm total protein loading.

**Cell Labeling and Detection by Confocal Microscopy.** Human skin fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine (2 mM), adjusted to contain sodium bicarbonate (1.5 g/L), and supplemented with penicillin (100 u/mL)/streptomycin (100 $\mu$g/mL) and FBS (10%). Cells were maintained in a humid 5% $CO_2$ atmosphere at 37 °C. Cells (50 000 cells) were grown on coverslips in the presence of $Ac_4$ManNAz (25 $\mu$M final concentration) for 2 days to metabolically incorporate SiaNAz into their glycoproteins. Fibroblast cells bearing azides were treated with the biotinylated compounds **4** and **8** (30 $\mu$M) in labeling buffer (PBS, pH 7.4 containing 1% FBS) for 1 h at room temperature or 4 h at 37 °C in the absence or presence of chloroquine (50 $\mu$M; Sigma), followed by fixation with formaldehyde (3.7% in PBS) for 15 min at room temperature. Cells were washed four times and permeabilized for 10 min at room temperature with Triton X-100 (0.2%) in PBS. The cells were incubated with rabbit antigiantin polyclonal antibody (1:2000) in Triton X-100 (0.2%) in PBS for 1 h at room temperature. Cells were washed four times and incubated with goat antirabbit antibody conjugated with Alexa Fluor 488 (1:500; Abcam) and Alexa Fluor 568-conjugated streptavidin (10 $\mu$g/mL; Molecular Probes) for 1 h at room temperature. Cells were washed three times with PBS and mounted with PermaFluor (Thermo Electron Corp.) before imaging. Initial analysis was performed on a Zeiss Axioplan2 fluorescent microscope. Confocal images were acquired on an Olympus FV-1000 laser scanning confocal microscope using a 60× (N.A. 1.42) oil objective. Stacks of optical sections were collected in the *z* dimensions. The step size, based on the calculated optimum for each objective, was between 0.25 and 0.5 $\mu$m. Subsequently, each stack was collapsed into a single image (*z*-projection). Analysis was performed offline using ImageJ 1.39f software (National Institutes of Health, U.S.) and Adobe Photoshop CS3 Extended Version 10.0 (Adobe Systems Inc.), whereby all images were treated equally.

## ■ ASSOCIATED CONTENT

### Supporting Information

Synthetic procedures, kinetic data, stability HPLCs, Figures S1 and S2, and NMR spectra of synthesized compounds. 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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