Glycoproteins

Glyco-SeS: Selenenylsulfide-Mediated Protein Glycoconjugation—A New Strategy in Post-Translational Modification**

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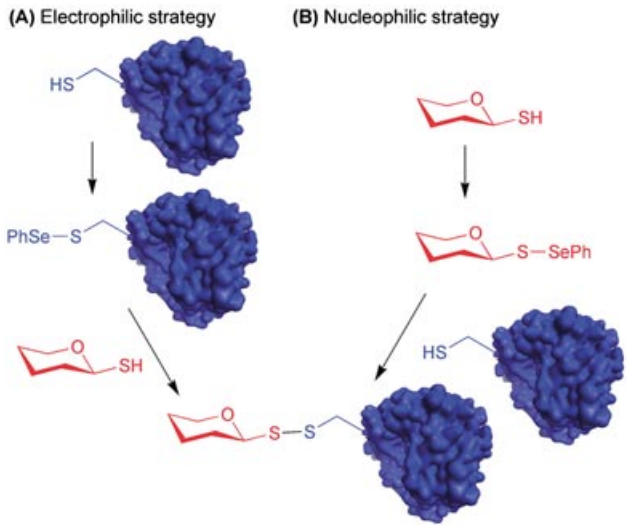
The co- and post-translational glycosylation[1] of proteins[2,3] is a key factor in protein folding and stability,[4] and plays a major role in essential biological processes, such as cell signaling and regulation,[5,6] development,[7] and immunity.[8] The study of these events is made difficult by the fact that glycoproteins occur naturally as mixtures of so-called glycoforms,[9] which possess the same peptide backbone but differ in both the nature and the site of glycosylation. Furthermore, since protein glycosylation is not under direct genetic control, the expression of therapeutic glycoproteins in mammalian cell cultures leads to heterogeneous mixtures of glycoforms.[10] The ability to synthesize homogeneous glycoprotein glycoforms is therefore a prerequisite not only for purposes of accurate investigation, but is of increasing importance for the preparation of therapeutic glycoproteins, which are currently marketed as multiglycoform mixtures (e.g. erythropoietin[11,12] and interleukins[13]).

Several chemical synthetic strategies have been developed to this effect.[14–18] We showed previously that the combined use of site-directed mutagenesis and chemoselective glycoconjugation could be used for site-selective protein glycoconjugation.[19,20] In this approach, a cysteine residue is introduced through mutagenesis to generate a protein nucleophile with a single free thiol, which is subsequently modified chemoselectively with electrophilic thiol-specific carbohydrate reagents, such as glycosyl methanethiosulfonates[19,20] or glycosyl phenylthiosulfonates.[21] This method makes possible the introduction of spacer-linked free and protected glycans alike, but only allows the preparation of directly linked free glycosides after enzymatic deprotection on protein,[22] thus potentially limiting its utility. Several other methods have been developed for the synthesis of glycoproteins based on cysteine modification to create a linkage, for example, the use of a 5-nitropyridine-2-sulfenyl-activated *N*-acetylglucosamine (GlcNAc),[23] or glycosyl iodoacetamides.[16] However, these methods have so far been limited to the addition of a single GlcNAc monosaccharide[16,23] and can be plagued by a lack of selectivity (modification of histidine residues) and/or incomplete reactions.[16] Furthermore, no multiple site-selective glycoconjugation has been demonstrated, a weakness that was highlighted recently.[24]

Inspired by the occurrence, albeit rare, of selenenylsulfide proteins in Nature as selective electrophilic moieties,[25,26] we report herein a selenenylsulfide-mediated protein glycoconjugation,[27] which allows glycoconjugation with mono- and oligosaccharides of up to seven saccharide units in size at single and multiple sites in a variety of proteins. Two parallel strategies were investigated in which the protein cysteine residue plays potentially contrasting electrophilic and nucleophilic roles (Scheme 1). In the first approach **A**, a cysteine-containing protein is converted into the corresponding (phenylselenenyl)sulfide; the electrophilic character of the sulfur atom in the resulting S–Se bond[28] renders it susceptible to nucleophilic substitution by 1-thio mono- or oligosaccharides. In the opposite approach **B**, 1-thio mono- or oligosaccharides are first converted into their selenenylsulfide analogues, which can subsequently be coupled to a cysteine residue. Thus, the cysteine residue this time acts as a nucleophile. Importantly, the exquisite selectivity of S–Se chemistry would obviate all need for protecting groups during glycoconjugation.



Scheme 1. Two potential parallel glycoconjugation strategies. The protein cysteine plays either an electrophilic role (**A**) or a nucleophilic role (**B**).

The representative monosaccharides glucose (Glc), galactose (Gal), and *N*-acetylglucosamine (GlcNAc), and oligosaccharides (trisaccharides) Glcα(1,4)-Glcα(1,4)-Glc (**7**) and Glcα(1,4)-(Glcα(1,4))$_5$-Glc (**10**) were chosen for the glycoconjugation reactions. To evaluate the feasibility of approach **A**, the glycosyl halides **1a–c** were converted into the

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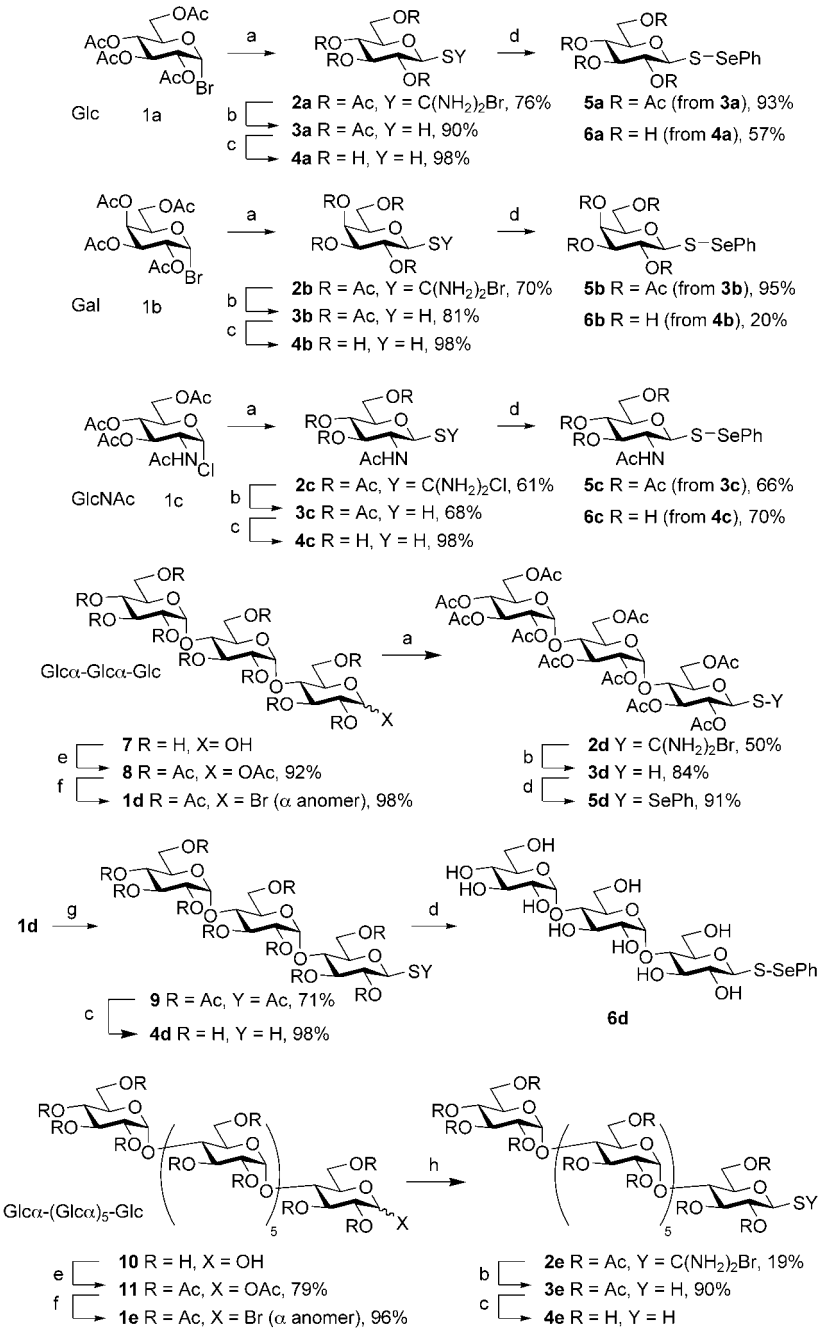
corresponding monosaccharide glycosyl-β-thiols **4a** (Glc), **4b** (Gal), and **4c** (GlcNAc) through treatment with thiourea to afford the corresponding isothiouronium salts,[29] followed by mild hydrolysis with sodium metabisulfite and Zémplen deacetylation (Scheme 2).[30] Following essentially similar procedures to those for the monosaccharides, the deprotected oligosaccharide thiols **4d** and **4e** were prepared from the maltotriose **7** and the maltoheptaose **10**, respectively (Scheme 2).



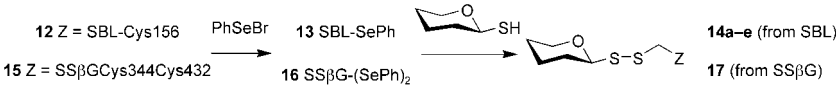
Scheme 2. Synthesis of protected and deprotected glycosylation reagents: a) thiourea, acetone, reflux; b) $Na_2S_2O_5$, CH_2Cl_2, water, 50 °C; c) NaOMe, MeOH; d) PhSeBr; e) NaOAc, Ac_2O, reflux; f) HBr (33%), AcOH, CH_2Cl_2; g) KSAc, acetone; h) thiourea, acetone, Bu_4NI (0.1 equiv), reflux.

Single-site glycoconjugation was explored by using the model cysteine-containing protein serine protease subtilisin *Bacillus lentus* mutant S156C[19,31] (SBL-Cys156, **12**; Table 1). The protein **12** was treated with phenylselenenyl bromide (PhSeBr)[32,33] to give the corresponding selenenylsulfide **13**, which was subsequently treated with the deprotected 1-thio monosaccharides **4a–c** to afford the corresponding glycosylated proteins **14a** (SBL-Glc), **14b** (SBL-Gal), and **14c** (SBL-GlcNAc) in quantitative yields as confirmed by ESI-MS (Table 1).[34] The power of this method was such that we were also able to glycosylate **12** quantitatively with the bulky trisaccharide **4d** and even with the heptasaccharide **4e**. To our knowledge the latter carbohydrate is the largest to have been used in a convergent site-selective protein glycoconjugation to date (Figure 1). In all cases quantitative protein glycoconjugation occurred rapidly (within 60 min)[35] with a low ratio of reagent to protein (typically 10–20 equiv of thiol, and as little as 1 equiv in some cases).[36] Moreover, the compatibility of this procedure with deprotected thio sugars removes the need for a postmodification deprotection step.

To demonstrate the applicability of this methodology to other proteins, and to carry out multiple-site glycoconjugations, we constructed a mutant of the thermophilic β-glycosidase from the archeon *Sulfolobus solfataricus*, which contains two cysteine residues (SSβG-Cys344Cys432, **15**). The doubly glycosylated protein **17** (SSβG-$[Glc]_2$) was obtained upon activation of **15** with PhSeBr, followed by treatment with the Glc thiol **4a**, as shown by ESI-MS (Table 1; m/z: calcd: 57775; found: 57760[37]). The three glycosylation sites in **12** (SBL-Cys156) and **15** (SSβG-Cys344Cys432) are found in a wide variety of protein structures and environments with different levels of exposure. This approach based on the modification of electrophilic cysteine residues can thus be used to prepare glycoproteins from very different proteins, and is not limited to single-site glycoconjugation, but is also amenable to multiple site-selective glycoconjugation. The site-selective modification of electrophilic protein residues is a rare but potentially powerful approach given the scarcity of other competing electrophiles.[15,38]

To probe approach **B** (Scheme 1) and the potential of a cysteine residue as a nucleophile, a series of protected glyco-SeS reagents were prepared readily from the thiols **3a–c** used above (Scheme 2). The protected glycosyl (phenylselenenyl)sulfides (glyco-SeS) **5a–c** were obtained from the reaction of the acetylated thiols **3a–c** with

Table 1: Glycoconjugation of **12** and **15** after activation with PhSeBr.[a]



Sugar thiol		Protein	Product		Conversion [%][b]	ESI-MS Found (calcd)
Glcβ-SH **4a**	SH	SBL-SSePh	**14a**		>95	26908 (26909)
Galβ-SH **4b**	SH	SBL-SSePh	**14b**		>95	26908 (26909)
GlcNAcβ-SH **4c**	SH	SBL-SSePh	**14c**		>95	26944 (26950)
GlcαGlcαGlcβ-SH **4d**		SBL-SSePh	**14d**		>95	27228 (27233)
Glcα(Glcα)$_5$Glcβ-SH **4e**		SBL-SSePh	**14e**		>95	27878 (27881)
Glcβ-SH **4a**	SH	SSβG-(SSePh)$_2$	**17**		>95	57760 (57775)

[a] Reagents: thiol (20 equiv), CHES (70 mM), MES (5 mM), $CaCl_2$ (2 mM), pH 9.5. [b] Conversion determined by ESI-MS.

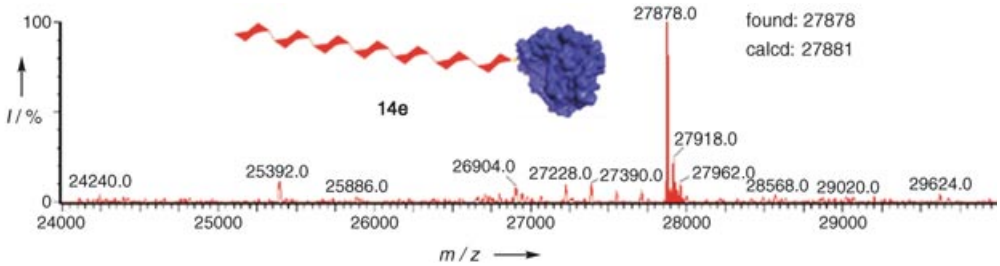


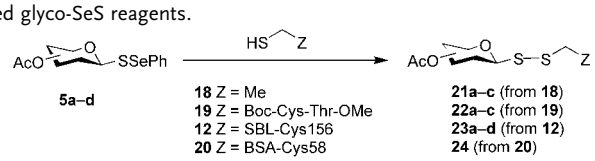
Figure 1. Deconvoluted ESI mass spectrum of **14e** (SBL-Glc(Glc)$_5$Glc).

PhSeBr. Their deprotected counterparts **6a–c** were obtained by using the same method following Zémplen deacetylation.[39] The protected and deprotected trisaccharides glyco-SeS **5d** and **6d**, respectively, were also prepared by this method (Scheme 2).

The protected and deprotected glyco-SeS reagents **5a–c** and **6a–c** were investigated in the glycoconjugation of the representative thiol EtSH (**18**) and dipeptide **19**. The corresponding protected glycoconjugate mixed disulfides **21a–c** and **22a–c** and their deprotected counterparts **25a**, **25c**, **26a**, and **26c** were all obtained in excellent yields, with complete retention of the anomeric β stereochemistry (Table 2 and Table 3). These novel glyco-SeS reagents were also investigated for their ability to glycosylate the model protein **12**. Again the glycoconjugation was quantitative, and the high purity of the eight glycoproteins **14a–d** and **23a–d** formed was confirmed by ESI-MS and Ellman titration.[40] The glycoprotein products **14a–d** were identical to those synthesized by using strategy **A**. To demonstrate the versatility of this methodology, we extended it to a larger cysteine-containing protein, bovine serum albumin (BSA-Cys58, **20**). The protein **20** was glycosylated successfully with both the protected and the deprotected Glc-SeS reagents **5a** and **6a** to afford **24** and **27**, respectively, and thus a third family of glycoproteins.

To further elucidate the mechanism of glycoconjugation with these glyco-SeS reagents, a time–course study of the reaction of **12** with the deprotected triose reagent **6d** was conducted, and the reaction conditions were explored (Figure 2). To our surprise, analysis by mass spectrometry

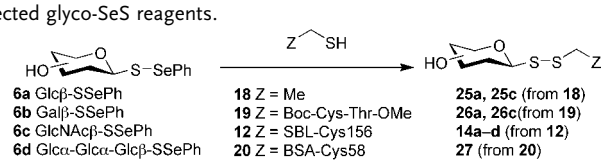
Table 2: Glycoconjugation with protected glyco-SeS reagents.



	EtSH (**18**)[a]		Dipeptide **19**[b]		SBLCys156 (**12**)[c]		BSA-Cys58 (**20**)[c]	
	Product	Yield [%]	Product	Yield [%]	Product	Conv. [%][d]	Product	Conv. [%][d]
Glc(Ac)$_4$-SSePh **5a**	**21a**	82	**22a**	75	**23a**	>95	**24**	>95
Gal(Ac)$_4$-SSePh **5b**	**21b**	82	**22b**	93	**23b**	>95	[e]	–
GlcNAc(Ac)$_3$-SSePh **5c**	**21c**	93	**22c**	88	**23c**	>95	–	–
Glc(Ac)$_4$Glc(Ac)$_3$Glc(Ac)$_3$-SSePh **5d**	–	–	–	–	**23d**	90	–	–

[a] Et_3N, CH_2Cl_2, room temperature, glyco-SeS/**18** 1:1. [b] Et_3N, CH_2Cl_2/MeOH (20:1), room temperature, glyco-SeS/**19** 3:1. [c] CHES (70 mM), MES (5 mM), $CaCl_2$ (2 mM), pH 9.5, 10–75 equivalents of glyco-SeS. Different quantities of the reagent were used typically to increase rate and/or convenience. Although prolonged exposure of glyco-SeS reagents to aqueous conditions caused decomposition in some cases, the reactions described were fast enough to minimize decomposition. In all cases no side reactions with protein were observed. [d] Conversion (Conv.) was determined by ESI-MS. [e] Dash (–) indicates reaction was not studied.

Table 3: Glycoconjugation with deprotected glyco-SeS reagents.



	EtSH (**18**)[a]		Peptide **19**[b]		SBLCys156 (**12**)[c]		BSA-Cys58 (**20**)[c]	
	Product	Yield [%]	Product	Yield [%]	Product	Conv. [%]	Product	Conv. [%]
Glc-SSePh **6a**	**25a**	90	**26a**	91	**14a**	>95	**27**	>95
Gal-SSePh **6b**	[d]	–	–	–	**14b**	>95	–	–
GlcNAc-SSePh **6c**	**25c**	77	**26c**	77	**14c**	>95	–	–
GlcGlcGlc-SSePh **6d**	–	–	–	–	**14d**	>95	–	–

[a] Et_3N, CH_2Cl_2, room temperature, glyco-SeS/**18** 1:1. [b] Et_3N, CH_2Cl_2/MeOH (20:1), room temperature, thioselenide/**19** 3:1. [c] 70 mM CHES, 5 mM MES, 2 mM $CaCl_2$, pH 9.5, 10–150 equivalents of glyco-SeS, conversion determined by ESI-MS. Different quantities of the reagent were used typically to increase rate and/or convenience. Although prolonged exposure of glyco-SeS reagents to aqueous conditions caused decomposition in some cases, the reactions described were fast enough to minimize decomposition. In all cases no side reactions with protein were observed. [d] Dash (–) indicates reaction was not studied.

showed the rapid formation of **13** (SBL-SePh; less than 1 min), followed by the subsequent formation of the glycosylated protein **14d** (Figure 2). This result suggests that an electrophilic glycoconjugation mechanism dominates regardless of the strategy (**A** or **B**) used. Thus, these two strategies may be used in a complementary manner, thereby increasing the flexibility of the glyco-SeS approach. We suggest that in strategy **B** an initial nucleophilic attack at the selenium center by the protein thiol generates **13** and liberates the sugar thiol **4d**, which is subsequently able to proceed exactly as in strategy **A** to displace SePh and form the glycosylated protein **14d** (SBL-GlcGlcGlc). Consistent with this mechanism, the use of a large excess of the glyco-SeS reagent was detrimental to the reaction, as the thiols released were then trapped by this reagent to give symmetrical disulfides. The reaction is pH-dependent: when carried out at pH 7.5 or pH 8.5 formation of the SBL-SePh intermediate **13** is observed, but just 10% conversion into the glycosylated protein **14d** occurs. Higher conversion is only observed at pH 9.5.[41] This observation is also consistent with the proposed mechanism of nucleophilic attack by the thiol **4d** (or the corresponding thiolate). These results show that glyco-SeS reagents in strategy **B** effectively act as the source of both the selenating reagent and the sugar thiol, thereby offering a convenient alternative to the two-step use of PhSeBr and a sugar thiol in strategy **A**.

Finally, to show the stability of the newly formed disulfide linkage towards enzymatic carbohydrate extension and to show that disulfide-linked glycoproteins may be processed by glycosyltransferases, this new glycoconjugation method was coupled with enzymatic carbohydrate extension (Figure 3). Inhibited GlcNAc-S-S-SBL was incubated with UDP-galactose (UDP-Gal) in the presence of β-1,4-galactosyltransferase,[42] which is known to catalyze the selective formation of the Galβ(1,4)-GlcNAc linkage.[43] The full conversion of the GlcNAc-S-S-SBL protein **14c** into the Galβ(1,4)-GlcNAc-S-S-SBL **28** was confirmed by ESI-MS (Figure 3), thereby further extending the utility of the glyco-SeS method.

In conclusion, we have described the synthesis of glycosyl selenenylsulfides (glyco-SeS), a novel class of glycosylating agents, and their use not only for the glycoconjugation of simple thiols and peptides (EtSH and dipeptides), but also of

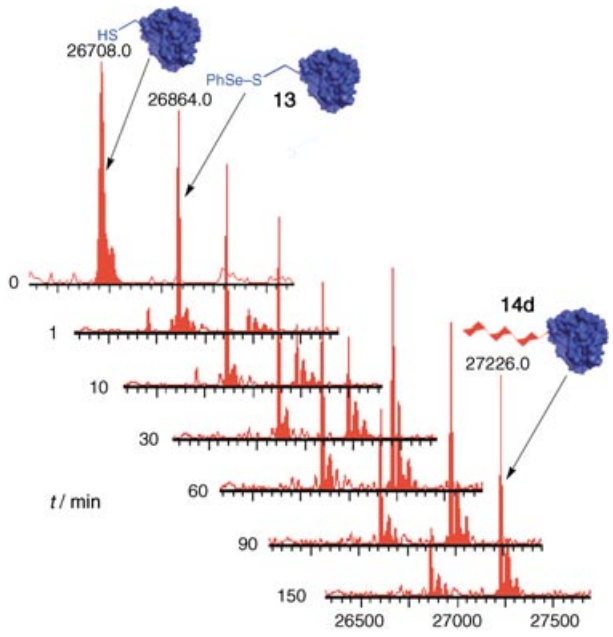


Figure 2. Deconvoluted ESI mass spectra showing glycoconjugation of **12** (SBLCys156; 26708 Da) with **6d** (GlcGlcGlc-SeS; 20 equiv) in a buffer solution (CHES (70 mM), MES (5 mM), $CaCl_2$ (2 mM); pH 9.5) after 1 min, 10 min, 30 min, 60 min, 90 min, 150 min. The selenenylated protein **13** (SBL-SePh; 26864 Da) is formed rapidly, followed by the glycoprotein **14d** (SBL-GlcGlcGlc; 27226 Da).

proteins. Whereas most site-specific glycoconjugation methods take advantage of the nucleophilic character of cysteine thiols (for example with glycosyl maleimides[44] or iodoacetamides[16,45]), our methodology is based upon a rarely exploited[15,38] but nonetheless powerful electrophilic glycoconjugation mechanism. In particular, this approach allows for the preparation of fully deprotected glycoconjugates and glycoproteins. We have demonstrated multiple site-selective glycoconjugation, which potentially provides access to polyvalent neoglycoproteins with control over valency,[24] the coupling of a heptasaccharide, and enzymatic elongation on a disulfide-linked glycoprotein after cysteine modification.[15] This methodology, combined with site-directed mutagenesis, allows the rapid, site-selective glycoconjugation of very different proteins with different fully deprotected carbohydrates, and the use of a low reagent-to-protein ratio. We are currently investigating the application of this novel selenenylsulfide methodology to other kinds of post-translational modification.

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Keywords: carbohydrates · glycoproteins · glycosylation · protein modifications · selenium

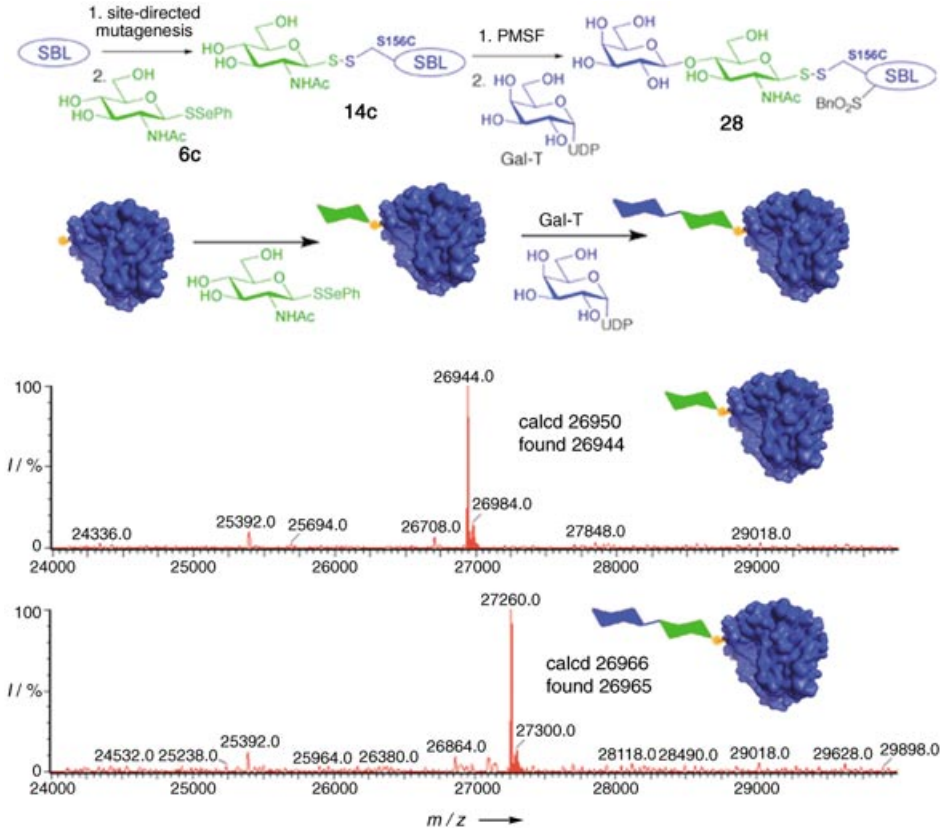

Figure 3. Chemical glycoconjugation of **12** (SBLCys156) with GlcNAc to form **14c** (GlcNAc-SBL; 2 mM), followed by the addition of a second monosaccharide unit (Gal) mediated by galactosyltransferase (Gal-T) to give **28** (Galβ(1,4)-GlcNAc-SBL): deconvoluted ESI mass spectra showing **14c** (GlcNAc-SBLCys156) before (top spectrum) and after (bottom spectrum) inactivation with phenylmethanesulfonyl fluoride (PMSF) and enzymatic galactosylation.

[1] The Gene Ontology Consortium has defined biological-process term-number GO:0006486 (protein amino acid glycosylation) as "The addition of a sugar unit to a protein amino acid, for example, the addition of glycan chains to protein." (see *Genome Res.* **2001**, *11*, 1425). Herein we use the broader term glycoconjugation to refer to the general process of addition of a glycosyl-unit-containing moiety to another moiety through a covalent linkage.

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[27] The reactions of thiols with unsymmetrical disulfides, in contrast to selenenylsulfides, are often slow and nonselective, typically require a large excess of the thiol, and lead to the release of thiol(ate), which may serve to cleave any disulfide formed. Reactions with selenenylsulfides are typically fast, favor S–S formation from S–Se through disproportionation, and release a selenate, which does not compete in the reaction.

[28] For examples of the behavior of S as an electrophile in S–Se-containing compounds, see: G. Bergson, G. Nordstrom, *Ark. Kemi* **1961**, *17*, 569; J. L. Kice, T. W. S. Lee, *J. Am. Chem. Soc.* **1978**, *100*, 5094; H. Fischer, N. Dereu, *Bull. Soc. Chim. Belg.* **1987**, *96*, 757. This reaction may compete with reaction at the electrophilic Se atom. However, reaction at the Se center would simply lead to in situ generation of glyco-SeS reagents, which would then feed into the same glycosylation sequence and ultimately lead to the required disproportionation of S–Se to S–S.

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[34] Representative glycosylation procedure: The protein **12** (SBLCys156; 1 mg) was dissolved in a buffer solution (1 mL; CHES (70 mM), MES (5 mM), $CaCl_2$ (2 mM); pH 9.5). The thio sugar **4a** (Glc-SH; 20 equiv) was added as a solution in water to the solution of the protein, and the mixture was placed on an end-over-end rotator. After 1 h the reaction was analyzed by mass spectrometry.

[35] A comparable glycosylation with iodoacetamides may take as long as 24 h: see reference [16].

[36] Considerably smaller quantities of the reagent (1–20 equiv) are required than typically used in protein glycosylation or protein modification (often of the order of 1000 equiv); see: B. G. Davis, *Curr. Opin. Biotechnol.* **2003**, *14*, 379. For more information, see Supporting Information.

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