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A triply-divergent reagent for glycoprotein synthesis

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Abstract: Chemical synthesis of glycoproteins through three different strategies, from the same reagent, is herein described. The flexibility of this system, which allows the comparison of different linkage motifs between the same glycan and protein, is shown by one-step diversification for a GlcNAc-ylating reagent, and its application in both site-specific and non-site-specific glycoprotein synthesis to create different conjugates from common representative protein scaffolds.

Keywords: protein modifications • glycoprotein synthesis • GlcNAc • tag-and-modify • glycoconjugates

Synthesis of glycoproteins through chemical modification is a powerful tool for the synthesis of new glycoconjugates.[1] Here we report that this can be achieved from one single versatile chemical entity, through a divergent derivatization approach.

Protein modification plays a key role at the frontier between chemistry, chemical biology and biochemistry. New strategies have been developed to increase control over site-selective modifications and to enable the multi-functionalization of natural and artificially-engineered proteins.[2] Expanding the post-translational modification toolbox available to scientists has become of primary importance in many fields, from basic research to therapeutic applications. The ability to efficiently and specifically modify complex systems such as proteins, under mild aqueous conditions, is a necessary requirement to explore the potential of such entities. Among all the post-translational modifications, glycosylation is the most diverse[3] and hence potentially intriguing. The associated kaleidoscopic variety of modifications that it creates, optimized by nature through evolution, imparts to similar basic biomolecular scaffolds a huge number of different and potentially highly specific functions. Synthetic glycoproteins can recapitulate and modulate such function and their construction can be achieved both enzymatically and chemically, by reacting selected protein functionalities with appropriately pre-activated carbohydrates.[1]

While non-specific protein modification generates a heterogeneous mixture of conjugates, site-specific modification of proteins enables access to precisely defined structures. Both methods have their differing utilities in terms of practicality and outcome and ready flexible comparison may be of increasing use as e.g. synthetic biologics[4] find increasing therapeutic application. One flexible, convergent protein synthesis methodology (that has the potential for both non- and site-specific application) is the "tag-and-modify" approach.[5] In this strategy, a chemical functionality (the 'tag') is positioned within a protein, often within the side chain of amino acid residues. This moiety can then be subsequently selectively chemically targeted ('modified') as desired to create a desired alteration in the protein. Using this method, a number of chemical modifications of proteins have been developed by our group, including several first applications in synthetic glycoprotein construction: from triazole formation,[6] thiol-ene,[7] and Suzuki-Miyaura couplings,[8] to traceless Staudinger ligation[9] and cross metathesis,[10] allowing incorporation of both natural and usefully unnatural sugars (e.g. ^{18}F-fluorosugars[11]) and even the creation of synthetic glycoproteins in living cells.[12]

Such chemistry often requires strategic flexibility. The consequent linkage between protein and sugar can often be determined by the tag and reagent identities at an early stage when the appropriate tag is installed in the protein and/or when the corresponding sugar reagent has been assembled. This can preclude diversification at a later stage to, for example, explore the effect of linkage type, site or copy number. A flexible method that would allow more ready later stage diversification would be useful. Here we describe such a strategy, which uses both a common sugar reagent intermediate and common protein platform intermediates. In this work we thus demonstrate that diverse glycoprotein syntheses can be achieved using a single model sugar reagent intermediate (here containing the natural sugar GlcNAc) that can be readily activated in three different ways from this common precursor and then used in forming conjugates containing three linkage types from single protein partners. Such an approach that uses a 'triply-divergent' reagent for glycoprotein synthesis should allow the use of multiple modification strategies

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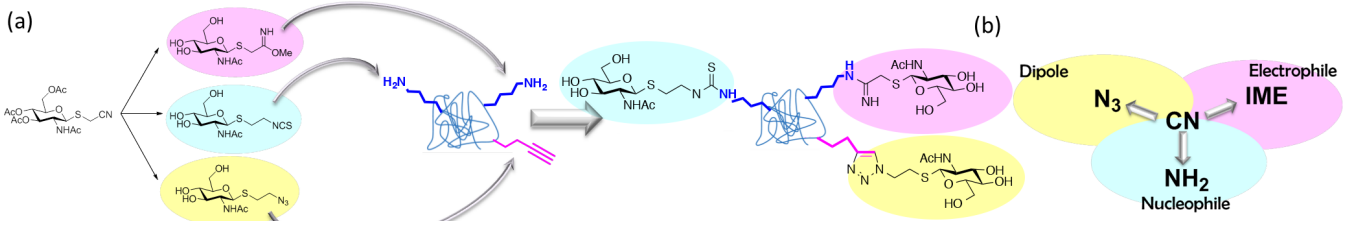


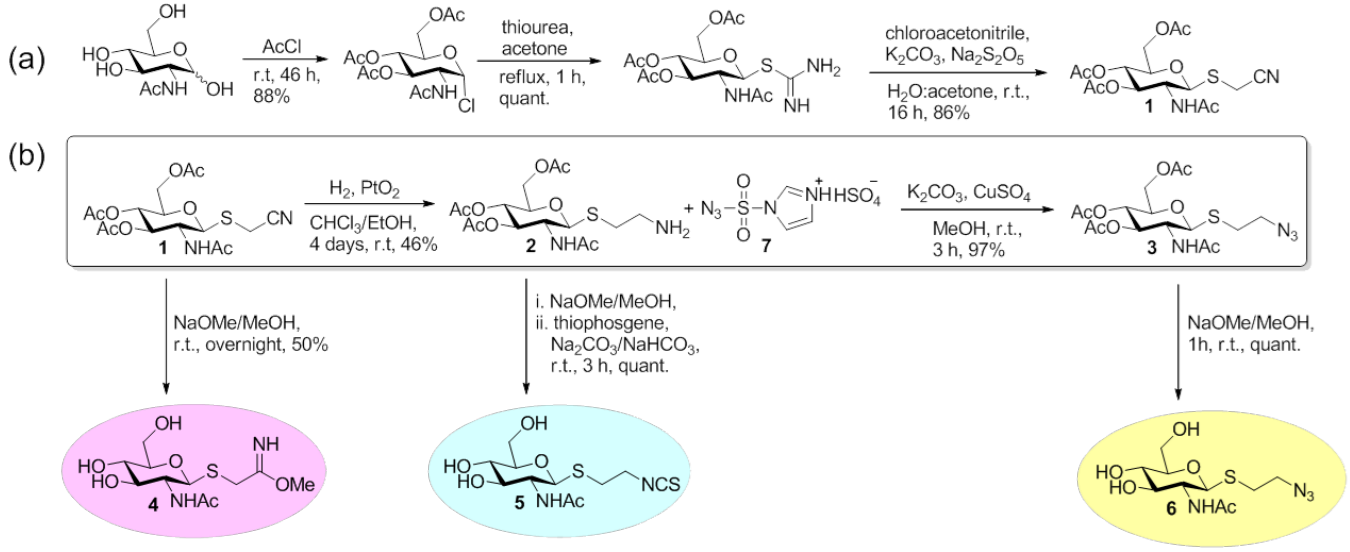
Figure 1. Triply-divergent glycoprotein synthesis. From the triple diversification of the same reagent, three different types of glycoproteins can be constructed on the same scaffold, either on natural or unnatural amino acids.

to maximise the efficiency and diversity of chemical glycoprotein synthesis and to potentially enable the introduction of different sugars on different glycosylation sites at different tags (Figure 1a).

In the design of the triply-divergent sugar reagent (Figure 1b) we envisaged that the nitrile group could provide a single precursor functional group that could then be readily elaborated to act potentially as a nucleophile, or an electrophile or a 1,3-dipole potentially even in a one-pot manner. Thus, a single sugar bearing a nitrile attached at the reducing (anomeric) terminus of the glycan reagent would potentially allow creation of three different linkages that would mimic the typical natural presentation of glycans on proteins (where the reducing terminus is attached to amino acid side chains). We envisaged a cascade of reactions (Scheme 1b) from this nitrile with activation at each stage of this cascade to create imidate, isothiocyanate and azide, respectively – each one ready for direct glycoprotein synthesis selectively on natural (Lys) or unnatural (homopropargylglycine, Hpg) amino acids in a single protein scaffold to create amidine,[13] thiourea,[14] and triazole[6] linkages respectively. In this we could envisage the modification of proteins either non-specifically or site-specifically depending upon chosen conditions, tag density and accessibility and desired copy number or linkage distribution.

To assess the readiness of this cascade of interconversions to create different reactive coupling groups, the peracetylated GlcNAc derivative **1**† was used as the common triply-divergent precursor and was readily prepared from the parent sugar (Scheme 1a). In the first step of the cascade (Scheme 1b) the cyano group of **1** was reduced to the primary amine **2** by hydrogenation[15] over PtO_2; this heterogeneous system has been shown to be less sensitive to poisoning by the thioether moiety in the sugar[16] than palladium or other catalysts and proved optimal here. A mixed chloroform-ethanol solvent system avoided the use of relatively harsh acidic conditions frequently employed in such reductions and we anticipate that these mild conditions would also be appropriate for more complex and acid-sensitive sugars. Amine **2** was then transformed into the corresponding azido-sugar **3** by diazo-transfer reagent **7**.[17]

Before modification of the proteins, each sugar was deprotected and activated as appropriate (Scheme 1b). GlcNAc-CN **1** was simultaneously deprotected and converted into the 2-imido-2-methoxy-ethyl (IME)[18] derivative **4** by stirring overnight with sodium methoxide in methanol. Whilst complete deprotection was rapid (5 min), the activation reaction is an equilibrium process and required more time to reach the maximum conversion; at a concentration of 0,1 M with 0,04 M NaOMe in MeOH this typically reaches 50% equilibrium position. Following glycoprotein synthesis recovered nitrile can be recovered, reactivated and re-used. Similarly, GlcNAc-NH_2 **2** was also deprotected



Scheme 1. A cascade of reactions from a common sugar intermediate (here **1**) allows the synthesis of activated imidate, isothiocyanate and azido sugars, ready to create glycoproteins in a triply diverse manner. †See ESI for full details.

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under Zemplén deacetylation conditions[19] and then activated with thiophosgene to form the isothiocyanate **5**.[14] The reaction was performed in a mixture of $NaHCO_3$ and Na_2CO_3 (0.3 M), and could be used crude or easily purified by size-exclusion (Biogel P2). Lastly, peracetylated GlcNAc-N_3 **3** was deprotected with NaOMe to afford azido reagent **6**.

Two representative protein scaffolds were selected for modification: Np276 is a right-handed quadrilateral β-helix protein scaffold from the genome of *Nostoc punctiforme*[20] and Qβ is a coat protein scaffold from the genome *Allolevivirus Q-beta*[21] that readily forms a 180-copy homomultimer that is an icosahedral protein particle. Reassignment of the Met sense codon to control the position of unnatural amino acid Hpg as an alkyne tag was achieved through heterologous expression in *E. coli* B834(DE3) auxotroph and allowed the creation of variants of both that contained both Lys and Hpg residues: Np276-Hpg61[22] and Qβ-Hpg16[11b] (Figure 2). These protein scaffolds, ready for diverse glycoprotein synthesis with **4**-**6** contain 3×Lys+1×Hpg and 6×Lys+1×Hpg, respectively, which together with the *N*-terminal amines were expected to allow varied modifications.

GlcNAc imidate reagent **4** was reacted with proteins under mildly basic conditions (pH 9). Use of varying levels of reagents allowed variation of Lys modification levels; use of 1000 equivalents for 2 h, for example, consistently gave a mixture of di- and tri-GlcNAc-ylated protein from Np276-Hpg61 (TAPS buffer 0.6 M, see Table 1, entry 3) whereas reaction of more bulky $(Qβ\text{-}Hpg16)_{180}$ (phosphate buffer 50 mM) with 1000 equivalents for 1 h gave a mixture of mono- and di-GlcNAc-ylated protein along with unreacted protein (see Table 1, entry 4). GlcNAc-isothiocyanate **5** reacted more slowly with both proteins and even after 16 h at pH 8 (bicarbonate buffer 0.3 M) to give only mono-GlcNAc-ylated protein from Np276-Hpg61 (see Table 1, entry 2), while $(Qβ\text{-}Hpg16)_{180}$ gave mono-GlcNAc-ylated protein along with unreacted protein (see Table 1, entry 5). GlcNAc azide **6** was reacted with Np276-Hpg61 for 3 h at pH 8 (phosphate buffer, 50 mM) with preformed Cu(I)•TTMA complex (preformed in acetonitrile from Cu(I)Br)[6a] to give only mono-GlcNAc-ylated product. $(Qβ\text{-}Hpg16)_{180}$, under the same conditions, gave fully mono-GlcNAc-ylated protein in 16 h. The generally more sluggish reactivity of homomultimer $(Qβ\text{-}Hpg16)_{180}$, compared to monomeric Np276-Hpg16, may be logically attributed to slower reaction of the Qβ 'particle', since non-denaturing conditions were used here. Under these conditions residues (including some occluded Lys residues) are predicted to be less accessible from molecular mechanics analysis.

In this way, we were able to access proteins with, in many cases, similar copy number (mono-GlcNAc-ylation) but each attached through different linkage types (amidine, thiourea and triazole). Linkages have logically been suggested to sometimes affect presentation and function in such conjugates. Given the differing properties (length, distance from protein surface, pK_a) diverse functional comparison could be of utility. To compare the functional variance of these proteins we evaluated the binding of the synthetic

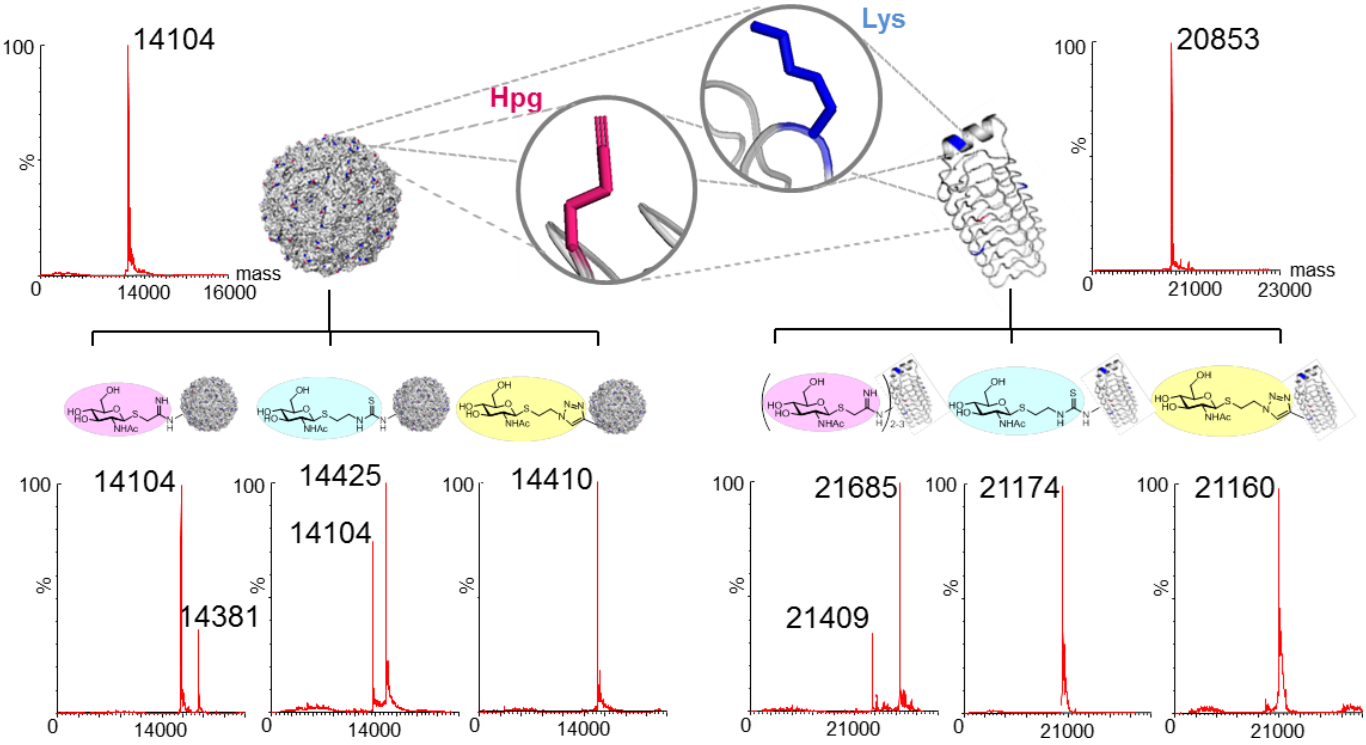
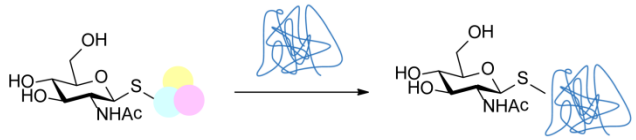


Figure 2. Construction of glycoproteins from of Qβ capsid protein (left) and NP276 (right) used in this study. The reaction with the three glycans intermediates resulted in different glycoconjuates with three different linkages (Hpg, magenta the unnatural amino acid, Lys, blue).

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glycoproteins to the model carbohydrate-binding[23] lectin wheat germ agglutinin (WGA) by surface plasmon resonance (SPR).

Table 1. Glycoprotein construction and conversions obtained using the triply-divergent approach.



Entry	Protein	Sugar	Glycans Added[a]	Conversion %[b]
1	Np276-Hpg61	**4**	2:3	28:72
2	Np276-Hpg61	**5**	1	>95
3	Np276-Hpg61	**6**	1	>95
4	Qβ-Hpg16	**4**	1	30
5	Qβ-Hpg16	**5**	1	58
6	Qβ-Hpg16	**6**	1	>95

[a] Number of sugars added to the protein during each conjugation. [b] Conversion obtained (in %), determined by MS analysis.

WGA was immobilized on the sensor chip and the diverse synthetic glycoproteins were used as the flow analytes (Fig. 3). The level of binding observed for the synthetic glycoproteins derived from Np276 as a platform proved to be consistent with the level of glycosylation, with the more heavily GlcNAc-ylated protein derived from modification of Lys by GlcNAc-imidate **4** (2-3 glycans) binding more strongly than those with single GlcNAc (derived from **5** or **6**). All bound more strongly that non-glycosylated Np276, which also showed some non-specific interaction with WGA. Interestingly, although the site of glycosylation is different in the latter two, no consequent significant difference was observed in binding. All three Np276

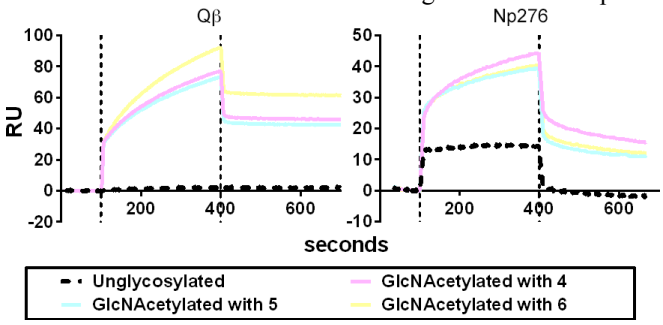


Figure 3. Binding of Synthetic Glycoprotein to Lectin WGA. Biotinylated WGA was captured onto streptavidin-coated SPR biosensor chip and the binding of glycoproteins as analytes (20 μg/mL) was followed for 5 min followed by a 5 min dissociation period. Dashed vertical lines indicate start and stop of the sample

glycoproteins dissociated relatively rapidly.

The three synthetic glycoproteins derived from multimeric Qβ showed strong binding, with no detectable non-specific binding for nonglycosylated protein (Fig. 3). Although the level of glycosylation per monomer was lower for Qβ, the total level of glycan display in the 180-mer homomultimeric particle, which Qβ assembles to form, provides a higher level of valency. The observed binding enhancement is consistent with our previous observations in highly-valent protein-derived glycodendrinanoparticles.[24] Again, the level of this interaction was consistent with the overall level of glycan loading, with the binding of the synthetic glycoprotein particle derived from modification of Hpg with **6** giving the highest binding. Interactions were observed for all that persisted throughout the dissociation period.

In conclusion, we have demonstrated that a 'triply-divergent' protein glycosylating reagent can be iteratively generated from a single divergent intermediate by a cascade of reactions, allowing formation of glycoconjugates containing three different linkage types, from single protein partners. It has allowed here diversified modulation of both the site of glycosylation and the copy number of glycosylation as well as the protein platform, which in turn generates diverse data to allow evaluation of associated differences in biological function (here binding to a lectin). This glycoprotein construction approach will allow the use of multiple modification strategies, in order to maximise efficiency and diversity, via the formation of different linkage types, allowing strategic flexibility. One example of the usefulness of this new methodology, could be in the ready creation and then comparison of glycoconjugates as vaccines, where some linkages, but not others, may generate so-called 'anti-linker' responses.[25] The ability to take for example the same glycan immunogen from a pathogen and to rapidly create similar copy number for protein loading but differing linkage type, as we have shown here, may be highly advantageous in the study of synthetic glycoconjugates as vaccines.[26]

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