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Glycoprotein Vaccines

A Coordinated Synthesis and Conjugation Strategy for the Preparation of Homogeneous Glycoconjugate Vaccine Candidates**

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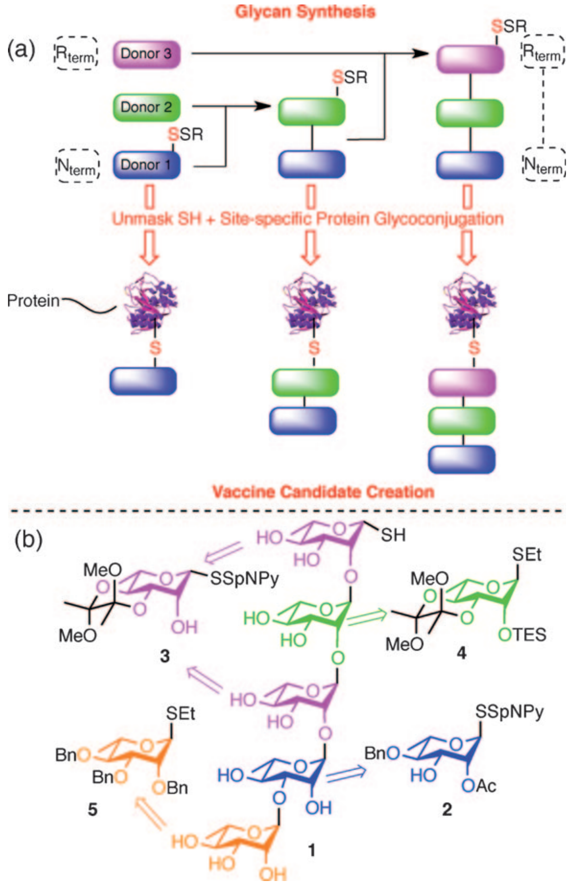
Glycoconjugates are the center of many therapeutic strategies[1–3] and carbohydrate-based vaccines in particular hold great promise.[4–8] The development of glycovaccines, however, can be hindered by the limited access offered by natural sources to homogeneous antigenic carbohydrates; efficient chemical synthesis offers an attractive route to pure samples of these carbohydrates. Furthermore, for an optimal immune response, the carbohydrate antigen should be conjugated to an immunogenic carrier, usually a protein.[9,10] The synthesis and use of well-defined glycoprotein therapeutics and glycovaccines—uniform in sugar, site, and level of protein attachment—is rare and most constructs are prepared and administered as complex mixtures.[4,9,10] Even strategies that utilize pure synthetic glycan may employ non-selective methods for subsequent conjugation to a protein carrier.[4,6,7,11,12] Given the unknown influence of conjugation site on immunogenic response, it is remarkable that, to our knowledge, no homogeneous glycovaccine has been studied. To fully evaluate the structure–activity relationships (SARs) between glycoprotein and immunogenicity, we have initiated a program for the construction of such "pure" or uniform glycoconjugate vaccines. We report a coherent strategy for homogenous glycoprotein construction that coordinates both carbohydrate synthesis and conjugation methodology. This approach features glycosyl disulfides as versatile donors in complex carbohydrate synthesis, providing strategic access to glycosyl thiols that can be site-specifically attached to a protein carrier through a well-defined thioether linkage (Scheme 1).



Scheme 1. a) Coordinated synthesis and conjugation strategy for the construction of glycoconjugate vaccines. Non-reducing (N_{term}) to reducing terminus (R_{term}) segmental assembly allows flexible building block use and ready attachment of "growing" carbohydrate antigen fragments to carriers during "growth". b) Retrosynthetic analysis of target O-antigenic repeating motif of 22 535 *Klebsiella pneumonia*.

We have previously reported the use of glycosyl disulfides as donors in the synthesis of mono- and disaccharides.[13,14] These novel donors exhibit reactivity similar to their thioglycoside counterparts,[15] but with the advantage that the disulfide linkage can be readily cleaved and exchanged at any stage, thereby tuning reactivity[16] through aglycon alteration. Competition experiments have demonstrated that "armed"[17] (more reactive) disulfide donors, can be activated preferentially over "disarmed" disulfides (Figure 1).[14]

Disarmed disulfides can be activated under more strenuous conditions (e.g. higher temperature) or through ready conversion to an "armed" disulfide. The differential reactivity

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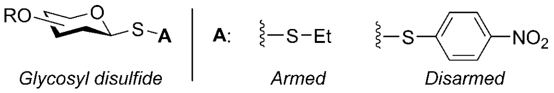
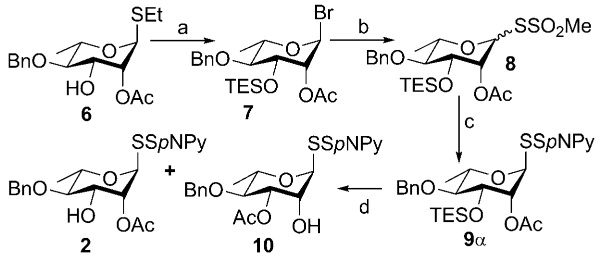


Figure 1. Examples of armed and disarmed glycosyl disulfides.

of mixed disulfide donors has not yet been explored in complex oligosaccharide synthesis. Since aglycon alteration of disulfides is mild, flexible, and compatible with many protecting groups, and so can be used to fine-tune a glycan synthesis even mid-way, we anticipated a strategic advantage in carbohydrate construction.[18] Also, the disulfide products can be easily reduced to thiols and then chemospecifically conjugated to a protein scaffold in a controlled manner,[19–22] allowing glycoconjugation at all stages of glycan synthesis (Scheme 1). The dual role of disulfides both as donors and as masked precursors to glycosyl thiols is the centerpiece of a unified approach to homogenous glycoprotein synthesis that we disclose here.

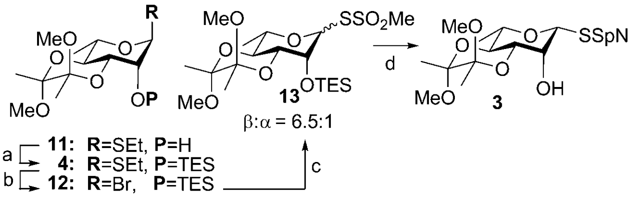
Rhamnosyl pentasaccharide **1** (Scheme 1) constitutes the repeating unit of the O-antigenic polysaccharide of the 22535 strain of the pathogen *Klebsiella pneumonia*[23,24] and therefore represents a model glycan moiety for the generation of glycoprotein vaccines.[25] Rhamnosyl donors, as 6-deoxy sugars, are generally more reactive than their glucose (hexose) counterparts[26] and thus constituted a good initial platform for examining donor reactivity tuning by aglycon alteration. Preliminary investigations revealed that orthogonal activation of the thiorhamnosides was best achieved with 5-nitro-2-pyridyl (*p*NPy)[27] as the deactivating aglycon. This ability to alter aglycon reactivity highlights a strategic advantage of disulfide donors: the aglycon can be exchanged to attenuate or promote reactivity as required and does not require an entirely new synthesis of the building block.

We initiated the synthesis of **1** from the building blocks **2**–**5**. Compound **2** was prepared from thioglycoside **6**[28] (Scheme 2). After conversion of **6** to the anomeric bromide **7**, reaction with sodium methanethiosulfonate in the presence of Bu_4NBr gave methanethiosulfonate **8** as an inseparable mixture of anomers (α:β = 5:1). Treatment with nitropyridyl thiol *p*NPySH gave **9** as a separable mixture of anomers (74% **9**α, 15% **9**β). Desilylation of **9**α with excess $Et_3N{\cdot}3HF$ in THF gave the required building block **2** in 81% yield with 4% formation of regioisomer **10**, a product of acetyl migration.



Scheme 2. Synthesis of disulfide donor **2**: a) TESOTf, Py, CH_2Cl_2, 96% then Br_2, CH_2Cl_2, 0°C, 100%; b) $NaSSO_2Me$, Bu_4NBr, dioxane, 70°C, 79%; c) *p*NPySH, Et_3N, CH_2Cl_2, 74% **9α**; d) $Et_3N{\cdot}3HF$, THF, 81% **2**, 4% **10**. TES = triethylsilyl.

Block **3** was prepared from butane-2,3-diacetal (BDA)-protected thioglycoside **11** (Scheme 3).[29] Silylation and bromination provided protected α-bromide **12**. Reaction with sodium methanethiosulfonate in the presence of Bu_4NBr gave **13** as a separable anomeric mixture in which, unlike **8**, the β-anomer was predominant (β:α = 6.5:1).[30] This difference in anomeric selectivity in the formation of **8** and **13** may be associated with the rigidity conferred to the rhamnose ring in **13** by the BDA protecting group.[31] **13β** was used in the next step and reaction with 5-nitro-2-pyridylthiol (*p*NPySH) followed by desilylation gave target building block **3** (Scheme 3).

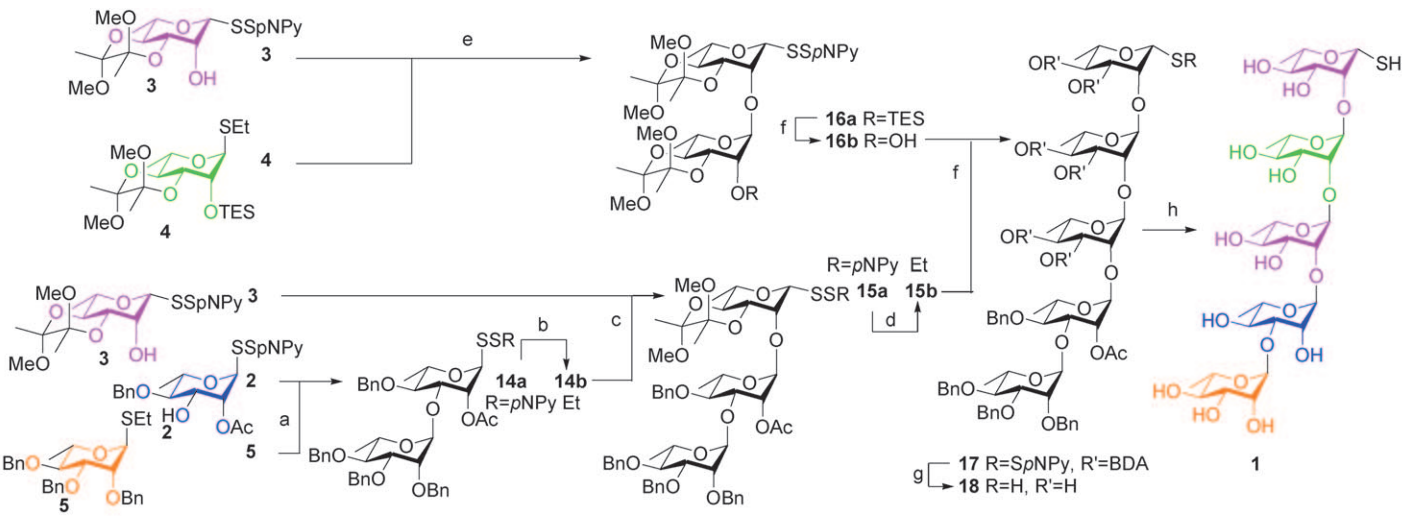


Scheme 3. Synthesis of disulfide donor **3**: a) TESOTf, Py, CH_2Cl_2, 91%; b) Br_2, CH_2Cl_2, 0°C, 100%; c) $NaSSO_2Me$, Bu_4NBr, dioxane, 80°C, 75%; d) *p*NPySH, Et_3N, CH_2Cl_2, 73% then $Et_3N{\cdot}3HF$, THF, 100%.

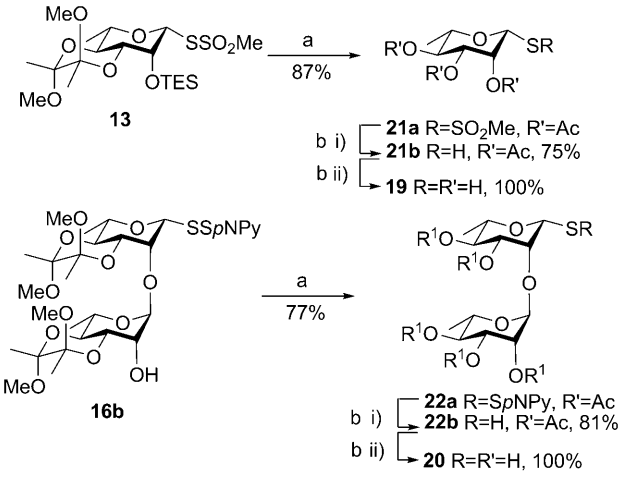
The first key glycosylation paired thioglycoside donor **5**[32] with acceptor **2** (Scheme 4). Using the mild activator *N*-phenyl thiocaprolactam/Tf_2O[33] at −10°C, the disaccharide **14a** was isolated in 68% yield with exclusive α selectivity and, as a result of reactivity tuning, without any concomitant activation of **2**. **14a** was then “aglycon altered”: converted into armed donor **14b** by disulfide reduction followed by reaction with ethyl methanethiosulfonate. **14b** was then reacted with acceptor **3** to give rhamnotrisaccharide **15a** in 66% yield, again with exclusive α selectivity and again without activation of **3** (Scheme 4).

Final fragment assembly was achieved by activation of this trisaccharide. Thus, **15a** was converted/altered into the active donor **15b**, and then coupled with the disaccharide acceptor **16b**. The precursor to **16b**, protected disaccharide building block **16a**, had itself been prepared by reaction of **3** with **4** using *N*-phenyl thiocaprolactam/Tf_2O at −20°C; desilylation furnished disarmed disulfide acceptor **16b**. [3+2] coupling of **16b** with trisaccharide building block **15b** gave pentasaccharide **17** which was then partially deprotected and reduced to **18**. Finally, debenzylation was accomplished by Birch reduction. For ease of handling, the crude thiol was globally acetylated, purified, and then subjected to Zemplén deprotection to give the pentasaccharide **1** (Scheme 4). Exclusive α-configurations in all glycosidic linkages were consistent with the absence of nOe interactions in the ^1H NMR spectra between the signals for H-1 and H-3 or H-5. Regiochemistry was established through COSY, ROESY, nOe, and HMBC analysis.[34]

Deprotected mono- and disaccharide thiols **19** and **20**, as smaller fragments of the antigen pentasaccharide **1**, were also synthesized from intermediate building blocks **13** and **16b**, respectively (Scheme 5). Their syntheses from intermediate glycosyl disulfide donors that had been used to create **1**



Scheme 4. Synthesis of pentasaccharide **1**: a) *N*-phenyl thiocaprolactam, Tf_2O, TTBP, CH_2Cl_2, −20 to −10°C, 68%; b) 1. PBu_3, $CHCl_3$/dioxane/H_2O; 2. $EtSO_2Me$, Et_3N, CH_2Cl_2; 85% over 2 steps; c) *N*-phenyl thiocaprolactam, Tf_2O, TTBP, CH_2Cl_2, 10°C–RT, 66%; d) 1. PBu_3, $CHCl_3$/dioxane/H_2O; 2. $EtSO_2Me$, Et_3N, CH_2Cl_2; 77% over 2 steps; e) *N*-phenyl thiocaprolactam, Tf_2O, TTBP, CH_2Cl_2, −20 to −10°C, 66%; f) 1. $Et_3N\cdot3HF$, THF; 2. *N*-phenylthiocaprolactam, Tf_2O, TTBP, CH_2Cl_2, 10°C–RT; 45% over steps; g) 1. TFA/H_2O (9:1), 79%; 2. PBu_3, dioxane/H_2O, 78%; h) 1. Na liq. NH_3/THF then Ac_2O, Py, 69%; 2. NaOMe, MeOH, 100%. TTBP = 2,4,6-tri-*tert*-butylpyrimidine, TFA = trifluoroacetic acid.



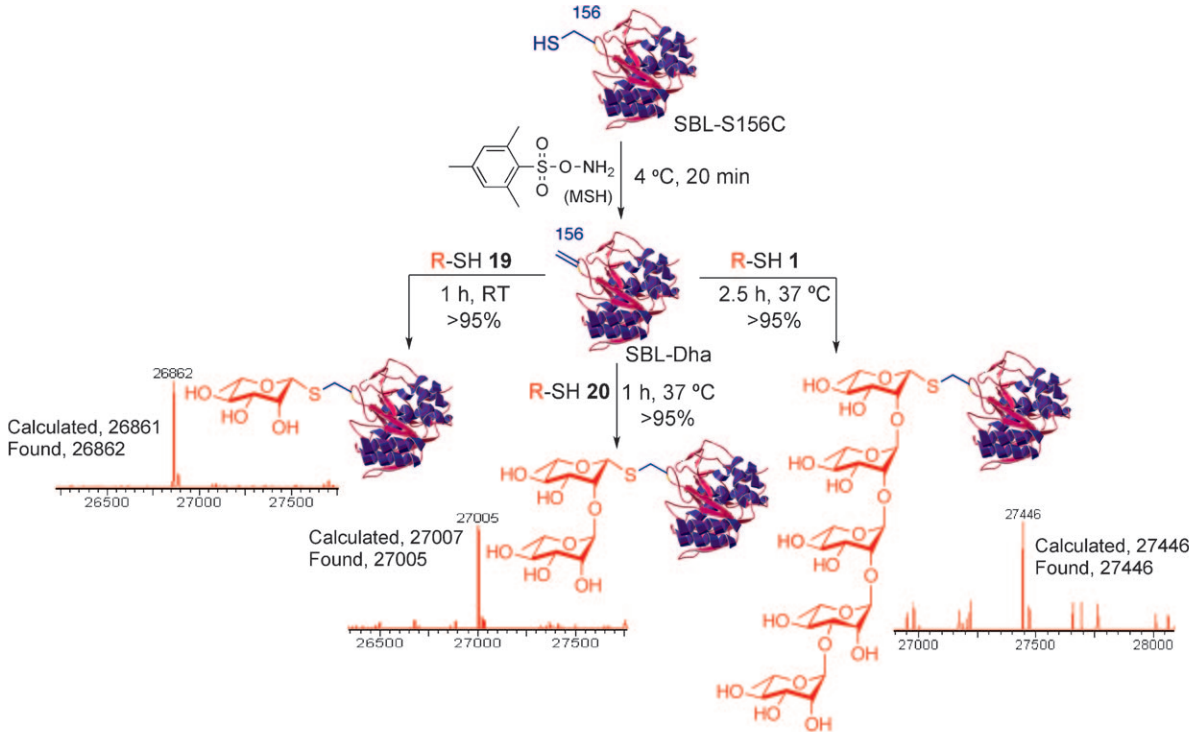
Scheme 5. Synthesis of **19** and **20**: a) TFA/H_2O (9:1) then Ac_2O, Py; b) 1. PBu_3, $CHCl_3$/dioxane/H_2O; 2. NaOMe, MeOH.

illustrates strategic access mid-route to key segments of the final carbohydrate that bear an anomeric thiol, the tag needed for site-specific conjugation.

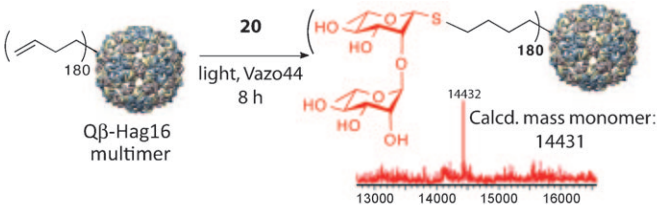
Next these glycan epitope fragments of increasing complexity, **19**, **20** and **1**, were site-specifically conjugated to a model *Bacillus* protein, as a potential immunogenic carrier, by conjugate addition to dehydroalanine (Dha) residue. Dha was chemically installed[21] by oxidative elimination of the single cysteine of the subtilisin protein (SBL) mutant S156C using Tamura's reagent,[35] *O*-mesitylenesulfonylhydroxylamine (MSH).[21] Reaction of SBL-Dha as a divergent intermediate with **1**, **19**, **20** gave the corresponding desired protein–epitope conjugates with greater than 95% conversion. The synthesis of these single-glycoform glycoprotein antigen carriers creates opportunities for SAR studies of glycan structure in candidate vaccines (Scheme 6).

A single copy of a carbohydrate displayed on a synthetic lipopeptide has proved successful in the generation of antibodies,[36] but this construct is quite different from typical protein glycoconjugates and in most cases multiple sugar loading has been shown to influence (and be necessary for) the efficacy of the vaccine.[37,38] Moreover, the method used in Scheme 6 generates diastereomers in the protein backbone as a result of the Michael-type addition to dehydroalanine. As a result (and to stay consistent to our goals of creating pure multivalent glycoconjugates as putative vaccines, see above), we decided to extend our glycoprotein vaccine technology to the construction of a pure, single glycoform of a larger protein carrier bearing multiple antigen copies as single diastereomers at well-defined sites. Virus-like bacteriophage particle Qβ was chosen as a known immunogenic carrier[39,40] and non-natural amino acid homoallylglycine (Hag) was site-specifically introduced into these protein systems as a "tag" for thiyl–ene conjugation[22] through expression of corresponding gene sequences in an auxotrophic strain of *E. coli* (B834 (DE3)).[41] A pure glycoprotein vaccine candidate displaying 180 copies of antigen **20** at well-defined sites was created (Scheme 7). The conjugation chemistry proceeded with greater than 95% conversion at pH 4.0 through the combined use of light and an initiator, Vazo44.[34] The synthesis of multivalent, pure glycovaccines with defined number of antigen copies at precise sites will enable the determination of where and how many antigen copies are necessary for improved vaccine efficacy.

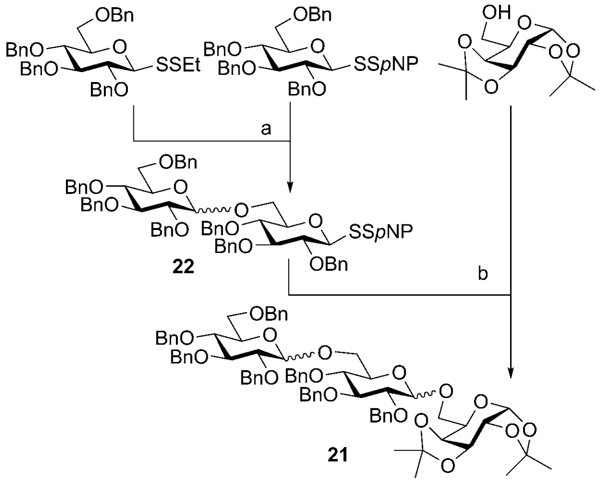
Finally, to explore the potential for this method to be applied to other oligosaccharides another sequential armed/disarmed glycosyldisulfide glycosylation was explored. This is important given that the inherent reactivities (as well as reactivity differences) may be drastically different in other systems (see above). In particular, fully oxygenated hexoses, such as D-glucose, are typically less reactive as glycosyl donors



Scheme 6. Site-specific conjugation of **19**, **20**, and **1** to model protein.



Scheme 7. Synthesis of well-defined conjugate bearing 180 copies of sugar antigen by thiyl–ene chemistry on immunogenic protein platform. Modification was successfully performed on Qβ multimer. The mass spectrum insert shows analysis of monomer protein formed after exhaustive reduction and denaturation.



Scheme 8. Synthesis of hexose oligomer **21**: a) DMTST, TTBP, CH_2Cl_2, −10 °C, 68 %; b) DMTST, TTBP, CH_2Cl_2, RT, 55 %. DMTST = dimethylsulfonium triflate, *p*NP = *para*-nitrophenyl.

than 6-deoxyhexoses, such as the L-rhamno units used above. We were pleased to observe that model oligomer **21** could be assembled using an essentially identical disulfide reactivity-tuning approach (Scheme 8). Again, as for the 6-deoxyhexose system under the appropriate reaction conditions disaccharide **22** was isolated, as a result of reactivity tuning, without any concomitant activation of the disarmed donor motif.

In summary, a strategy has been developed for the construction of glycoconjugate vaccines in which the site of glycan attachment is well-defined, allowing preparation of more precisely defined candidates. This strategy coordinates oligosaccharide synthesis with site-specific protein conjugation. Glycosyl disulfides are demonstrated as useful donors for the synthesis of oligosaccharides. Their aglycon flexibility allows straightforward iterative assembly of complex carbohydrates and protecting group freedom since the “armed” or “disarmed” status is altered through the aglycon rather than the protecting groups of the sugar. Moreover, the use of glycosyl disulfides advantageously delivers glycosyl thiol products that are suitable for site-specific protein ligation, here applied using two different site-selective and complementary methods.[21,22] Finally the use of pure, well-defined glycoproteins as potential immunogens is rare, if not unprecedented, and will allow insight into the effect of epitope positioning and immune response. For example, the role and importance of linkers between sugar epitope and protein carrier is a matter for debate, with some examples highlighting usefully increased accessibility, while others show that the linker can confound and modulate immune response.[42,43] Valuably, through the two methods that we demonstrate here, identical sugar epitopes (starting from the same sugar thiol direct from the synthetic route) can be installed at different distances to test this hypothesis (in one case through a one-carbon side chain and in the other through a four-carbon). The use of this coordinated strategy for therapeutic glycovaccine candidates is currently underway.

Experimental Section

General glycosylation: A solution of thiorhamnoside acceptor (0.45 mmol), disulfide donor (0.45 mmol), *N*-phenylthiocaprolactam (0.47 mmol), and TTBP (2,4,6-tri-*tert*-butylpyridine) (0.50 mmol) in dry dichloromethane (15 mL) was stirred with molecular sieves (0.30 g) under argon for 1 h. The mixture was cooled to −20 °C using a cryocooler. Trifluoromethanesulfonic anhydride (0.50 mmol) was added. After TLC showed complete consumption of starting materials, the reaction mixture was cooled in a dry ice/acetone bath and diluted with dichloromethane, and passed through a silica plug which was washed with dichloromethane and then ethyl acetate. The eluent was evaporated and the residue purified by flash column chromatography.

General aglycon alteration: Argon was bubbled through a stirred mixture of the disulfide (0.45 mmol), dioxane (2.7 mL), chloroform (5.4 mL), and water (0.9 mL) for 30 min. Tributylphosphine (0.91 mmol) was added. A deep orange color was produced, which rapidly faded to yellow. After 1 h TLC indicated the reaction was complete. The reaction mixture was concentrated and purified by flash column chromatography. The resulting thiol (0.45 mmol) was redissolved in dichloromethane (50 mL) and the solution added dropwise to a solution of ethyl methanethiosulfonate (0.49 mmol) and triethylamine (0.45 mmol) in dichloromethane (20 mL) at 0 °C over 1.5 h. The ice bath was then removed and after an additional hour TLC showed complete comsumption of starting material. The solvent was evaporated and the residue purified by flash column chromatography.

General protein conjugation to Dha procedure: A 100 μL aliquot of 0.3 mg mL^{-1} SBL-C156Dha (pH 8.0 sodium phosphate, 50 mM) was prepared as previously described.[21] A 60 μL aliquot of a 40 mM solution of the sugar thiol in sodium phosphate buffer (50 mM, pH 8.0) was added to SBL-C156Dha and vortexed to homogenize. After shaking at room temperature or 37 °C for 1 to 3 h, LC-MS analysis of the reaction mixture revealed complete conversion to the corresponding glycoprotein.

Typical thiyl–ene addition procedure:[22] Di-rhamno-SH **20** (1.38 mg, 4.22 μmol) and Vazo44 (0.28 mg, 0.84 μmol) were added to a solution of Qβ-M16Hag protein (100 μL of 1.19 mg mL^{-1}, 8.44 nmol) in 250 mM ammonium acetate buffer (pH 4.0). The reaction mixture was placed in a cuvette and irradiated with a medium pressure 125 W Hg-lamp with borosilicate filter at room temperature for 8 h. Small molecules were removed from a 50 μL reaction mixture aliquot by loading the sample onto a PD minitrap desalting column (GE Healthcare). An aliquot (20 μL) was mixed with 1 M DTT (dithiothreitol) in H$_2$O (10 μL) and incubated at 60 °C for 5 min to allow the protein to denature prior to analysis by LC-MS which revealed complete conversion to the corresponding glycoprotein.

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