VIP Live-Cell Imaging

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Visualizing Metabolically Labeled Glycoconjugates of Living Cells by Copper-Free and Fast Huisgen Cycloadditions**

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Dedicated to Sir J. Fraser Stoddart on the occasion of his 65th birthday

Azides, which are extremely rare in biological systems, are emerging as attractive chemical handles for bioconjugation.[1–5] In particular, the Cu^I-catalyzed 1,3-dipolar cycloaddition of azides with terminal alkynes to give stable triazoles[6,7] has been employed for the tagging of a variety of biomolecules,[8–12] activity-based protein profiling,[13] and the chemical synthesis of microarrays and small-molecule libraries.[14]

An attractive approach for installing azides into biomolecules is based on metabolic labeling, whereby an azide-containing biosynthetic precursor is incorporated into biomolecules by using the cells' biosynthetic machinery.[15] This approach has been employed for tagging proteins, glycans, and lipids of living systems with a variety of reactive probes. These probes can facilitate the mapping of saccharide-selective glycoproteins and identify glycosylation sites.[16] Alkyne probes have also been used for cell-surface imaging of azide-modified biomolecules, and a particularly attractive approach involves the generation of a fluorescent probe from a nonfluorescent precursor by a [3+2] cycloaddition.[17]

The cellular toxicity of the Cu^I catalyst has precluded applications wherein cells must remain viable,[18] and hence there is a great need for the development of Cu^I-free [3+2] cycloadditions.[19–21] In this respect, alkynes can be activated by ring strain, and, for example, constraining an alkyne within an eight-membered ring creates 18 kcal mol^{-1} of strain, much of which is released in the transition state upon [3+2] cyclcoaddition with an azide.[19,20] As a result, cyclooctynes such as **1** react with azides at room temperature without the need for a catalyst (Figure 1). The strain-promoted cycloaddition has been used to label biomolecules without observable cytotoxicity.[20] The scope of the approach has, however, been limited because of the slow rate of reaction.[22] Appending electron-withdrawing groups to the octyne ring can increase the rate of strain-promoted cycloadditions; however, currently Staudinger ligation with phosphine **2** offers the most attractive reagent for cell-surface labeling with azides.

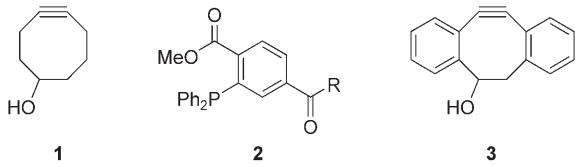
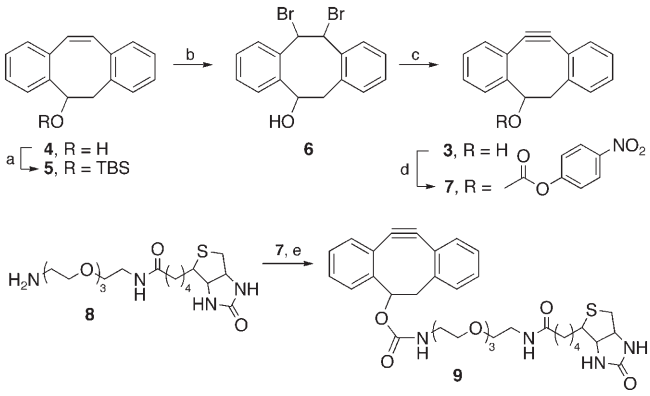


Figure 1. Reagents for labeling of azido-containing biomolecules.

It was envisaged that 4-dibenzocyclooctynols such as compound **3** would be ideal for labeling living cells with azides because the aromatic rings are expected to impose additional ring strain and conjugate with the alkyne, thereby increasing the reactivity of the alkyne in metal-free [2+3] cycloadditions with azides. The compound should, however, have excellent stability because the *ortho* hydrogen atoms of the aromatic rings shield the alkyne from nucleophilic attack. Furthermore, the hydroxy group of **3** provides a handle for the incorporation of tags such as fluorescent probes and biotin.

Compound **3** could be prepared easily from known[23,24] 3-hydroxy-1,2:5,6-dibenzocycloocta-1,5,7-triene (**4**) by protection of the hydroxy group as a TBS ether to give **5**, which was brominated to provide dibromide **6** in a yield of 60% (Scheme 1). The TBS protecting group was lost during the latter transformation, but the bromination was low yielding when performed on alcohol **4**. Dehydrobromination of **6** by treatment with LDA in THF at 0°C[25] gave the target cyclooctyne **3** in a yield of 45%.



Scheme 1. Reagents and conditions. a) TBSCl, pyridine; b) Br_2, $CHCl_3$; c) LDA, THF; d) 4-nitrophenyl chloroformate, pyridine, CH_2Cl_2; e) DMF, Et_3N. LDA = lithium diisopropylamide, TBS = *tert*-butyldimethylsilyl.

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Compound **3** has an excellent, long shelf life and after treatment did not react with nucleophiles such as thiols and amines. However, upon exposure to azides a fast reaction took place and gave the corresponding triazoles in high yield. For example, triazoles **10**–**13** were obtained in quantitative yields as mixtures of regioisomers by reaction of the corresponding azido-containing sugar and amino acid derivatives with **3** in methanol for 30 min (Figure 2). The progress

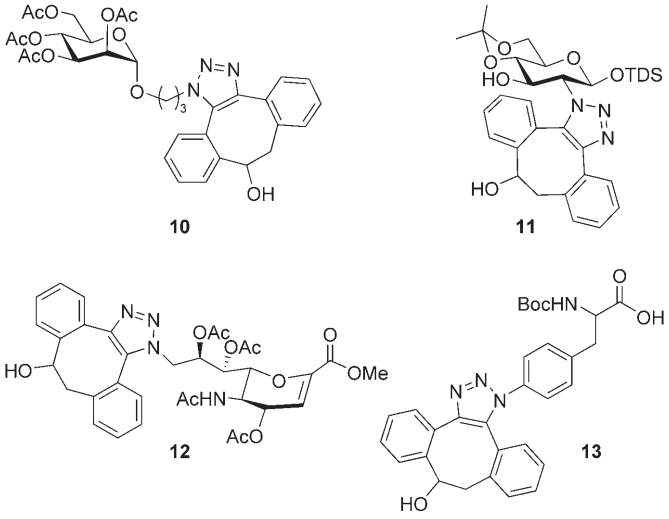

Figure 2. Metal-free cycloadditions of compound **3** with azido-containing amino acid and saccharides. Boc = *tert*-butoxycarbonyl, TDS = thexyldimethylsilyl.

of the reaction of **3** with benzyl azide in methanol and in a mixture of water/acetonitrile (1:4 v/v) was monitored by ^{1}H NMR spectroscopy by integration of the benzylic proton signals, and second-order rate constants of 0.17 and 2.3 $M^{-1}s^{-1}$, respectively, were determined. The rate constant of the reaction with **3** in acetonitrile/water is approximately three orders of magnitude greater than that with cyclooctyne **1**.

Having established the superior reactivity of **3**, we focused our attention on the preparation of a derivative of 4-dibenzocyclooctynol (**9**; Scheme 1), which is modified with biotin. Such a reagent should make it possible to visualize biomolecules after metabolically labeling cells with an azido-containing biosynthetic precursor, followed by cycloaddition with **9** and treatment with avidin modified with a fluorescence probe. Alternatively, biotinylation of glycoconjugates with **9** should make it possible to isolate these derivatives for glycocomics studies using avidin immobilized on a solid support. Compound **9** could easily be prepared by a two-step reaction involving treatment of **3** with 4-nitrophenyl chloroformate to give activated intermediate **7**, followed by immediate reaction with **8**.

Next, Jurkat cells were cultured in the presence of 25 μM *N*-azidoacetylmannosamine (Ac_4ManNAz) for three days to metabolically introduce *N*-azidoacetylsialic acid (SiaNAz) moieties into glycoproteins.[26] As a negative control, Jurkat cells were employed that were grown in the absence of Ac_4ManNAz. The cells were exposed to a 30 μM solution of compound **9** for various time periods, and after washing, the cells were stained with avidin–fluorescein isothiocyanate (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. For comparison, the cell-surface azido moieties were also labeled by Staudinger ligation with biotin-modified phosphine **2** followed by treatment with avidin–FITC. The labeling with **9** was almost complete after an incubation time of 60 min (Figure 3a).

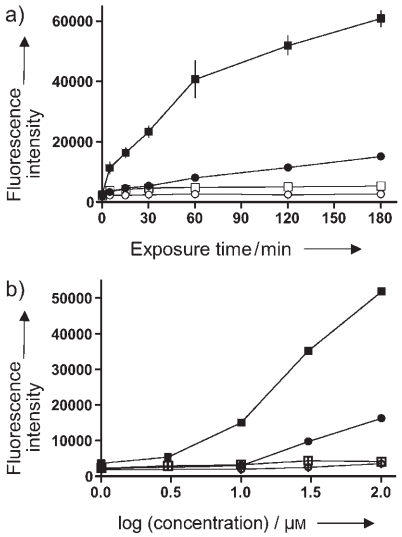

Figure 3. Cell-surface labeling with compounds **2** and **9**. Jurkat cells grown for three days in the absence or presence of Ac_4ManNAz (25 μM) were incubated a) with compounds **2** and **9** (30 μM) for 0–180 min or b) with compounds **2** and **9** (0–100 μM) for 1 h at room temperature. Next, the cells were incubated with avidin–FITC for 15 min at 4 °C, after which cell lysates were assessed for fluorescence intensity. Samples are indicated as follows: blank cells incubated with **2** (○) or **9** (□), and Ac_4ManNAz cells incubated with **2** (●) or **9** (■).

Interestingly, under identical conditions phosphine **2**[22] gave significantly lower fluorescent intensities, indicating that cell-surface labeling by Staudinger ligation is slower and less efficient. In each case, the control cells exhibited very low fluorescence intensities, demonstrating that background labeling is negligible. It was found that the two-step labeling approach with **9** had no effect on cell viability, as determined by morphology and exclusion of trypan blue.

The concentration dependence of the cell-surface labeling was studied by incubation of cells with various concentrations of **2** and **9** followed by staining with avidin–FTIC (Figure 3b). As expected, cells displaying azido moieties showed a dose-dependent increase in fluorescence intensity. Reliable fluorescent labeling was achieved at a 3 μM concentration of **9**; 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 owing to the limited solubility of **9**.

Next, attention was focused on visualizing azido-containing glycoconjugates of living cells by confocal microscopy. Thus, adherent Chinese hamster ovary (CHO) cells were cultured in the presence of Ac_4ManNAz (100 μM) for three days. The resulting cell-surface azido moieties were treated with **9** (30 μM) for 1 h and then with avidin–Alexa Fluor 488 for 15 min at 4 °C. As expected, staining was observed only at

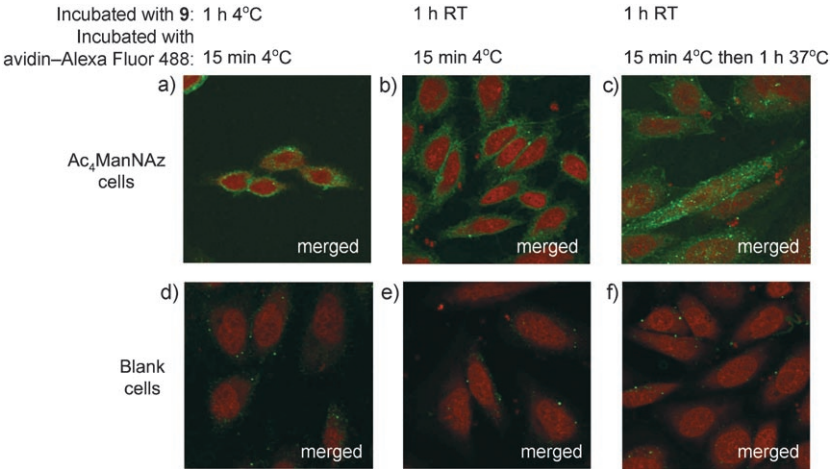

Figure 4. Fluorescence images of cells labeled with compound **9** and avidin–Alexa Fluor 488. CHO cells grown for three days in the absence (d–f) or presence (a–c) of $Ac_4ManNAz$ (100 μM) were incubated with compound **9** (30 μM) for 1 h at 4 °C (a, d) or room temperature (b, c, e, f). Next, cells were incubated with avidin–Alexa Fluor 488 for 15 min at 4 °C and, after washing, fixing, and staining for the nucleus with the far-red-fluorescent dye TO-PRO, imaged (a, b, d, e) or after washing incubated for 1 h at 37 °C before fixing, nucleus staining, and imaging (c, f). Merged indicates that the images of cells labeled with Alexa Fluor (488 nm) and TO-PRO-3 iodide (633 nm) are merged and shown in green and red, respectively.

the cell surface (Figure 4), and importantly, the labeling procedure was equally efficient when performed at either ambient temperature or 4 °C. Furthermore, blank cells exhibited very low fluorescence staining, confirming that background labeling is negligible.

Cell-surface glycoconjugates are constantly recycled by endocytosis, and to monitor this process, metabolically labeled cells were reacted with **9** and avidin–Alexa Fluor 488 according to the standard protocol and incubated at 37 °C for 1 h before examination by confocal microscopy. We observed that a significant quantity of labeled glycoproteins had been internalized into vesicular compartments.

At the completion of these studies, Bertozzi and co-workers reported a difluorinated cyclooctyne (DIFO) that reacts with azides at almost the same reaction rate as compound **3**.[27] DIFO linked to Alexa Fluor was employed to investigate the dynamics of glycan trafficking. It was found that after incubation for 1 h, labeled glycans colocalized with markers for endosomes and Golgi.

4-Dibenzocyclooctynols such as **3** and **9** have several advantageous features for researchers such as ease of chemical synthesis and the possibility to further enhance the rate of cycloaddition by functionalization of the aromatic moieties. Modifying the aromatic rings may also offer an exciting opportunity to obtain reagents that become fluorescent upon [3+2] cycloaddition with azido-containing compounds, which will make it possible to monitor in real time the trafficking of glycoproteins and other biomolecules in living cells.

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