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Strain-Promoted Alkyne–Azide Cycloadditions (SPAAC) Reveal New Features of Glycoconjugate Biosynthesis

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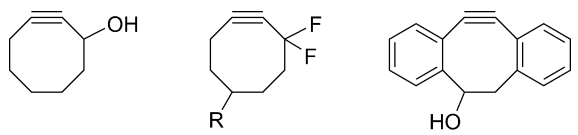
We have shown that 4-dibenzocyclooctynol (DIBO), which can easily be obtained by a streamlined synthesis approach, reacts exceptionally fast in the absence of a Cu^{I} catalyst with azido-containing compounds to give stable triazoles. Chemical modifications of DIBO, such as oxidation of the alcohol to a ketone, increased the rate of strain promoted azide–alkyne cycloadditions (SPAAC). Installment of a ketone or oxime in the cyclooctyne ring resulted in fluorescent active compounds whereas this property was absent in the corresponding cycloaddition adducts; this provides the first example of a metal-free alkyne–azide fluoro-switch click reaction. The alcohol or ketone functions of the cyclooctynes offer a chemical handle to install a variety of different tags, and thereby facilitate biological studies. It was found that DIBO modified with biotin combined with metabolic labeling with an azido-containing monosaccharide can determine relative quantities of sialic acid of living cells that have defects in glycosylation (Lec CHO cells). A combined use of metabolic labeling/SPAAC and lectin staining of cells that have defects in the conserved oligomeric Golgi (COG) complex revealed that such defects have a greater impact on *O*-glycan sialylation than galactosylation, whereas sialylation and galactosylation of *N*-glycans was similarly impacted. These results highlight the fact that the fidelity of Golgi trafficking is a critical parameter for the types of oligosaccharides being biosynthesized by a cell. Furthermore, by modulating the quantity of biosynthesized sugar nucleotide, cells might have a means to selectively alter specific glycan structures of glycoproteins.

Introduction

Metal-free cycloadditions between cyclooctynes and azides that give stable 1,2,3-triazoles, have found wide utility in labeling glycans in proteins and lipids of living cells, glycoprotein enrichment for proteomics, protein and oligonucleotide modification, and tissue reengineering.[1] These reactions, which have been coined "strain-promoted alkyne–azide cycloadditions" (SPAAC) have also made an entry in material sciences and have, for example, been employed for the assembly, cross-linking and surface modification of dendrimers,[2] derivatization of polymeric nanostructures,[3] and patterning of surfaces.[4] The attraction of SPAAC is that it does not require a toxic metal, is highly efficient even in a very complex milieu and proceeds efficiently at ambient temperature. Density functional theory (B3LYP) calculations of the transition states of cycloadditions of phenyl azide with acetylene and cyclooctyne indicate that the fast rate of the strain promoted cycloaddition is due to a lower energy required for distorting the 1,3-dipole and alkyne into the transition-state geometry.[5]

The first generation of cyclooctynes (**1**) suffered from relatively slow reaction rates and as a consequence the scope of these reagents is rather limited (Scheme 1). It has, however, been found that electron-withdrawing fluorine groups at the propargylic position of a cyclooctyne (DIFO, **2**) dramatically increase the rate of strain-promoted cycloaddition with azides.[6] The attractiveness of this methodology has, for example, been demonstrated by visualization of glycans in vivo at subcellular resolution during the development of zebrafish embryos.[7]

We have found that derivatives of 4-dibenzocyclooctynol (DIBO, **3**) react fast with azido-containing saccharides and amino acids, and can be employed for visualizing metabolically labeled glycans of living cells.[8] While the fluorine atoms of DIFO (**2**) influence rate enhancement by increasing interaction energies, the aromatic rings of **3** accomplish a similar increase in reaction rate through conformational effects that result in decreasing the distortion energy. Attractive features of DIBO (**3**) include easy access to the compounds by a simple synthesis approach, nontoxicity and straightforward attachment of a variety of probes. Furthermore, dibenzocyclooctynes can be generated photochemically by short irradiation by UV light of corresponding cyclopropenones, and thereby provide opportunities for the spatially and temporally controlled labeling of



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Scheme 1. Reagents for labeling azido-containing biomolecules.

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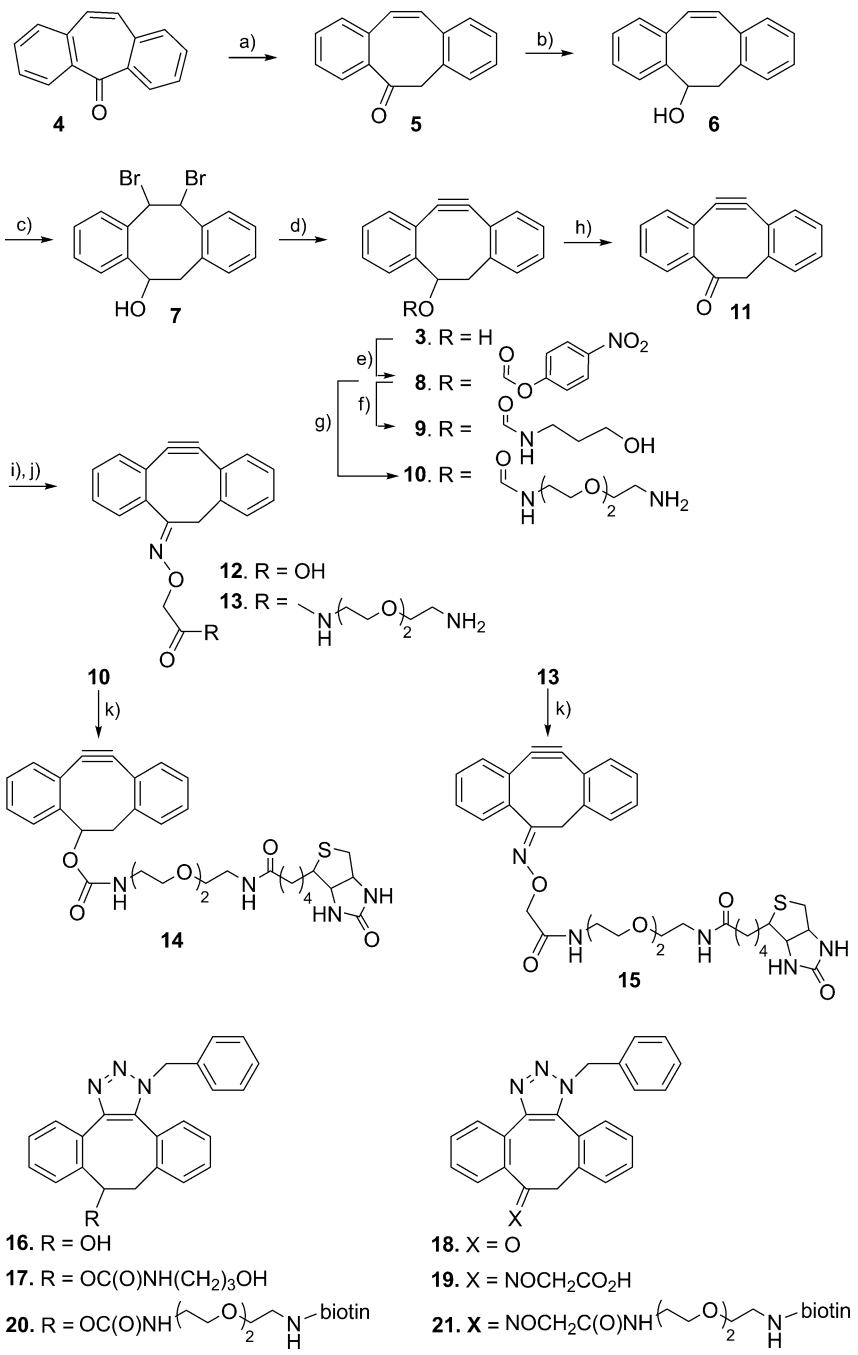
the target substrates.[9] We have also shown that by employing nitrones and nitrile oxides as 1,3-dipoles, the rate of cycloaddition can be further enhanced and this technology has, for example, made it possible to selectively tag proteins at the N terminus or perform sequential modifications of complex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

We report here a streamlined approach for the preparation and modification of DIBO, and show that modification of the eight-membered ring by, for example, installment of a ketone, affects the rate of cycloaddition. The presence of a ketone or oxime resulted in compounds that are fluorescent. Interestingly, the corresponding cycloaddition products are nonfluorescent, and hence compounds such as **11** and **12**, provide a novel metal-free alkyne–azide fluoro-switch click reaction, which, for example, can be exploited in monitoring reactions in real time. Metabolic labeling combined with SPAAC of wild-type cells and those that have known defects in their glycosylation machinery showed that relative quantities of sialylation of glycoconjugates can easily and reliably be established. Furthermore, a combined use of metabolic labeling/SPAAC and lectin staining revealed that a defect in the conserved oligomeric Golgi (COG) complex affects terminal processing of *N*-glycans to a greater extent than modification of *O*-glycans.



Scheme 2. Reagents and conditions: a) $TMSCHN_2$, $BF_3{\cdot}OEt_2$, DCM, −10 °C, 3 h, 71%; b) $NaBH_4$, EtOH/THF, 7 h, 100%; c) Br_2, $CHCl_3$, 0.5 h, 58%; d) LDA, THF, 0.5 h, 57%; e) 4-nitrophenyl chloroformate, pyridine, DCM, 18 h, 92%; f) propanol amine, Et_3N, DCM, 3 h, 89%; g) tris(ethylene glycol)-1,8-diamine, Et_3N, DCM, 3 h, 80%; h) Dess–Martin, DCM, 0.5 h, 92%; i) (carboxymethyl)hydroxyamine, DCM/MeOH, 61%; j) *N*-{2-[2-(2-amino-ethoxy)ethoxy]ethyl}-2-aminooxy-acetamide, AcOH, DCM/MeOH, 63%; k) biotin-OSu, Et_3N, DCM/MeOH, 89% for **14**, 92% for **15**.

Results and Discussion

Chemical synthesis and physical properties of DIBO

4-Dibenzocyclooctynol (**3**) was easily prepared starting from commercially available dibenzosuberenone (**4**, Scheme 2), which was treated with $TMSCHN_2$ in the presence of $BF_3{\cdot}Et_2O$; this resulted in ring expansion by carbene insertion to give trieneone **5**.[12] The latter compound was reduced with $NaBH_4$ (→**6**), brominated with bromine in chloroform (→**7**), and then treated with LDA in THF[13] to give target compound **3** in a yield of 57%. In an alternative approach, intermediate **6** was obtained by base-mediated ring opening of Kagan's ether, which was prepared by a double Friedel–Crafts alkylation phenylacetaldehyde.[14] The former synthesis route provides a

higher overall yield (71% vs. 42%), requires fewer chemical steps, and is more scalable.

The hydroxyl of **3** provides an opportunity for further functionalization and, for example, can be activated with 4-nitrophenyl chloroformate to give activated carbonate **8**, which can be treated with amines, such as propanol amine and tris(ethylene glycol)-1,8-diamine, to give carbamates **9** and **10**, respectively. Alternatively, the alcohol of **3** can be oxidized to ketone **11** by using Dess–Martin periodate, which can then be modified by aminooxy derivatives. This procedure was employed for the preparation of compounds **12** and **13**. It was observed that oxime formation was rather sluggish and the rate of reaction could not significantly be increased by the addition of aniline.[15] Finally, acylation of the amino groups of compounds **10** and **13** with *N*-(biotinyloxy)succinimide (biotin-OSu) and Et_3N gave biotin-labeled derivatives **14** and **15**, respectively.

It was observed that compound **3** has an excellent shelf life and remained intact after treatment with nucleophiles, such as thiols and amines. However, upon exposure to azides a fast reaction took place to give the corresponding triazoles in high yield. Rate measurements of cycloaddition of compounds **3**, **9**, **11** and **12** were conducted by UV spectroscopy at 25±0.1 °C. A calculated amount of 0.25 M solution of benzyl azide, which was required to achieve a desired azide concentration (6×10^{-4}–1.5×10^{-2} M), was added to a thermally equilibrated 6×10^{-5} M solution of cyclooctyne in methanol. The progress of the reactions was monitored by the decay of the characteristic absorbance of acetylenes at approximately 317 nm. Consumption of starting material followed a first-order equation and pseudo-first-order rate constants were obtained by least-square fitting of the data to a single exponential equation. The rate dependence as a function of the concentration of azide was linear and least-square fitting of the data to a linear equation produced the bimolecular rate constants summarized in Table 1.

Table 1. Bimolecular rate constants for the reactions of acetylenes with benzyl azide in methanol at 25±0.1 °C.

Cyclo-octyne	Product	Rate [$M^{-1}s^{-1}$]	Cyclo-octyne	Product	Rate [$M^{-1}s^{-1}$]
3	**16**	0.0567±0.0027	9	**17**	0.0696±0.0019
11	**18**	0.2590±0.0067	12	**19**	0.0611±0.0035

The rate constant of cycloaddition of DIBO (**3**) with benzyl azide is two orders of magnitude higher than that of cyclooctyne **1**, and similar to that of DIFO. Conversion of the alcohol of DIBO into a carbamate, as in compound **9**, did not influence the rate of cycloaddition. However, ketone **11** reacted three-times faster than the parent alcohol **3**. Probably, oxidation of **3** to **11** induces a small change in the conformation of the cyclooctyne ring, which might be responsible for the observed enhancement of reaction rate. In this respect, density functional theory (B3LYP) calculations of the transition states of cycloadditions of phenyl azide with acetylene and cyclooctyne indicate that the fast rate of the strain promoted cycloaddition is due to a lower energy required for distorting the 1,3-dipole and alkyne into the transition-state geometry.[5] This finding has been exploited in the design of more reactive cyclooctynes and, for example, it has been found that installment of an amide or cyclopropanation in the eight membered ring leads to higher rates of cycloaddition.[16] Surprisingly, oxime **12** did not exhibit a faster rate of reaction than alcohol **3**. Computational studies will be required to provide a rationale for these observations.

The acid labile oxime linkage of compound **15** provides opportunities for catch and release strategies, which, for example, are required for glycoproteomic applications. In this respect, treatment of triazole **21**, which was formed by treatment of **15** with benzyl azide, at pH 2 for 10 h resulted in hydrolysis of the oxime linkage. On the other hand, compound **20**, which was formed by treatment of derivative **14** with benzyl azide, was stable under these conditions.

It was observed that compound **11** exhibits fluorescent properties and in methanol emits light with a maximum fluorescent intensity of 436 nm and a quantum yield of 36% when excited at 375 nm. Oxime **12** showed a maximum fluorescent intensity of 380 nm and a quantum yield of 34% when excited at 313 nm (Figure 1). Interestingly, triazoles **18** and **19**, which were formed by treatment of **11** and **12**, respectively, with

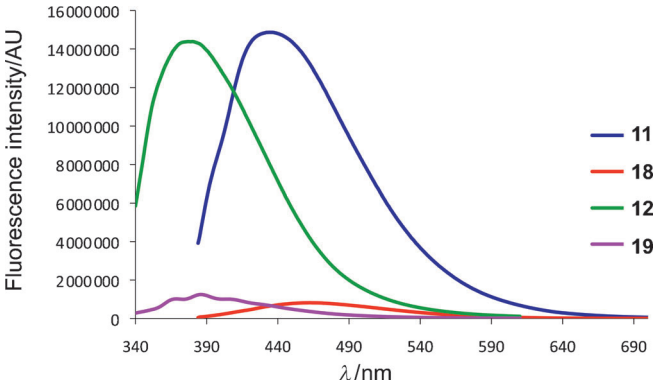


Figure 1. Fluorescence spectra of compounds **11** and **18** (λ_{ex}=375 nm) and **12** and **19** (λ_{ex}=313 nm) in methanol (10^{-5} M); AU: arbitrary fluorescence units.

benzyl azide, showed only very weak fluorescence. Previously, a number of Cu^{I}-mediated fluorogenic alkyne–azide click (CuAAC) reactions were described, which were based on the formation of a fluorescent 1,3-triazole from a nonfluorescent precursor, such as azide or alkyne substituted coumarins, 1,8-naphthalimides or anthracenes.[17] Treatment of **11** and **12** with benzyl azide is the first example of a metal-free alkyne–azide fluoro-switch click reaction. A unique feature of this reaction is that the triazole moiety is responsible for fluorescent quenching whereas this effect is not observed for the parent cyclooctyne. It is to be expected that fluorogenic SPAAC will provide opportunities to monitor the progress of reactions in real time. Such an approach will in particular be advantageous for applications in which a fluorescent cyclooctyne is attached to a surface or solid support.[4,18]

Evaluation of DIBO for labeling glycoconjugates of living cells

The bioorthogonal chemical reporter strategy is emerging as a versatile method for labeling biomolecules, such as nucleic acids, lipids, proteins, and carbohydrates.[1a,c,d] In this approach, an abiotic chemical functionality (reporter) is incorporated into a target biomolecule, which can then be reacted with a complementary bioorthogonal reagent linked to a probe. Azide is commonly employed as a reporter and can be installed into biomolecules by using azido-containing biosynthetic precursors that can be accepted by the cell's native or engineered biosynthetic machinery. For example, azido-containing glycoconjugates can be biosynthesized by metabolic labeling with peracetylated *N*-α-azidoacetylmannosamine (Ac_4ManNAz), which is an appropriate substrate for the cell's glycosylation machinery.[19] A subsequently bioorthogonal reaction can then covalently attach a probe to the azido function, which in turn makes it possible to conduct a multitude of functional studies. A number of bioorthogonal reactions have been described for reactions with azides, however, SPAAC is emerging as a particularly attractive approach as it can be performed under physiological conditions and does not require a toxic metal catalyst.

To establish biotin-modified DIBO derivatives **14** and **15** as appropriate bioorthogonal reagents, we employed these compounds to determine relative quantities of cell surface sialylation of wild-type and mutant cells, and compared the results with traditional lectin staining. It is well established that Ac_4ManNAz can be employed by the glycosylation machinery to install azido-containing sialic acid in various glycoconjugates and a subsequent reaction with **14** or **15** was expected to provide quantitative data on cell surface sialylation.[19] Thus, Jurkat cells were cultured in the presence of Ac_4ManNAz (25 μM) for 3 days to metabolically introduce *N*-azidoacetyl-sialic acid (SiaNAz) moieties into glycoproteins and glycolipids. As a negative control, Jurkat cells were employed that were grown in the presence of peracetylated *N*-acetylmannosamine (Ac_4ManNAc). A time-course experiment was conducted by exposing the cells to 30 μM of **14** and **15** for different time periods at room temperature, washed and then 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. Gratifyingly, the ManNAz-labeled cell exhibited strong fluorescent readings after being stained with the two different DIBO derivatives, and the cell labeling was almost complete after a reaction time of 60 min (Figure 2), whereas the control cells gave a very low fluorescence intensity showing that background labeling is negligible. Similar results were obtained when Chinese hamster ovary (CHO)-K1 cells were subjected to the same procedure.

Metabolically labeled cells were also examined by confocal microscopy (Figure 3 and Figure S1 in the Supporting Information). Thus, adherent Chinese hamster ovary (CHO) cells were cultured in the presence of Ac_4ManNAz (100 μM) for two days.[20] Next, cell surface azido moieties were treated with **14** or **15** (30 μM) for 1 h at ambient temperature, and then visualized with avidin–AlexaFluor488 for 15 min at 4 °C. Staining was mainly observed at the cell surface, and as expected, blank cells exhibited very low fluorescence staining confirming that background labeling is negligible. We also found that the two-step labeling approach with **14** and **15** had no effect on cell viability as determined by morphology and exclusion of trypan blue (data not shown).

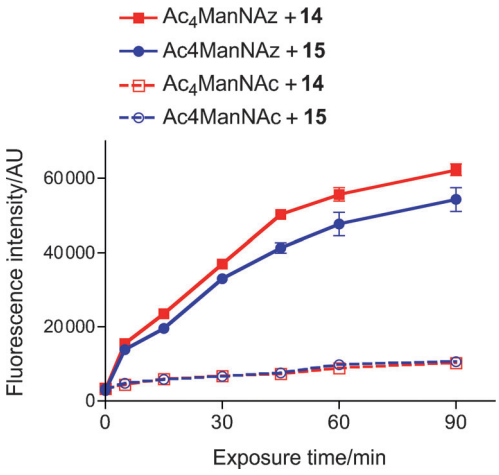


Figure 2. Time course of cell surface labeling with compounds **14** and **15**. Jurkat cells grown for 3 days in the presence of Ac_4ManNAc or Ac_4ManNAz (25 μM) were incubated with compounds **14** or **15** (30 μM) for 0–90 min at room temperature. Next, cells were incubated with avidin–FITC for 15 min at 4 °C, after which cell lysates were assessed for fluorescence intensity. AU indicates arbitrary fluorescence units. Data ($n = 3$) are presented as mean ± SD.

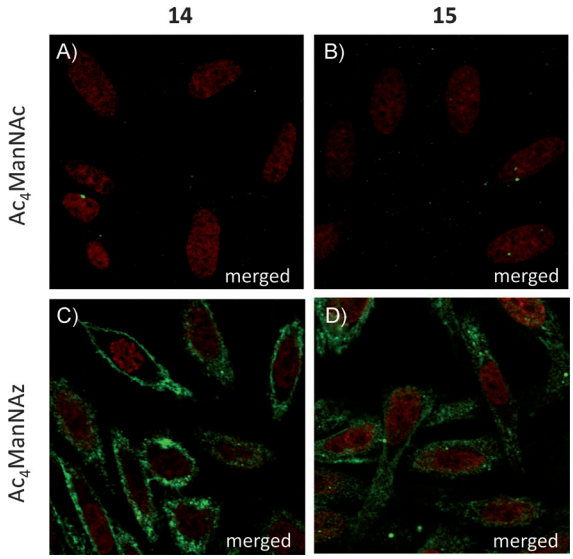


Figure 3. Fluorescence images of cells labeled with compounds **14** and **15** and avidin–AlexaFluor488. CHO cells grown for 2 days in the presence of: A), B) Ac_4ManNAc, or C), D) Ac_4ManNAz (100 μM) were incubated with compounds: A), C) **14**, or B), D) **15** (30 μM) for 1 h at room temperature. Next, cells were incubated with avidin–AlexaFluor488 for 15 min at 4 °C, and imaged after being washed, fixed, and stained for the nucleus with the far-red-fluorescent dye TO-PRO-3 iodide. "Merged" indicates that the images of cells labeled with AlexaFluor (488 nm) and TO-PRO (633 nm) are merged and shown in green and red, respectively.

The concentration dependency of the cell surface labeling was studied by incubating Jurkat and CHO-K1 cells with various concentrations of **14** or **15** followed by staining with avidin–FITC (Figure S2 in the Supporting Information). As expected, cells displaying azido moieties showed a dose-dependent increase in fluorescence intensity and reliable labeling was achieved at a concentration of 3 μM of **14** or **15**, however, optimal results were obtained at concentrations ranging from 30 to 100 μM. No increase in labeling was observed at concentrations higher than 100 μM due to limited solubility.

Jurkat and CHO-K1 cells were also metabolically labeled with peracetylated *N*-α-azidoacetylgalactosamine (Ac_4GalNAz, 100 μM), which can be metabolized by a number of cells and installed on mucin type glycoproteins.[21] Subsequent treatment of the CHO-K1 cells with **14** followed by avidin–FITC resulted in strong fluorescent labeling whereas weak labeling was observed for Jurkat cells (Figure S3 in the Supporting Information). These results are in agreement with the well-known fact that CHO cells produce significant quantities of mucins whereas this is not the case for Jurkat cells.[22]

Having established optimal conditions for SPAAC of azido-modified glycoconjugates of living cells with DIBO reagents, labeling studies were performed with a panel of lectin-resistant (Lec) mutant CHO cells. These cell lines (Lec2, Lec13 and Lec32), which exhibit unique structural changes in surface carbohydrates that reflect specific defects in glycosylation reactions, were expected to be ideally suited for validation of the SPAAC methodology. Lec2 cells have a mutation in the open reading frame of the CMP-sialic acid transporter, and therefore, are unable to translocate CMP-sialic acid into the lumen of the Golgi apparatus, resulting in a marked reduction in glycoprotein and ganglioside sialylation.[23] Although very small amounts of sialic acid containing glycoconjugates are made by these cells,[24] the major class of glycans are asialo, core fucosylated *N*-glycans having LacNAc moieties.[25] Lec32 mutants also exhibit a defect in sialylation due to a reduced expression of CMP-sialic acid synthetase. As a result, these cells have an increase in terminal β-galactoside residues on cell surface glycoproteins.[26] Lec13 mutants exhibit a reduced expression of GDP-Man-4,6-dehydratase activity; this results in a decrease in GDP-fucose biosynthesis and underfucosylation of glycoproteins and glycolipids.[27] The *N*-glycan profiles of these cells show increased levels of core nonfucosylated *N*-glycans with the most abundant *N*-glycans being asialo-, mono-, or di-sialylated structures.

Wild-type CHO-K1 and Lec2, Lec13 and Lec32 mutant cells were cultured in the presence of Ac_4ManNAz or Ac_4ManNAz (100 μM) for 2 days and then exposed to biotin-modified DIBO (**14**) for 1 h at room temperature. Next, the cells were washed and labeled with avidin–FITC at 4 °C and the fluorescence intensity measured. As expected, the wild-type and Lec13 cells gave similar and strong fluorescence intensity readings (Figure 4A). On the other hand, the Lec2 and Lec32 mutants showed a significant reduction in staining intensity and, in the case of the Lec2 cells, the readings were barely above the control, which indicates that these cells express very low levels of surface sialosides. Thus, the results of these studies are in agreement with the previously described defects in the mutant cell lines and hence support the notion that the chemi-

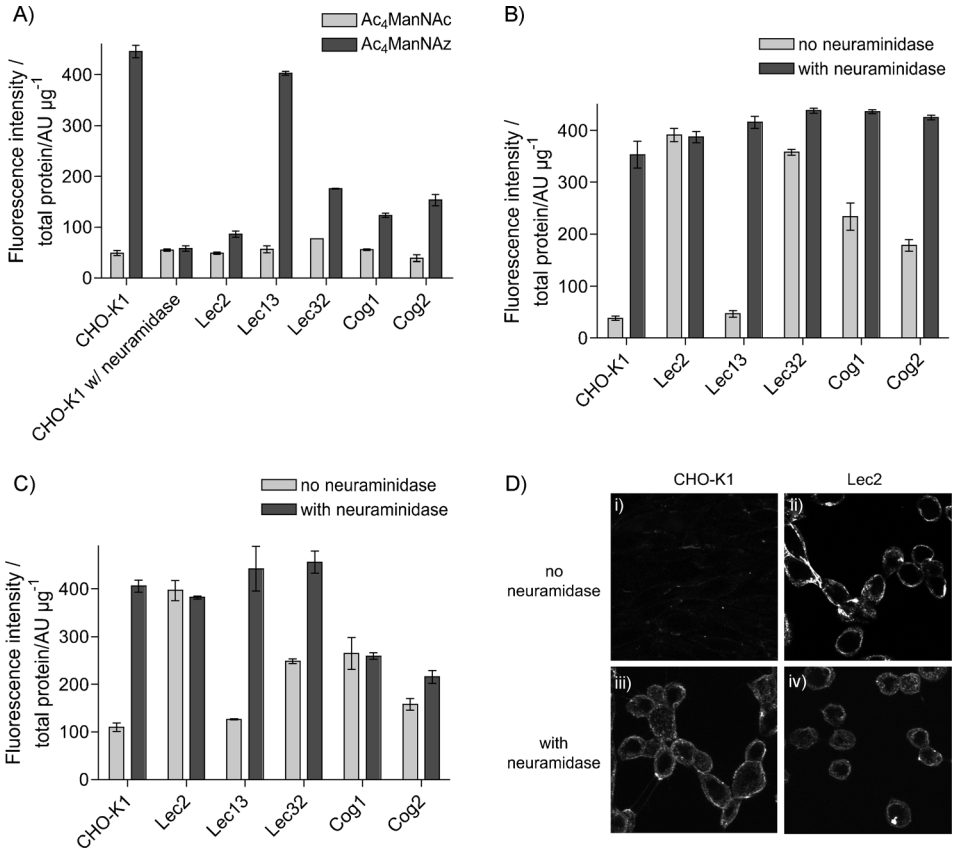


Figure 4. SiaNAz expression in CHO-K1 and CHO glycosylation mutant cells and the effect of neuraminidase treatment on cell surface labeling. A) CHO-K1 and CHO mutant cells grown for 2 days in the presence of Ac_4ManNAc or Ac_4ManNAz (100 μM) were incubated, either directly or after treatment with *V. cholerae* neuraminidase (50 mU mL^{-1}) in serum-free culture medium for 2 h at 37 °C, with compound **14** (30 μM) for 1 h at room temperature. Next, cells were incubated with avidin–FITC for 15 min at 4 °C, after which cell lysates were assessed for fluorescence intensity. To assess the effects of desialylation of CHO-K1 and CHO glycosylation mutants on their recognition by PNA and RCA1, cells were incubated, either directly or after treatment with *V. cholerae* neuraminidase (50 mU mL^{-1}), in serum-free culture medium for 2 h at 37 °C with: B) PNA–FITC (50 μg mL^{-1}), or C) RCA1–FITC (50 μg mL^{-1}) for 45 min on ice in the dark. Next cell lysates were assessed for fluorescence intensity. AU indicates arbitrary fluorescence units. Data ($n=3$) are presented as mean ± SD. Similarly, D) CHO-K1 (i, iii) and Lec2 (ii, iv) cells were imaged, either directly (i, ii) or after treatment with *V. cholerae* neuraminidase (iii, iv) with PNA–FITC.

cal reporter strategy can be employed to determine relative quantities of sialylation of glycoconjugates of living cells. Furthermore, treatment of the wild-type cells with *Vibrio cholerae* neuraminidase led to a similar fluorescent reading as control cells, confirming selective azide incorporation into sialic acid.

The results of the metabolic labeling studies were compared with traditional lectin staining by using FITC-labeled peanut (*Arachis hypogaea*) agglutinin (PNA) and FITC-labeled *Ricinus communis* (castor bean) agglutinin type 1 (RCA1), which mainly recognize terminal β-Gal-(1-3)-GalNAc residues of O-linked structures and β-Gal-(1-4)-GlcNAc (LacNAc) found on N-linked glycoproteins, respectively. Cells that have intact sialylation machinery modify β-galactosyl residues with sialic acid and hence display low reactivity against PNA and RCA1 lectins. Indeed, the wild-type and Lec13 cells gave fluorescent intensities just above background whereas the Lec2 and Lec32 mutants, which have a defect in sialylation, showed strong staining (Figure 4B–D).

Treatment of the wild-type and Lec13 cells with *V. cholerae* neuramidase resulted in fluorescent intensities similar to that of the Lec2 and Lec32 cells; this indicates that the various cell types express similar quantities of galactosyl-containing glycoproteins. Furthermore, a similar neuraminidase treatment of Lec2 cells followed by staining with PNA–FITC or RCA1–FITC did not lead to a significant increase in fluorescent intensity; this demonstrates that these cells do not significantly modify their cell surface glycoconjugates with sialic acid. On the other hand, neuraminidase treatment of Lec32 cells resulted in an increase in fluorescence staining with RCA1–FITC, whereas it did not impact the reading of PNA–FITC. These results indicate that the N-linked glycans of Lec32 contain some sialosides whereas this modification is absent in O-linked residues.

The lectin staining (Figure 4B–D) and metabolic labeling followed by SPAAC (Figure 4A) gave similar results and in particular both approaches showed that Lec2 cells express very small quantities of sialosides whereas the Lec32 mutant attach some sialic acid to their glycoconjugates. Surprisingly, a shortage of CMP-Neu5Ac as in Lec32 cells resulted in differential sialylation of N- and O-linked glycans and it appears that N- but not O-linked glycans are modified by some sialic acid. Finally, both approaches showed that a defect in fucosylation does not impact the level of glycoconjugate sialylation. Attempts were made to directly assess differences in sialylation within the Lec mutants by utilizing lectins that recognize α(2,3)- or α(2,6)-linked terminal sialic acid residues (MAA and LFA). Surprisingly, we found fluorescence intensity for both lectins to be comparable in wild-type and Lec2 mutants (data not shown); this suggests that these lectins might recognize additional sugar structures other than terminal sialic acid and are not suitable for this study. Thus, we believe that the chemical reporter strategy provides a more reliable approach to determine relative differences in glycoprotein sialylation.

Having established biotin-modified DIBO (**14**) as a reliable reagent for detection of cell surface sialosides, attention was focused on sialylation of the CHO mutants Cog1 (*ldlB*) and Cog2 (*ldlC*). These cell lines were identified in a genetic screen for mutations that block low-density lipoprotein receptor (LDLR) activity.[28] Further examinations have shown that these cell lines have defects in the conserved oligomeric Golgi (COG) complex, which is a protein complex consisting of eight subunits (Cog1–8) that play a critical role in retrograde vesicle transport and intra-Golgi trafficking. Malfunctions in the COG complex impact Golgi integrity and result in defects in protein sorting and glycosylation.[29] Mutations in COG subunits have also been observed in humans and result in severe congenital disorders of glycosylation (CDG).[30]

Metabolic labeling of Cog1 and Cog2 cells with ManNAz followed by SPAAC with **14** and staining with avidin–FITC showed that these cells produce sialylated glycoconjugates, however, at a significantly reduced level compared to wild-type CHO-K1 cells (Figure 4A). Staining with PNA–FITC demonstrated that that the cells expose terminal galactosyl residues on their O-linked glycans (Figure 4B). Furthermore, treatment of the cells with *V. cholerae* neuraminidase led to a similar staining intensity as for wild-type cells. This indicates that both cell types express similar quantities of galactosyl moieties, and thus it appears that in Cog1 and -2 cells, glycoprotein sialylation of *O*-glycans is more severely imparted than galactosylation. Interestingly, a different staining profile was obtained when RCA1–FITC was employed (Figure 4C) and in this case untreated and neuraminidase exposed cells gave similar but reduced fluorescent intensities highlighting that *N*-glycan sialylation and galactosylation are both affected in the Cog1 and Cog2 mutants. These results suggest that loss of COG complex function might affect the localization and/or stability of glycosyltransferases involved in terminal processing of *N*-glycans to a greater extent than those enzymes that modify *O*-glycans. In support of this hypothesis, recent studies have shown that the stability of β(1,4)-galactosyltransferase is altered in COG-depleted HeLa cells due to altered trafficking and proteasomal degradation.[31] At this point, we can, however, not rule out the possibility that these differences are due to the type and amount of glycoprotein cargo that is modified in the Golgi of these cells.

Conclusions

The past several years has seen a rapid development of the bioorthogonal chemical reporter methodology for the labeling of glycoconjugates of living cells and whole organisms. In this paper, we present a streamlined chemical approach for the preparation and derivatization of DIBO, which is an ideal bioorthogonal reagent for the chemical reporter strategy. Attractive features of DIBO include easy access by a simple and scalable synthesis approach, nontoxicity and straightforward attachment of a variety of probes. The use of several cell lines with known defects in glycoconjugate glycosylation validated DIBO as a reagent for determining relative quantities of cell surface glycoconjugate sialylation. The chemical reporter strategy in combination with lectin staining revealed that *O*-glycan sialylation of Cog1 and -2 cells is more severely impacted than galactosylation. Surprisingly, sialylation and galactosylation of *N*-glycans were similarly affected in these mutant cell lines. These results suggest that loss of COG complex function might differ-

ently affect the localization and/or stability of glycosyltransferases involved in terminal processing of *N*- and *O*-glycans. Differential modulation of N- and O-linked sialylation was also observed in Lec32 cells, which exhibit a reduced expression of CMP-sialic acid synthetase, and in this case N-linked oligosaccharides acquire some sialic acid moieties whereas this is not the case for O-linked structures. It is well known that the various cell types express different ensembles of glycans. The results of this study indicate that a limited availability of sugar nucleotides is one way for a cell to selectively modulate the structures of glycoprotein glycans. We anticipate multiple applications of the described chemical reporter methodology in glycobiology and glycomedicine, including the tagging and isolation of glycoproteins from cell and tissue extracts as well as the investigation of trafficking and turnover of glycoconjugates in healthy and diseased cells.

Experimental Section

General methods and materials: Chemicals were purchased from Aldrich or Fluka and used without further purification. Dichloromethane was distilled from CaH_2 and stored over molecular sieves 4 Å. Pyridine was distilled from P_2O_5 and stored over molecular sieves 4 Å. THF was distilled from sodium. All reactions were performed under anhydrous conditions under an atmosphere of argon. Reactions were monitored by TLC on Kieselgel 60 F254 (Merck). Detection was carried out under UV light (254 nm). Flash chromatography was performed on silica gel (Merck, 70–230 mesh). Iatrobeads (60 μm) were purchased from Bioscan. ^{1}H NMR (1D, 2D) and ^{13}C NMR spectra were recorded on a Varian Merc 300 spectrometer equipped with Sun workstations. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ or CD_3OD, and chemical shifts are given relative to solvent peaks ($CDCl_3$: ^{1}H, $\delta = 7.24$, ^{13}C, $\delta = 77.0$; CD_3OD: ^{1}H, $\delta = 3.31$, ^{13}C, $\delta = 49.0$) as internal standard for compounds. High-resolution mass spectra were obtained by an Applied Biosystems 4700 MALDI mass spectrometer in positive ion reflective mode by using 2,5-dihydroxyl-benzoic acid in CH_3CN as matrix.

6*H*-Dibenzo[*a,e*]cyclooctatrien-5-one (5): A solution of trimethylsilyl diazomethane (10.5 mL, 21.9 mmol) in CH_2Cl_2 (20 mL) was added dropwise to a stirred solution of dibenzosuberenone **4** (2.88 g, 14.0 mmol) and $BF_3{\cdot}OEt_2$ (2.59 mL, 21.0 mmol) in CH_2Cl_2 (30 mL) at $-10\,^\circ$C over a period of 1 h. The reaction mixture was stirred at $-10\,^\circ$C for 2 h, and then poured into ice water. The aqueous layer was extracted with CH_2Cl_2 (3×100 mL) and the combined organic layers washed with brine, dried ($MgSO_4$), filtered and the filtrate was concentrated under reduced pressure. The crude product was purified by flash chromatography over silica gel (2:1→1:2, *v/v*, hexanes/CH_2Cl_2) to give compound **5** as an amorphous solid (2.22 g, 72%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.26$ (q, $J = 1.4$, 6.6 Hz, 1H), 7.13–7.43 (m, 7H), 7.05 (q, $J = 3.8$, 12.9 Hz, 2H), 4.06 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 196.6$, 136.9, 136.3, 135.4, 133.8, 133.1, 132.4, 131.4, 130.6, 129.3, 128.8, 128.0, 127.3, 126.9, 48.4; MALDI HRMS: *m/z* 243.0767 [*M*+Na$^+$]; calcd for $C_{16}H_{12}NaO^+$: 243.0780.

5,6-Dihydro-dibenzo[*a,e*]cycloocten-5-ol (6): Sodium borohydride (0.757 g, 20 mmol) was slowly added to a stirred solution of **5** (2.20 g, 10 mmol) in a mixture of EtOH and THF (1:1, *v/v*, 120 mL). The reaction mixture was stirred for 7 h, after which TLC analysis indicated completion of the reaction. The reaction was quenched by slow addition of acetic acid (1 mL) and the solvents were evaporated. The residue was dissolved in CH_2Cl_2 (100 mL) and the resulting solution was washed with brine (100 mL), which was back extracted with CH_2Cl_2 (4×100 mL). The combined organic phases were dried ($MgSO_4$), filtered and concentrated under reduced pressure to give **6** as a white solid (2.22 g), which was directly used in the next step without further purification. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.50$ (m, 1H), 7.14–7.30 (m, 7H), 6.90 (q, $J =$ 2.7, 12.0 Hz, 2H), 5.31 (q, 1H, $J = 6.3$, 10.0 Hz), 3.41 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 141.7$, 136.7, 136.2, 134.5, 131.7, 131.5, 130.1, 129.9, 129.3, 128.7, 127.4, 127.2, 126.9, 125.9, 74.4, 42.7. MALDI HRMS: *m/z* 245.0949 [*M*+Na$^+$]; calcd for $C_{16}H_{14}NaO^+$: 245.0937.

11,12-Dibromo-5,6,11,12-tetrahydro-dibenzo[*a,e*]cycloocten-5-ol (7): Bromine (0.51 mL, 10 mmol) as added dropwise to a stirred solution of **6** (2.22 g, 10 mmol) in $CHCl_3$ (50 mL). After stirring the mixture for 0.5 h, TLC analysis indicated completion of the reaction. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography over silica gel (2:1→1:2, *v/v*, hexanes/CH_2Cl_2) to give **7** as a light-yellow oil (2.22 g, 58%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.54$–7.47 (2H, aromatics), 7.31–6.72 (6H, aromatics), 5.77 (d, $J = 5.4$ Hz, 1H, C*H*Br), 5.22 (dd, $J = 3.6$, 15.9 Hz, 1H, C*H*OH), 5.19 (d, $J = 5.4$ Hz, 1H, C*H*Br), 3.50 (dd, $J = 3.6$, 15.9 Hz, 1H, CH_2), 2.75 (dd, $J = 3.6$, 15.9 Hz, 1H, CH_2); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 141.3$, 140.0, 137.2, 134.0, 133.4, 131.5, 131.3, 130.9, 127.8, 126.2, 123.7, 121.3, 76.5, 70.0, 62.3, 32.2. MALDI HRMS: *m/z* 402.9313 [*M*+Na$^+$]; calcd for $C_{16}H_{14}Br_2NaO^+$: 402.9304.

5,6-Dihydro-11,12-didehydro-dibenzo[*a,e*]cycloocten-5-ol (3): Lithium diisopropylamide in tetrahydrofuran (2.0 M; 8 mL, 16 mmol) was added dropwise to a stirred solution of **7** (1.53 g, 4.0 mmol) in tetrahydrofuran (40 mL) under an atmosphere of argon. The reaction mixture was stirred for 0.5 h, after which it was quenched by the dropwise addition of water (0.5 mL). The solvents were evaporated under reduced pressure, and the residue was purified by flash chromatography on silica gel (hexanes/CH_2Cl_2 2:1→0:1, *v/v*) to give **3** as a white amorphous solid (0.50 g, 57%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.67$ (1H, aromatics), 7.37–7.18 (7H, aromatics), 4.57 (dd, $J = 2.1$, 14.7 Hz, 1H, C*H*OH), 3.04 (dd, $J = 2.1$, 14.7 Hz, 1H, CH_2), 2.86 (dd, $J = 2.1$, 14.7 Hz, 1H, CH_2); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 154.5$, 150.6, 128.6, 127.1, 1127.0, 126.0, 125.8, 125.1, 124.7, 123.0, 122.7, 121.7, 111.9, 109.6, 74.2, 47.7.

Carbonic acid, 5,6-dihydro-11,12-didehydro-dibenzo[*a,e*]cycloocten-5-yl ester, 4-nitrophenyl ester (8): 4-Nitrophenyl chloroformate (0.4 g, 2 mmol) and pyridine (0.4 mL, 5 mmol) were added to a solution of **3** (0.22 g, 1 mmol) in CH_2Cl_2 (30 mL). After being stirred for 4 h at room temperature, the mixture was washed with brine (2×10 mL) and the organic layer was dried ($MgSO_4$). The solvents were evaporated under reduced pressure, and the residue was purified by silica gel column chromatography (hexane/ethyl acetate, 10:1, *v/v*) to afford **8** (0.34 g, 89%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.23$–8.18 (2H, aromatics), 7.56–7.54 (2H, aromatics), 7.46–7.18 (8H, aromatics), 5.52 (dd, $J = 3.9$, 15.3 Hz, 1H, C*H*OH), 3.26 (dd, $J = 3.9$, 15.3 Hz, 1H, CH_2), 2.97 (dd, $J = 3.9$, 15.3 Hz, 1H, CH_2); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 154.5$, 150.7, 149.1, 148.7, 129.0, 127.4, 127.3, 126.7, 126.5, 125.5, 125.2, 124.3, 124.0, 122.6, 122.4, 120.8, 120.6, 120.2, 112.2, 108.5, 80.6, 44.8; MALDI HRMS: *m/z* 408.0852 [*M*+Na$^+$]; calcd for $C_{23}H_{15}NNaO_5^+$: 408.0842.

3-Hydroxypropyl-carbamic acid 5,6-dihydro-11,12-didehydro-dibenzo[*a,e*]cycloocten-5-yl ester (9): 3-Aminopropan-1-ol (15 mg, 0.2 mmol) and triethylamine (10 μL) were added to a stirred solution of **8** (38 mg, 0.1 mmol) in CH_2Cl_2 (15 mL). The reaction mixture was stirred at room temperature for 12 h, after which the solvents

were evaporated under reduced pressure and the residue was purified by silica gel column chromatography (CH_2Cl_2/CH_3OH, 20:1, *v/v*) to afford **9** (25 mg, 77%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 6.94–7.43 (m, 8H, aromatics), 5.42 (m, 1H, Ph-C*H*-O), 3.61(m, 2H, CH_2OH), 3.30 (m, 2H, CH_2NH), 3.08 (dd, *J* = 15.0, 1.8 Hz, 1H, PhHC*H*), 2.84 (dd, *J* = 15.0, 3.9 Hz, 1H, PhHC*H*), 1.53–1.68 (m, 2H, CH_2CH$_2$OH); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 150.8, 149.1, 128.9, 128.0, 127.0, 126.1, 126.0, 125.9, 125.8, 125.3, 125.1, 125.0, 122.8, 122.6, 120.3, 111.9, 108.9, 58.6, 45.2, 36.8, 36.7, 31.6. MALDI HRMS: *m/z* 344.1246 [*M*+Na$^+$]; calcd for $C_{20}H_{19}O_3NNa^+$: 344.1257.

{2-[2-(2-Amino-ethoxy)ethoxy]ethyl}carbamic acid 5,6-dihydro-11,12-didehydro-dibenzo[*a,e*]cyclooctен-5-yl ester (10): Et_3N (0.139 mL, 1.0 mmol) was added to a stirred solution of **8** (77 mg, 0.2 mmol) and tris(ethylene glycol)-1,8-diamine (0.293 mL, 2 mmol) in CH_2Cl_2 (20 mL). The reaction mixture was stirred for 3 h, after which the solvent was removed under reduced pressure. The residue was purified by flash chromatography over latrobeads (MeOH/CH_2Cl_2, 8→30%, *v/v*) to give **10** as a light-yellow amorphous solid (0.063 g, 80%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.51 (d, 1H, *J* = 7.3 Hz), 7.24–7.37 (m, 7H), 5.81 (s, 1H, N*H*), 5.48 (br s, 1H), 3.50–3.68 (m, 8H), 3.39 (m, 2H), 3.16 (d, *J* = 14.8 Hz, 1H), 2.91 (br s, 2H), 2.88 (d, *J* = 14.8 Hz, 1H), 2.57 (br s, 2H, NH_2); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 155.7, 152.2, 151.1, 130.0, 128.1, 128.0, 127.2, 127.1, 126.3, 126.0, 123.9, 123.8, 121.3, 113.0, 110.0, 76.7, 72.8, 70.3, 70.2, 70.1, 70.0, 46.2, 41.5, 41.0. MALDI HRMS: *m/z* 417.1766 [*M*+Na$^+$]; calcd for $C_{23}H_{26}N_2NaO_4^+$: 417.1785.

6*H*-11,12-Didehydro-dibenzo[*a,e*]cyclooctatrien-5-one (11): Dess–Martin reagent (0.40 g, 0.94 mmol) was added to a stirred solution of **3** (0.172 g, 0.78 mmol) in CH_2Cl_2 (40 mL). The mixture was stirred for 0.5 h after which TLC analysis indicated completion of the reaction. The reaction mixture was filter through a short pad of silica gel, which was washed with CH_2Cl_2. The filtrate was concentrated, and the residue was purified by flash chromatography over silica gel (hexanes/CH_2Cl_2, 1:1→0:1, *v/v*) to give **11** as a white amorphous solid (0.158 g, 92%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.29–7.57 (m, 8H), 4.17 (d, *J* = 10.6 Hz, 1H), 3.64 (d, *J* = 10.6 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 200.4, 154.7, 148.2, 131.21 (2C), 131.18, 129.3, 128.2, 127.8, 126.3, 125.9, 122.2, 111.1, 109.4, 49.3. MALDI HRMS: *m/z* 241.0638 [*M*+Na$^+$]; calcd for $C_{16}H_{10}NaO^+$: 241.0624.

(6*H*-11,12-didehydro-dibenzo[*a,e*]cyclooctен-5-ylideneamino-oxy)acetic acid (12): A solution of 6*H*-11,12-didehydro-dibenzo-[*a,e*]cyclooctatrien-5-one (**11**; 21.8 mg, 0.1 mmol) and (carboxymethyl)hydroxylamine hemihydrochloride (21.8 mg, 0.2 mmol) in MeOH/CH_2Cl_2/HOAc (1:1:0.02, *v/v/v*, 8 mL) was stirred for 2 days. The solvents were removed under reduced pressure, and the residue was purified by flash chromatography on silica gel (EtOAc) to give **12** as an amorphous white solid (17.8 mg, 61%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.54 (d, *J* = 7.4 Hz, 1H), 7.46 (d, *J* = 7.4 Hz, 1H), 7.18–7.39 (m, 6H), 4.53 (m, 2H), 4.23 (d, *J* = 12.8 Hz, 1H), 3.16 (d, *J* = 12.8 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 175.2 and 173.6, 154.1 153.2, 130.7,129.5, 19.3, 129.2, 129.1, 128.1, 128.0, 127.1, 126.9, 125.5, 125.2, 122.7, 113.9, 111.2, 84.7, 68.3 and 67.1, 35.0 and 33.2. MALDI HRMS: *m/z* 314.0770 [*M*+Na$^+$]; calcd for $C_{18}H_{13}NNaO_3^+$: 314.0788.

***N*-{2-[2-(2-Amino-ethoxy)ethoxy]ethyl}-2-(6*H*-11,12-didehydro-dibenzo[*a,e*]cyclooctен-5-ylideneaminooxy)acetamide (13):** A solution of **11** (46 mg, 0.211 mmol), *N*-{2-[2-(2-amino-ethoxy)ethoxy]ethyl}-2-aminooxy-acetamide (84 mg, 0.251 mmol) and acetic acid (0.1 mL) in MeOH/CH_2Cl_2 (1:1, *v/v*, 4 mL) was stirred for 2 days. The solvents were evaporated under reduced pressure, and the residue was purified by flash chromatography over latrobeads (MeOH/CH_2Cl_2, 4→15%, *v/v*) to give **13** as a light-yellow solid (56 mg, 63%). ^{1}H NMR (300 MHz, CD_3OD): δ = 7.73 (d, *J* = 7.6 Hz, 1H), 7.30–7.56 (m, 7H), 4.48 (m, 2H), 4.35 (d, *J* = 14.7 Hz, 1H), 3.50–3.68 (m, 8H), 3.24 (d, *J* = 13.6 Hz, 1H), 3.08 (d, *J* = 5.3 Hz, 2H), 3.02 (d, *J* = 5.3 Hz, 2H); ^{13}C NMR (75 MHz, CD_3OD): δ = 172.2, 162.0, 150.0, 149.0, 133.2, 131.0, 130.6, 129.8, 129.3, 128.5, 127.1, 126.8, 124.9, 124.3, 111.2, 110.5, 73.8, 71.4, 71.3, 70.4, 67.8, 40.6, 39.7, 39.1. MALDI HRMS: *m/z* 444.1875 [*M*+Na$^+$]; calcd for $C_{24}H_{27}N_3NaO_4^+$: 444.1894.

{2-[2-(2-Biotinylamino-ethoxy)ethoxy]ethyl}carbamic acid 5,6-dihydro-11,12-didehydro-dibenzo[*a,e*]cyclooctен-5-yl ester (14): Three drops of Et_3N were added to a solution of **10** (15.8 mg, 0.04 mmol) and biotin-OSu (20.5 mg, 0.06 mmol) in MeOH/CH_2Cl_2 (1:1, *v/v*, 7 mL). The reaction mixture was stirred for 2 h, and then the solvents were removed under reduced pressure. The residue was purified by column chromatography over latrobeads (MeOH/CH_2Cl_2, 5→15%, *v/v*) to give **14** as a light-yellow amorphic solid (22.1 mg, 89%). ^{1}H NMR (300 MHz, CD_3OD): δ = 7.59 (1H, aromatics), 7.42–7.33 (7H, aromatics), 5.44, (dd, *J* = 5.0, 14.1 Hz, 1H, ArC*H*OH), 4.60, 4.46 (m, 2H, C*H*NH), 4.24 (s, 4H, OCH_2CH_2O), 3.72 (m, 4H, OCH_2), 3.64 (m, 2H, CH_2NH), 3.55 (m, 1H, C*H*S), 3.33 (dd, *J* = 4.8, 12.0 Hz, 1H), 3.23 (t, *J* = 6 Hz, 2H, CH_2NH$_2$), 3.22, (dd, *J* = 5.0, 14.1 Hz, 1H, CH_2), 2.88, (dd, *J* = 5.0, 14.1 Hz, 1H, CH_2), 2.68 (d, *J* = 12.45 Hz, 1H), 2.20 (t, *J* = 7.5 Hz, 2H, CH_2CO), 1.4 (m, 6H, biotin-CH_2); ^{13}C NMR (75 MHz, CD_3OD): δ = 175.0, 164.9, 156.9, 152.5, 151.3, 129.9, 128.2, 128.1, 127.2, 127.1, 126.0, 125.7, 123.8, 121.2, 112.7, 109.8, 76.8, 70.2, 70.1, 69.8, 69.4, 62.1, 60.4, 55.8, 54.6, 46.0, 42.6, 40.6, 39.9, 39.1, 35.5, 28.6, 28.3, 25.6, 17.5, 16.1, 12.0; MALDI HRMS: *m/z* 643.2575 [*M*+Na$^+$]; calcd for $C_{33}H_{40}N_4NaO_6S^+$ 643.2561.

***N*-{2-[2-(2-Biotinylamino-ethoxy)ethoxy]ethyl}-2-(6*H*-11,12-didehydro-dibenzo[*a,e*]cyclooctен-5-ylideneaminooxy)acetamide (15):** Two drops of Et_3N were added to a solution of **13** (8.4 mg, 0.02 mmol) and biotin-OSu (10.2 mg, 0.03 mmol) in MeOH/CH_2Cl_2 (1:1, *v/v*, 4 mL). The reaction mixture was stirred for 2 h, after which the solvents were removed under reduced pressure. The residue was purified by column on latrobeads (MeOH/CH_2Cl_2, 5→15, *v/v*) to give **15** as a light-yellow solid (11.9 mg, 92%). ^{1}H NMR (300 MHz, CD_3OD): δ = 7.64 (1H, aromatics), 7.54–7.30 (7H, aromatics), 4.47 (m, 3H), 4.34 (m, 1H), 3.67 (s, 4H, OCH_2), 3.60–3.40 (m, 3H), 3.30 (m, 2H), 3.20 (m, 3H), 2.96–2.86 (m, 2H), 2.20 (t, *J* = 7 Hz, 2H, biotin-CH_2CO), 1.80–1.50 (m, 4H, biotin-CH_2) 1.48–1.38 (m, 2H, biotin-CH_2). MALDI HRMS: *m/z* 670.2665 [*M*+Na$^+$]; calcd for $C_{34}H_{41}N_5NaO_6S^+$ 670.2670.

General procedure for the preparation of triazoles (16–19): A solution of dibenzocyclooctyne derivative (**3**, **9**, **11** or **12**, 0.1 mmol) and benzyl azide (0.1 mmol) in MeOH (10 mL) was stirred for 3 h. The solvent was evaporated under reduced pressure, and the residue was purified by silica gel column chromatography to give the desired product (**16**, **17**, **18** or **19**, respectively) in a quantitative yield.

*Compound **16**:* ^{1}H NMR (300 MHz, CD_3OD): δ = 8.29–7.98 (1H, aromatic), 7.70–6.80 (12H, aromatics), 5.84–5.25 (2H, PhCH_2), 5.31–4.70 (1H, C*H*OH), 3.77–2.55 (2H, ArCH_2). MALDI HRMS: *m/z* 377.1507 [*M*+Na$^+$]; calcd for $C_{23}H_{20}N_3NaO^+$ 377.1499.

*Compound **17**:* ^{1}H NMR (300 MHz, CD_3OD): δ = 8.00–6.90 (13H, aromatics), 6.03–5.26 (2H, PhCH_2), 5.10–4.77 (1H, C*H*OCO), 3.58 (2H, CH_2OH), 3.29 (2H, NHCH_2), 3.20–2.64 (2H, ArCH_2), 1.62 (2H, CH_2CH$_2$OH). MALDI HRMS: *m/z* 477.1889 [*M*+Na$^+$]; calcd for $C_{27}H_{26}N_4NaO_3^+$ 477.1897.

*Compound **18**:* ^{1}H NMR (300 MHz, CD_3OD): $\delta = 8.09–6.92$ (13H, aromatics), 5.63 (2H, PhCH_2), 3.70 (2H, COCH_2). MALDI HRMS: *m/z* 375.1328 [*M*+Na^+]; calcd for $C_{23}H_{18}N_3NaO^+$ 375.1342.

*Compound **19**:* ^{1}H NMR (300 MHz, CD_3OD): $\delta = 7.59–7.06$ (11H, aromatics), 7.04–6.97 (2H, aromatics), 5.89–5.32 (2H, PhCH_2), 4.68–4.43 (2H, CH_2CO_2H), 4.30–4.03 (1H), 3.07–2.96 (1H). MALDI HRMS: *m/z* 447.1439 [*M*+Na^+]; calcd for $C_{25}H_{20}N_4NaO_3^+$: 447.1428.

Fluorescence measurement: The fluorescence spectra of compounds **11** and **18** (excited at 375 nm) and **12** and **19** (excited at 313 nm) in methanol (10^{-5} M) were recorded on a spectrofluorometer FluoroMax-3 from Horiba Jobin Yvon.

Kinetics of the cycloaddition reaction: The rate measurements of cycloaddition of cyclooctynes with benzyl azide were conducted by UV spectroscopy at 25±0.1 °C. A calculated amount of solutions of benzyl azide required to achieve desired azide concentration (6×10^{-4}–1.5×10^{-2} M) was added to a thermally equilibrated solution of acetylene in MeOH (6×10^{-5} M). Reactions were monitored by following the decay of the characteristic absorbance of acetylenes at about 317 nm. 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 rate dependence as a function of the concentration of azide was linear. Least-squares fitting of the data to a linear equation produced the bimolecular rate constants summarized in Table 1. In this respect, the UV spectroscopic method can be performed under pseudo-first-order conditions over a wide range of reagent concentrations making the analysis of second-order kinetic curves more reliable.

Reagents for biological experiments: Synthetic compounds **14** and **15** were reconstituted in DMF and stored at −80 °C. Final concentrations of DMF never exceeded 0.56% to avoid toxic effects. Ac_4ManNAc, Ac_4ManNAz, Ac_4GalNAc and Ac_4GalNAz were synthesized as reported,[32] and reconstituted in ethanol. Avidin–FITC and avidin–AlexaFluor488 were obtained from Molecular Probes, *V. cholerae* neuraminidase was from Sigma–Aldrich, and PNA–FITC and RCA1–FITC were from EY Laboratories.

Cell culture conditions: 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^{-1}), glucose (4.5 g L^{-1}), HEPES (10 mM), and sodium pyruvate (1 mM). CHO cells (clone K1; ATCC) were cultured in Kaighn's modification of Ham's F12 medium (ATCC) with L-glutamine (2 mM), adjusted to contain sodium bicarbonate (1.5 g L^{-1}). Mutant CHO cells (Lec2, Lec13, Lec32 mutants were obtained from Dr. Pamela Stanley and Cog1 and Cog2 mutants (*ldlB* and *ldlC*) obtained from Dr. Monty Kreiger) were cultured in minimum essential medium Alpha 1X (Cellgro) with Earle's salts, ribonucleosides, deoxyribonucleosides and L-glutamine (2 mM). All media were supplemented with penicillin (100 U mL^{-1})/streptomycin (100 μg mL^{-1}; Mediatech) and fetal bovine serum (FBS, 10%; Hyclone). Cells were maintained in a humid 5% CO_2 atmosphere at 37 °C.

Cell surface azide labeling: Jurkat cells were seeded at a density of 75000 cells per mL in a total volume of 40 mL culture medium in the presence of Ac_4ManNaz or Ac_4GalNaz (25 μM final concentration) and grown for 3 days; this led to the metabolic incorporation of the corresponding *N*-azidoacetyl sialic acid (SiaNAz) into their cell surface glycoproteins. Control cells were grown in the presence of Ac_4ManNac or Ac_4GalNac (25 μM final concentration) for 3 days. CHO cells were plated in 12-well plates (250000 cells per well) and grown in medium that contained Ac_4ManNaz or Ac_4GalNaz (100 μM) and as control cells Ac_4ManNAc or Ac_4GalNac (100 μM) for 2 days. Expected cell number on day of click chemistry was approximately 1×10^6 per well.

Sialidase pretreatment: Cells were washed twice with serum-free culture medium and incubated with *V. cholerae* neuraminidase (50 mU mL^{-1}) in serum-free culture medium for 2 h at 37 °C, washed in PBS and subjected to the respective assay.

Click chemistry and detection by fluorescence intensity: Jurkat cells bearing azides and control cells were washed with labeling buffer (DPBS, pH 7.4 containing 1% FBS and 1% BSA) and transferred to round-bottom tubes (1×10^6 cells per sample). CHO cells (untreated or sialidase pretreated) were left in the 12-well plates (~1×10^6 cells per sample) and washed with labeling buffer. Next, cells were incubated with the biotinylated compounds **14** or **15** (0–100 μM) in labeling buffer for 0–90 min at room temperature. The cells were washed three times with cold labeling buffer and then incubated with avidin–FITC (5 μg mL^{-1}) for 15 min at 4 °C in the dark. Following three washes and cell lysis in passive lysis buffer (Promega), lysates were analyzed for fluorescence intensity ($\lambda_{em}=520/\lambda_{ex}=485$) by using a microplate reader (BMG Labtech). Data points were collected in triplicate and are representative of three separate experiments. Fluorescence of Jurkat cell lysates was expressed as fluorescence (arbitrary units; AU) per 800000 cells. CHO cell lysates were assayed for total protein by using the bicinchoninic acid assay (BCA; Pierce Biotechnology) and fluorescence intensity was expressed as fluorescence (AU) per μg total protein.

Lectin binding assay: Untreated or sialidase pretreated cells (~1×10^6) were washed twice in cold PBS and subsequently incubated in 300 μL PBS containing PNA–FITC (50 μg mL^{-1}) or RCA1–FITC (50 μg mL^{-1}) for 45 min on ice in the dark. After being washed with cold PBS, the cells were lyzed in passive lysis buffer (Promega) and lysates were analyzed for fluorescence intensity ($\lambda_{em}=520/\lambda_{ex}=485$) by using a microplate reader.

Detection of cell labeling and lectin staining by fluorescence microscopy: For cell surface labeling, CHO-K1 cells labeled with Ac_4ManNAc or Ac_4ManNAz (100 μM) for 2 days were seeded at a density of 50000 cells per coverslip (22 mm) and allowed to adhere, overnight, in their original medium. After two washes with wash buffer (DPBS, supplemented with 1% FBS), live cells were incubated with biotinylated compounds **14** or **15** (30 μM) in wash buffer for 1 h at room temperature, followed by three washes in wash buffer (10 min per wash). Next, the cells were incubated with avidin conjugated with AlexaFluor488 (5 μg mL^{-1}) for 15 min at 4 °C. Cells were washed three times with wash buffer and fixed with formaldehyde (3.7% in PBS) at room temperature for 15 min. After the coverslips were washed four times in PBS (5 min per wash), the nucleus was labeled with the far red-fluorescent TO-PRO-3 iodide dye (Molecular Probes). The cells were mounted with PermaFluor (Thermo Electron Corporation) before being imaged.

For lectin staining of cell surface glycans, CHO-K1 and Lec2 cells were seeded at a density of 50000 cells per coverslip (22 mm) and allowed to adhere, overnight. After two washes with serum-free culture medium, live cells were treated with *V. cholerae* neuraminidase (50 mU mL^{-1}) in serum-free culture medium for 2 h at 37 °C. Coverslips were washed with DPBS and incubated with PNA–FITC (50 μg mL^{-1}) in PBS supplemented with BSA (1%) for 45 min at 4 °C. Cells were washed with PBS and fixed and mounted as above.

Initial analysis was performed on a Zeiss Axioplan2 fluorescent microscope. Confocal images were acquired by using a 60× (NA1.42) oil objective. Stacks of optical sections were collected in the *z* di-

mensions. The step size, based on the calculated optimum for each objective, was between 0.25 and 0.5 μm. Subsequently, each stack was collapsed into a single image (*z* projection). Analysis was performed offline by using ImageJ 1.39f software (National Institutes of Health, USA) and Adobe Photoshop CS3 Extended Version 10.0 (Adobe Systems Incorporated), whereby all images were treated equally.

Statistical analysis: Statistical significance between groups was determined by two-tailed, unpaired Student's *t* test. Differences were considered significant when $P < 0.05$.

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