A unique and highly facile method for synthesising disulfide linked neoglycoconjugates: a new approach for remodelling of peptides and proteins

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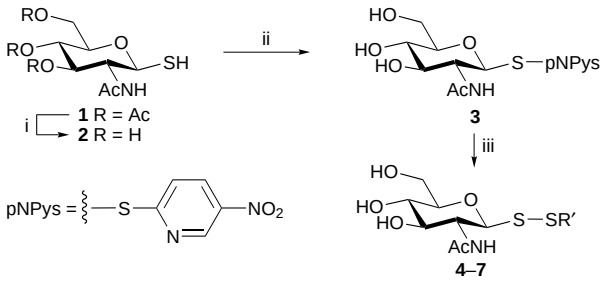
An asymmetric disulfide linkage, formed by conjugation of a 5-nitropyridine-2-sulfenyl activated thioglycoside and a protein or pre-assembled peptide sequence, represents a good structural mimic of natural asparagine glycosylation.

Most eukaryotic cellular proteins with the exception of some hormones and enzymes are reliant on the sugar units bound to them to confer a broad range of important biological functions, including immunogenicity, solubility, recognition, protection from proteolytic attack, and induction and maintenance of the protein conformation in a biologically active form.[1] The biosynthesis of *N*-linked glycoproteins is complex and results in micro-heterogeneity in the carbohydrate structure (formation of glycoforms).[2] Several studies have highlighted that each glycoform should be seen as a unique population of molecular species with particular biological properties.[3]

Much effort has been directed to alter the constitution of the carbohydrate protion of particular glycoproteins.[4] Saccharide glycoprotein remodelling (GPR) originally relied on cumbersome and inefficient means of adding carbohydrate structures, either by chemical or enzymatic methods, to proteins.[5] This approach has been supplanted by a technique using recombinant proteins to alter the intrinsic glycosylation pattern of cells.[6] However, the drawback of this technology is that it has the propensity to produce either inactive, undesired or missing glycoforms as part of the protein product.[7]

We report here an approach to the GPR problem which is based on asymmetric disulfide conjugation (an S–S glycosidic linkage) between a 5-nitropyridine-2-sulfenyl activated thioglycoside (*e.g.* **3**, Scheme 1) and a protein or pre-assembled peptide sequence having its natural asparagine glycosylation site replaced with a cysteine residue (R′SH). The merit of such an asymmetric disulfide linkage is that it represents a good structural mimic for natural *N*-linked glycoproteins[9] since the disulfide linkage is flexible enough to adopt conformations imposed by natural amide linkages at glycosylation sites.[10]

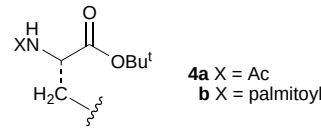
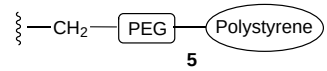
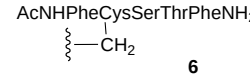
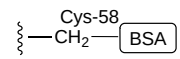
The synthesis of compound **3** was executed in five steps (25% yield overall) from commercially available *N*-acetylglucosamine *via* a modified procedure precedent for preparing 2-acetamido-2-deoxy-1-thio-β-D-glucose **2**.[11] Compound **1** decomposed during both Zemplén and KOBu^t mediated deacetylation conditions, but quantitatively gave thiol sugar **2** by a mild saponification procedure (Et_3N–MeOH–H_2O). The 5-nitropyridine-2-sulfenyl (pNpys) function was easily introduced onto mercapto-sugar **2** by treating the latter with 2,2′-dithiobis(5-nitropyridine) (DTNP) in AcOH–water[12] to yield compound **3** in a satisfactory yield of 58%. We anticipate the synthesis to be amenable for preparing oligosaccharide analogues thereof as judged by the mildness of the procedure.



Scheme 1 *Reagents and conditions*: i, Et_3N–MeOH–H_2O (2 : 5 : 5), 0.5 h, quant.; ii, DTNP, AcOH–water (12 : 1), 6 h, 58%; iii, R′SH, NH_4OAc (1 M) pH 5, 2–15 min

The activated thio-*N*-acetylglucosamine **3** under mild buffered conditions (1 M NH_4OAc, pH 5) is able to form asymmetric disulfide conjugates with a variety of free thiol-containing substrates. Reaction times are in the range of 2–15 min and yields are quantitative with excesses of **3** (*e.g.* product **7**). The generality thus far encompasses peptide, protein and polymer substrates, as shown in Table 1. Certainly, model conjugate **6** is of high immunological importance, in that the pentapeptide moiety represents that of a human IgG2 sequence[13] with Cys in place of Asn-297. Unfortunately, the ^{1}H NOESY spectra of both conjugate **6** and the natural glycopentapeptide equivalent (in this case Cys is replaced by Asn and thus becomes a structural motif for the natural *N*-glucoasparagine linkage found in wild-type human IgG2) did not reveal any cross peaks between sugar-peptide protons. Therefore the structural similarities between the disulfide and natural amide glycopeptide linkages of the neoglycotripeptide AcPhe(1-thio-β-GlcNAc-1→*S*)CysSerNHCH$_3$ **8** were examined by molecular modelling. Thus, a starting structure of compound **8** was built based on the tripeptide moiety of the same IgG2 sequence[13] (*vide supra*) with Cys in place of Asn-297 [AcPhe(β-GlcNAc-1→*N*)AsnSerNHCH$_3$ **9**] and subjected to Monte Carlo multi-

Table 1 Generality of synthesis of disulfide linked neoglycoconjugates

Mole ratio **3**/R′SH	Product R′	Yield (%)
1 : 1	 **4a** X = Ac **b** X = palmitoyl	53–65[a]
5 : 1	 **5**	63[b]
2 : 1	 **6**	77
50 : 1	 BSA = Bovine serum albumin **7**	quant.[c]

[a] Yields are representative for two different *N*-acylated cysteine derivatives prepared, X = Ac (65%) and X = Palmitoyl (53%). [b] Determined by the amount of **1** cleaved from the support (1,2-dithioerythritol–CH_2Cl_2) after treatment with Ac_2O–pyridine. [c] Absolute yield determined by HPAEC-PAD analysis of thio-*N*-acetylglucosamine **2** deconjugated from **7** using 1,2-dithioerythritol–water.

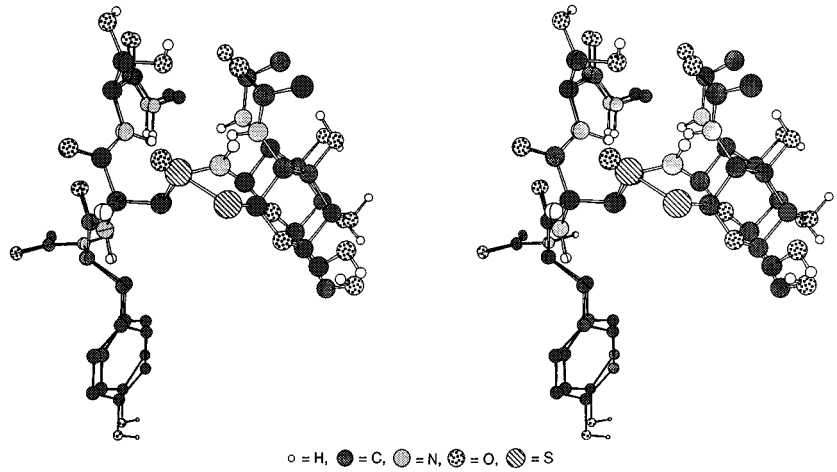


Fig. 1 Stereoviews showing superimposition of neoglycotripeptide **8** and neoglycotripeptide **9** as present in human IgG2

conformer analysis in combination with molecular mechanics calculations.[14] The global minimum of neoglycotripeptide **8** as well as all conformations lying within a 50 kJ mol^{-1} energy window were investigated using both filtering and clustering analysis resulting in families of conformers. Overlays of the resulting families of these conformations with glycopeptide **9** demonstrate that a similar spatial orientation as present at the glycosylation site of human IgG2[15] can be adopted by disulfide analogue **8** (see Fig. 1).

Bovine serum albumin (BSA) was chosen as a model protein on the merit of its availability and the convenience of having a single cysteinyl function at Cys-58 of its protein sequence. After 15 min of reacting **3** with BSA, the BSA-containing fractions were quickly isolated free from the excess amount of **3** and other low molecular weight thiol by-products (Sephadex G-15; eluent, water). Ellman analysis[16] of the isolated conjugate **7** against a blank sample of non-reacted BSA gave a zero absorbance reading at 412 nm, showing the conjugation to be complete. Further spectroscopic corroboration of conjugate **7** came from electrospray mass spectrometry. The mass increase for conjugate **7** over the BSA sample was 160 ± 100 while the theoretical difference is 235.[17]

The conjugate **7** was treated with a 50-fold excess of glutathione. After 7 days, the MALDI-TOF spectra of test sample aliquots showed only molecular ion peaks of the conjugate and no exchange products, which supports the idea that the disulfide bond contained in the conjugates are of considerable stability.

Currently, we are involved in the construction of an aglycosylated IgG recombinant antibody by site-directed mutagenesis which bears a cysteine residue in lieu of Asn-297. Covalent introduction of defined and activated oligosaccharide thiol derivatives will provide neoglycoconjugates that will enable us to study in detail the relationship between carbohydrate microstructure and biological properties of IgG molecules.

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Notes and References

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