Protein Modification

DOI: 10.1002/anie.200704381

From Disulfide- to Thioether-Linked Glycoproteins**

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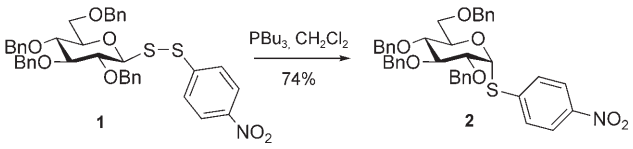
The presence of carbohydrate structures on proteins has been estimated to occur in 50% of eukaryotic cells[1] and is linked to several biological events,[2] such as the regulation of cell signaling,[3] cellular differentiation,[4] and immune response.[5] In nature, glycoproteins are found as heterogeneous mixtures, which complicates their characterization and functional determination.[6] Better access to homogeneous glycoproteins and their mimics is likely to improve our understanding of their roles.

Naturally occurring protein and peptide glycans are predominantly linked to an asparagine or serine/threonine residue, and many glycopeptide syntheses are based on the introduction of mimics of such tethers.[2] It was not until 1971 that a natural S-glycosidic linkage was identified on a peptide:[7] Löte and Weiss isolated octa- and decapeptides in which galactose and glucose, respectively, were attached to the side chain of an N-terminal cysteine residue.[8] Several methods have since been developed for the synthesis of S-linked glycopeptides:[9] the conjugate addition of a glycosyl thiol to a dehydroalanine-containing peptide,[10] the reaction of a glycosyl thiol with a β-bromoalanine moiety,[11] and the rearrangement of an allylic selenenylsulfide.[12] However, to date no chemical method has been applied to the synthesis of S-linked glycoproteins.[13] Importantly, S-linked glycopeptides display enhanced chemical[14] and enzymatic[15] stability relative to their native congeners. A process for desulfurizing disulfide-linked glycoproteins to provide thioether-linked homologues would allow ready access not only to this class of natural products but also to novel glycoproteins.

We have described previously the use of glycomethanethiosulfonates (glycoMTS),[16] glycophenylthiosulfonates (glycoPTS),[17] and glycoselenenylsulfides (glycoSeS)[18,19] as efficient chemoselective reagents for the synthesis of disulfide-linked glycopeptides and glycoproteins. Herein we present the synthesis of thioether-linked glycoconjugates from these readily synthesized disulfide-linked precursors.

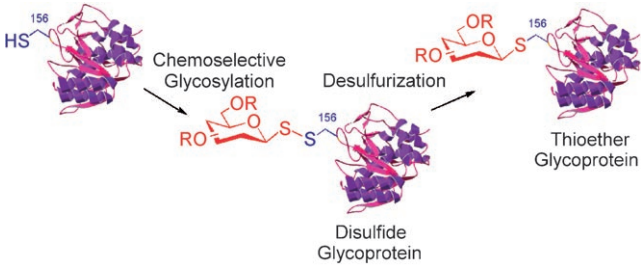
Important examples of the contraction of disulfide and peroxide linkages upon treatment with sources of P^{III} are known;[20] however, the generality of such transformations remains to be established. Our research in this area was motivated by a then surprising reaction of the sugar disulfide **1** with tributylphosphine to afford thioglycoside **2** in 74% yield (Scheme 1). Inversion at the anomeric center suggested



1 2

Scheme 1. Desulfurization of a thioglycoside. Bn = benzyl.

attack at the disulfide bond followed by an S_N2-like reaction of the resulting thiophosphonium salt (see Scheme S1 in the Supporting Information).[21] We considered that phosphines might mediate an analogous reaction in which a single sulfur atom is lost from a disulfide[22] glycoprotein to give the corresponding S-linked glycoprotein (Scheme 2). This transformation would provide controlled access to a thioether linkage resistant to reduction and less prone to enzymatic degradation than the corresponding disulfide linkage.



Scheme 2. Strategy for the site-selective synthesis of thioether-linked glycoproteins.

Disulfide-linked glycosyl amino acid and glycopeptide model substrates were constructed by using glycoPTS[17] and glycoSeS[18] methods, the reagents for which were prepared from the parent carbohydrates (Glc, Gal, Fuc, GlcNAc) in good yields (see Supporting Information). Initially, we used the glucosyl amino acid **3** as a model substrate with tributylphosphine (1.2 equiv) as the reducing agent and methanol as the solvent. The desulfurized product **4** was obtained in 8% yield as a mixture of diastereoisomers

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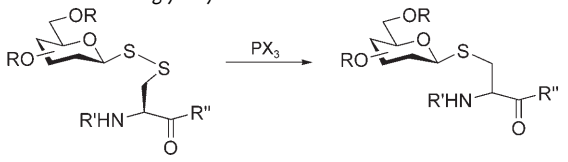
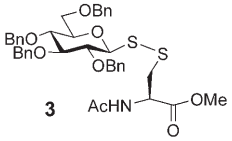
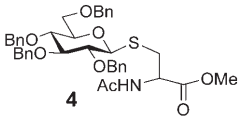
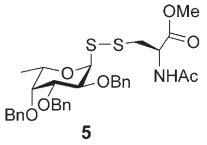
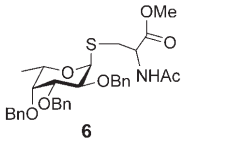
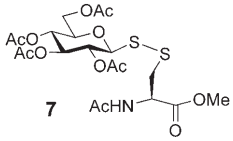
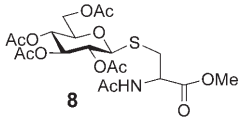
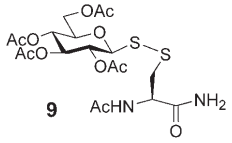
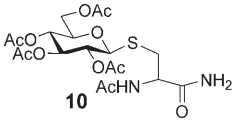
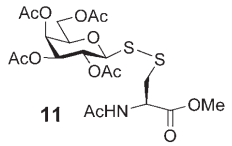
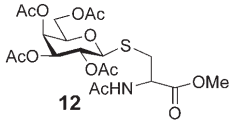
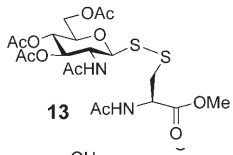
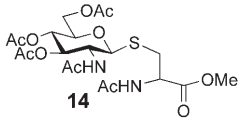
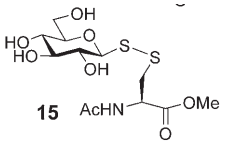
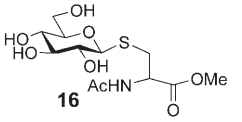
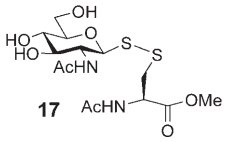
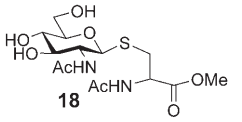
[**] We thank the Fundação para a Ciência e a Tecnologia, Portugal (G.J.L.B.), the Rhodes Trust (J.M.C), and the European Commission (F.E.O.) for financial support and the Daphne Jackson Trust for a returner's fellowship funded by the Royal Commission for the Exhibition of 1851 (E.J.G.).

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

Table 1: Desulfurization of disulfide glycosyl amino acids.[a]



Entry	Substrate	PX_3 (equiv)	t [h]	Product	Yield [%][b]
1	 **3**	PBu_3 (1.2)	6	 **4**	8
2		$P(NMe_2)_3$ (1.2)	3		41
3		$P(NMe_2)_3$ (2.2)	3		71
4		$P(NEt_2)_3$ (2.2)	3		73
5	 **5**	$P(NMe_2)_3$ (2.2)	3	 **6**	61
6	 **7**	$P(NMe_2)_3$ (2.2)	3	 **8**	69
7		$P(NEt_2)_3$ (2.2)	3		72
8		$P(NMe_2)_3$ (2.2)	5		47[c]
9	 **9**	$P(NMe_2)_3$ (2.2)	3	 **10**	75
10		$P(NMe_2)_3$ (2.2)	7		9[d]
11	 **11**	$P(NMe_2)_3$ (2.2)	3	 **12**	70
12	 **13**	$P(NMe_2)_3$ (2.2)	3	 **14**	73
13	 **15**	$P(NMe_2)_3$ (2.0)	3	 **16**	68
14	 **17**	$P(NMe_2)_3$ (2.0)	3	 **18**	74

[a] Unless otherwise noted, reactions were carried out in degassed anhydrous methanol at room temperature; see the Supporting Information for details. [b] The product was obtained as a 1:1 mixture of diastereomers in all cases, except in entry 12 (d.r. 6:5). [c] The reaction was carried out in methanol/water (2:1, v/v). [d] The reaction was carried out in methanol/water (1:1, v/v).

(Table 1, entry 1). Thus, the mechanism for the reaction of **3** appeared to differ from that observed for the reaction of **1**. The retention of the anomeric configuration suggests that the stereodivergence arises from the racemization of cysteine.[23] This hypothesis can be rationalized mechanistically by invoking a dehydroalanine intermediate formed by the disulfide cleavage of **3** and β elimination of the resulting thiophosphonium salt. The redelivery of the glycosyl thiol to dehydroalanine would provide **4** (see Scheme S3 in the Supporting Information).

The recovery of reduced cysteine and glycosyl thiols prompted us to use more polarized, electron-rich phosphines, which would attack the disulfide to give a phosphonium ion that was less susceptible to hydrolysis. Accordingly, the use of hexamethylphosphorous triamide (HMPT) afforded thioether **4** in an improved yield of 41%. Under optimized reaction conditions (HMPT (2.2 equiv), degassed anhydrous methanol, room temperature),[24] **3** was converted into the S-glycosyl amino acid **4** in 71% yield.[25]

The reaction was found to be broadly tolerant with respect to the sugar moiety (Glc, Gal, Fuc, GlcNAc) and the protecting group (Bn/Ac) and afforded the desired desulfurized products in good yields (61–75%; Table 1, entries 3–12) with retention of the anomeric configuration. The reaction could even be performed in aqueous solvents (Table 1, entries 8 and 10). The standard reaction conditions were fully compatible with the use of unprotected glycosyl amino acids: Compounds **15** and **17**, accessed by the glycoSeS method,[18] underwent desulfurization to give **16** and **18** in good yields (Table 1, entries 13 and 14).

The compatibility of the method with glycopeptides and other amino acid residues was also shown. The glucotripeptide **19** and galactotetrapeptide **21**, both of which were prepared by using glycoPTS[17] reagents, were desulfurized successfully with HMPT to give the corresponding S-linked glycopeptides **20** and **22** in 72 and 67% yield, respectively (Scheme 3).[26]

Next, we investigated the applicability of this novel ligation strategy to the conversion of disulfide-linked glycoproteins into thioether-linked glycoproteins. The disulfide-linked glucosyl protein **23** was constructed from a mutant of the serine protease subtilisin *Bacillus lentus* SBL-S156C, which contains a single cysteine residue, by using the glycoPTS method.[17] HMPT-mediated desulfurization converted **23** into the thioether-linked glycoprotein **24** (Figure 1).[27,28] The treatment of the newly formed thiother-containing protein **24** with tris(2-

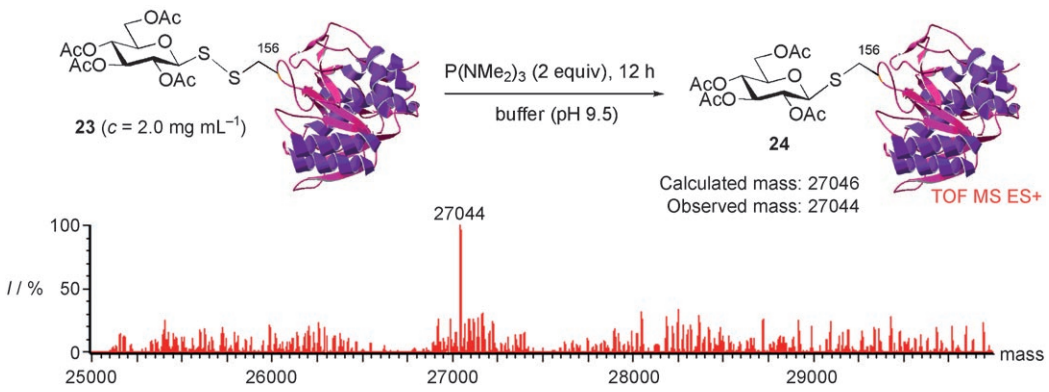
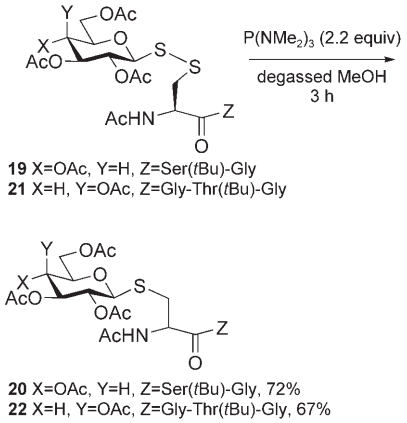


Figure 1. ESIMS spectrum of S156C-S-Glc(OAc)$_4$ (see the Supporting Information for full reaction details).



Scheme 3. Extension of desulfurization to the synthesis of S glycopeptides.

carboxyethyl)phosphine (TCEP) showed it to be resistant to reduction. When the disulfide-linked protein **23** was treated with TCEP, rapid reduction to the free thiol protein SBL-S156C was observed.

In summary, we have developed a desulfurization reaction that enables the conversion of readily synthesized disulfide-linked glycosyl amino acids, glycopeptides, and glycoproteins into the corresponding thioether-linked glycoconjugates. The reaction is compatible with a variety of protected and unprotected sugar residues, different amino acid residues, and an aqueous environment, and proceeds with retention of the anomeric configuration. We also applied this ligation method to protein modification in the first example of the chemical conversion of a surface-exposed cysteine residue to provide the corresponding S-linked glycoprotein.

Received: September 22, 2007
Published online: February 14, 2008

Keywords: disulfides · glycoproteins · glycosylation · protein modifications · thioethers

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[21] The electron-withdrawing nitro group was necessary for the formation of the product in useful yields. When unsubstituted thiophenol was used instead of *p*-nitrothiophenol as a component of the disulfide substrate, the thioglycoside was obtained in low yield, and a large amount of the anomeric thiol was formed (see the Supporting Information).

[22] A disulfide contraction of this type was observed recently for a disulfide derivative of a protected cysteine residue: D. Crich, V. Krishnamurthy, F. Brebion, M. Karatholuvhu, V. Subramanian, K. T. Hutton, *J. Am. Chem. Soc.* **2007**, *129*, 10282 – 10294.
[23] Cysteine epimerization, as opposed to carbohydrate anomerization, was established through the complete assignment of the NMR spectrum, including the critical observation of two distinct signals for 1-H^{β}.
[24] Similar yields were observed with hexaethylphosphorus triamide; however, the purification of the product was more cumbersome than when HMPT was used.
[25] Crossover experiments on differentially protected glycosyl cysteine derivatives under the optimized reaction conditions revealed that glycosyl exchange takes place and thus lent support for a dehydroalanine intermediate. ^{1}H NMR spectroscopic analysis allowed the direct observation of dehydroalanine formation and consumption (see the Supporting Information for details). The low diastereoselectivity in the subsequent conjugate addition is consistent with that previously reported in reference [10].
[26] Other α centers in the peptide did not undergo epimerization, which suggests that the observed epimerization at the α center of cysteine derives largely, if not exclusively, from dehydroalanine formation and not from deprotonation by HMPT.
[27] The diastereoselectivity of the reaction in Figure 1 will depend on the stereochemical environment of C156. Conjugate addition to dehydroalanine-containing peptides has been shown to be largely sequence dependent, with a diastereomeric ratio of $>$85:15 estimated for certain scaffolds: a) Ref. [10]; b) U. Schmidt, E. Öhler, *Angew. Chem.* **1976**, *88*, 54; *Angew. Chem. Int. Ed. Engl.* **1976**, *15*, 42.
[28] Modification at C156 was corroborated by trypsin digestion and MALDI MS analysis (see the Supporting Information).

