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Chemical Glycobiology

Two-Color Glycan Labeling of Live Cells by a Combination of Diels–Alder and Click Chemistry**

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Protein glycosylation is a complex form of posttranslational modification and has been shown to be crucial for the function of many proteins. Sialic acid is prominently positioned at the outer end of membrane glycoproteins. It plays a critical role for the regulation of a myriad of cellular functions and it forms a shield around the cell. Furthermore, it constantly interacts with the environment of cells and contributes to histocompatibility.[1] This makes studying sialylation an interesting field of research, but monitoring sialic acid in vivo is challenging. While proteins are routinely labeled by genetic methods, such as expression as GFP fusion proteins, comparable methods are not available for secondary gene products, such as glycans of glycoconjugates. Metabolic oligosaccharide engineering (MOE) is a successful new strategy to visualize the localization of glycans in vitro and in vivo.[2] In this approach, cells are cultivated in the presence of non-natural monosaccharide derivatives that carry a chemical reporter group and are nonetheless accepted by the biosynthetic machinery of a cell. For instance, peracetylated *N*-azidoacetylmannosamine (Ac_4ManNAz) is taken up by the cell, deacetylated by cellular esterases, and owing to the promiscuity of the enzymes of sialic acid biosynthesis, is converted into *N*-azidoacetyl neuraminic acid and incorporated into sialoglycoconjugates.[3] Once presented on the cell surface, the azide-containing sialylated glycan can be visualized through a bioorthogonal ligation reaction.[4] Besides Ac_4ManNAz, several monosaccharide derivatives of *N*-acetylgalactosamine,[5] *N*-acetylglucosamine,[6] and L-fucose[7] are suitable for MOE providing further insights into the role of cellular structures and functions of glycans in the cell.

Currently, mainly Staudinger ligation[3] and azide–alkyne [3+2] cycloaddition (copper-catalyzed[8] or strain-promoted,[9] also known as the click reaction) are applied as ligation reactions in MOE. However, both of them rely on the reaction of azides and thus cannot be used for the concurrent detection of two different metabolically incorporated carbohydrates. A labeling strategy that can be carried out in the presence of azides and alkynes would significantly expand the scope of chemical labeling reactions in living cells and is thus highly desirable.

Recently, it was shown that the Diels–Alder reaction with inverse electron demand (DARinv) of 1,2,4,5-tetrazines[10] with strained dienophiles, such as *trans*-cyclooctenes,[11] cyclobutenes,[12] norbornenes,[11d,f,13] cyclooctynes,[11d,f] and substituted cyclopropenes,[14] fulfills the requirements of a bioorthogonal ligation reaction and furthermore is orthogonal to the azide–alkyne cycloaddition. However, these cyclic alkenes or kinetically stable tetrazines[15] are expected to be too large for being efficiently metabolized by the sialic acid biosynthetic pathway, starting from the corresponding *N*-acylmannosamine derivative. In search for smaller dienophiles suitable for MOE, we identified monosubstituted (terminal) alkenes as a new class of chemical reporters. We recently reported the successful application of the DARinv between terminal alkenes and 1,2,4,5-tetrazines in the preparation of carbohydrate microarrays.[13c] The fact that terminal alkenes are hardly found in biological systems and are completely absent in proteins makes them a promising reporter group. Herein, we show that ManNAc derivatives containing a terminal alkene in the acyl side chain are metabolically incorporated into cell-surface sialic acids and can subsequently be labeled by the DARinv (Figure 1). Moreover, we demonstrate that double labeling of two differently modified, metabolically incorporated monosaccharides is possible by combining the DARinv with strain-promoted azide–alkyne cycloaddition (SPAAC).

As the reaction rate of the DARinv of acyclic olefins with tetrazines is very sensitive to steric hindrance, double bonds with more than one substituent react very slowly.[16] Terminal alkenes, on the other hand, can react rapidly without any further activation. This prompted us to design mannosamine derivatives **2** and **4** (Figure 2) that were synthesized in three steps from mannosamine hydrochloride (see the Supporting Information). Based on previous work by Keppler et al.,[2c] we expected both derivatives to be accepted by cells with *N*-pentenoylmannosamine **2** (owing to the shorter acyl side chain) being incorporated with higher efficiency. On the other

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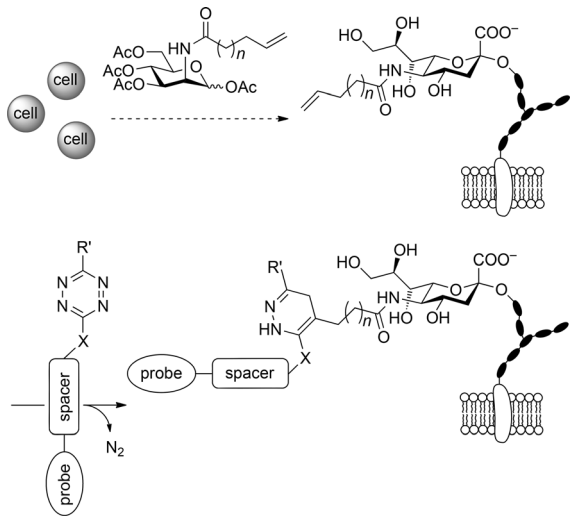
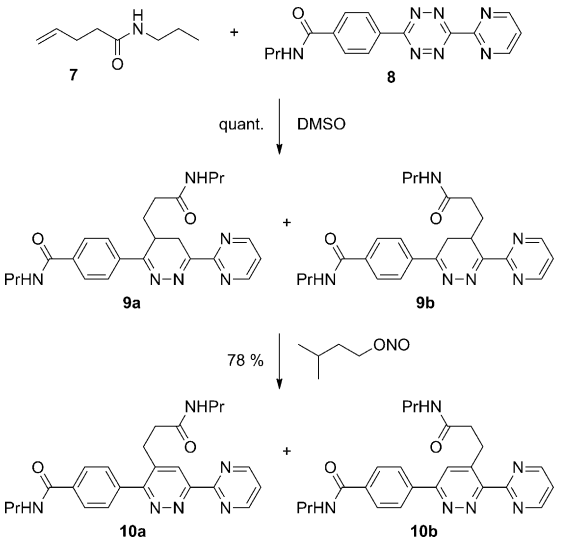


Figure 1. Strategy for MOE using terminal alkenes as chemical reporter groups.



Scheme 1. DARinv between pentenamide **7** and diaryltetrazine **8**. Only one tautomeric form of **9a/b** is shown.

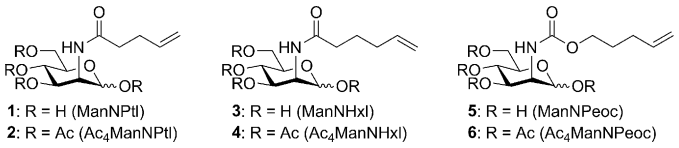


Figure 2. Mannosamine derivatives employed in MOE. Hxl = hexenoyl, Peoc = pentenyloxycarbonyl, Ptl = pentenoyl.

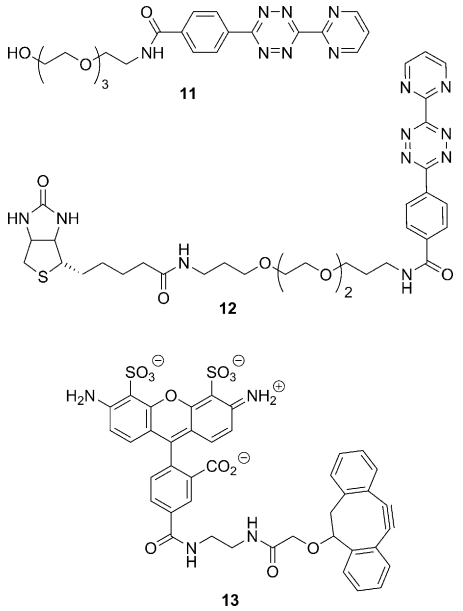


Figure 3. Tetrazine and dibenzocyclooctyne derivatives.

hand, the hexenoyl side chain of mannosamine derivative **4** was expected to react faster in the DARinv owing to the longer distance between the double bond and the electron-withdrawing carbonyl group.

As a reaction partner for the alkenes, we selected a diaryltetrazine because these compounds have been previously shown to be sufficiently stable against hydrolysis which is an important prerequisite for bioorthogonal chemistry. In a model reaction, pentenamide **7** was reacted in DMSO with tetrazine **8** to give dihydropyridazines **9a/b** that each exist in several tautomeric forms in virtually quantitative yield (Scheme 1 and the Supporting Information). LC-MS analysis revealed that the products are easily oxidized to pyridazines **10a/b**. To complete this conversion, we treated **9a/b** with isopentyl nitrite and obtained pyridazines **10a/b** in a yield of 78% after chromatographic purification.

Having shown that the coupling between a terminal alkene and a tetrazine proceeds in excellent yield, we investigated the kinetics of this ligation reaction. It is known that the DARinv is much faster in water than in organic solvents.[17] Thus, we attached a tri(ethylene glycol) linker to obtain water-soluble tetrazine **11** (Figure 3). Employing **11**, quantitative kinetic measurements were carried out in aqueous buffer with ManNPtl (**1**) and ManNHxl (**3**; see the Supporting Information), and second-order rate constants were determined to be 0.021 $L\,mol^{-1}\,s^{-1}$ for the pentenoyl and 0.041 $L\,mol^{-1}\,s^{-1}$ for the hexenoyl compound. Although these numbers indicate a lower reaction rate compared to strained cyclic alkenes, they are comparable to the rate constants of the Staudinger ligation ($k = 0.002\ L\,mol^{-1}\,s^{-1}$ in CD_3CN/MeOH 95:5)[18] and SPAAC of a dibenzocyclooctyne ($k = 0.0565\ L\,mol^{-1}\,s^{-1}$ in MeOH),[19] which have both been demonstrated to be extremely valuable bioorthogonal labeling reactions.[20]

To test the suitability of the new ManNAc derivatives for MOE, different cell lines were cultured in the presence of Ac_4ManNPtl (**2**) and Ac_4ManNHxl (**4**), respectively. Subsequently, the cells were labeled with tetrazine–biotin conjugate **12** followed by incubation with AlexaFluor-647-labeled streptavidin (AF647-streptavidin). Figure 4a shows the results obtained with HEK293T cells grown in the presence of Ac_4ManNPtl (**2**). Confocal fluorescence microscopy clearly showed intense labeling of the cell membrane, as expected for

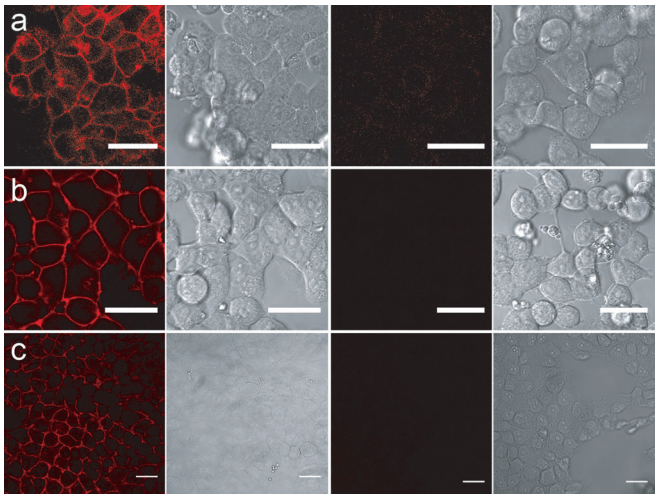


Figure 4. a) HEK293T cells were grown for two days with 100 μM Ac_4ManNPtl (**2**; left) or without **2** (right). Living cells were labeled with 1 mM tetrazine biotin **12** at 37°C for 6 h followed by incubation with AF647-streptavidin for 20 min at RT. b) HEK293T cells treated in the same way as described under (a) but with Ac_4ManNHxl (**4**) instead of Ac_4ManNPtl (**2**). c) HeLa S3 cells grown for 3 days with 100 μM Ac_4ManNPeoc (**6**; left) or without **6** (right), fixed with paraformaldehyde, and then treated as described under (a). Scale bar: 30 μm.

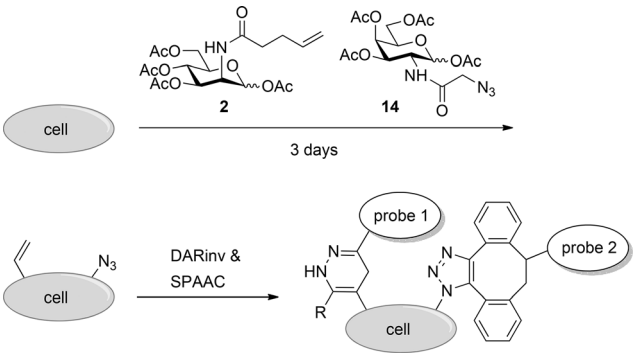


Figure 5. Dual-labeling strategy for MOE employing alkene- and azide-labeled monosaccharides followed by DARinv and SPAAC with two different labels.

this significantly sialylated cell line. Control experiments in which cells were cultured in absence of **2** but otherwise treated in the same way showed a negligible background staining. This is an important result confirming that tetrazine–biotin conjugate **12** does not react with other cellular components, such as unsaturated cell-membrane lipids, under these conditions. Corresponding results obtained with HEK293T cells and Ac_4ManNHxl (**4**) are shown in Figure 4b. Interestingly, both sugars **2** and **4** lead to about the same intensity of cell-membrane staining. Similar results were obtained with HeLa S3 cells (see the Supporting Information, Figures S15 and S16).

So far, all mannosamine derivatives employed for MOE were equipped with an acyl chain connected to the 2-amino group.[21] To investigate whether the biosynthetic machinery also accepts other functional groups, we employed pentenyl carbamate **6**. A carbamate group has the advantage that it facilitates the synthetic access to functionalized mannosamines derived from alcohols, such as pentenol. MOE experiments carried out with HeLa S3 cells showed that Ac_4ManNPeoc (**6**) leads to similar staining intensity compared to the experiments with sugars **2** and **4** (Figure 4c). The second-order rate constant for DARinv of ManNPeoc (**5**) with tetrazine **11** was determined to be 0.017 $L\,mol^{-1}\,s^{-1}$.

We next turned our attention to the possibility to employ DARinv in combination with SPAAC for the concurrent detection of two different metabolically incorporated sugars. HeLa S3 cells were grown in presence of both alkene-labeled Ac_4ManNPtl (**2**) and peracetylated *N*-azidoacetylgalactosamine (Ac_4GalNAz, **14**; Figure 5). Whereas *N*-acylmannosamine derivatives, such as **2**, are biosynthetically converted into sialic acids that are found as terminal structures of glycoproteins, Ac_4GalNAz (**14**) has been shown to be incorporated into mucin-type O-glycans.[5a] As control experiments, only one sugar or no sugar was added to the culture medium. After three days the cells were stained by both DARinv (with tetrazine-biotin conjugate **12** followed by incubation with AF647-streptavidin) and SPAAC (with AlexaFluor 488-dibenzocyclooctyne **13**, AF488-DIBO) and investigated by confocal fluorescence microscopy.

When both sugars were present in the culture medium, we detected a distinct labeling of the cell membrane in both channels (Figure 6b), whereas omission of one of the sugars

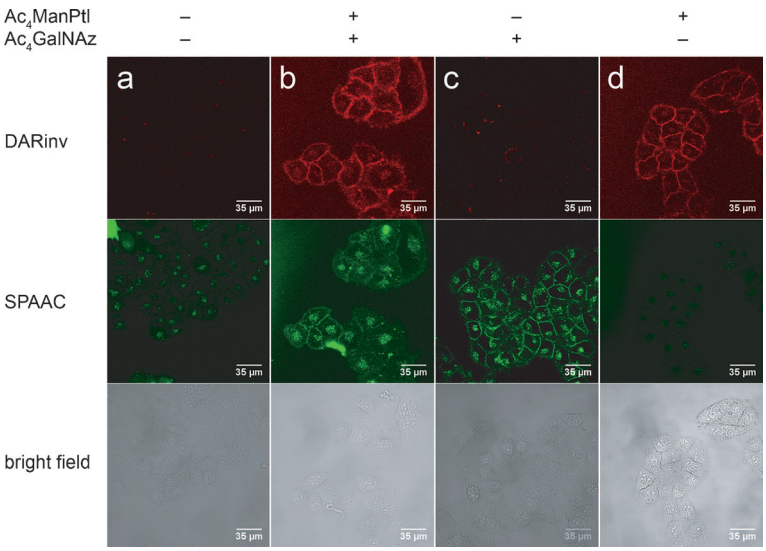


Figure 6. a) HeLa S3 cells were grown for three days. Living cells were labeled with tetrazine–biotin **12** (1 mM) for 6 h at 37°C followed by incubation with AF647–streptavidin for 20 min at RT and with AF488-DIBO (4 μM) for 30 min at RT. b) Cells were grown with 100 μM Ac_4ManNPtl (**2**) and 25 μM Ac_4GalNAz (**14**) and then stained in the same way as described under (a). c) Cells were grown with 25 μM Ac_4GalNAz (**14**) and then stained in the same way as described under (a). d) Cells were grown with 100 μM Ac_4ManNPtl (**2**) and then stained in the same way as described under (a). Scale bar: 35 μm.

resulted in the absence of membrane labeling in the corresponding channel (Figure 6c,d). Interestingly, the azide channel revealed some intracellular staining that we attribute to non-specific staining as it is also visible in the control experiments without addition of $Ac_4GalNAz$ (**14**; Figure 6a,d). Non-specific staining of cyclooctynes has been reported previously[13e] and may result from addition of SH groups to the triple bond[22] or from non-specific binding of the dye. In the DARinv channel, on the other hand, hardly any background staining was observed (Figure 6a,c). These findings show that the application of alkenes and azides as chemical reporter groups allows detecting two different metabolically incorporated sugars in the same experiment.

In summary, we have demonstrated that terminal alkenes in *N*-acylmannosamine are suitable chemical reporter groups for metabolic oligosaccharide engineering. Owing to their small size, which is comparable to that of well-established azide reporters, they are accepted by the enzymes involved in sialic acid biosynthesis and incorporated into cell-surface glycoconjugates. We could further show that this pathway is also permeable for a mannose derivative containing a carbamate functionality. Terminal alkenes can be labeled in a bioorthogonal manner using a Diels–Alder reaction with inverse electron demand that does not require catalysis by toxic metals. Moreover, we have shown that the DARinv can be carried out in the presence of azides, which allows the simultaneous detection of two different sugars. Such detection strategies are important for advanced multiplexed glycan analysis. As terminal alkenes are also easily incorporated into other biomolecules, for example into proteins by genetic code expansion, our labeling approach is not only confined to metabolic carbohydrate engineering but is broadly applicable.

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