Protein Modification

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Thiyl Glycosylation of Olefinic Proteins: S-Linked Glycoconjugate Synthesis**

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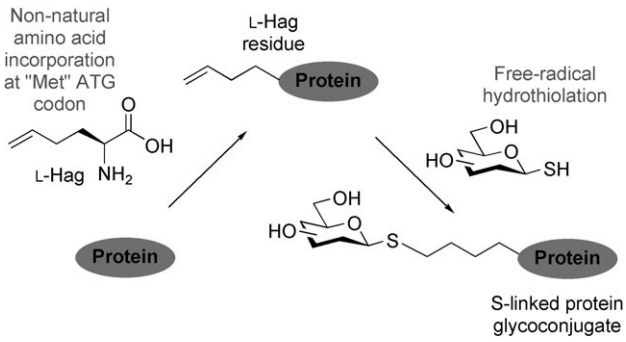
Over half of all proteins in nature are estimated to be glycosylated,[1] and these biomolecules play key roles in protein expression, folding and stability[2–7] and are fundamental to various biological processes.[8–13] In recent years, synthetic homogenous glycoforms[14–20] of glycoproteins have been one of the primary targets in glycobiology,[11,21] not only to allow function determination, but also to create glycoprotein mimetics useful as, e.g., therapeutic agents. Beyond the preference for the more abundant native O- and N-linked glycoproteins, S-linked glycoproteins are also attractive synthetic targets as a result of their enhanced chemical stability and enzymatic resistance.[22] Following the discovery of the first natural S-glycosidic linkage by Lote and Weiss in 1971,[23,24] methods have been developed for the synthesis of S-linked glycopeptides[25] and more recently S-linked glycoproteins.[26,27]

Here, we describe the development of a convergent approach for the synthesis of a novel class of S-linked glyconjugate proteins through the site-specific ligation of 1-glycosyl thiols to proteins (Scheme 1). The strategy exploits non-natural amino acid incorporation[28,29] for the introduction of L-homoallylglycine (L-Hag) into a protein and free-radical addition hydrothiolation reactions, under conditions mild enough to retain protein activity throughout (Scheme 1). In this way, Hag functions as a new "tag" combined here with a new modification as part of a general "tag–modify" strategy for synthetic-protein construction.[30] Using this strategy previously we have, for example, been able to demonstrate the successful use of azide or alkyne tags.[31,32] Prior, elegant conjugations have shown that radical-addition reactions may be successfully applied to proteins.[33–35] Whilst 1-thioglycoside formation by the free-radical addition of 1-glycosyl thiols to alkenes has been reported for the synthesis of small molecules,[36–38] to date this method has not been applied to the synthesis of S-linked glycoproteins or bioconjugates. The unique reactivity profile of L-Hag, with an olefinic side-chain compared to the natural amino acids characteristically found in proteins, allows for a chemoselective chemical reaction. Amongst these selectivity advantages is the inertness of Hag to almost all common protein modification reactions, thereby allowing the potential for orthogonal use in combined, multireaction protein chemistry strategies.[31] This inertness to other reagents would not be shared by strategies that would use a protein-thiyl radical, perhaps derived from Cys, although this "reverse" approach would allow the potential for use of proteins containing only natural amino acids. In this context, during the final stages of this work,[39] we became aware of usefully complementary methods employed by Dondoni, Massi and co-workers for glycosylation of protein-thiyl radicals.[35]



Scheme 1. Summarized strategic approach.

The free-radical reaction of glycosyl thiyls was investigated and optimized initially on representative amino acid model systems containing Hag (Table 1). As a prerequisite for later protein stability, solubility and viability, the reaction was developed to comply with aqueous solution chemistry. Significantly, all prior protocols[36–38] for glycosyl thiyl generation have until now employed organic solvents. Since the stability and reactivity of glycosyl thiyls can vary, and to demonstrate substrate breadth and broad applicability of the method, we utilized a wide range of 1-glycosyl thiols, in both protected and unprotected form, as starting materials including 1-thio-β-D-glucose (β-GlcSH), 1-thio-α-D-glucose (α-GlcSH), 2-acetamido-2-deoxy-1-thio-β-D-glucose (GlcNAc-SH), 1-thio-β-D-galactose (GalSH), 1-thio-β-D-mannose (ManSH), and disaccharide 4-*O*(β-D-galactosyl)-1-thio-β-D-glucose (Gal(β1,4)GlcSH). Together reactions (Table 1) probed the effect of bulk, differing configuration, protecting groups, and anomeric stereochemistry in the thiols; variation around the N- and C-termini of Hag and varying conditions, including alternative modes of initiation (using water soluble initiator Vazo44 (VA044, 2,2′-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride)[40] and/or photochemical

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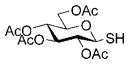
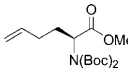
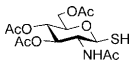
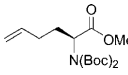
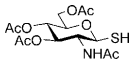
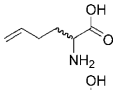
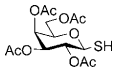
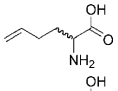
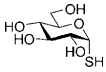
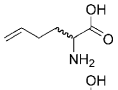
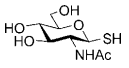
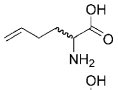
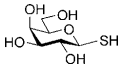
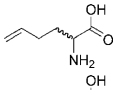
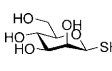
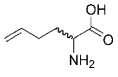
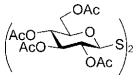
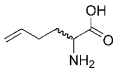
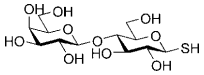
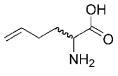
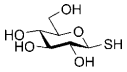
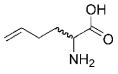
Table 1: Amino acid glycoconjugation reactions.

Entry	1-Glycosyl thiol[a]	Olefin	Solvent	Yield[b] [%]
1			H_2O/MeOH (1:1)	79
2			H_2O/MeOH (1:1)	71[c]
3			H_2O	81
4			H_2O/MeOH (1:1)	57
5			pH 4 acetate	42 (≈50[d])
6			pH 4 acetate	63 (>98[d])
7			pH 4 acetate	70 (>80[d])
8			pH 4 acetate	28 (≈50[d])
9			pH 4 acetate	0
10			pH 4 acetate	56 (>98[d])
11			pH 4 acetate	91[b] (>98[d])

[a] 1.2 Equivalents. [b] Yield of isolated compounds after 8 h reaction time. [c] Mono-Boc formed as product olefin. Boc = *tert*-butoxycarbonyl. [d] Conversion determined by crude ^{1}H NMR spectroscopy.

(medium-pressure 125 W Hg lamp with borosilicate vessel giving maximum emission at 365 nm)).

Optimization studies with β-GlcSH (Table S2) indicated that both forms of initiation allowed successful conjugation; interestingly, enhancements in rate and yields were observed through photoinitiation in the presence of Vazo44. pH proved important with lower pHs (4–7) proving superior to the use of pH 10 CAPS buffer (*N*-cyclohexyl-3-aminopropanesulfonic acid). Importantly, ambient conditions in aqueous solutions potentially compatible with protein stability (without the need for, e.g., degassing)[41] proved effective. Control reactions with L-Hag and representative thiols β-GlcSH and GlcNAcSH confirmed all to be individually stable (e.g., to racemization/anomerization) under the reaction conditions and, in particular, to UV irradiation. Good regioselectivity (>98% ε-thiolation) and retention of anomeric configuration in product were observed. Use of extended reaction times or excess thiol (3–10 equiv) allowed essentially complete conversion.

Pleasingly, extension of the reaction to the full range of glycosyl thiols revealed successful coupling in all cases (Table 1). Notably, rates and yields for *cis*-1,2-hydroxythiols β-ManSH and α-GlcSH were significantly lower (entries 5 (42%) and 8 (28%)), whilst those for less hindered *trans*-hydroxythiols were good to excellent. For these more reactive thiols, conversions were essentially complete even for larger sugars such as disaccharide Gal(β1,4)GlcSH; the isolated yields largely reflect difficulties handling amphiphilic products. Although disulfides have been suggested as putative thiyl precursors previously,[42] use of disulfide $(\beta\text{-GlcS})_2$ gave no product (entry 9). This suggests that not only thiols provide an important hydride source in the conjugation reaction but that the lower yields observed at higher pH (10) may be a consequence of more rapid competitive oxidation to unreactive disulfide. The chemoselectivity of the glycoconjugation reaction for Hag over each of the 20 naturally occurring natural amino acids was also investigated (see Supporting Information): when β-GlcSH was exposed to each amino acid under optimal conditions no conjugation products with any amino acid other than Hag were observed.[43]

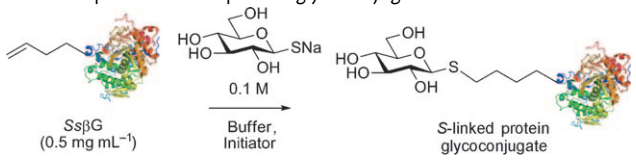
Next, the thiyl glycoconjugation procedure was applied to a number of model protein systems: a TIM-barrel protein (*Ss*βG),[44] a "cuboid", right-handed quadrilateral β-helix protein (Np276),[45] and self-assembled homomultimer virus-like bacteriophage particle Qβ.[46–48] Non-natural amino acid Hag was site-specifically introduced into these protein systems as an olefinic "tag" through expression of corresponding gene sequences in an auxotrophic strain of *E. coli* (B834(DE3)).[29] Gene sequences were designed to create proteins displaying olefin at a single site, the position of which could simply be controlled by the "Met" triplet codon ATG.[49]

Model protein substrate *Ss*βG-Hag43, with an olefin uniquely positioned at site 43, is formally a twelve-point mutant of the wild-type *Sulfolobus solfataricus* β-glycosidase in which all cysteine (Cys344Ser) and methionine (Met21Ile, Met73Ile, Met148Ile, Met206Ile, Met236Ile, Met275Ile, Met280Ile, Met383Ile, Met439Ile) residues occurring in the wild-type protein, except Met43, have been mutated to serine and isoleucine, respectively. Importantly, the elucidation of key structural and sequence features associated with an unique, serendipitous photocleavage reaction in certain TIM-barrel proteins such as *Ss*βG-WT[39,50] allowed us to also introduce a key stabilizing Pro152Ala mutation, in order to make the protein stable to UV light.

Use of *Ss*βG-Hag43 as a substrate in reactions with β-GlcSH allowed optimization (See Table 2 and Supporting Information for time course) of the protein chemistry and revealed conditions consistent with those determined in amino acid systems: fastest, complete conversions (>95%) were seen at pH < 7 through the combined use of Vazo44 and photoinitiation. Relatively high concentrations of thiol (up to 100 mM) also proved important for rapid reaction. Initial results suggest that hydride abstraction may be rate limiting;[51,52] unreacted thiol is readily recovered as its disulfide post-reaction. Importantly, all glycoconjugated *Ss*βG products were functionally (enzymatically) active, thereby confirming the benign nature of the reaction.

Having established conditions for the successful site-selective glycoconjugation to the single olefinic side-chain in *Ss*βG-Hag43 we explored extension to other glycans and proteins (Table 3). These revealed that, although slightly slower, glycoconjugations at pH 6 (entries 2, 6 and 9) were

Table 2: Optimization of protein glycoconjugations.[a]



Conditions	0.25 M acetate (pH 4)	0.25 M sodium phosphate (pH 7)	0.25 M CAPS (pH 10)
hν	>95% (5 h)	26% (8 h)	NR (8 h)
55 °C	≈10% (8 h)	NR (8 h)	NR (8 h)
Vazo44 (0.2 equiv), *hν*	>95% (3 h)	45% (9 h)	25% (9 h)
Vazo44 (0.2 equiv), 55 °C	>95% (3 h)	25% (7 h)	NR (9 h)

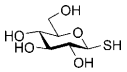
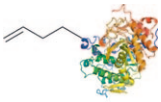
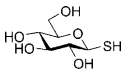
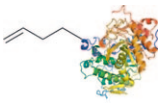
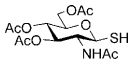
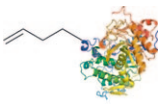
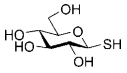
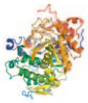
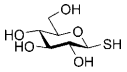
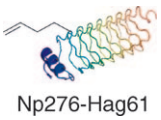
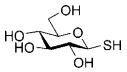
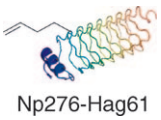
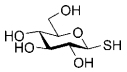
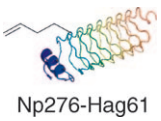
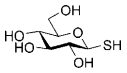
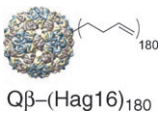
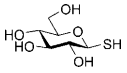
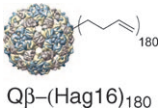
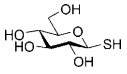
[a] NR = No reaction.

equally successful, yielding >95% product in 5 h or less. Although no degradation was seen here with any of the proteins at pH 4, this more mild pH should allow glycoconjugation to even highly acid-sensitive proteins. Rates of reaction for different thiols paralleled those seen in Table 1; for example, β-GlcNAcSH (Table 3, entry 3) was slightly slower than β-GlcSH (entry 1). Importantly, use of *Ss*βG-Met43, as a negative control substrate that does not contain Hag, showed no coupling (entry 4), further confirming the chemoselectivity of the reaction for the olefinic "tag" and the lack of reaction with other amino acids in *Ss*βG.

Cuboid protein Np276–Hag61 was also successfully glycoconjugated. Incorporation of Hag as the olefinic "tag" was again achieved through the mutation of the native *np276* gene to position a single ATG triplet codon appropriately, here allowing display of a single olefin at position 61. Reactions under optimal conditions were even more rapid than for *Ss*βG-Hag43. Again, reactions at pH 4 and 6 gave >95%; those at pH 7 proved sluggish and gave only 40% conversion after 3 h and did not reach completion.

Finally, we extended the glycoconjugation reaction to the self-assembled multimeric, virus-like particle Qβ (Table 3, entries 8–10).[46] Creation of a protein (Qβ-Hag16) with Hag at position 16 and self-assembly and purification allowed the creation of a virus-like particle displaying 180 olefins. Glycoconjugation under optimal conditions at either pH 4 or 6 gave rapid formation of product with complete consumption of starting protein suggesting complete glycoconjugation at all 180 sites (entries 8 and 9).[53–55] Again, use of a negative control substrate that does not contain Hag, showed no coupling (entry 10). Furthermore, reductive disassembly of the Qβ virus-like particle after reaction and alkylative labelling (see Supporting Information) confirmed that structurally key intramolecular disulfide bonds mediated by Cys 75 and Cys 81, which covalently link each assembled monomer, remained untouched by the hydrothiolation glycoconjugation reaction. This further confirmed the excellent chemoselectivity of the reaction for the Hag olefinic site, even with Cys-containing proteins. The Qβ particle is composed of 180 copies of a coat protein assembled into a icosahedral virion of average diameter 27 nm.[56] To our knowledge this precise and apparently complete site-selective glycoconjugation at multiple sites in a "protein nanoparticle" is unprecedented,[57,58] and we believe may find utility in the creation of precise glycoconjugate vaccines in which high levels of loading may be achieved whilst precisely controlling the position of epitope display.[48,59,60]

Table 3: Protein glycoconjugation reactions.

Entry	Glycosyl thiol[a]	Olefinic protein	pH	*t* [h]	Product [%]
1		 *Ss*βG-Hag43	4	3	>95
2		 *Ss*βG-Hag43	6	5	>95
3		 *Ss*βG-Hag43	4	3	>95
4		 *Ss*βG-Met43	6	12	0
5		 Np276-Hag61	4	2	>95
6		 Np276-Hag61	6	3	>95
7		 Np276-Hag61	7	3	40
8		 Qβ–(Hag16)$_{180}$	4	2	>95
9		 Qβ–(Hag16)$_{180}$	6	3	>95
10		 Qβ–WT	4	8	0

[a] 20-100 mM thiol, 0.1–0.5 mg mL^{-1} protein, Vazo44 (0.2 equiv), *hν*, room temperature.

In summary, we have developed an effective protein glycoconjugation method based on hydrothiolation that allows rapid and complete reaction under mild conditions (ambient temperature, pH 4–6) that have been shown to be compatible with a representative selection of different proteins. Combined use of this reaction with an olefinic "tag" that may be positioned using the non-natural amino

acid Hag allows site-selective glycoconjugation at pre-determined sites using chosen sugars and proteins through a "tag–modify"[30] approach (Hag "tag"–hydrothiolation "modify"). The reaction is compatible with all other natural amino acids, which are inert under these conditions; this chemoselectivity should allow its use in orthogonal, multi-modification approaches.[31] In addition, the glycosyl thiols needed as glycoconjugation partners may be readily accessed in a number of ways, even directly from unprotected reducing sugars.[61] Here, the reaction has been applied to proteins with different structures, which retain full function, for precise, site-selective glycoconjugation at single and even high levels of multiple sites. We are currently exploring the potential of this method to create glycoconjugate immunogens as putative vaccines.

Experimental Section

Representative glycoconjugation reaction: To a solution of Qβ-Hag16 (0.5 mg mL^{-1}, 500 μL) in 0.25 M sodium acetate buffer (pH 4) was added 1-thio-β-D-glucose (100 mM) and Vazo44 (20 mM). The reaction mixture was placed in a cuvette and irradiated with a medium-pressure 125 W Hg-lamp with borosilicate filter. The progress of the reaction was monitored by mass spectrometry (ESI-MS (ES+) and MALDI-TOF). After reaction virus-like particle samples (50 μL, 0.5 mg mL^{-1}) were mixed with urea (75 μL, 8 M) and DTT (dithiothreitol) (7.5 μL, 1 M), and incubated at 37 °C for 1 h to allow the protein to denature prior to analysis by ESI-MS. The samples were further incubated with iodoacetamide (7.5 μL, 1 M) at 37 °C for up to 16 h to cap any free cysteines prior to analysis by ESI-MS. *m*/*z* for monomer of Qβ-Hag16-S-β-Glc: calcd. 14301 (14417 with iodoacetamide); found 14303 (14416 with iodoacetamide). After 2 h these revealed essentially complete conversion (> 95 %) of Qβ-Hag16 into Qβ-$(CH_2)_5$-S-Glcβ, which could be isolated through dialysis, sucrose gradient and/or size-exclusion membrane purification.

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