# Chemical Reporter for Visualizing Metabolic Cross-Talk between Carbohydrate Metabolism and Protein Modification

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S *Supporting Information*

ABSTRACT: Metabolic chemical reporters have been largely used to study posttranslational modifications. Generally, it was assumed that these reporters entered one biosynthetic pathway, resulting in labeling of one type of modification. However, because they are metabolized by cells before their addition onto proteins, metabolic chemical reporters potentially provide a unique opportunity to read-out on both modifications of interest and cellular metabolism. We report here the development of a metabolic chemical reporter 1-deoxy-*N*-pentynyl glucosamine (1-deoxy-GlcNAlk). This small-molecule cannot be incorporated into glycans; however, treatment of mammalian cells results in labeling of a variety proteins and enables their visualization and identification. Competition of this labeling with sodium acetate and an acetyltransferase inhibitor suggests that 1-deoxy-GlcNAlk can enter the protein acetylation pathway. These results demonstrate that metabolic chemical reporters have the potential to isolate and potentially discover cross-talk between metabolic pathways in living cells.
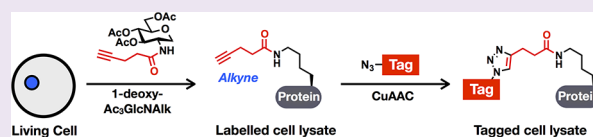
An increasing number of posttranslational modifications (PTMs) have been discovered that can have dramatic effects on the function (i.e., activity, localization, stability, etc.) of substrate proteins. To catalog and investigate these important modifications, a variety of chemical approaches have been developed to visualize and identify PTMs in cell lysates, living cells, and *in vivo*.[1,2] One of the most successful chemical technologies involves the biosynthetic incorporation of synthetic analogs of endogenous PTMs onto proteins in living cells or animals.[3] Typically, these metabolic chemical reporters (MCRs) contain unique chemical-functionalities that can undergo bioorthogonal reactions to install visualization or affinity tags. Until recently, research using MCRs has primarily focused on the end-point of their biosynthetic incorporation, namely, the specific PTM of interest. However, because MCRs must be metabolically transformed, typically into high-energy donor substrates [e.g., uridine-diphosphate (UDP) mono-saccharides or acetyl-CoA], they provide a direct opportunity to chemically track cellular metabolism. For example, we and others demonstrated that after the azide-containing MCR *N*-azidoacetyl glucosamine (GlcNAz) is metabolized into UDP-GlcNAz, it can be enzymatically converted to UDP *N*-azidoacetyl galactosamine (UDP-GalNAz), resulting in the incorporation into at least three classes of glycoproteins.[4,5] While this "metabolic crosstalk" is less than ideal for the analysis of a single type of glycosylation, it raises the possibility that MCRs could be used to isolate, analyze, and potentially discover different branching biosynthetic-pathways from common metabolic intermediates (Figure 1). For example, one recently discovered branching pathway involves metabolism from the *N*-acetyl glucosamine (GlcNAc) salvage pathway[6] to protein acetylation.[7] Specifically, Varki and co-workers demonstrated that the previously uncharacterized enzyme

amidohydrolase-domain-containing 2 (AMDHD2) converts GlcNAc-6-phosphate into glucosamine-6-phosphate and acetate.[8] This acetate might then be activated on CoA and subsequently used for protein acetylation. While it had been previously demonstrated that acetyl-CoA was required for de novo synthesis of UDP-GlcNAc from glucose through the hexosamine biosynthetic pathway,[9,10] these data reveal that under certain nutrient or metabolic conditions, cells may utilize scavenged GlcNAc for not only for the biosynthesis of glycans but also for other posttranslational modifications.

Here, we report the development of a MCR that isolates the metabolism of GlcNAc into posttranslational modifications that are not glycosylation. This MCR, termed 1-deoxy-GlcNAlk, builds upon our published chemical reporter for glycosylation, *N*-pentynyl glucosamine (GlcNAlk),[5] but structurally lacks the 1-hydroxyl group that is absolutely required for biosynthesis into the corresponding UDP-monosaccharide and subsequent incorporation into glycans. Treatment of a variety of cells with 1-deoxy-GlcNAlk, followed by copper-catalyzed azide–alkyne cycloaddition (CuAAC) with a fluorescent tag, resulted in differential labeling that is detectable in a majority of cell-lines. Notably, the intensity of this signal was inhibited by the addition of the acetyl-transferase inhibitor curcumin and competition with sodium acetate, suggesting that some of the protein labeling is a result of lysine acetylation. Furthermore, proteomic analysis using 1-deoxy-GlcNAlk identified 60 known acetylated proteins. Finally, labeling of the acetylated-proteins histones H1.1 and H2B was confirmed using in-gel fluorescence
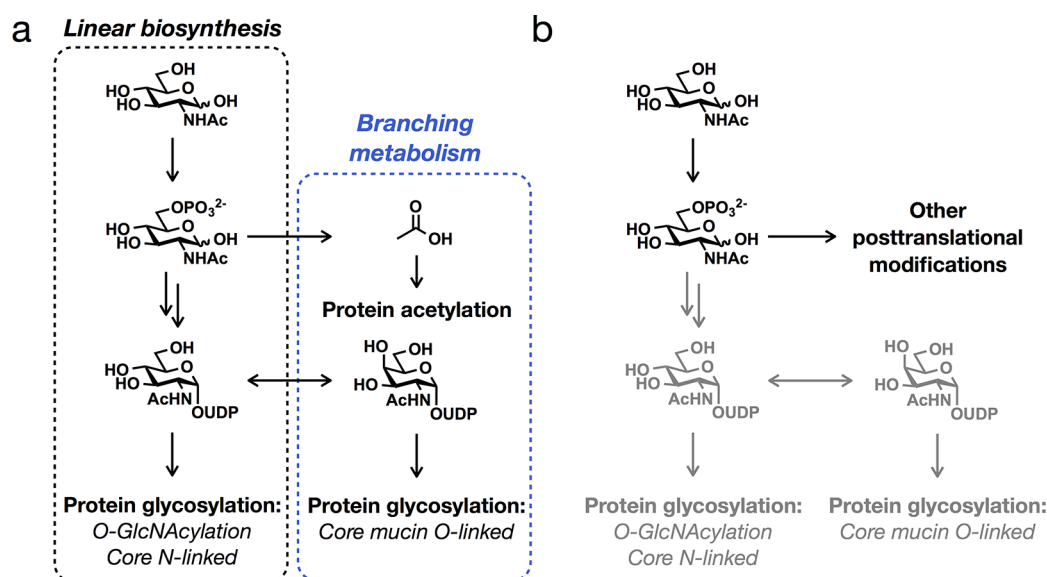
Figure 1. Using metabolic chemical reporters (MCRs) to detect cellular metabolism. (a) Salvaged *N*-acetyl glucosamine (GlcNAc) can enter a linear biosynthetic pathway that yields UDP-GlcNAc that can be directly incorporated onto glycoproteins. Additionally, GlcNAc metabolic intermediates can enter branching pathways to generate acetate and other monosaccharides. (b) MCRs have the potential to isolate branching metabolic pathways, such as the transformation of GlcNAc into acetate and subsequent acetylation of proteins.
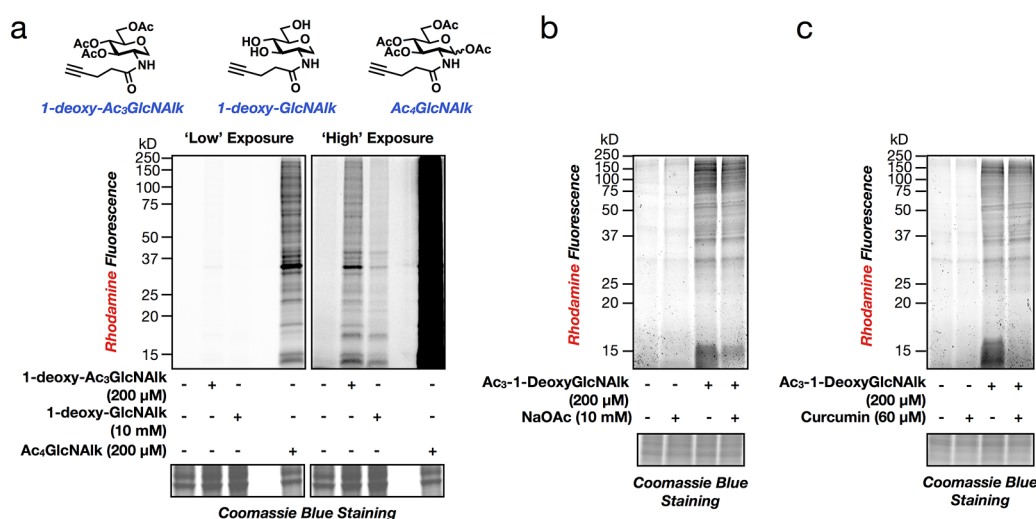
Figure 2. Characterization of proteins that are labeled by the MCR 1-deoxy-GlcNAlk. (a) NIH3T3 cells were treated with the indicated MCRs for 16 h before the corresponding lysates were subjected to CuAAC with az-rho and analyzed by in-gel fluorescence scanning. (b) NIH3T3 cells were treated with 1-deoxy-Ac₃GlcNAlk with or without sodium acetate for 6 h before CuAAC az-rho and in-gel fluorescence scanning. (c) NIH3T3 cells were pretreated with the protein acetyltransferase inhibitor curcumin for 30 min before addition of 1-deoxy-Ac₃GlcNAlk for an additional 5.5 h. Labeled proteins were then visualized using in-gel fluorescence scanning following CuAAC with az-rho. Coomassie blue staining shows equal loading.

scanning. These data demonstrate that 1-deoxy-GlcNAlk is a MCR of protein modification and more importantly suggest that MCRs can be used to characterize and potentially discover branching metabolic-pathways in living cells.

To create a MCR capable of isolating the cellular metabolism of GlcNAc into protein modifications that are not glycosylation, we synthesized a structural analog of our previously published glycoprotein MCR, GlcNAlk (Supporting Information Scheme 1).[5] This analog, 1-deoxy-GlcNAlk, lacks the 1-hydroxyl group and therefore cannot enter any glycosylation metabolic-pathways. Additionally, we generated the per-acetylated derivative, 1-deoxy-Ac₃GlcNAlk, as the acetates allow for passive diffusion of the MCR into living cells where they are

subsequently removed by esterases.[11] NIH3T3 cells were treated with either 1-deoxy-Ac₃GlcNAlk (200 μM), 1-deoxy-GlcNAlk (10 mM), or Ac₄GlcNAlk (200 μM) as a positive control. After 16 h, the corresponding cell-lysates were subjected to the bioorthogonal reaction copper-catalyzed azide−alkyne cycloaddition (CuAAC) with a fluorescent tag, azido-rhodamine (az-rho). In-gel fluorescence scanning revealed that both versions of the 1-deoxy MCR were robustly incorporated onto proteins (Figure 2A), albeit at a lower level than the highly efficient GlcNAlk. The per-acetylated MCR, 1-deoxy-Ac₃GlcNAlk, was incorporated more efficiently than 1-deoxy-GlcNAlk, consistent with other MCRs,[11] and was therefore used in all our subsequent experiments.

We and others have previously demonstrated that protein labeling by certain MCRs can be competed by the availability of specific nutrients in cell culture. For example, MCRs that largely read out on the intracellular glycosylation O-GlcNAc modification can be competed by increasing glucose concentrations,[5] and increasing the amount of serum can inhibit the incorporation of radio-labeled glucosamine.[12] To investigate the sensitivity of 1-deoxy-GlcNAlk to different cell-culture conditions, NIH3T3 cells were treated with 1-deoxy-Ac₃GlcNAlk (200 μM) in the presence of low or high glucose concentrations (1.0 vs 4.5 g/mL) or three different amounts of serum (0, 2 or 10% v/v). In-gel fluorescence scanning, following lysis and CuAAC with az-rho, demonstrated that 1-deoxy-GlcNAlk labeling is largely insensitive to these different culture conditions (Supporting Information Figure 1A).

To determine if any 1-deoxy-GlcNAlk labeling could be attributable to protein acetylation, we used sodium acetate and the p300-specific acetyltransferase inhibitor curcumin.[13] NIH3T3 cells were treated with or without sodium acetate (10 mM) and 1-deoxy-Ac₃GlcNAlk (200 μM) for 6 h. In-gel fluorescence showed that sodium acetate was able to compete 1-deoxy-GlcNAlk labeling (Figure 2B). To investigate whether any observed protein acetylation by 1-deoxy-GlcNAlk is enzymatic in nature, NIH3T3 cells were pretreated with curcumin (60 μM) for 30 min prior to treatment with 1-deoxy-Ac₃GlcNAlk (200 μM) for 5.5 h. Cell lysates were then subjected to CuAAC with az-rho and analyzed by in-gel fluorescence scanning (Figure 2C). Curcumin-treated cells also showed reduced 1-deoxy-GlcNAlk labeling compared to controls. Notably, in both of these experiments, the effect on the labeling of different proteins was not uniform. For example, labeled proteins in the region of histones and other small proteins (∼15 kDa) are more sensitive to both competition by sodium acetate and curcumin treatment. Together, these data suggest that pentynoic acid is likely removed from 1-deoxy-GlcNAlk, where it is know to be enzymatically incorporated into protein acetylation[14] and potentially other protein modifications. We next directly compared 1-deoxy-Ac₃GlcNAlk to the known acetylation reporter sodium pentynoate.[14] Specifically, NIH3T3 cells were treated with 1-deoxy-Ac₃GlcNAlk (200 μM) or sodium pentynoate (200 or 5000 μM) for 8 h. Visualization of the labeled proteins by in-gel fluorescence showed that sodium pentynoate is a more efficient MCR, even at equal concentrations (Supporting Information Figure 1B). Notably, the pattern of proteins that are labeled by 1-deoxy-GlcNAlk and pentynoate are also different. Together, these data suggest that while at least some of the proteins that become modified by 1-deoxy-Ac₃GlcNAlk treatment are acetylated, its metabolism and/or distribution into different types of posttranslational modification (e.g., acetylation vs long-chain fatty acylation) are different than sodium pentynoate.

To further characterize this MCR, NIH3T3 cells were treated with various concentrations of 1-deoxy-Ac₃GlcNAlk for 16 h prior to lysis and CuAAC with az-rho. In-gel fluorescence scanning showed that proteins are dose-dependently labeled by 50−200 μM 1-deoxy-Ac₃GlcNAlk treatment (Supporting Information Figure 2A). To determine the kinetics and dynamics of 1-deoxy-GlcNAlk labeling, we next performed pulse and pulse-chase experiments. We first treated NIH3T3 cells with 1-deoxy-Ac₃GlcNAlk (200 μM) for different lengths of time. After lysis and CuAAC with az-rho, in-gel fluorescence scanning revealed protein labeling in as little as 2 h, with similar kinetics to other direct MCRs of protein acetylation

(Supporting Information Figure 2B).[14] NIH3T3 cells were then treated with 1-deoxy-Ac₃GlcNAlk (200 μM) for 16 h, after which time the growth medium was replaced with fresh media containing 1-deoxy-N-acetyl glucosamine (1-deoxy-Ac₃GlcNAc, 200 μM). In-gel fluorescence scanning after CuAAC revealed a time-dependent loss of signal (Supporting Information Figure 2C). To ascertain the generality of 1-deoxy-GlcNAlk as a MCR, a small panel of cell-lines were treated with 1-deoxy-Ac₃GlcNAlk (200 μM) for 16 h before lysis and reaction with az-rho using CuAAC. In-gel fluorescence scanning showed labeling of proteins in each of the cell-lines tested (Figure 3).
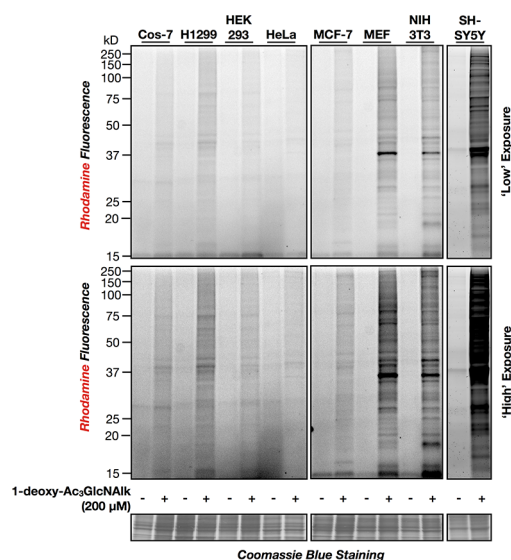
Figure 3. Generality of 1-deoxy-GlcNAlk labeling. The indicated cell lines were treated with 200 μM 1-deoxy-Ac₃GlcNAlk for 16 h before modified proteins were subjected to CuAAC with az-rho and in-gel fluorescent scanning.

To determine if treatment of cells with the MCR resulted in any toxicity, NIH3T3 cells were treated with either 1-deoxy-Ac₃GlcNAc (200 μM), 1-deoxy-Ac₃GlcNAlk (200 μM), or DMSO vehicle. After 24 or 48 h of treatment, the viability of the cells was measured using a commercially available MTS assay (Supporting Information Figure 3). No toxicity was observed with 1-deoxy-Ac₃GlcNAlk treatment, despite some toxicity with the control compound 1-deoxy-Ac₃GlcNAc.

Finally, we performed a large-scale mass spectroscopy experiment to identify proteins labeled by 1-deoxy-GlcNAlk and directly compare them to those modified by our published MCR GlcNAlk. NIH3T3 cells were treated in triplicate with 1-deoxy-GlcNAlk (200 μM), GlcNAlk (200 μM), or GlcNAc (200 μM) as a negative control for 16 h. Treated cells were pelleted and lysed with a denaturing buffer (4% SDS). Protein concentration was normalized, and 10 mg of protein was subjected to CuAAC with an azide-functionalized biotin affinity-tag. The biotinylated samples were enriched with streptavidin beads, washed extensively, and subjected to on-bead trypsin digestion, and the recovered peptides were subjected to LC-MS/MS analysis. Proteins were identified using Proteome Discover and Mascot and curated using the following criteria to identify "hits": (1) Proteins must be identified in all 3 runs (at least 1 spectral count per run) with a sum of at least 4 spectral-counts overall. (2) The sum of the spectral counts must be 4-fold greater in the 1-deoxy-GlcNAlk or GlcNAlk samples than the GlcNAc-treated samples. (3) The
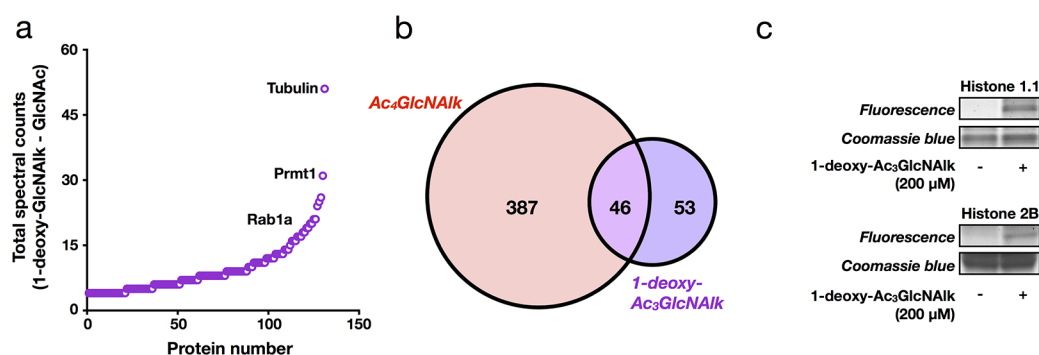
Figure 4. Identification of posttranslationally modified proteins using 1-deoxy-GlcNAlk. (a) NIH3T3 cells were treated with 1-deoxy-Ac$_3$GlcNAlk, Ac$_4$GlcNAlk, or Ac$_4$GlcNAc (all at 200 μM concentration) for 16 h. Proteins identified by LC-MS/MS are graphically presented as total number of positive minus total number of control spectral counts. Three known acetylated proteins are annotated in black. (b) Overlap between proteins identified using 1-deoxy-Ac$_3$GlcNAlk and Ac$_4$GlcNAlk. (c) Enriched histones from NIH3T3 cells labeled with 1-deoxyAc$_3$GlcNAlk or DMSO vehicle were subjected to CuAAC with az-rho. In-gel fluorescence reveals modification of histones H1.1 and H2B.

number of spectra counts in the MCR-treated sample compared to the control must be statistically significant (p-value <0.05, t test). Following these requirements, we identified 99 proteins modified by 1-deoxy-GlcNAlk (Figure 4A and Supporting Information Table 1) and 433 proteins modified by GlcNAlk (Supporting Information Figure 4 and Table 2). Of the 1-deoxy-GlcNAlk labeled proteins, 60 have been previously identified as acetylated proteins, including those annotated in Figure 4A, supporting this MCR as a reporter of acetylation. Forty-six proteins identified using 1-deoxy-GlcNAlk were also present in the GlcNAlk treated samples (Figure 4B and Supporting Information Tables 1 and 2), suggesting that these proteins are either simultaneously modified by O-GlcNAc glycosylation or that GlcNAlk can also be metabolized through an "off-target" pathway. Notably, 16 of these overlapping proteins are known acetylated proteins, suggesting that both MCRs may be metabolized into the protein acetylation pathway. The proteins identified using 1-deoxy-GlcNAlk also contained 39 previously uncharacterized substrates (Supporting Information Table 1), suggesting that this MCR can be used to find new modification (e.g., acetylation) events. To confirm 1-deoxy-GlcNAlk labeling of known acetylated proteins, histones H1.1 and H2B,[7] histones were enriched from NIH-3T3 cells treated with 1-deoxy-Ac$_3$GlcNAlk (200 μM) or 1-deoxy-Ac$_3$GlcNAc (200 μM) as negative control for 16 h using acid precipitation.[15] Purified histones were then subjected to CuAAC with az-rho and in-gel fluorescence scanning confirmed labeling of H1.1 and H2B (Figure 4C and Supporting Information Figure 5).

Bioorthogonal chemistries have enabled the creation of MCRs for the visualization and enrichment of a wide array of PTMs[3] including glycosylation,[5,16,17] lipidation,[18] methylation,[19] and different forms of acetylation.[14,20] Because MCRs must be metabolized by living cells, they provide unique opportunities to simultaneously interrogate a certain PTM and the upstream metabolic and biosynthetic pathways. We have demonstrated that alterations in the chemical structure of a MCR can impact its acceptance into different glycosylation pathways.[5] Building upon those results, we synthesized and characterized an MCR (1-deoxy-GlcNAlk) that reports on the metabolic crosstalk between the GlcNAc salvage pathway and nonglycosylation modifications on proteins. Using a fluorescent azide-tag, in combination with CuAAC, we demonstrated that 1-deoxy-GlcNAlk treatment results in labeling of a range of proteins in different cell lines. The labeling intensities in these

cell-lines varies dramatically, raising the possibility that MCRs could be used to classify metabolic flux in different cells. Co-treatment with different nutrient sources and an inhibitor of protein acetyltransferases showed that 1-deoxy-GlcNAlk labeling is competed by exogenous acetate and acetyltransferase inhibition. This demonstrates that at least some 1-deoxy-GlcNAlk enters the protein acetylation pathway. This is further supported by our proteomic identification of 60 previously identified, acetylated proteins, which account for ~60% of the total proteins identified. The most likely pathway responsible for these observations is the one identified by Varki and co-workers mentioned above.[8] In this case, 1-deoxy-GlcNAlk would be phosphorylated and then deacetylated by the enzyme AMDHD2 to generate pentynoic acid, although this remains to be experimentally confirmed.

However, not all of the labeled proteins were equally susceptible to competition by sodium acetate or inhibition of the p300 acetyltransferase (Figure 2B and C). In the case of sodium acetate competition, the intensity of all of the labeled proteins is reduced; however, the proteins at ~15 kDa molecular weight display a more dramatic effect. This difference could be attributable to acetylation dynamics. Rapidly cycling acetylation marks, such as those on the core histones that are found around 15 kDa,[21,22] could be more sensitive to competition by excess sodium acetate. In contrast, any long-lived pentynyl-modification events could persist throughout the experiment. Likewise, treatment with curcumin resulted in dramatic reduction of the labeling of proteins at low molecular weights but less-so for other proteins. Since curcumin is a specific inhibitor of the p300 acetyltransferase,[13] the proteins that show no change in labeling intensity might be modified by other acetyltransferases. We next directly compared 1-deoxy-GlcNAlk with pentynoic acid. At equal concentrations, 1-deoxy-GlcNAlk is significantly less efficient at labeling proteins, and pentynoate-labeling can be performed at higher concentrations to maximize incorporation (Supporting Information Figure 1B). Interestingly, 1-deoxy-GlcNAlk and pentynoate treatment resulted in the visualization of different patterns of proteins. This demonstrates that 1-deoxy-GlcNAlk is not a simple replacement of a known MCR of protein acetylation.[14] The differences between the two MCRs could simply arise from changes in their metabolism. For example, if the two MCRs are metabolized at different rates, a different subset of proteins could be modified after the same length of labeling. It is also possible that the two MCRs are incorporated into different

types of posttranslational modifications. For example, short-chain fatty acid reporter could be metabolized into the corresponding lipid-reporter (e.g., palmitoylation).[23] It is also possible that either pentynoate or 1-deoxy-GlcNAlk is metabolized into an unknown, nonacetylation pathway that contributes to some of the signal, or results in nonenzymatic modification of proteins.[24,25]

Finally, to compare 1-deoxy-GlcNAlk to a glycoprotein MCR, we performed a proteomics experiment using 1-deoxy-GlcNAlk and GlcNAlk. Enrichment with 1-deoxy-GlcNAlk resulted in the identification of 99 proteins. Treatment with GlcNAlk resulted in the identification of a large number of O-GlcNAc modified proteins and 64 proteins that overlapped with the 1-deoxy-GlcNAlk sample. Notably, 16 of these proteins were also previously identified as being acetylated. This raises the likely possibility that any glycoprotein MCR bearing its chemical functionality at the N-acetyl position will read-out on some acetylated proteins. Therefore, care should be taken to confirm the glycosylation of candidate proteins identified using these reporters.

In summary, our competition, inhibition and proteomics experiments support the conclusion that a large fraction of 1-deoxy-GlcNAlk is metabolized into the protein acetylation pathway. We cannot definitively rule out the incorporation of our MCR into other types of protein modifications but believe that our data demonstrates the unique utility of chemical synthesis to develop new MCRs that can be used to visualize cellular metabolism in addition to their traditional roles as probes of posttranslational modifications. Given the resurgent importance of cellular metabolism in human disease (e.g., diabetes and cancer), we believe that these tools can provide important and exact information on the transformation of metabolites to PTMs where they can directly effect protein function.

■ METHODS

See Supporting Information for detailed description of experimental methods.

■ ASSOCIATED CONTENT

⑤ Supporting Information

Synthetic and biochemical methods for the generation and characterization of the metabolic chemical reporter, additional data figures, and proteomic identification of labeled proteins. 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.

■ ACKNOWLEDGMENTS

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