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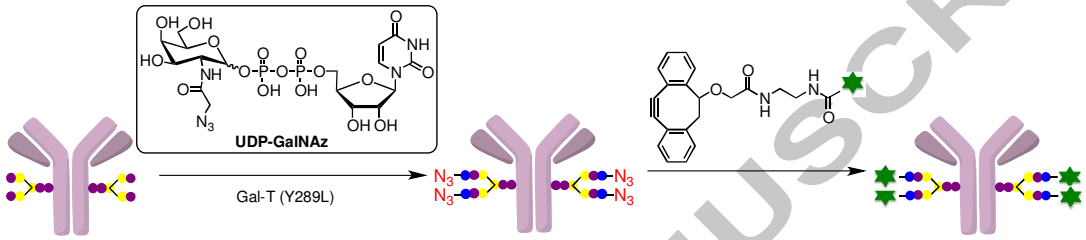

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A chemoenzymatic approach toward the preparation of site-specific antibody-drug conjugates

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ABSTRACT

An efficient chemical synthesis of UDP-*N*-azidoacetylgalactosamine (UDP-GalNAz) is presented, while the value of this molecule was demonstrated through its attachment to an antibody Fc domain. Thus, the antibody was first degalactosylated, which was followed by loading of the UDP-GalNAz with a recombinant galactosyltransferase. This engineered Azide-Fc-*N*-glycan antibody was subsequently "clicked" by a strain-promoted alkyne-azide cycloaddition reaction for site-specific attachment of a fluorescent probe. The principles detailed will allow for the facile preparation of chemically defined homogeneous antibody-drug conjugates (ADCs).

Bio-orthogonal chemical reporting has emerged as an important strategy for the labeling and probing of glycoproteins *in vitro* or *in vivo*.[1-3] The premise of this method is the enzymatic or metabolic labeling of a glycan with a monosaccharide precursor that has been modified with a functional group that is readily "clickable".[4,5] Azides, which are absent in the biological sphere, are virtually inert *in vivo*; however, they are effective partners with reagents such as phosphines [6] and alkynes.[7-9] Augmenting their biotherapeutic value can come via an azido-sugar; hence, utilizing such reagents glycans can be covalently labeled in a second step with appendages bearing imaging agents or epitope tags. Using a two-step logic, i.e., azido-sugar incorporation followed by chemical labeling, allows for the preparation of structurally defined and homogenous glycoproteins as therapeutic agents or critical tools for glycobiology. Herein, we explored this two-step strategy for the preparation of antibody-drug conjugates with the goal of precise control over the site and stoichiometry of the bio-conjugation.

Antibody-drug conjugates (ADCs) have been proven clinically to be more effective anti-cancer agents than native antibodies.[10-12] ADC technology exploits the specificity of a monoclonal antibody against a tumor-specific antigen for the targeted delivery of highly potent, yet, non-discriminate cytotoxic agent. The tumor target, the cytotoxic agent, and the manner in which the agent is attached to the antibody are key determinants for clinical activity and tolerability of ADCs. Specifically, the method of attachment of the linker and cytotoxic agent to the monoclonal antibody (mAb) have been shown to influence ADC efficacy, including aggregation, antigen binding, pharmacokinetics and tolerability.[13-15] In the past, classical conjugation chemistries have been engaged to prepare ADCs, typically through targeting primary amines or hinge disulfides found with an antibody Fc region, yet, these strategies have a number of shortcomings including linkage instability and heterogeneous product profiles that differ in the site and stoichiometry of modification.[16-21] A goal then is to identify broadly applicable, site-specific conjugation strategies that can reduce product heterogeneity and improve the therapeutic properties of ADCs.

We began our chemoenzymatic approach to prepare well-defined antibody-drug conjugates through the targeting of native glycosylation sites found within an immunoglobulin G antibody (IgG) (Figure 1). Thus, all IgGs contain a singularly conserved *N*-linked glycosylation site (Asn297) within each heavy chain of the Fc region. Since IgG glycosylation sites are well removed from the antigen-binding domain, they have been examined as a popular site for antibody-chemical modification.[22] Indeed, one of the most common modification strategies is based upon the oxidation of vicinal alcohols embedded within the sugar chain to an aldehyde; this then followed by subsequent labeling via reductive amination or hydrazide condensation.[23,24] The liabilities associated with this approach include harsh reaction conditions, low conversion yields and non-specific labeling. This then has contributed to the stifling of oligosaccharide modification as a general strategy for the preparation of ADCs.

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A strategy that has revitalized antibody-oligosaccharide ADCs is based on enzymatic labeling of the native glycans with unnatural sugars, especially pertinent has been the azide-bearing *N*-azidoacetylgalactosamine (GalNAz). The versatility of the GalNAz can be seen through its application for metabolic detection, proteomic analysis of *O*-GlcNAc proteins, or for our purposes the enzymatic modification of glycopeptides/ glycoproteins using an engineered β-1,4-galactosyltransferase enzyme (Y289L GalT).[25-27] When using this latter approach, the azide bearing chemical handle has allowed strain-promoted alkyne-azide cycloaddition reactions to ensue for introduction of a florescent probe or a cytotoxic drug.[28] In total, GalNAz is one of the most common sugars for the introduction of a bioorthogonal azido functional group through glycosylation.[29,30]

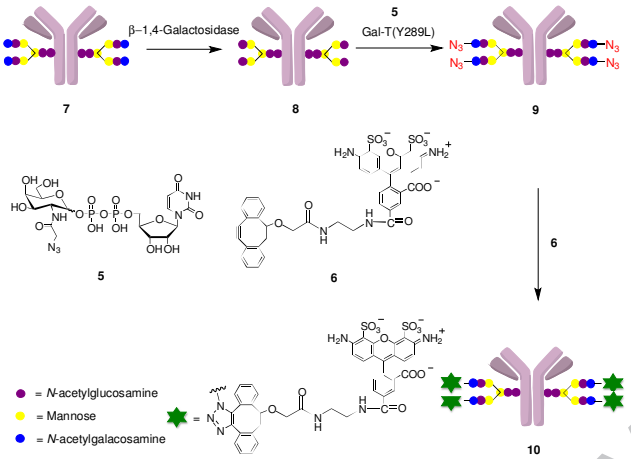
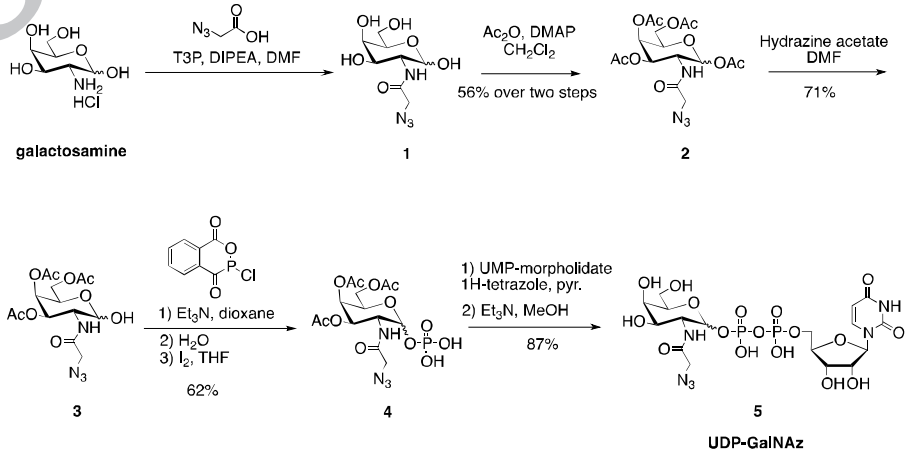


Figure 1. Strategy for the generation of site-specific antibody-drug conjugates.

Based upon GalNAz adaptability we looked to use it for selective antibody modification, and because enzymatic addition would be tested we required an activated nucleotide sugar donor building block; UDP-*N*-azidoacetylgalactosamine (UDP-GalNAz) based on previous reports appeared to be a sound choice.[28] In scouring the literature, the preferred method to prepare UDP-GalNAz was through a biosynthetic pathway. This recourse has been taken to circumvent the difficulties inherent in the formation of a pyrophosphate bond.[31] While enzymatic and chemo-enzymatic approaches provide attractive access to UDP-GalNAz and UDP-sugars in general, chemical approaches are still valued, particularly for non-natural compounds. A fundamental advantage of a chemistry-centered approach can be found in its versatility, wherein enzymatic methods can be limited by enzyme availability and substrate specificity. In short, we sought to investigate a practical and efficient route to UDP-*N*-azidoacetylgalactosamine (UDP-GalNAz) using a purely chemical approach, which if successful would be scalable and readily amenable to the facile preparation of structural analogues.

Our synthesis of UDP-GalNAz is illustrated in Scheme 1. A formidable challenge for a fully synthetic route to UDP-GalNAz required efficient pyrophosphate bond formation. Most commonly, the pyrophosphate bond is formed from two monophosphate precursors, which requires the activation of a phosphoric acid in one of the two building blocks, usually the nucleoside monophosphate.[32] Accordingly, the target sugar-nucleotide would be prepared from glycosyl phosphate **4** and an activated nucleoside monophosphate (UMP-morphlidate), using Khorana's morpholidate chemistry.[33] With this mindset, the synthesis started from acetylation of D-(+)galactosamine with azidoacetic acid,[34] followed by treatment of acetic anhydride and DMAP in CH_2Cl_2 to provide per-*O*-acetylated GalNAz **2** in a 56% yield over two steps. Selective removal of the 1-α-acetyl protecting group with hydrazine acetate in DMF provided compound **3** in 71% yield. Azide **3** was treated with 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one, followed by hydrolysis in water. The resulting phosphite intermediate was oxidized directly using I_2 in THF to produce monophosphate **4** in 62% yield over three steps. In the final step, phosphate **4** was coupled with UMP-morpholidate in pyridine using tetrazole as the promoter, followed by deacetylation with triethylamine in methanol, which provided the target compound **5** in 87% yield.

With the nucleotide-sugar UDP-GalNAz in hand, we investigated the two-step chemoenzymatic strategy for the modification of a generic mAb **7**, which would be predicated upon labeling the four terminal galactose residues residing within the Fc domain of an IgG (Figure 1). To create four acceptor sites for the attachment of UDP-GalNAz, mAb **7** was first treated with β-1,4-glactosidase in phosphate-buffered saline to produce mAb **8**.[28] Next, UDP-GalNAz was incorporated into mAb **8** using a recombinant galactosyltransferase, GalT(Y289L) at 30 °C for 16 h to yield azide-bearing mAb **9**. Electrospray-ionization mass spectrometry (ESI-MS) confirmed a complete addition of four GalNAz molecules per antibody (Figures 2A&2B). To obtain such an efficient ligation, various conjugation conditions were performed, focusing on reaction time, buffer composition, and



Scheme 1. Chemical synthesis of UDP-*N*-azidoacetylgalactosamine (UDP-GalNAz).

concentration of UDP-GalNAz (data not shown). Our final optimized conditions consisted of 25 mM Tris-Cl, 10 mM NaCl and 5 mM $MnCl_2$, at pH 7.2 with 4.5 μM GalT (Y289L) and 0.6 □M UDP-GalNAz in the presence of 6.7 μM mAb.

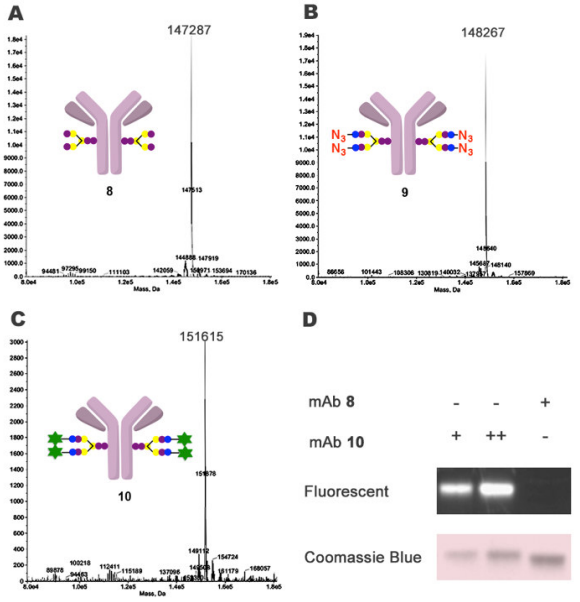


Figure 2. Site-specific conjugation of an antibody with a dibenzylcyclooctynol (DIBO)-fluorescein derivative. (**A**) ESI-MS characterization of mAb before; (**B**) after modification by the azido-sugar; (**C**) conjugation with the DIBO-Alexa Fluor® 488. (**D**) SDS/PAGE gel of mAb **10** labeled with four Alexa Fluor® 488 molecules, and comparison with unlabeled mAb **8**.

Finally, with the enlistment of UDP-GalNAz **5** specifically embedded within the glycan sites of the IgG, dibenzylcyclooctynol (DIBO) derivative **6**, which contains a green fluorescent dye (Alexa Fluor® 488), was attached to mAb **9** in Tris-Cl buffer. The reaction took place over 16 h at 25 °C, and then was subjected to a microspin column to remove excess reagents. Excitingly, SDS-PAGE presented us with a unique fluorescent band at 150 kDa, which indicated the attachment of the fluorescent probe to the mAb (Figure 2D). ESI-MS analysis revealed a molecular mass of 151,615 corresponding to the attachment of four Alexa Fluor® 488 probes per mAb (Figure 2C). Again, we sought to identify the most efficient coupling conditions, and research here demonstrated how a coupling buffer (25 mM Tris-Cl, 150 mM NaCl) at pH 7.2 with 6 equivalents of DIBO- Alexa Fluor® 488 when added to the mAb afforded quantitative labeling of four fluorescent probes per mAb.

In summary, an efficient solely chemical synthesis of an azide-modified galactose-nucleotide (UDP-GalNAz) is described. The heavy chain glycans of an antibody were enzymatically remodeled to introduce in a specific fashion terminal *N*-azidoacetylgalactosamine monosaccharide. The incorporated azide functionality was subsequently reacted with a strain-promoted alkyne-derivative to achieve site-specific attachment of a fluorescent probe. We anticipate scaling of the chemically prepared UDP-GalNAz will lead to new ADCs with the intent of applying our methodologies toward next generation targeted therapies.

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Supplementary Material

Supplementary materials associated with synthetic protocols, compound characterizations and biological assays can be found in the online version.