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Bifunctional Unnatural Sialic Acids for Dual Metabolic Labeling of Cell-Surface Sialylated Glycans

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Supporting Information

ABSTRACT: Sialic acid analogues containing a unique chemical functionality or chemical reporter have been metabolically incorporated into sialylated glycans. This process, termed metabolic glycan labeling, has emerged as a powerful tool for studying sialylation as well as other types of glycosylation. Currently, this technique can install only a single functionality. Here we describe a strategy for dual labeling of sialylated glycans using a new class of bifunctional sialic acid analogues containing two distinct chemical reporters at the *N*-acyl and C9 positions. These bifunctional unnatural sialic acids were metabolically incorporated into cellular glycans, where the two chemical reporters exerted their distinct functions. This approach expands the capability of metabolic glycan labeling to probe sialylation and glycan−protein interactions.

Sialic acids are often the outermost monosaccharides of cell-surface glycans in vertebrates.[1] Sialylated glycans linked on glycoproteins or glycolipids play important roles in diverse biological and pathological processes. For example, sialyl Lew^X, a sialic acid-containing tetrasaccharide that serves as a ligand for selectins, is crucial for mediating leukocyte homing.[2] Sialylated glycolipids such as gangliosides regulate cell−cell and cell−pathogen interactions.[3] In addition, a high level of sialic acid expression is usually found in tumor cells and has been shown to correlate with metastatic potential in several cancer types.[4] Despite the fundamental significance of sialylated glycans, it remains challenging to understand fully the functions of sialylation using conventional biochemical and genetic methods.[5]

In recent years, metabolic glycan labeling has emerged as a powerful approach for chemical probing of sialylation as well as several other types of glycosylation in live cells and within living animals.[6] In this strategy, the cell's sialic acid biosynthetic pathway is harnessed to incorporate unnatural sialic acids modified with a bioorthogonal functional group either on the *N*-acyl side chain or at the C9 position (Figure 1A). Metabolic labeling of sialic acids has also been achieved by feeding cells with analogues of *N*-acetylmannosamine (ManNAc), the biosynthetic precursor of sialic acid. For unnatural ManNAc analogues, the bioorthogonal functional group can be installed on the *N*-acyl side chain but not at the C6 position, which corresponds to the C9 position of sialic acid. The C6 hydroxyl group of ManNAc is enzymatically modified by ManNAc 6-kinase during its biosynthetic conversion to sialic acid, so modification at C6 is not tolerated. In a second step, the cell-surface bioorthogonal group is bioorthogonally reacted with a fluorescent probe bearing a complementary functional group for imaging of sialic acids or with an affinity tag for enrichment and identification of sialylated glycoproteins. Azide and alkyne are the two most popular bioorthogonal groups that have been exploited for metabolic labeling of sialic acids. The Bertozzi group demonstrated that azide can be incorporated into cell-surface glycans using *N*-azidoacetylmannosamine[7a] or 5-azido sialic acid.[7b] Our group recently used 9-azido sialic acid (9AzSia) for metabolic labeling of sialylated glycans.[8] The Wong group reported metabolic incorporation of an alkyne using *N*-(4-pentynoyl)mannosamine, which was converted to cell-surface alkynyl sialic acid (SiaNAl).[9]

Another type of interesting functional group installed on unnatural sugars are photo-cross-linkers. The Bertozzi group[7b] showed that aryl azides can be efficiently incorporated into sialylglycoconjugates using a sialic acid analogue bearing an *N*-acyl aryl azide. The Paulson group later developed a sialic acid analogue containing an aryl azide at C9 (9AAzSia) to capture the glycan ligand of CD22, a key regulator of B-cell signaling,[10] and

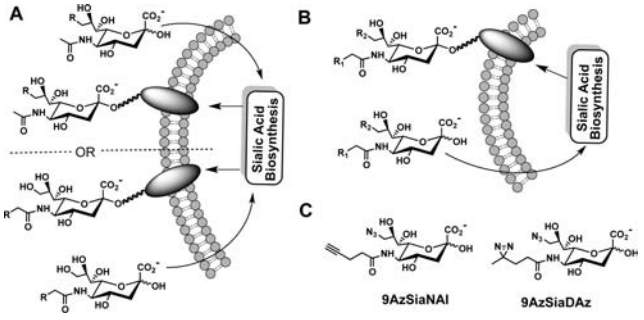

Figure 1. (A) Metabolic labeling of sialylated glycans using sialic acid analogues containing an unnatural functional group (e.g., a bioorthogonal group or photo-cross-linker) on the *N*-acyl side chain or at the C9 position. (B) Dual labeling strategy for simultaneously installing unnatural functionalities on both the *N*-acyl side chain and at the C9 position using bifunctional unnatural sialic acids. (C) Two bifunctional sialic acid analogues used in this study.

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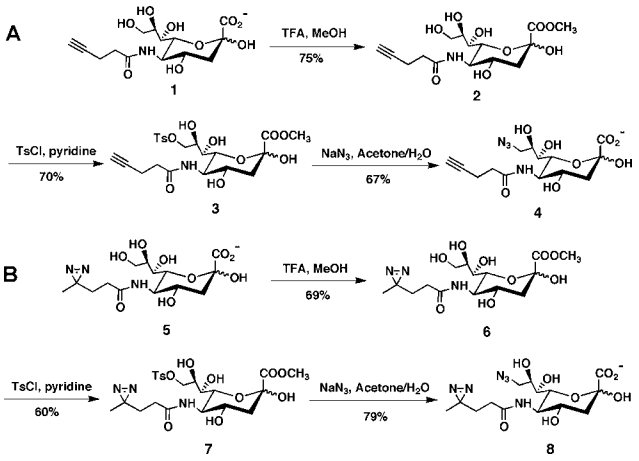
the Kohler group developed sialic acid and ManNAc analogues bearing a diazirine on the *N*-acyl side chain (i.e., SiaDAz and ManNDAz, respectively).[11]

All of the currently available sialic acid analogues can endow sialylated glycans with only one functional group. We were interested in expanding the metabolic labeling strategy to incorporate two functionalities into sialic acids simultaneously (Figure 1B). We envisioned that the bifunctional sialic acids, once incorporated, would enable biological applications that are not possible with the monofunctional analogues. For example, two bioorthogonal functional groups could be used to conjugate two probes for dual-color imaging, or a bioorthogonal group could be used to enrich sialic acid-binding proteins when combined with a photo-cross-linker. We reasoned that the biosynthetic machinery would tolerate sialic acid analogues with two distinct chemical reporters simultaneously installed on the *N*-acyl side chain and at the C9 position on the basis of its promiscuity for modification at either position.

To test our hypothesis, we designed and synthesized two classes of bifunctional sialic acid analogues: 9AzSiaNAl (**4**) contains two bioorthogonal functional groups, an azide at C9 and an alkyne on the *N*-acyl side chain, while 9AzSiaDAz (**8**) contains an azide at C9 and a photo-cross-linker, diazirine, on the *N*-acyl side chain (Figure 1C). To synthesize **4**, we first generated SiaNAl (**1**) as previously reported.[12] The azido group was then installed at the C9 position of **1** to give **4** in an overall yield of 35% (Scheme 1A). Similarly, **8** was synthesized from SiaDAz (**5**)[11] in an overall yield of 33% (Scheme 1B).

Scheme 1. Synthesis of 9AzSiaNAl (4) and 9AzSiaDAz (8)



First, we determined whether 9AzSiaNAl and 9AzSiaDAz could be metabolically incorporated into cellular sialylglycoconjugates in various cell lines, including CHO, HeLa, Daudi, and BJA-B K20 cells. The cells were treated for 24 h with 9AzSiaNAl, 9AzSiaDAz, or 9AzSia as a positive control, and cell-surface azides were then detected using the copper-free click reaction[13] with azadibenzocyclooctyne–biotin conjugate (DBCO–biotin).[14] In this study, we used a DBCO–biotin conjugate containing a sulfo group (sulfo-DBCO–biotin) for better water solubility. The cells were then stained with streptavidin–Alexa Fluor 647 and analyzed by flow cytometry [Figure 2A and Figures S1–S4 in the Supporting Information (SI)]. Both the 9AzSiaNAl- and 9AzSiaDAz-treated CHO cells exhibited robust fluorescence labeling in a dose-dependent manner, indicating the metabolic incorporation of the bifunctional sialic acid analogues

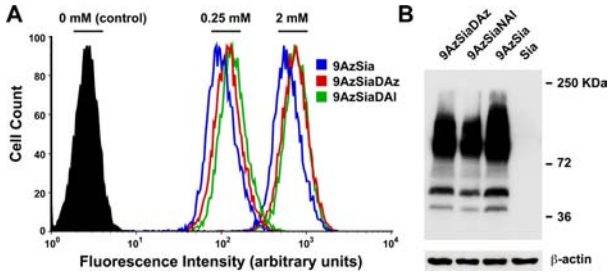


Figure 2. Evaluation of metabolic incorporation of bifunctional sialic acid analogues. (A) CHO cells were treated with 9AzSia, 9AzSiaNAl, and 9AzSiaDAz at various concentrations for 24 h. The treated cells were labeled with sulfo-DBCO–biotin and streptavidin–Alexa Fluor 647 and analyzed by flow cytometry. (B) Immunoblot analysis of glycoproteins from CHO cells metabolically labeled with unnatural sialic acids. The cells treated with 9AzSia, 9AzSiaNAl, 9AzSiaDAz, and sialic acid (Sia) were reacted with sulfo-DBCO–biotin and then lysed. The cell lysates were probed with HRP-conjugated anti-biotin (top panel). Equal protein loading was confirmed using an anti-β-actin antibody (bottom panel).

(Figures 2A and S1). The incorporation efficiencies of the bifunctional sialic acid analogues were comparable to that of 9AzSia, indicating that the simultaneous incorporation of chemical reporters on both the *N*-acyl group and C9 is well-tolerated by the sialic acid biosynthetic machinery. Similar results were obtained using HeLa cells (Figure S2), Daudi cells (Figure S3), and K20 cells (Figure S4). The relative incorporation efficiencies of the three sialic acid analogues varied slightly in different cell lines and at different concentrations but had the same order of magnitude. These results show that the bifunctional sialic acid analogues can be used in various cell lines.

As a further confirmation that the bifunctional sialic acids can be incorporated into glycoproteins, CHO cells treated with 9AzSiaNAl, 9AzSiaDAz, and 9AzSia were reacted with sulfo-DBCO–biotin and then lysed. The cell lysates were then characterized by anti-biotin Western blot. Like 9AzSia, both 9AzSiaNAl and 9AzSiaDAz were metabolically incorporated into a wide repertoire of glycoproteins (Figure 2B).

When we detected the cell-surface azides using Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC) assisted by the ligand BTTAA, a biocompatible variant of click chemistry with improved reaction kinetics developed by the Wu group,[15] similar levels of fluorescence were observed in cells treated with 9AzSiaNAl and 9AzSia (Figure S5). This implies that the cross-reaction between cell-surface 9AzSiaNAl moieties was insignificant, probably because of steric hindrance between the sialylated glycans.

In addition, we evaluated the metabolic incorporation of the peracetylated analogue Ac_4-9AzSiaDAz (**9**) (see the SI for the synthesis). Peracetylation has been shown to provide a significant improvement (up to hundreds-fold) in the metabolic efficiency of ManNAc analogues by facilitating the passive diffusion across the plasma membrane.[16] By contrast, peracetylation of sialic acid analogues has shown much less improvement.[7b] In agreement with the previous reports, Ac_4-9AzSiaDAz exhibited only a 3–5-fold increase in the incorporation efficiency relative to free 9AzSiaDAz at different concentrations (Figure S6). We therefore used the free sialic acid analogues for the following studies.

We next performed detailed investigations of the biological effects of the bifunctional sialic acid analogues. We first assessed the time-dependent incorporation of the bifunctional sialic acid analogues in comparison with the monofunctional counterpart.

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CHO cells were incubated with 3 mM 9AzSia, 9AzSiaNAl, and 9AzSiaDAz for various periods of time, and the incorporation of the unnatural sialic acids was analyzed by flow cytometry. The metabolic incorporation of all three analogues increased over time and reached a similar maximal level after ~20 h (Figure S7A). The incorporation of 9AzSia ascended more steeply than that of the two bifunctional analogues in the first 12 h, after which it rose more slowly until reaching saturation. We next evaluated the effects of the sialic acid analogues on cell growth. At the highest concentration used (3 mM), 9AzSiaNAl and 9AzSiaDAz exhibited significant inhibitory effects on cell growth of CHO cells (Figure S7B) and HeLa cells (Figure S7C). Reduced cell growth was also previously observed when cells were fed with a high concentration (>100 μM) of peracetylated ManNAc analogues bearing diazirine.[17] Furthermore, we evaluated the cytotoxicity of the sialic acid analogues using 7-amino-actinomycin D (7-AAD) cell viability assays. No apparent toxic effects were observed at any concentration for either 9AzSiaNAl or 9AzSiaDAz (Figure S7D,E). These results indicate that the bifunctional sialic acid analogues impair cell growth, probably by imposing cellular stress, but do not affect cell viability.

The introduction of dual or multiple bioorthogonal functional groups into biomolecules, including DNA,[18a] proteins,[18b,c] glycans,[18d,e] and lipids,[18f] has recently drawn great attention. We further demonstrated the bifunctional nature of cell-surface 9AzSiaNAl using two-color fluorescence imaging (Figure 3). 9AzSiaNAl-treated HeLa cells were first reacted with DBCO–Fluor 488 to label cell-surface azides using copper-free click chemistry. The cell-surface alkynes were then reacted with Alexa Fluor 647–azide using BTTAA-assisted CuAAC. As expected, the cells showed two-color labeling, and the two fluorescent dyes exhibited perfect colocalization, showing exclusively the merged yellow color (Figure 3A). Cells treated with 9AzSia or SiaNAl were labeled with only a single color. As a further confirmation that the dual labeling of azide and alkyne occurred simultaneously on the same 9AzSiaNAl molecules, we compared cells treated with a mixture of 3 mM 9AzSia and 3 mM SiaNAl by two-color colocalization and Förster resonance energy transfer (FRET) imaging. As expected, the merged yellow color, though seemingly to a lesser extent, was also observed in cells treated with the mixture of two monofunctional sialic acid analogues, since some sialic acids may reside closely on cell surfaces (Figure 3B). FRET is extremely sensitive to the donor–acceptor distance, and the range of effective FRET is 1–10 nm. We reasoned that dual labeling of the azide and alkyne of 9AzSiaNAl results in intramolecular FRET, which should be more effective than intermolecular FRET occurring in cells treated with the mixture of 9AzSia and SiaNAl. We observed significant FRET-induced fluorescence in HeLa cells treated with 9AzSiaNAl but only a very weak signal in cells treated with the 9AzSia/SiaNAl mixture (Figure 3C, left column). Quantification of the FRET efficiency by donor dequenching after acceptor photobleaching yielded FRET efficiencies of ~48% and ~6% in 9AzSiaNAl- and 9AzSia/SiaNAl-treated cells, respectively (Figure 3C, right column). These results indicate that the cell-surface 9AzSiaNAl was effectively dually labeled.

9AzSiaDAz is designed to endow sialic acid-containing glycans with a bioorthogonal functional group and a photo-cross-linker simultaneously. Once metabolically incorporated, the diazirine upon UV light activation can covalently cross-link sialic acid-binding proteins while the azide simultaneously enables enrichment of the target proteins by conjugation with an affinity tag functionalized with an alkyne. To demonstrate the feasibility

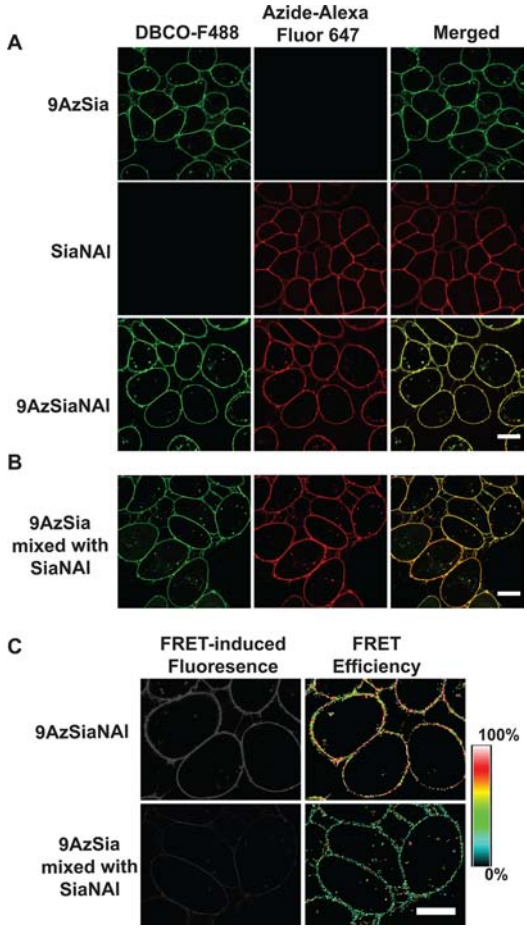

Figure 3. Two-color fluorescence imaging and FRET analysis of 9AzSiaNAl-incorporated glycans on cell surfaces. (A) HeLa cells were treated with 3 mM 9AzSia, SiaNAl, or 9AzSiaNAl for 24 h. The treated cells were reacted with DBCO–Fluor 488 and Alexa Fluor 647–azide. (B) HeLa cells were treated with a mixture of 3 mM 9AzSia and 3 mM SiaNAl, with subsequent conjugation with DBCO–Fluor 488 and Alexa Fluor 647–azide. (C) FRET-induced fluorescence imaging (left column) and FRET efficiency imaging (right column) of HeLa cells treated with 9AzSiaNAl or the 9AzSia/SiaNAl mixture. FRET-induced fluorescence was excited using a 488 nm laser and collected with a 640 nm long-pass filter. The FRET efficiency for each pixel was measured by donor dequenching after acceptor photobleaching. The color-coded scale is for the FRET efficiencies. Scale bars represent 20 μm.

of using 9AzSiaDAz to identify sialic acid-binding proteins, we chose to capture the dimerization of CD22 (sialic acid-binding immunoglobulin-like lectin 2), which has been shown to be mediated by sialic acid–CD22 interactions, via photo-cross-linking[10,11] as a proof-of-principle experiment. Daudi cells, a human B lymphoma cell line expressing CD22, were cultured with 2 mM 9AzSiaDAz for 24 h and subsequently UV-irradiated for 20 min to cross-link sialic acid with its binding partners. The cells were also treated with SiaDAz and natural sialic acid for comparison. The cells were lysed and reacted with alkyne–biotin. The tagged, photo-cross-linked complexes were then purified using streptavidin beads and analyzed by Western blot using an anti-CD22 antibody. We observed the photo-cross-linked CD22 in 9AzSiaDAz-treated cells (Figure S8). The absence of UV irradiation resulted in only monomeric CD22, indicating the UV-induced photo-cross-linking via the diazirine. For cells treated with SiaDAz, no CD22 was observed because of the lack of the affinity tag. These results collectively show that

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once 9AzSiaDAz is incorporated into cell-surface sialylated glycans, the diazirine and azide simultaneously exert their photo-cross-linking and affinity enrichment functions.

In summary, the bifunctional sialic acid analogues 9AzSiaNAl and 9AzSiaDAz have been developed for simultaneous incorporation of two distinct chemical reporters into cellular sialylated glycans. We have demonstrated that 9AzSiaNAl can be used for two-color and FRET imaging. Further applications include probing the biosynthesis and turnover dynamics of sialylated glycans as a function of time by conjugating distinct probes to the azide and alkyne at different time points. This could be used to categorize glycoproteins by their turnover rates.

9AzSiaDAz has been metabolically incorporated into cell-surface glycans, and the installed diazirine and azide were used for photo-cross-linking of binding proteins and affinity enrichment, respectively. When SiaDAz is used to capture glycoprotein interactions, detecting its cell-surface incorporation is not straightforward. Lectins recognizing sialic acids or core oligosaccharide structures that are masked by the addition of sialic acids have been used to detect the incorporation of SiaDAz on live cells.[11,19] This method is only applicable in cell lines with deficiencies in sialic acid biosynthesis such as K20 cells,[20] a subclone of the human B lymphoma cell line BJA-B, which is deficient in UDP-GlcNAc 2-epimerase activity. However, quantification of the incorporation efficiency can be complicated by the fact that the diazirine modification might influence the lectin binding. Notably, our bifunctional analogue 9AzSiaDAz enables the direct detection of the cell-surface display of photo-cross-linking diazirine-containing sialic acids, even in cells with intact sialic acid biosynthetic machinery, as demonstrated in CHO, HeLa, Daudi, and K20 cells. More importantly, this photo-cross-linking bifunctional sugar will find invaluable applications in identifying sialic acid-binding proteins. For monofunctional photosugars such as SiaDAz and 9AAzSia, one would have to tag a sialylated protein of interest, so only binding partners of a specific protein could be identified at a time. In our strategy, the sialic acid is directly tagged, offering the possibility of global identification of all sialic acid-binding proteins. In addition, other bifunctional sialic acid analogues with desired properties can be developed, given the generic nature of the synthetic process.

Notably, bifunctional ManNAc analogues that contain functionalities on both the *N*-acyl chain and the C6 position cannot be metabolically converted into the corresponding sialic acids because of the participation of the C6 hydroxyl group in the enzymatic transformation of ManNAc to sialic acid. Recently, the Hakenberger group reported that a ManNAc analogue bearing an azide at C4 can be metabolically converted to the corresponding C7-substituted sialic acid and incorporated into cell-surface sialylated glycans.[21] It will be interesting to test whether ManNAc analogues bearing functionalities on both the *N*-acyl chain and the C4 position can be used for metabolic glycan labeling. This is currently under investigation in our laboratory.

■ ASSOCIATED CONTENT

Supporting Information

Chemical synthesis and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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