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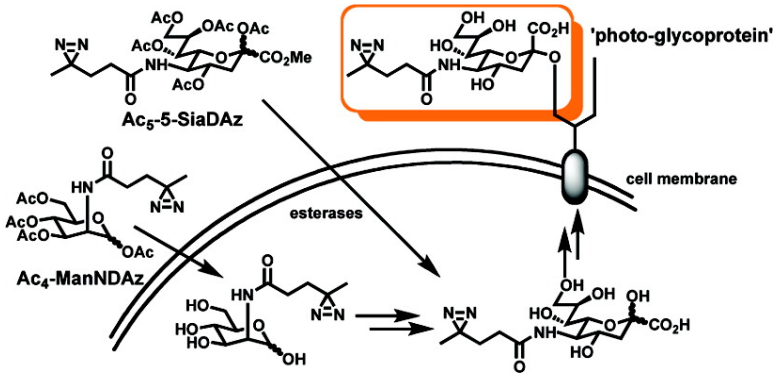

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Photoactivatable Crosslinking Sugars for Capturing Glycoprotein Interactions

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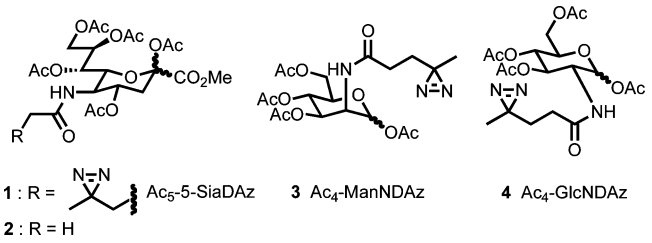
Carbohydrates are essential mediators of many extracellular interactions, particularly developmental, immunological, and metastatic processes. Despite critical roles in physiology, carbohydrate−protein interactions are often transient and of low affinity.[1] As a result, identification of the molecular species involved in these extracellular binding events remains challenging. In recent, pioneering work, the Paulson group reported the metabolic incorporation of a sialic acid analogue bearing a photoactivatable aryl azide (9-AAz-NeuAc) and its use to covalently capture carbohydrate−protein interactions.[2] Once a covalent bond is formed between a carbohydrate and its interaction partner, the binding partner can potentially be identified using biochemical or analytical techniques.

The use of photocrosslinking sugars represents a powerful strategy to interrogate glycan-mediated interactions in their native context, but the utility of 9-AAz-NeuAc is likely to be limited. First, metabolic incorporation of 9-AAz-NeuAc has only been demonstrated in a modified cell line that is incapable of sialic acid biosynthesis,[3] restricting the biological questions that can be addressed. Second, 9-AAz-NeuAc bears its photocrosslinking group at the 9 position, precluding 9-*O*-acetylation, 9-*O*-lactylation, or 9-phosphorylation, physiologically relevant modifications that can dramatically alter binding properties of the sugar.[4] Furthermore, functionality at the 9-position exerts a dramatic impact on sialic acid's affinity for protein binding partners, particularly members of the large Siglec family, which includes CD22.[5] Aromatic modifications at the 9-position are notable for enhancing affinity by several orders of magnitude,[6] while acetylation often abrogates binding.[7,8] Therefore, crosslinks that are formed with 9-AAz-NeuAc may not accurately reflect interactions mediated by naturally occurring sialic acids.

Here we report the syntheses of monosaccharides containing diazirine crosslinkers, the metabolic incorporation of these "photo-sugars" onto cell surfaces, and their utility in detecting the multimerization of a cell surface glycoprotein. We chose to use diazirine crosslinkers because their small size should minimize interference with the interactions we wish to study, and because of their outstanding crosslinking characteristics. Indeed, recent reports[9−11] have demonstrated the ability of diazirine crosslinkers to capture protein oligomerization and protein-peptide interactions.

We introduced diazirines on the *N*-acyl side chains of sialic acid and mannosamine because previous work showed that the biosynthetic machinery that incorporates these sugars into cell surface proteins is relatively permissive for the addition of substituents at the *N*-acyl position.[12−15] ManNAc and sialic acid analogues bearing an aryl azide at the *N*-acyl position have been reported, but incorporation levels for the ManNAc analogue were extremely poor and neither molecule has been demonstrated to function in crosslinking applications.[15] We reasoned that analogues bearing an *N*-acyl diazirine would exhibit improved utility.

Our diazirine-containing ManNAc analogue, ManNDAz, was synthesized by coupling 4,4-azo-pentanoic acid[16] with mannosamine. ManNDAz was converted enzymatically to 5-SiaDAz using NeuAc aldolase,[15] followed by methyl esterification. The control molecule, GlcNDAz, was synthesized in the same manner as ManNDAz. All photo-sugars were fully acetylated to improve their cell permeability.[15] We expected that the acetyl groups would be removed by nonspecific cellular esterases.



First, we wished to demonstrate that Ac_5-5-SiaDAz (**1**) and Ac_4-ManNDAz (**3**) could be metabolically incorporated into cellular glycoproteins in the form of sialic acid. To do so, we took advantage of the BJAB K20 cell line, which is impaired in sialic acid biosynthesis (Scheme S1, Supporting Information). BJAB K20 cells lack the UDP-GlcNAc 2-epimerase that converts UDP-GlcNAc to ManNAc. This enzyme is essential for biosynthesis of sialic acid.[3]

K20 cells were cultivated for 72 h with no additional sugar or with **1**, **2**, **3**, or **4**. Cell surface sialic acid was detected by flow cytometry using FITC-labeled *Sambucus nigra* agglutinin (FITC-SNA), a lectin that recognizes sialic acid (Figure 1A). Untreated K20 cells display low FITC-SNA binding; supplementation with natural sialic acid increases FITC-SNA binding. Similarly, we observed that supplementation with Ac_5-5-SiaDAz resulted in high levels of FITC-SNA binding. Incubation with Ac_4-ManNDAz, but not Ac_4-GlcNDAz, also yielded increased FITC-SNA binding, consistent with the fact that only ManNAc analogues can be efficiently converted to their sialic acid counterparts. As a control, we also examined wild-type BJAB cells (K88), which are capable of sialic acid biosynthesis. K88 cells displayed FITC-SNA binding that was similar to K20 cells supplemented with Ac_5-5-SiaDAz. This experiment indicated that both Ac_5-5-SiaDAz and Ac_4-ManNDAz are incorporated onto cell surfaces in the form of a sialic acid analogue.

Next, we demonstrated that photo-sugars could be used to capture a carbohydrate-mediated interaction. As a test case, we examined the sialic acid-dependent multimerization of CD22, which has been previously studied in K20 cells using an aryl azide crosslinker, 9-AAz-NeuAc.[2] K20 cells were cultivated with various concentrations of Ac_5-5-SiaDAz for 24 h, then irradiated with 350 nm light for 20 min and their contents analyzed by Western blot using an anti-CD22 antibody (Figure 1B). We observed higher

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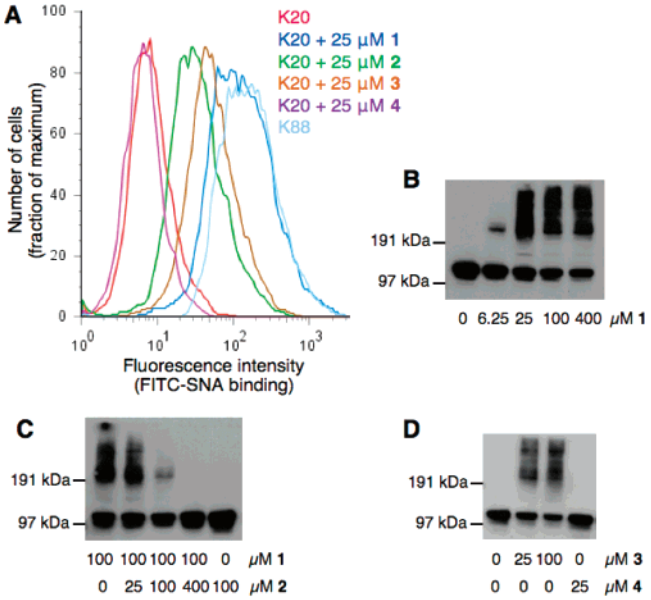

Figure 1. Ac_5-5-SiaDAz and Ac_4-ManNDAz are incorporated into cell surface glycoproteins in K20 cells. (A) Detection of cell surface sialic acids by flow cytometry using FITC-SNA. (B) CD22 crosslinking observed at various concentrations of **1**. (C) CD22 crosslinking is reduced by competition with **2**. (D) CD22 crosslinking is observed with **3** but not **4**.

molecular weight crosslinked bands in response to UV irradiation (Figure S1, Supporting Information). These bands were observed at Ac_5-5-SiaDAz concentrations as low as 6.25 μM.

Two experiments confirm that crosslinking depended on the incorporated 5-SiaDAz. First, we performed a competition experiment where K20 cells were cultivated with 100 μM of Ac_5-5-SiaDAz and increasing concentrations of acetylated natural sialic acid (**2**) for 24 h (Figure 1C). We observed decreased crosslinking as the concentration of **2** increased, suggesting that these two molecules are competitive in the same biosynthetic pathway. Next, K20 cells were cultivated with Ac_4-ManNDAz (**3**), or Ac_4-GlcNDAz (**4**) for 72 h (Figure 1D). After UV irradiation, we observed CD22 crosslinking for cells treated with **3**, but not **4**. This experiment, along with the flow cytometry data, indicated that only photo-sugars that can be metabolically converted to a sialic acid analogue are capable of mediating CD22 crosslinking.

To demonstrate the broad utility of Ac_5-5-SiaDAz and Ac_4-ManNDAz, we conducted CD22 crosslinking experiments in BJAB K88 cells, a cell line with an intact sialic acid biosynthetic pathway. K88 cells were cultivated with increasing concentrations of Ac_5-5-SiaDAz for 24 h (Figure 2A). After UV irradiation, we observed CD22 crosslinking for all Ac_5-5-SiaDAz concentrations examined; crosslinking was apparent even at concentrations as low as 25 μM Ac_5-5-SiaDAz. Similarly, we cultivated K88 cells with various concentrations of Ac_4-ManNDAz for 72 h (Figure 2B). Again, crosslinking was observed at all Ac_4-ManNDAz concentrations, including as low as 25 μM Ac_4-ManNDAz. These experiments indicate 5-SiaDAz and ManNDAz are able to compete effectively with endogenous sialic acid for incorporation into cell

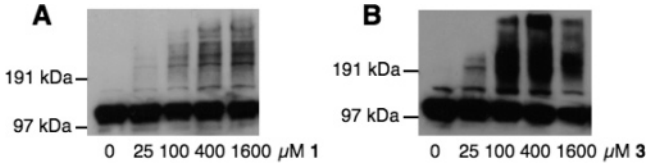

Figure 2. Ac_5-5-SiaDAz and Ac_4-ManNDAz are incorporated into CD22 in K88 cells. (A) CD22 crosslinking observed at various concentrations of **1**. (B) CD22 crosslinking observed at various concentrations of **3**.

surface glycoproteins. Our results in K88 cells suggest that these photo-sugars may find utility in a variety of cell types.

In summary, diazirine-containing photo-sugars can be metabolically incorporated into cell surface glycoproteins. Once incorporated, these molecules can be photoactivated and used to covalently trap interactions among glycoproteins. Employing the small diazirine crosslinker at the *N*-acyl position enhances metabolic incorporation relative to larger crosslinkers and minimizes perturbation of the interactions under study. As a result, our molecules have significantly enhanced utility as compared to previously reported crosslinking sugars. In addition, we anticipate using analogous molecules to metabolically incorporate crosslinkers into GlcNAc- and GalNAc-containing glycans.

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Supporting Information Available: Detailed experimental section, syntheses, and spectral characterization of new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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