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Investigating Cellular Metabolism of Synthetic Azidosugars with the Staudinger Ligation

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Abstract: The structure of sialic acid on living cells can be modulated by metabolism of unnatural biosynthetic precursors. Here we investigate the conversion of a panel of azide-functionalized mannosamine and glucosamine derivatives into cell-surface sialosides. A key tool in this study is the Staudinger ligation, a highly selective reaction between modified triarylphosphines and azides that produces an amide-linked product. A preliminary study of the mechanism of this reaction, and refined conditions for its in vivo execution, are reported. The reaction provided a means to label the glycoconjugate-bound azidosugars with biochemical probes. Finally, we demonstrate that the cell-surface Staudinger ligation is compatible with hydrazone formation from metabolically introduced ketones. These two strategies provide a means to selectively modify cell-surface glycans with exogenous probes.

Introduction

The intricate process of cell-surface glycosylation is becoming increasingly amenable to in vivo manipulation by use of metabolic and chemical tools.[1] Analogues of naturally occurring monosaccharides are known to traverse select biosynthetic pathways, resulting in cell-surface display of unnatural oligosaccharides.[2,3] Sialic acid biosynthesis is particularly amenable to this approach. Metabolically generated unnatural sialosides have been shown to selectively alter native glycosylation patterns such as polysialylation, which plays a key role in cancer cell metastasis.[4,5] Viral infection can also be modulated by the introduction of novel sialosides onto cells.[6,7] If the unnatural biosynthetic substrate possesses a selectively reactive functional group, its cell-surface product can undergo chemoselective ligation with an exogenously delivered reaction partner, resulting in further modification (Figure 1A).

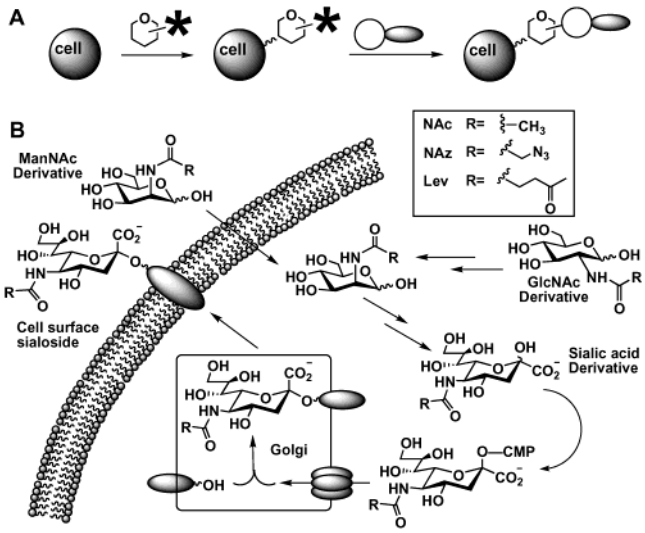

Figure 1. (A) Metabolism of a sugar bearing a selectively reactive functional group (*) results in its cell-surface display. Chemoselective ligation with an exogenously delivered reaction partner further modifies the cell-surface glycan. (B) Sialic acid biosynthetic pathway. Cell-surface sialosides are biosynthesized in a series of enzymatic steps. The process normally begins with either ManNAc or GlcNAc; however, exogenously added unnatural analogues of these compounds (or more advanced intermediates) are able to intercept the pathway and produce cell-surface sialosides with novel functionality.

The first committed intermediate in sialic acid biosynthesis is *N*-acetylmannosamine (ManNAc), which can be generated in cells from *N*-acetylglucosamine (GlcNAc) or taken up from outside the cell (Figure 1B). To deliver a selectively reactive functional group to the cell surface, we previously substituted

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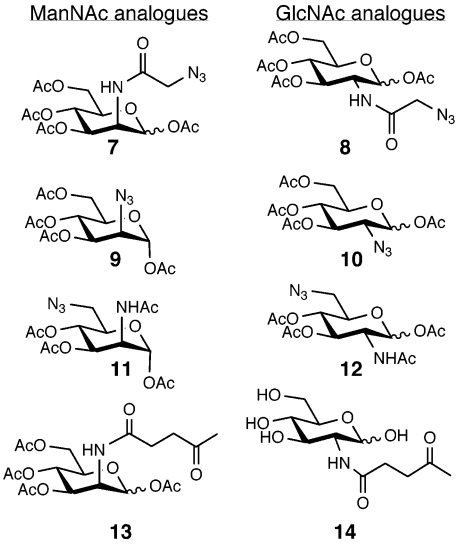
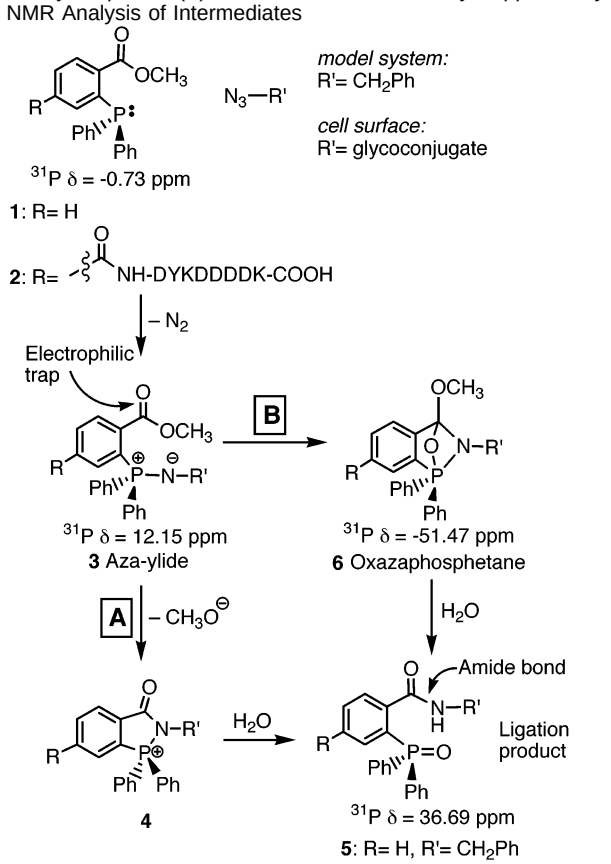


Figure 2. Structures of ManNAc and GlcNAc analogues used in this study.

ManNAc with *N*-levulinoylmannosamine (ManLev, Figure 1B).[8] ManLev acts as a substrate for the enzymes in the sialic acid biosynthetic pathway, and is converted into a ketone-bearing cell-surface sialoside. The ketone functional group reacts selectively with hydrazides or aminooxy groups to produce hydrazones or oximes, respectively. The use of biotin hydrazide offered a means to quantify reactive ketone groups by staining the cells with FITC-avidin, rendering them fluorescent. The corresponding GlcNAc analogue, GlcLev (**14**, Figure 2), was also prepared as a potential metabolic precursor for cell-surface sialosides; however, it was metabolized at much lower levels than ManLev.[9] Since the initial demonstration of cell-surface engineering with ManLev, the technology has been applied to the development of targeted imaging reagents[10] and methods for gene transfer.[11] The efficiency of ManLev uptake was greatly increased by peracetylation to form the tetra-*O*-acetyl compound, Ac_4ManLev (**13**, Figure 2).[12] Acetylation increases the membrane permeability of the monosaccharide; once the molecule is inside the cell, the acetyl groups are readily cleaved by cytosolic esterases. Thus, treatment with a peracetylated sugar results in the same density of unnatural cell-surface epitopes as 200-fold higher concentrations of the corresponding free sugar.

Here we present a study of a panel of azide-derivatized sugars (**7**−**12**, Figure 2) that were tested for their ability to mimic either GlcNAc or ManNAc, resulting in metabolic conversion to unnatural cell-surface sialosides. The recently developed Staudinger ligation has proven to be an effective tool for selective tagging of azide-functionalized biomolecules with probes, even within a complex mixture of cellular components.[13,14] Covalent modification of azide-derivatized glycoconjugates via the Staudinger ligation allowed their identification and quantification on the cell surface.[13] The cellular products

Scheme 1. Staudinger Ligation: (A) Reaction Pathway as Previously Proposed; (B) Revised Reaction Pathway Supported by ^{31}P NMR Analysis of Intermediates



of the azidosugars were confirmed as sialic acids by a series of metabolic competition experiments, and their residence within glycoconjugate subclasses on various cell types was established with glycosylation inhibitors.

In addition, we examined the mechanism by which the Staudinger ligation occurs by NMR analysis of intermediates formed during the reaction. We also determined the optimal pH, reagent concentrations, and time required for maximal ligation on cells. Finally, we directly compared the metabolism of the most efficient cell-surface azide delivery vehicle, *N*-azidoacetylmannosamine (Ac_4ManNAz, **7**, Figure 2)[13] with that of Ac_4ManLev. Using these two substrates, we demonstrated that the reactivity of the corresponding sialic acid derivatives permits simultaneous labeling by two mutually orthogonal chemoselective ligation reactions.

Results and Discussion

Investigation of Intermediates Formed during the Staudinger Ligation. The Staudinger ligation as we had initially formulated the reaction is shown in Scheme 1, pathway A.[13] The key functionality is a triarylphosphine in which a carboxymethyl group is situated adjacent to phosphorus on one of the aryl rings (**1**). A biochemical probe of interest can be appended, as shown for compound **2**, which possesses the eight-residue FLAG peptide.[14] We initially proposed[13] that these phosphines react with azides to form aza-ylide intermediates (**3**, Scheme 1), which in turn react in an intramolecular fashion to generate phosphonium analogues (**4**; pathway A, Scheme 1). Hydrolysis then provides the ligation product (**5**).

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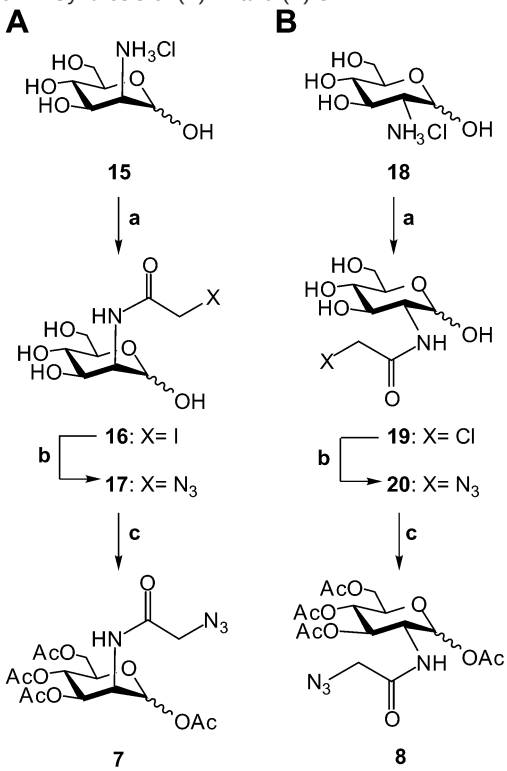
To directly characterize the reaction intermediates, we performed ^{31}P NMR analysis during the reaction in the presence of substoichiometric amounts of water. Compound **1** (Scheme 1) and benzyl azide were dissolved in wet CD_3CN. After 20 min the reaction mixture was examined by ^{31}P NMR, revealing resonances at −51.47, −0.73, 12.15, 33.01, and 36.69 ppm (Figure 1, Supporting Information). Two of these correspond to the known (vide infra) ^{31}P chemical shifts of **1** (−0.73 ppm) and the ligation product **5** (36.69 ppm). The literature value for the ^{31}P resonance of the aza-ylide formed from triphenylphosphine and benzyl azide is 12.6 ppm.[15] Aza-ylide **3** is structurally similar to the literature compound, which led us to conclude that the presence of **3** in the reaction gave rise to the peak at 12.15 ppm. The peak at −51.47 ppm is within the range reported for oxazaphosphetanes (−35 to −55 ppm),[15] suggesting that an intermediate such as **6** is present and that the reaction proceeds via pathway B rather than pathway A (Scheme 1). We saw no evidence for compound **4** by ^{31}P NMR, which would be expected to display a ^{31}P resonance further downfield than any we observed.[16] If the aza-ylide does form oxazaphosphetane intermediate **6**, as suggested by the NMR results, then this strained bicyclic structure could form the ligation product **5**, either via direct hydrolysis or through another short-lived intermediate that was not observed.

After approximately 50 min, the only resonances observed were at −51.47, −0.73, and 36.69 ppm (Figure 1, Supporting Information). The addition of 10 μL of water resulted in the rapid conversion of remaining **1** and **6** to product (**5**). The synthesis of substituted analogues of **1** is currently underway in order to confirm the proposed reaction mechanism.

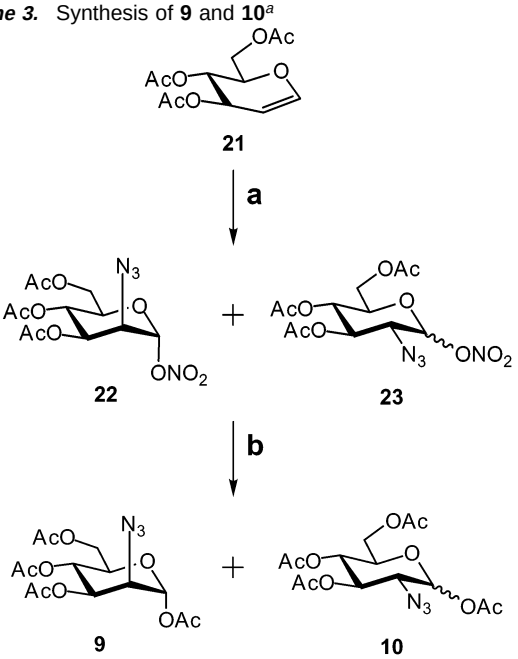
Synthesis of ManNAc and GlcNAc Analogues. Ac_4ManNAz (**7**) and Ac_4GlcNAz (**8**, Figure 2) were synthesized as substrates for unnatural sialic acid biosynthesis in cells,[3,12] starting from mannosamine hydrochloride and glucosamine hydrochloride, respectively (Scheme 2). The monosaccharides were deprotonated with sodium methoxide, followed by acylation with either iodo- or chloroacetic anhydride in methanol. Displacement of the halide with either sodium or lithium azide, followed by acetylation, provided the target compounds. The synthesis of four new azide-bearing ManNAc and GlcNAc analogues (compounds **9**−**12**, Figure 2) was undertaken in an effort to identify other sites tolerant of modification. Compounds **9** and **10** were synthesized in two steps starting from peracetylated D-glucal (**21**, Scheme 3). Azidonitration[17] provided a mixture of *manno*- and *gluco*-epimers (**22** and **23**) that were converted to a mixture of the desired products by displacement of the nitrate group with sodium acetate in acetic acid. Recrystallization, silica gel chromatography, and reverse-phase HPLC permitted the separation of compounds **9** and **10** to provide these products in pure form. The synthesis of compounds **11** and **12** is depicted in Scheme 4. ManNAc was selectively tosylated at the 6-position and the tosylate was displaced with lithium azide to afford compound **11** after peracetylation. Compound **12** was synthesized in an analogous fashion from GlcNAc as the starting material.

Scheme 2. Synthesis of (A) **7**[a] and (B) **8**[b]



[a] (a) NaOMe, iodoacetic anhydride, MeOH. (b) NaN_3, MeOH. (c) Ac_2O, pyridine. [b](a) NaOMe, chloroacetic anhydride, MeOH. (b) LiN_3, DMF. (c) Ac_2O, pyridine.

Scheme 3. Synthesis of **9** and **10**[a]



[a] (a) NaN_3, CAN, MeCN. (b) NaOAc, AcOH.

Optimization of Cell-Surface Staudinger Ligation. We previously reported that Ac_4ManNAz (**7**, Figure 2) is efficiently metabolized by Jurkat cells to produce cell-surface azidosialosides that can react with exogenously delivered phosphines.[13] When the azide-bearing cells are reacted with phosphine-FLAG (**2**), a covalently bound cell-surface conjugate is formed that renders these cells immunoreactive toward anti-FLAG antibod-

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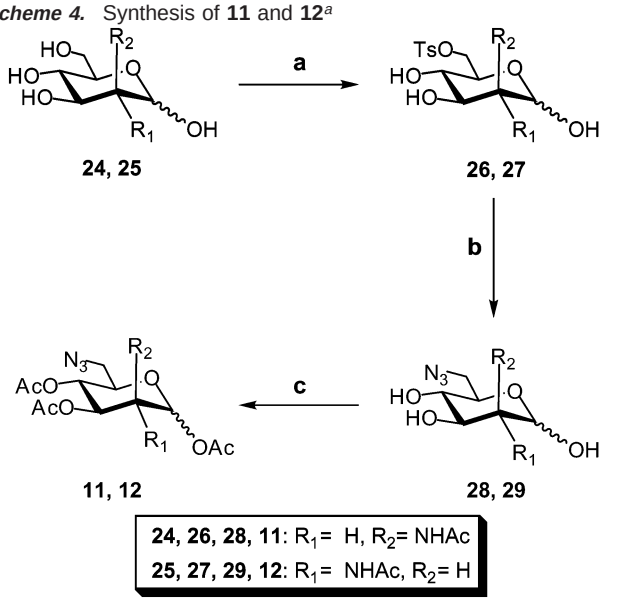
Scheme 4. Synthesis of **11** and **12**[a]



[a] (a) TsCl, pyridine. (b) LiN_3, DMF. (c) Ac_2O, pyridine.

ies, which can be detected in FITC-labeled form by flow cytometry.

To facilitate the use of the Staudinger ligation as a tool for detecting cell-surface azidosugars, we set out to identify optimal reaction conditions. The model reaction of compound **1** with benzyl azide (Scheme 1) is complete after 1 h when both reagents (phosphine and azide) are present at a concentration of 100 mM. However, cell-surface azides are present at low concentration and are restricted to a two-dimensional surface rather than dispersed in solution. Therefore, we sought to establish the time necessary to maximize the observed fluorescence signal in a cell-based experiment. Ac_4ManNAz-treated (20 μM, 3 days) and untreated Jurkat cells were incubated with **2** (0.25 mM) for various periods of time (1 to 8 h) followed by antibody labeling. A maximal fluorescence signal for cells treated with Ac_4ManNAz was observed after 5 h (Figure 2, Supporting Information). In contrast, control Jurkat cells that were not incubated with Ac_4ManNAz showed no increase in fluorescence over background even after 8 h of exposure to phosphine-FLAG (**2**). It is important to note that, after only 1 h, the fluorescence signal from the azide-labeled cells was 60-fold greater than background. This suggests that a reaction time of 1 h is sufficient for robust cell-surface detection of azidosugars.

The second variable we examined was the concentration of **2** required to generate the maximum fluorescence signal. Ac_4ManNAz-treated Jurkat cells (20 μM, 3 days) were exposed to phosphine-FLAG for 1 h at concentrations ranging from 0.1 to 1 mM. The fluorescence signal increased with increasing concentrations of phosphine-FLAG up to 0.4 mM, at which point the signal reached saturation (Figure 3, Supporting Information). The fluorescence remained constant even when phosphine-FLAG concentrations were increased up to 5 mM (data not shown). Therefore, to obtain maximum fluorescence under these conditions, 0.4 mM phosphine-FLAG is required, whereas 0.25 mM phosphine-FLAG provides a sufficiently high fluorescence signal for cell-surface labeling.

On the basis of our proposed mechanism for the Staudinger ligation (pathway B, Scheme 1), we speculate that hydrolysis of the initial aza-ylide intermediate (**3**) should be the major competing side reaction. This side reaction should be favored at lower pH where the aza-ylide is more extensively protonated. We carried out the cell-surface Staudinger ligation in buffers at various pH values (5−8.5) within the bounds of cell viability. We predicted that the fluorescence signal would decrease at lower pH due to the increased hydrolysis of the aza-ylide. However, the yield of ligation reaction appeared independent of pH since the fluorescence signals were identical within the pH range tested (data not shown). Given the NMR evidence for an oxazaphosphetane intermediate, we conclude that the formation of this intermediate must be extremely rapid, thereby minimizing the competing hydrolysis reaction.

Azide Reduction during Metabolism and Staudinger Ligation of Azidosugars. The azide is considered relatively inert to biological nucleophiles, electrophiles, and oxidants.[18] Nonetheless, we sought to examine the fate of the azide during metabolism and after reaction with phosphine-FLAG. Reduction of azides to amines can occur on exposure to thiols, usually at high pH and concentration,[19−21] and there is some evidence that this reaction takes place in vivo, in certain cell types.[22] A second avenue by which azides could be converted to amines is by hydrolysis of the aza-ylide intermediate during the Staudinger ligation. Although model reactions in vitro have shown that the Staudinger ligation produces ligated product in essentially quantitative yield (vide infra), without evidence of the amine byproduct generated by aza-ylide hydrolysis, the products on cell surfaces had not been examined in detail.

To probe for the presence of amines generated during either Ac_4ManNAz metabolism or Staudinger ligation, we quantified cell-surface-exposed amino groups using sulfo-NHS-LC-LC-biotin (Pierce). The resulting cell-surface biotin conjugate was detected with FITC-avidin. Jurkat cells were grown in the presence or absence of Ac_4ManNAz and populations of each of these cells were treated with buffer, phosphine-FLAG (**2**), or tricarboxyethylphosphine (TCEP), a trialkylphosphine that rapidly reduces azides to amines (as well as reducing disulfide bonds to the corresponding thiols).[23,24] The resulting six populations of cells were exposed to sulfo-NHS-LC-LC-biotin, followed by FITC-avidin labeling, and analyzed by flow cytometry.

It appeared that the number of accessible cell-surface amines increased (Figure 3, column 1) upon incubation with Ac_4ManNAz alone. One explanation for this is that some fraction of azides are reduced to amines by intracellular thiols during metabolism. Indeed, we observed a similar process during the metabolic incorporation of azidoamino acids into proteins in *Escherichia coli*.[14] It is also possible that changes in glycosylation, brought about by the presence of the unnatural sugar, render more amines accessible to the labeling reagent. However, no additional reactive amines were observed after exposure of the Ac_4ManNAz-treated cells to phosphine-FLAG (Figure 3, column 2). We

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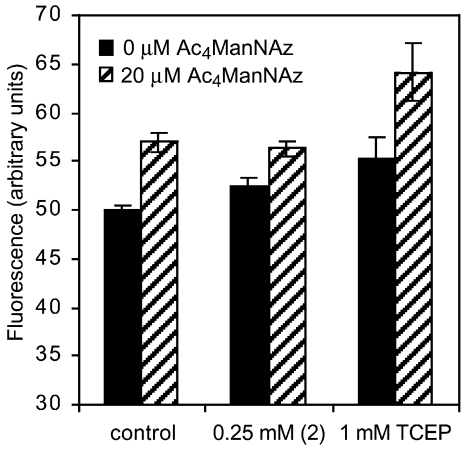

Figure 3. Quantification of cell-surface amines before and after Staudinger ligation. Jurkat cells were incubated in the presence or absence of 20 μM Ac_4ManNAz for 3 days, washed, and treated with 0.25 mM phosphine-FLAG or 1 mM TCEP or mock-treated with buffer for 1 h. The cells were washed again and labeled with sulfo-NHS-LC-LC-biotin, followed by staining with FITC-avidin. The fluorescence signal measured by flow cytometry is reported in arbitrary units, and error bars represent the standard deviation for three replicates.

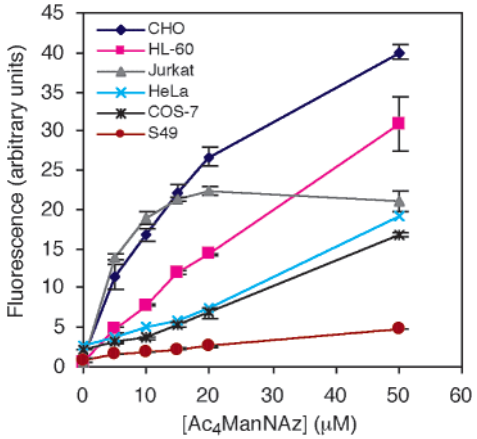

Figure 4. Metabolism of Ac_4ManNAz in various cell lines. Cell lines were incubated in the presence or absence of 20 μM Ac_4ManNAz for 3 days, washed, and treated with 0.25 mM phosphine-FLAG for 1 h. The cells were washed again and stained with FITC-anti-FLAG. The fluorescence signal measured by flow cytometry is reported in arbitrary units, and error bars represent the standard deviation for three replicates.

conclude that the Staudinger ligation proceeds in high yield on cells, with no detectable azide reduction. As a positive control, an increase in cell-surface amines was observed when the azides were intentionally reduced with TCEP (Figure 3, column 3). In the absence of Ac_4ManNAz, treatment with TCEP also produced a slight increase in accessible cell-surface amines. This may be due to changes in protein conformation brought about by disulfide reduction. Additional investigation of Ac_4ManNAz metabolism, with radiolabeled precursors, should resolve this issue. However, these data suggest that amines are not produced during the reaction with phosphine-FLAG, indicating that ligation, not hydrolysis, is the predominant reaction pathway on the cell surface.

Metabolism of Ac_4ManNAz by Various Cell Lines. The fluorescence observed after the Staudinger ligation and antibody labeling of Ac_4ManNAz-treated cells was dependent on the initial concentration of Ac_4ManNAz in the medium (Figure 4). In Jurkat cells, the fluorescence signal plateaued at concentrations of Ac_4ManNAz above 15 μM. Several scenarios could account for this observation. All available sites for the unnatural sialic acid may be occupied, the cell-surface topology might limit the accessibility of either the azides or the phosphine-FLAG conjugate, or enzymes in the pathway may become saturated. We next examined the efficiency of Ac_4ManNAz metabolism in two additional human cell lines (HL-60 and HeLa) as well as cell lines originating from other species: hamster (CHO), green monkey (COS-7), and mouse (S49). In these cell lines, the fluorescence signal did not plateau at concentrations of Ac_4ManNAz as high as 50 μM. Both CHO and HL-60 cells displayed higher fluorescence, and thus greater numbers of cell-surface azides, than Jurkat cells when each was treated with 50 μM Ac_4ManNAz. COS-7 and HeLa cells displayed lower fluorescence at comparable Ac_4ManNAz concentrations, and the murine cell line S49 showed almost no increase in fluorescence over background levels. It is evident that the ability to metabolize Ac_4ManNAz is highly cell-line-dependent. For comparative purposes, all further experiments were carried out in Jurkat cells unless stated otherwise.

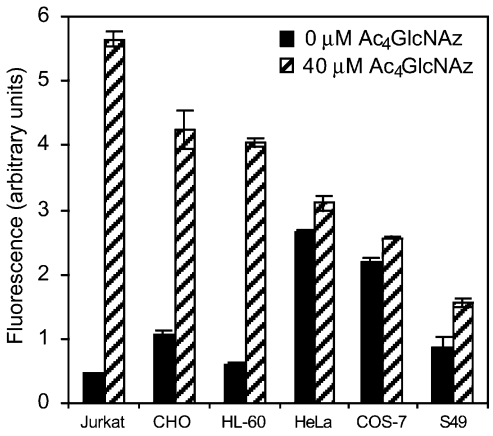

Figure 5. Metabolism of Ac_4GlcNAz in various cell lines. Cell lines were incubated in the presence or absence of 40 μM Ac_4GlcNAz for 3 days, washed, and treated with 0.25 mM phosphine-FLAG for 1 h. The cells were washed again and stained with FITC-anti-FLAG. The fluorescence signal measured by flow cytometry is reported in arbitrary units, and error bars represent the standard deviation for three replicates.

Metabolism of Ac_4GlcNAz by Various Cell Lines. As shown in Figure 1B, GlcNAc can also be metabolized to sialic acid, by first undergoing conversion to ManNAc.[25−27] We therefore investigated the metabolism of GlcNAc analogue **8** (Ac_4GlcNAz, Figure 2) to the corresponding unnatural cell-surface sialoside. It should be noted, however, that GlcNAc can also be incorporated directly into glycoproteins.[28] Therefore, Ac_4GlcNAz may produce multiple metabolic products. Figure 5 shows the results of treating various cell lines with Ac_4GlcNAz followed by subsequent reaction with **2** and antibody labeling. It is evident that Ac_4GlcNAz is metabolized to cell-surface products in Jurkat, HL-60, and CHO cells. However, the overall fluorescence was approximately 4-fold lower than that observed for similar concentrations of Ac_4ManNAz. In contrast, the

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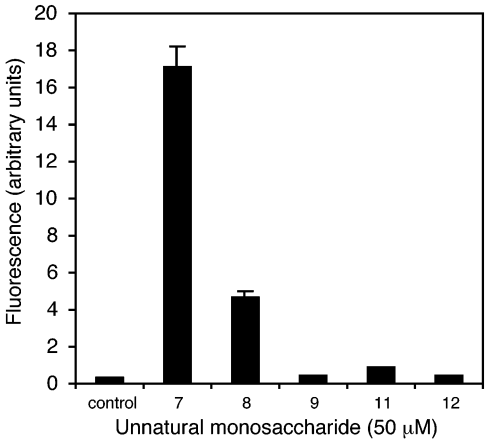

Figure 6. Metabolism of ManNAc and GlcNAc analogues to cell-surface products. Jurkat cells were incubated in the presence or absence of 50 μM azidosugar, washed, and treated with 0.25 mM phosphine-FLAG for 1 h. The cells were washed again and stained with FITC-anti-FLAG. The fluorescence signal measured by flow cytometry is reported in arbitrary units, and error bars represent the standard deviation for three replicates.

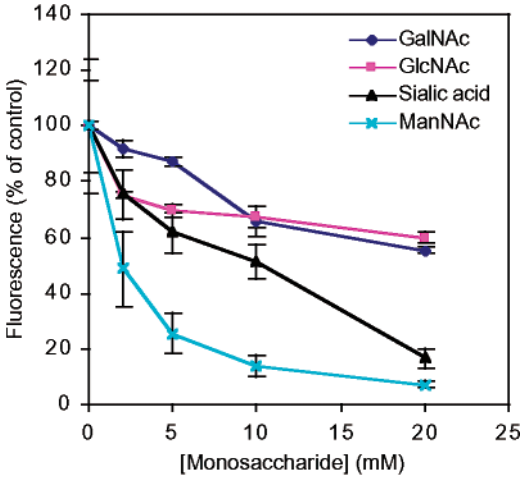

Figure 7. Ac_4ManNAz metabolism is strongly inhibited by ManNAc and sialic acid but only slightly by GlcNAc and GalNAc. Jurkat cells were incubated with 20 μM Ac_4ManNAz and various amounts of the appropriate monosaccharide for 3 days, washed, and treated with 0.25 mM phosphine-FLAG for 1 h. The cells were washed again and stained with FITC-anti-FLAG. The fluorescence signal measured by flow cytometry is reported as percent of control, where the control represents the fluorescence signal from Jurkat cells incubated with 20 μM Ac_4ManNAz alone. Error bars represent the standard deviation for three replicates.

number of cell-surface ketones resulting from *N*-levulinoylglucosamine (GlcLev) metabolism in Jurkat cells has previously been shown to be 15-fold lower than that arising from ManLev metabolism.[9]

The metabolism of Ac_4ManNAz and Ac_4GlcNAz varies greatly across cell lines. Acetylation of the compounds ensures efficient cellular uptake, and previous work has shown that the enzymes downstream of sialic acid biosynthesis are quite permissive for unnatural substrates.[29] Therefore, discrimination most likely takes place at one of the earlier steps in the pathway, between cell entry and conversion to sialic acid. Recent evidence suggests that the initial phosphorylation of ManNAc may be a "bottleneck" in the pathway.[30] It is possible that the responsible enzyme (ManNAc- or GlcNAc-6-kinase) is expressed at lower levels in cell lines that metabolize unnatural ManNAc or GlcNAc analogues poorly or that these cells naturally possess lower levels of sialic acid on their membrane glycoproteins.

Metabolism of Other ManNAc and GlcNAc Analogues by Jurkat Cells. We examined the metabolism of GlcNAc and ManNAc analogues in which the azido group replaced the 2-*N*-acyl substituent altogether (compounds **9** and **10**, Figure 2), or the C-6 hydroxyl group (compounds **11** and **12**, Figure 2). In order for compounds **11** and **12** to be converted to sialic acid they must bypass the 6-*O*-phosphorylation step mediated by ManNAc- (or GlcNAc-) 6-kinase and still be accepted as a substrate by the subsequent enzyme in the pathway, sialic acid synthase.[30] We found that compound **9** was not converted to cell-surface products at detectable levels, indicating that the *N*-acetamido group is essential for enzymatic conversion of ManNAc analogues (Figure 6). Compound **10** was found to be toxic to cells at all concentrations tested, perhaps due to interference with essential biosynthetic pathways as a glucose or GlcNAc analogue or by termination of glycan synthesis. The more conservatively modified analogue **11** produced very low levels of cell-surface azides, approximately 2-fold above background and 20-fold less than that observed upon treatment of Jurkat cells with Ac_4ManNAz, from which we conclude that the initial phosphorylation step is critical for subsequent processing. Incubation of Jurkat cells with **12** did not produce detectable cell-surface azides. It may be possible to engineer key enzymes in the pathway to improve the utility of certain substrates. Currently, however, Ac_4ManNAz remains the most efficient vehicle for delivery of azides to the cell surface through the sialic acid pathway.

Determining the Identity of Cell-Surface Azidosugars by Use of Competing Monosaccharides. The identity of the metabolic products of Ac_4ManNAz and Ac_4GlcNAz metabolism was ascertained by competition experiments with natural sugars. Coincubation of Jurkat cells with Ac_4ManNAz (20 μM) and various concentrations of ManNAc (0−20 mM) resulted in dose-dependent reduction in the fluorescence signal observed after 3 days (Figure 7). This suggests that ManNAc and ManNAz are competing for the same enzymes. A similar reduction in the fluorescence signal was observed when sialic acid was used as a competing substrate. It is possible that the later enzymes in the pathway are more tolerant of unnatural sialic acids than the earlier enzymes are with respect to unnatural ManNAc analogues.[12,29] Thus, sialic acid would be a weaker competitor than equal amounts of ManNAc. However, at the highest concentration of sialic acid tested, the amount of detectable cell-surface azides diminished nearly to background levels.

Addition of GlcNAc to the cell culture medium reduced the fluorescence signal from Ac_4ManNAz metabolism, which may result from conversion of the added GlcNAc to ManNAc. The negligible magnitude of this effect is likely due to the multiple biosynthetic fates of GlcNAc within the cell.[28] Therefore, increasing the intracellular concentration of GlcNAc results in only small changes in the output of any one pathway. The addition of GalNAc, which is not involved in sialic acid biosynthesis, resulted in a similar slight decrease in fluorescence. This may reflect a global change in the flux of monosaccharides through the various biosynthetic pathways within the cell. The

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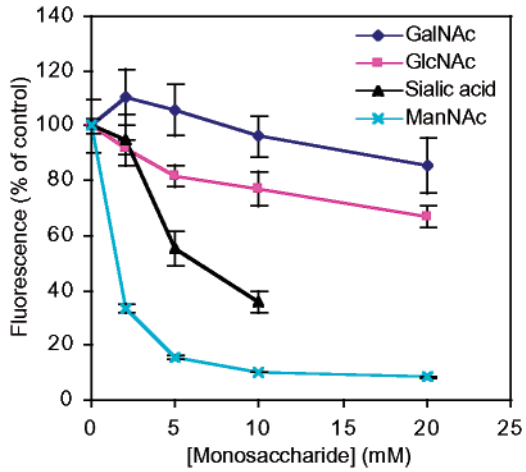

Figure 8. $Ac_4GlcNAz$ metabolism is inhibited by ManNAc and sialic acid but only slightly by GlcNAc and GalNAc. Jurkat cells were incubated with 40 μM $Ac_4GlcNAz$ and various amounts of the appropriate monosaccharide for 3 days, washed, and treated with 0.25 mM phosphine-FLAG for 1 h. The cells were washed again and stained with FITC-anti-FLAG. The fluorescence signal measured by flow cytometry is reported as percent of control, where the control represents the fluorescence signal from Jurkat cells incubated with 40 μM $Ac_4GlcNAz$ alone. Error bars represent the standard deviation for three replicates.

results of these four experiments suggest that $Ac_4ManNAz$ is converted to cell-surface SiaNAz by the sialic acid biosynthetic machinery.

Multiple biosynthetic pathways utilize GlcNAc as an intermediate and therefore we were curious to examine the effects of competing monosaccharides on the fluorescence signal resulting from metabolism of $Ac_4GlcNAz$. The most significant decrease in the fluorescence signal occurred upon the addition of ManNAc (Figure 8), an indication that ManNAc competes with $Ac_4GlcNAz$ in the same biosynthetic pathway. Sialic acid also inhibited the fluorescence signal, although to a lesser extent than ManNAc (Figure 8), as was observed for cells treated with $Ac_4ManNAz$ (Figure 7). In both the $Ac_4ManNAz$ competition experiment (Figure 7) and the $Ac_4GlcNAz$ competition experiment (Figure 8) the addition of 20 mM sialic acid was somewhat toxic to the cells, due to the accompanying decrease in pH. In the case of $Ac_4GlcNAz$ metabolism, insufficient viable cells were obtained at 20 mM sialic acid, preventing the acquisition of data at that concentration. However, at sialic acid concentrations below 20 mM, the compound was adequately buffered by the media and cytotoxicity was not observed. Only a slight decrease in fluorescence was observed when GlcNAc was added to the cell culture medium (Figure 8). From this result we conclude that $Ac_4GlcNAz$ does not act directly as a GlcNAc surrogate. As was observed with $Ac_4ManNAz$, GalNAc does not inhibit the display of cell-surface azides. We conclude that $Ac_4GlcNAz$ is most likely converted to ManNAz, followed by metabolism to cell-surface SiaNAz.

Determining the Location of Cell-Surface SiaNAz by Use of Glycosylation Inhibitors. We determined which types of glycoproteins host SiaNAz on the cell surface by using known inhibitors of glycosylation. Jurkat cells display sialic acids predominantly as terminal residues on *N*-linked glycoproteins.[31] Tunicamycin inhibits the production of *N*-linked glycans by blocking the biosynthesis of their dolichol-oligosaccharide precursor.[32] Coincubation of tunicamycin with $Ac_4ManNAz$ abrogated cell-surface azide expression relative to control (Figure 9A), indicating that the metabolic product of $Ac_4ManNAz$ is resident within *N*-linked oligosaccharides. This was further confirmed by the use of another more specific glycosylation inhibitor, deoxymannojirimycin. Unlike tunicamycin, which blocks *N*-linked glycosylation entirely, deoxymannojirimycin inhibits a downstream glycoconjugate processing enzyme (mannosidase I), resulting in truncated glycans that lack terminal residues.[33] The decrease in the fluorescence signal of deoxymannojirimycin-treated cells (Figure 9B) indicates that the cell-surface azides are present in terminal sugars of *N*-linked glycans such as sialic acid. Inhibition of *O*-linked glycosylation, by αBnGalNAc,[34,35] was not expected to have as dramatic an effect on the azidosugar expression in principally *N*-glycosylated Jurkat cells. This was reflected in the data shown in Figure 9C. In contrast, HL-60 cells are predominantly sialylated on *O*-linked glycoproteins.[36] Treatment of these cells with αBnGalNAc produced a dramatic decrease in the fluorescence signal resulting from $Ac_4ManNAz$ metabolism (Figure 9D), as expected.

Concurrent Labeling of the Cell Surface by Utilization of Two Separate and Compatible Chemistries. Jurkat cells were grown in the presence of one or both of the most efficient unnatural sialic acid precursors, $Ac_4ManLev$ (**13**, synthesized as previously described)[12] and $Ac_4ManNAz$, and then simultaneously treated with biotin hydrazide and phosphine-FLAG (**2**), followed by FITC-anti-FLAG and PE-avidin. The data in Figure 10 highlight the selectivity of these reactions. Although all of the cells were subjected to identical labeling conditions, only cells that were initially incubated with $Ac_4ManLev$ displayed red fluorescence (PE), and only cells incubated with $Ac_4ManNAz$ displayed green fluorescence (FITC). While hydrazone formation proceeds most readily under slightly acidic conditions (pH $= 5.5-6.5$), the Staudinger ligation tolerates a wider pH range without significant loss in rate or yield. Thus the Staudinger ligation may be performed at pH 7.4, which is optimal for cell viability, or at pH 6.5 if hydrazone formation is being executed at the same time.

Overall, the azide-derived fluorescence signal was higher than the ketone-derived fluorescence signal. There are multiple explanations for this observation. $Ac_4ManNAz$ may be converted to sialic acid more efficiently than $Ac_4ManLev$, producing more cell-surface azides than ketones. Consistent with this notion, the less sterically demanding *N*-propanoyl- and *N*-butanoylmannosamine are metabolized more efficiently to sialic acids than *N*-pentanoylmannosamine.[30] Alternatively, the rate of reaction could be faster for the Staudinger ligation than for hydrazone formation. It is unlikely that the difference is due to differential binding of anti-FLAG and avidin to their respective ligands as both interactions are known to take place rapidly and with high affinity. The amounts of the two unnatural monosaccharides were varied in order to compare their metabolic efficiencies. Although it is difficult to directly compare fluorescence signals for the aforementioned reasons, it was possible

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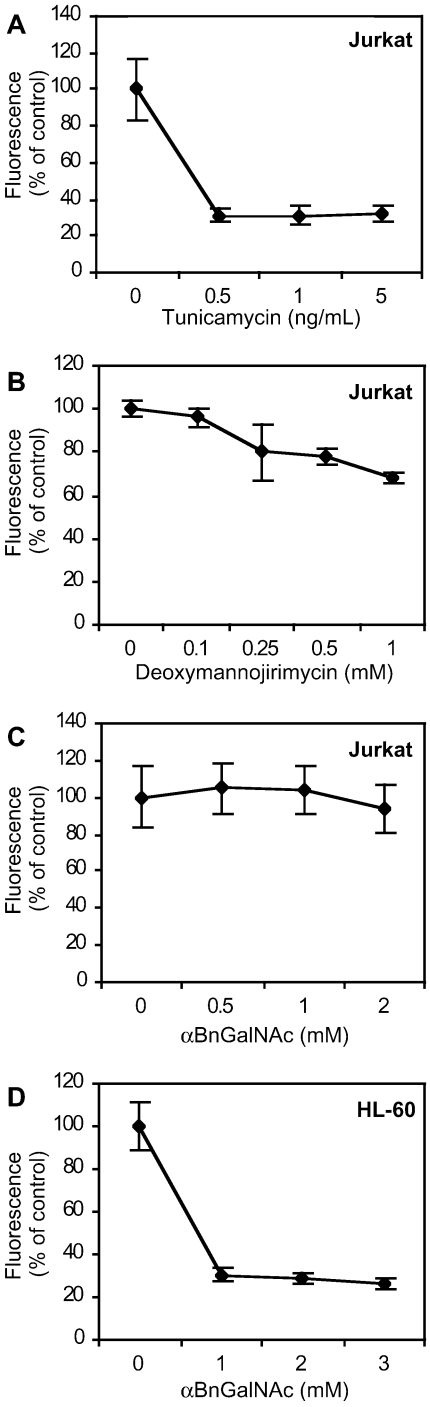

Figure 9. Effects of glycosylation inhibitors on azidosugar metabolism. The appropriate cell line was incubated in the presence or absence of 20 μM Ac_4ManNAz and various amounts of the appropriate inhibitor for 3 days, washed, and treated with 0.25 mM phosphine-FLAG for 1 h. The cells were washed again and stained with FITC-anti-FLAG. The fluorescence signal measured by flow cytometry is reported as percent of control, where the control represents the fluorescence signal from Jurkat cells incubated with 20 μM Ac_4ManNAz alone. Error bars represent the standard deviation for three replicate data points. (A) Treatment of Jurkat cells with tunicamycin. (B) Treatment of Jurkat cells with deoxymannojirimycin. (C) Treatment of Jurkat cells with αBnGalNAc. (D) Treatment of HL-60 cells with αBnGalNAc.

to compare changes in the two signals. The addition of 5 μM Ac_4ManLev caused the green fluorescence signal arising from 20 μM Ac_4ManNAz to decrease by 4%, whereas the reverse experiment reduced the red fluorescence signal by 43% (Figure 10). This suggests that Ac_4ManNAz is a better substrate for sialic acid biosynthesis than Ac_4ManLev, possibly due to its closer structural similarity to the native ManNAc. When the concentration of Ac_4ManLev was increased to 4 times that of Ac_4ManNAz, both red and green fluorescence were observed at comparable levels, confirming that the two ligation reactions are mutually orthogonal and may be performed concurrently on the same cell.

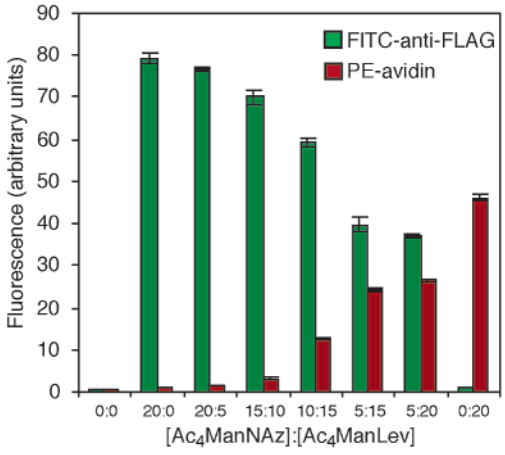

Figure 10. Ac_4ManNAz and Ac_4ManLev can be metabolized and labeled concurrently. Jurkat cells were incubated with various amounts of Ac_4ManNAz and Ac_4ManLev for 3 days, washed, and treated with 0.25 mM phosphine-FLAG and 2 mM biotin hydrazide for 1 h. The cells were washed again and stained with FITC-anti-FLAG and PE-avidin. The fluorescence signals measured by flow cytometry are reported in arbitrary units, and error bars represent the standard deviation for three replicates.

Our detailed understanding of the metabolism of Ac_4ManNAz to SiaNAz and the subsequent reaction with phosphine-FLAG sets the stage for the development of new in vivo applications. The structures and functions of native cell-surface glycans may be investigated via the selective installation of biochemical probes. Chemoselective ligation also provides a means to engineer the material properties of the cell surface, acting as a bridge between artificial materials and living cells. Artificial clustering of cell-surface epitopes to control intracellular signaling could be instigated by a bifunctional phosphine moiety. Together with ketone-based cell-surface engineering methodology, the Staudinger ligation provides a measure of chemical control over complex biological systems that was previously unknown.

Abbreviations

ManNAc, *N*-acetylmannosamine; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; ManNAz, *N*-azidoacetylmannosamine; GlcNAz, *N*-azidoacetylglucosamine; ManLev, *N*-levulinoylmannosamine; Ac_4ManNAz, peracetylated *N*-azidoacetylmannosamine; Ac_4GlcNAz, peracetylated *N*-azidoacetylglucosamine; Ac_4ManLev, peracetylated *N*-levulinoylmannosamine; SiaNAz, *N*-azidoacetylneuraminic acid; FBS, fetal bovine serum; PE, phycoerythrin; FITC, fluorescein isothiocyanate; TCEP, tricarboxyethylphosphine; P−S, penicillin−streptomycin; FLAG peptide, NH_2-DYKDDDDK-COOH; αBnGalNAc, α-benzyl-*N*-acetylgalactosamine.

Experimental Section

General. Phosphine-FLAG (**2**) was synthesized as previously reported.[14] Tunicamycin, deoxymannojirimycin, αBnGalNAc, GlcNAc, GalNAc, sialic acid, biotin hydrazide, Dulbecco's phosphate-buffered

saline (PBS), FITC-labeled avidin (stock concentration 2.8 mg/mL), and penicillin−streptomycin (P−S) were from Sigma. ManNAc was from Pfanstiehl. PE-labeled avidin (stock concentration 0.6 mg/mL) was from Caltag Laboratories. Sulfo-NHS-LC-LC-biotin and tricarboxyethylphosphine (TCEP) were from Pierce. FITC-labeled anti-FLAG M2 (stock concentration 1.8 mg/mL) was prepared according to a literature protocol[37] from anti-FLAG M2 (Sigma) and fluorescein isothiocyanate (Aldrich). RPMI medium 1640 was from Life Technologies, Inc., Dulbecco's modified Eagle's medium (DMEM) was from CellGro, and fetal bovine serum (FBS) was from HyClone Laboratory. Iscoves MEM medium and Ham F12 nutrient mixture were from Invitrogen. Cell densities were determined on a Coulter Counter-ZM. Flow cytometry analysis was performed on a Coulter Epics XL-MCL cytometer using a 488 nm argon laser. At least 10^4 viable cells were analyzed from each sample. Cell viability was ascertained by gating the samples on the basis of forward scatter (to sort by size) and side scatter (to sort by granularity). All cell-based experiments were performed in triplicate. The average of the mean fluorescence intensity obtained from each of the three replicate experiments was calculated to obtain a representative value in arbitrary units.

All chemical reagents were of analytical grade, obtained from commercial suppliers and used without further purification unless otherwise noted. Thin-layer chromatography was performed on Analtech Uniplate silica gel plates. Compounds were visualized by staining with ceric ammonium molybdate, by triphenylphosphine followed by ninhydrin (for azides), and/or by the absorbance of UV light. All ^{1}H and ^{13}C NMR spectra were measured with a Bruker AMX-300, AMX-400, or DRX-500 MHz spectrometer as noted. Chemical shifts are reported as δ relative to tetramethylsilane for ^{1}H and ^{13}C spectra and relative to H_3PO_4 for ^{31}P spectra. Coupling constants (J) are reported in hertz. Fast atom bombardment (FAB), chemical ionization (CI), and electrospray (ES) mass spectra were obtained at the UC Irvine and UC Berkeley Mass Spectrometry Laboratories. Elemental analyses were obtained at the UC Berkeley Microanalytical Laboratory. Infrared spectra were acquired on a Perkin-Elmer series Fourier transform infrared spectrometer.

Synthesis of Model Compounds. Methyl 2-(Diphenylphosphanyl)-benzoate (1).[38] To a flame-dried flask was added CH_3CN (21 mL), TEA (1.4 mL, 9.4 mmol), methyl 2-iodobenzoate (1.4 mL, 9.4 mmol), and palladium acetate (2.2 mg, 0.01 mmol). The mixture was degassed in vacuo before diphenylphosphine (1.6 mL, 9.4 mmol) was added to the flask via syringe under Ar. The resulting solution was heated at reflux for 4 h, at which point it was cooled to room temperature and concentrated. The residue was partitioned between 250 mL of 1:1 Et_2O/H_2O and the layers were separated. The organic layer was concentrated and dried over Na_2SO_4, and the product was purified via silica gel chromatography with elution by 50:1 EtOAc/hexanes. The pure product was obtained as 2.0 g (66%) of a white crystalline solid, mp 95−96 °C (lit. 96°C). IR (thin film): 3052, 3000, 2648, 2839, 1719, 1584 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.73 (s, 3H), 6.90−6.94 (m, 1H), 7.26−7.84 (m, 12H), 8.03−8.05 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 51.9, 128.2, 128.4, 128.5, 128.6, 130.7, 131.9, 133.8, 133.9, 134.2, 134.3, 137.8, 137.9, 140.3, 140.5, 167.2, 167.2. ^{31}P NMR (160 MHz, $CDCl_3$): δ −0.73. MS (CI) m/z 321.1 (MH^+). Anal. Calcd for $C_{20}H_{17}O_2P$: C, 74.99; H, 5.35. Found: C, 74.75; H, 5.42.

Benzyl Azide.[39] Benzylbromide (3.5 mL, 29 mmol) was dissolved in DMF (70 mL), and NaN_3 (2.9 g, 44 mmol) was added. After the solution was stirred at room temperature for 12 h, EtOAc (150 mL) was added. The solution was washed with H_2O (4 × 30 mL) followed by brine (2 × 30 mL), dried over Na_2SO_4, and concentrated. Remaining traces of DMF were removed by dissolving the crude product in H_2O and extracting with pentane. Evaporation of the pentane produced 3.5 g (91%) of a clear oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.35 (s, 2H), 7.31−7.42 (m, 5H). Lit. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.35 (s, 2H), 7.26−7.45 (m, 5H).

Compound 5. Methyl 2-(diphenylphosphanyl)benzoate (**1**, 32 mg, 0.099 mmol) and benzyl azide (13 mg, 0.099 mmol) were dissolved in CH_3CN/H_2O (3:1, 1 mL). The evolution of N_2 gas was observed immediately upon reactant mixing. The solution was stirred at room temperature for 2 h. Removal of the solvent provided the crude ligation product which was purified by SiO_2 column chromatography eluting with a gradient of 20−50% EtOAc in hexanes to provide 38 mg of a white solid (92%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.06 (d, 2H, J = 5.4), 7.06 (dd, 1H, J = 7.7, 14.4), 7.19−7.24 (m, 5H), 7.36 (t, 1H, J = 7.6), 7.45−7.48 (m, 4H), 7.54−7.66 (m, 7H), 8.00 (dd, 1H, J = 4.0, 7.6), 9.04 (br s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 44.1, 127.2, 128.0, 128.6, 128.7, 128.8, 129.8, 130.0, 130.5, 131.5, 131.7, 131.8, 131.8, 132.4, 132.4, 132.7, 132.7, 133.3, 133.4, 137.6, 141.0, 141.0, 167.3 (d, J_{C-P} = 3.92). ^{31}P NMR (160 MHz, $CDCl_3$): δ 39.56. HRMS calcd for $C_{26}H_{23}NO_2P$ (MH^+) 412.1466, found 412.1467.

^{31}P NMR Analysis of Reaction Intermediates. Methyl 2-(diphenylphosphanyl)benzoate (**1**, 32 mg, 0.099 mmol) and benzyl azide (13 mg, 0.099 mmol) were dissolved in wet CD_3CN (1 mL) and transferred to an NMR tube. The evolution of N_2 gas was observed immediately upon reactant mixing. The appearance of new ^{31}P resonances was monitored on a Bruker AMX-400 spectrophotometer. After 50 min of reaction time, water (10 μL) was added.

Cell Culture Conditions. All cells were cultured in media containing 2% P−S. Jurkat cells were grown in RPMI medium 1640 supplemented with L-glutamine and 5−10% FBS. HL-60 cells were grown in Iscoves MEM supplemented with L-glutamine, sodium pyruvate, and 20% FBS. CHO cells were grown in Ham F12 nutrient mixture supplemented with L-glutamine and 10% FBS. HeLa and COS-7 cells were grown in DMEM supplemented with 10% FBS. S49 cells were grown in RPMI medium 1640 supplemented with 1% sodium pyruvate and 10% FBS.

Treatment of Cells with ManNAc and GlcNAc Analogues. The appropriate amount of peracetylated compound in a solution of ethanol was added to 6-well tissue culture plates. After evaporation of the ethanol, cultures of cells were seeded at a density of 1.5×10^5 cells/mL in a total volume of 2 mL of culture medium. The cells were incubated for 3 days. Control wells containing no added sugar were also seeded with cells and incubated.

Azide Labeling Procedures. After growth in the presence of the appropriate monosaccharides, cells from each well were distributed among three wells of a 96-well V-bottom tissue culture plate. The cells were pelleted (3500 rpm, 3 min) and washed twice with 200 μL of labeling buffer (1% FBS in PBS, pH = 7.4). After the second wash, cells were typically resuspended in a volume of 50 μL of labeling buffer and 50 μL of **2** in solution (0.5 mM in PBS, pH = 7.4). After incubation at room temperature for 1 h, the cells were pelleted (3500 rpm, 3 min) and washed three times with ice-cold labeling buffer. Cells were then resuspended in 100 μL of FITC-anti-FLAG staining solution (1:900 dilution in labeling buffer). After a 30-min incubation in the dark at 4 °C, the cells were pelleted, washed with 200 μL of ice-cold labeling buffer, and then diluted to a volume of 400 μL for flow cytometry analysis. To determine optimal conditions, the concentration of **2**, incubation time, and pH of the reaction buffer were varied as described under Results and Discussion.

Labeling of Cell-Surface Amines. Cells were grown in the presence of the appropriate sugars, transferred to a 96-well V-bottom tissue culture plate, pelleted, and washed as described above. Reduction of cell-surface azides was accomplished by resuspending cells in 100 μL of TCEP solution (1 mM in labeling buffer) and incubating at room temperature for 1 h. Control populations of cells were exposed to either the normal azide labeling conditions or labeling buffer alone. After incubation, the cells were pelleted and washed twice with 200 μL of labeling buffer. Cells were then resuspended in 90 μL of labeling buffer,

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and 10 μL of sulfo-NHS-LC-LC-biotin solution (1 mg/mL in labeling buffer) was added. After a further 30-min incubation at room temperature, these cells were pelleted and washed twice with 200 μL of ice-cold labeling buffer. Cells were then resuspended in 100 μL of FITC-avidin staining solution (1:500 dilution in labeling buffer). After a 10-min incubation in the dark at 4 °C, the cells were pelleted, resuspended in 100 μL of FITC-avidin staining solution (1:500 dilution in labeling buffer) and incubated for an additional 10 min in the dark at 4 °C. This was followed by pelleting of the cells, washing with 200 μL of ice-cold labeling buffer, and dilution to a volume of 400 μL for flow cytometry analysis.

Inhibition Studies. The appropriate amount of **7** (20 μM) or **8** (40 μM) in a solution of ethanol was added to 6-well tissue culture plates. After evaporation of the ethanol, the desired amount of inhibitor was added to the wells. Tunicamycin, deoxymannojirimycin, and αBnGalNAc were added as ethanol solutions. Ethanol was allowed to evaporate before addition of cells to the plate. The monosaccharides (GlcNAc, GalNAc, ManNAc, and sialic acid) were added as PBS solutions followed by direct addition of the appropriate cells. Cultures of cells were seeded at a density of 1.5×10^5 cells/mL into a total volume of 2 mL of culture medium. The cells were incubated for 3 days. Control wells containing the peracetylated analogues alone were also seeded with cells and incubated.

Dual Labeling with Ac_4ManLev and Ac_4ManNAz. Cells were grown in the presence of the appropriate sugars (Ac_4ManLev and Ac_4ManNAz, 0−20 μM), transferred to a 96-well V-bottom tissue culture plate, pelleted, and washed as described above, with biotin buffer (0.1% FBS in PBS, pH = 6.5) in place of labeling buffer for the first wash. After the second wash, cells were resuspended in a volume of 50 μL of biotin hydrazide solution (4 mM in biotin buffer) and 50 μL of **2** in solution (4 mM in biotin buffer). After incubation at room temperature for 2 h, the cells were pelleted (3500 rpm, 3 min) and washed three times with 200 μL of ice-cold labeling buffer. Cells were then resuspended in 50 μL of FITC-anti-FLAG staining solution (1:450 dilution in labeling buffer) and 50 μL of PE-avidin staining solution (1:30 dilution in labeling buffer). After a 30-min incubation in the dark at 4 °C, the cells were pelleted, washed with 200 μL of ice-cold labeling buffer, and reexposed to the staining conditions for an additional 10 min. Finally the cells were pelleted, washed with 200 μL of ice-cold labeling buffer, and then diluted to a volume of 400 μL for flow cytometry analysis.

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Supporting Information Available: ^{31}P NMR spectra of model Staudinger ligation, graphs showing time course of cell-surface Staudinger ligation and dependence of cell-surface Staudinger ligation on the concentration of phosphine-FLAG (**2**), and experimental details for the synthesis of compounds **7**−**12** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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