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6″-Azido-6″-deoxy-UDP-*N*-acetylglucosamine as a glycosyltransferase substrate

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ABSTRACT

6″-Azido-6″-deoxy-UDP-*N*-acetylglucosamine (UDP-6Az-GlcNAc) is a potential alternate substrate for *N*-acetylglucosaminyltransferases. This compound could be used to generate various glycoconjugates bearing an azide functionality that could in turn be subjected to further modification using Staudinger ligation or Huisgen cycloaddition. UDP-6Az-GlcNAc is synthesized from α-benzyl-*N*-acetylglucosaminoside in seven-steps with an overall yield of 6%. It is demonstrated to serve as a substrate donor for the glycosyl transfer reaction catalyzed by the human UDP-GlcNAc:polypeptidyltransferase (OGT) to the acceptor protein nucleoporin 62 (nup62).

Carbohydrates bearing bioorthogonal chemical reporters have been extensively used to generate glycoconjugates or polysaccharides that can be probed or modified using specific chemical reactions.[1] In order to do this the modified sugar must first be activated as a sugar nucleotide, which may then serve as an alternate substrate for a glycosyltransferase. This has been accomplished in vivo using free sugars lacking the nucleotide diphosphate that are cell permeable.[2–11] Upon entering the cell, these sugars are converted into sugar nucleotide donors by the cell's catalytic machinery, and then transferred onto their appropriate cellular targets. Transfer by a glycosyltransferase has also been accomplished in vitro using non-cell permeable sugar nucleotide donors and either cellular lysates or purified substrates/enzymes.[12–17,8] The most common chemical reporter that has been employed is the azide functionality as it can be chemically modified via either the Staudinger ligation or the Huisgen's cyclization (click chemistry).[18,19] Much of the work to date has focused on amino sugars, such as *N*-acetylglucosamine and *N*-acetylneuraminic acid, as these are common components of many biologically important glycoconjugates. In these cases, the azide functionality has typically been incorporated into the *N*-acetamido functionality as an *N*-azidoacetyl substituent.[12,14,7–10] One potential problem with this approach is that the *N*-acetamido functionality may serve as a key recognition element for glycosyltransferases, and the extra steric bulk of the azide group may preclude such sugar nucleotides from acting as substrates. In order to expand the tool kit available to the chemical glycobiologist we decided to prepare 6″-deoxy-6″-azido-UDP-*N*-acetylglucosamine **1** (UDP-6Az-GlcNAc) and to test its suitability as an alternate *N*-acetylglucosaminyltransferase substrate (Scheme 1). We reasoned that the replacement of a hydroxyl group by an azide group is less sterically demanding than the replacement of a hydrogen atom, and that the 'remote' positioning of the label might be more forgiving in certain instances.

The glycosyltransferase reaction we chose to probe was that of the human UDP-GlcNAc:polypeptidyltransferase (OGT).[20–23] This enzyme is responsible for the glycosylation of many cellular protein targets at specific serine and threonine residues with an *O*-linked-*N*-acetylglucosamine residue (*O*-GlcNAc). This posttranslational modification has been identified on a wide variety of proteins including transcription factors, nuclear pore proteins, and enzymes. In certain cases, the site of glycosylation coincides with that of phosphorylation indicating that reversible glycosylation may play a role in cellular signaling. The protein substrate we chose to analyze is the nuclear pore protein p62 (nup62).[24–26] It is well established that this protein is extensively modified with as many as 10 *N*-acetylglucosamine residues by the action of OGT.

We chose to use a purely chemical route for the synthesis of UDP-6Az-GlcNAc **1** starting from the α-benzylglycoside of *N*-acetylglucosamine **2** (Scheme 1). A one-pot tosylation of the 6-hydroxyl followed by peracetylation gave compound **3** in a 75% yield. The anomeric center of compound **3** was deprotected via hydrogenolysis and the crude product was immediately subjected to phosphitylation with dibenzyl *N*,*N*-diisopropyl phosphoramidite and then oxidation with mCPBA. This gave the α-dibenzylphosphate **4** in an overall 70% yield. It should be noted that any β-dibenzylphosphate that forms in this procedure readily decomposes due to neighboring group participation from the acetamido func-

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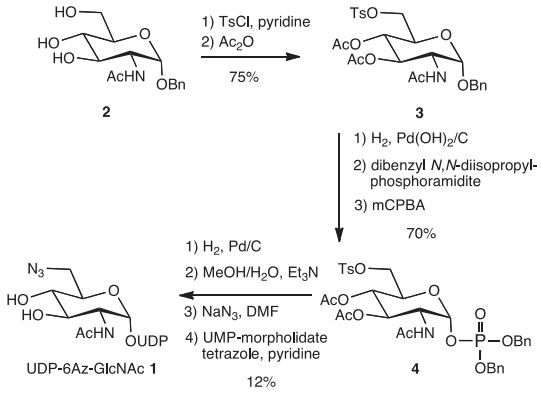

Scheme 1.

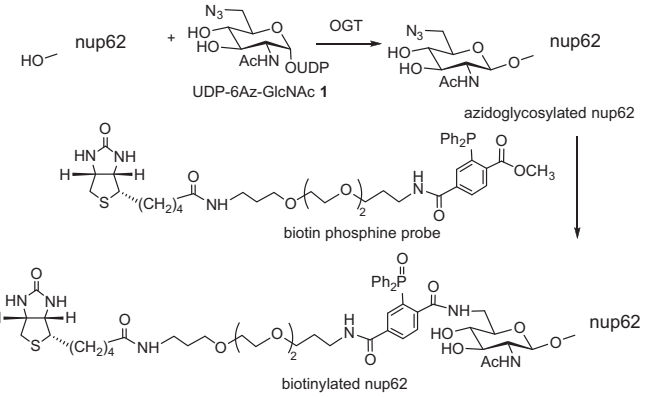

Figure 1. OGT can use UDP-6Az-GlcNAc as a substrate donor to transfer 6Az-GlcNAc onto the protein acceptor nup62. The azido moiety undergoes the Staudinger ligation upon treatment with a biotin phosphine probe. The biotin moiety can be detected by Western blot using horseradish peroxidase-conjugated streptavidin.

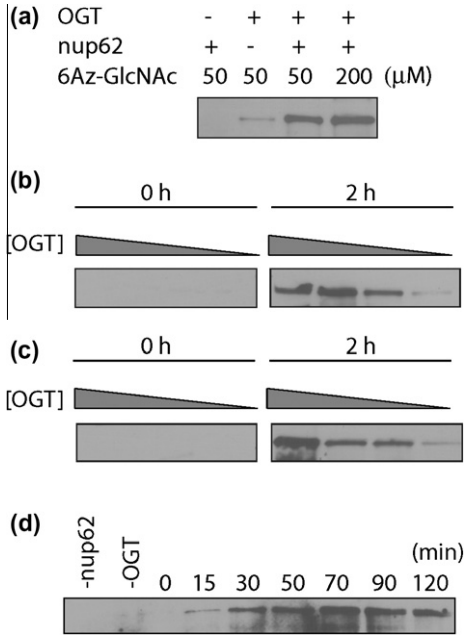

Figure 2. OGT can utilize UDP-6Az-GlcNAc as a substrate to modify nup62 with 6Az-GlcNAc in vitro. (a) Western blot analysis of OGT modification of nup62 with 6Az-GlcNAc (at a concentration of 50 μM or 200 μM). (b) Western blot analysis of OGT modification of nup62 with 6Az-GlcNAc at varying concentrations of OGT (from 288 nM to 36 nM, left to right). OGT activity was terminated using 100 mM UDP at either 0 h (left) or 2 h (right) prior to incubation with the biotin phosphine. (c) Western blot analysis of OGT modification of nup62 with 6Az-GlcNAc at varying concentrations of OGT (from 288 nM to 36 nM, left to right). OGT activity was terminated by boiling the sample for 5 min at either 0 h (left) or 2 h (right) prior to incubation with the biotin phosphine. (d) Western blot analysis of OGT modification of nup62 with 6Az-GlcNAc to demonstrate it occurs in a time-dependent manner. In all cases, the blots were probed with streptavidin, which binds to the biotin; the biotin is conjugated to a phosphine probe, which had undergone the Staudinger ligation with the azido group of the 6Az-GlcNAc prior to gel analysis.

tionality at C-2.[27] Thus, it is not necessary to separate anomeric phosphates. Dibenzylphosphate **4** was then converted to UDP-6Az-GlcNAc **1** in a four-step procedure without purification of the intermediate compounds. Hydrogenolysis followed by treatment with triethylamine in water/methanol was used to deprotect the phosphate and hydroxyl groups, respectively.[28] Treatment with sodium azide in DMF led to the displacement of the tosyl group and incorporation of the azide at C-6. This was left to a late stage in the synthesis to avoid problems with performing hydrogenolysis in the presence of an azide functionality. Finally, treatment with UMP-morpholidate and tetrazole generated UDP-6Az-GlcNAc **1**.[29] Following anion exchange and size exclusion chromatography, a sample of compound **1** was obtained that contained an impurity of 6Az-GlcNAc 1-phosphate due to incomplete coupling. Treatment with alkaline phosphatase was used to dephosphorylate the impurity and a second round of ion exchange chromatography provided compound **1** in a pure form with an overall yield of 12% from compound **4**. It should be noted that a recent report has described the chemoenzymatic synthesis of compound **1** from 6-deoxy-6-azido-*N*-acetylglucosamine.[30,31] Cai et al. used the *N*-acetylglucosamine 1-kinase NahK and ATP to introduce the anomeric phosphate and then used the *N*-acetylglucosamine 1-phosphate uridyltransferase GlmU and UTP to introduce the UDP functionality. While their chemoenzymatic approach is attractive due to the facile introduction of the phosphate functionalities, our purely synthetic approach may be preferable to researchers who do not wish to clone and express the NahK and GlmU enzymes. Overall, the two approaches require a similar number of overall steps and result in a similar overall yield of pure product.

UDP-6Az-GlcNAc was assessed for its ability to be used as a substrate donor for OGT in vitro using nup62 as an acceptor. Assays were performed at pH 7.4 and 37 °C and typically allowed to proceed for 1 h (over which time OGT activity has been shown to be linear). Following incubation of UDP-6Az-GlcNAc with OGT and nup62, the reaction was terminated, and then incubated with a biotin phosphine probe (Fig. 1).[10] The reactive azido group of the 6Az-GlcNAc undergoes the Staudinger ligation with the phosphine, to generate biotin-labeled nup62. Western blot analysis, where the biotin group could be probed with horseradish peroxidase-conjugated streptavidin, allowed visualization of the 6Az-GlcNAc transfer onto nup62.

Western blot analysis demonstrated that OGT was capable of utilizing UDP-6Az-GlcNAc as a substrate and does indeed transfer 6Az-GlcNAc onto nup62 (Fig. 2a), whereas in the absence of either OGT or nup62 there is little or no signal. Given that no signal is seen in lane 1 of Figure 2d (no nup62), the faint band in lane 2 of Figure 2a (no nup62) likely stems from trace sample spill-over from lane 3. The signal, which correlates with the amount of 6Az-GlcNAc transferred, increases when a higher concentration of UDP-6Az-GlcNAc is used in the assay. Two ways to terminate OGT activity prior to incubation with the biotin phosphine probe were tested; by boiling the assay mixture for 5 min to precipitate the proteins or by addition of 100 mM UDP (UDP has previously been shown to be a good inhibitor of OGT, with a reported IC_{50} of 1.8 μM).[32] Both methods were shown to work well to terminate OGT activity over a range of OGT concentrations (Fig. 2b,c, 0 h time points), with no detectable signal following incubation with the

biotin phosphine probe (which is performed at 37 °C for 1.5 h). Using these methods, we were able to demonstrate that the degree of 6Az-GlcNAc modification was dependent on the enzyme concentration (Fig. 2b,c, 2 h time points). In addition, we were able to show that the modification of nup62 with 6Az-GlcNAc by OGT occurred in a time-dependent manner (Fig. 2d), with maximum modification reached by approximately 70 min.

In summary, we were able to demonstrate that UDP-6Az-GlcNAc serves as an alternate substrate for a key human *N*-acetylglycosaminyltransferase, OGT. This provides an attractive alternative to the use of *N*-azidoacetamido-substituted sugars and could be particularly useful in cases where such compounds do not serve as substrates. While both types of azide-substituted sugar donors are accepted by OGT, it is likely that this will not be the case for all *N*-acetylglucosaminyltransferases, and that certain transferases will not tolerate modifications to the acetamido moiety. In fact, initial results with certain glycosaminoglycan synthases have indicated that UDP-6Az-GlcNAc serves as an alternate substrate, whereas UDP-*N*-azidoacetylglucosamine (UDP-GlcNAz) does not (P.L. DeAngelis and R.J. Linhardt, personal communication). One disadvantage to the approach of installing the azide at the C-6 position, however, is that one cannot feed the free 6Az-GlcNAc to mammalian cells and expect that the UDP moiety will be installed in vivo. This is because the conversion of GlcNAc into UDP-GlcNAc first requires a phosphorylation of the C-6 hydroxyl group by *N*-acetylglucosamine 6-kinase. Thus, this technology will be largely restricted to in vitro labeling studies.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.12.090.

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