

A New Ligation Strategy for Peptide and Protein Glycosylation: Photoinduced Thiol–Ene Coupling

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In memory of Professor Fernando Montanari

Protein glycosylation comprises a large family of post-translational modifications in eukaryotic organisms and is biosynthetically mediated by numerous glycosyltransferases.^[1] Notably, more than 50 % of proteins in human cells are glycosylated.^[2] The carbohydrate residues profoundly affect protein folding, immunogenicity, and stability toward proteases as well as control biological properties and activities.^[3] The latter functions operate mainly in cellular processes such as cellular recognition and adhesion, cell growth and differentiation.^[4] In this way glycoproteins are involved in vital biological events either detrimental (inflammation, cancer metastasis, viral and bacterial infection)^[5] or beneficial (immune response, fertilization). The isolation of glycopeptides and glycoproteins with a well-defined carbohydrate structure from natural sources is difficult and even with current techniques virtually impossible. This is due to two main factors, that is, the oligosaccharide microheterogeneity produced by the presence of various glycoforms and the very labile *N*- and *O*-acetal bonds which are the most diffuse connections between the terminal carbohydrate of the oligosaccharide fragment and asparagine or serine/threonine residue, respectively. Therefore it is difficult to identify the specific oligosaccharide structures that are responsible of an individual biological process or protein function. Hence there is an urgent need for the preparation of non-natural glycopeptides and glycoproteins exhibiting robust anomeric linkages and a well defined structure and composition. These

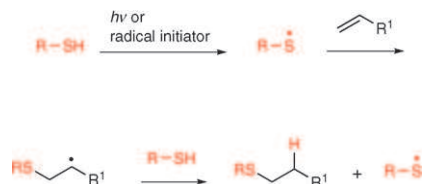
homogeneous materials are likely to improve our understanding of the role of the natural counterparts in biological events and can be interesting drug candidates in different areas of medicine. Methods for the synthesis of glycopeptides exploiting various peptide-ligation protocols based on chemoselective reactions have been chronicled in recent years.^[6] Notably, Shultz et al.^[7] in 2007 and Davis et al.^[8] in 2008 reported on the site-specific glycosylation of dehydroalanine-containing proteins by Michael additions of glycosyl thiol nucleophiles. Moreover, in the same year the Davis group described the first synthesis of an *S*-linked glycoprotein via phosphine-mediated desulfurization of a disulfide glycoprotein^[9] that in turn was prepared by glycosyl phenylthiosulfonate-mediated glycosylation of a protein containing a single cysteine residue.^[10] The same author and co-workers had previously combined the disulfide-based ligation approach with the copper(I)-catalyzed azide–alkyne cycloaddition for the dual glycosylation of proteins with different carbohydrate fragments.^[11] The availability of other ligation protocols for protein modifications^[12] are likely to extend the scope of single and dual protein glycosylation. While pointing out some specific requisites for a ligation method to be useful for protein modification, Davis et al. have very recently discussed the potential of olefin cross-metathesis for site-selective protein glycosylation.^[13] In this context, we would like to report herein on a new and straightforward ligation strategy that allows performing the glycopeptide and glycoprotein assembly in a site specific manner. The method is based on the photoinduced coupling of an alkenyl *C*-glycoside with a peptide or protein containing a free sulfhydryl group. Hence our method is essentially based on the alkene hydrothiolation reaction, the so called thiol–ene coupling (TEC), that being thermally or photochemically induced occurs by a radical mechanism in anti-Markovnikov regioselective fashion (Scheme 1).^[14] The great potential of this high yielding reaction that is not metal-based catalyst dependent and is compatible with oxygen and water has been amply documented in polymer chemistry and to a lesser

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extent in bioorganic chemistry.^[15] The potential role of this reaction in carbohydrate and protein chemistry has been validated, however, by significant applications such as heteroglycoclusters,^[16] vaccines,^[17] and *S*-disaccharides^[18] syntheses as well as by the site-specific immobilization and patterning of proteins.^[19]



Scheme 1. Mechanism of thiol-ene coupling (TEC).

We considered the study of the hydrothiolation of allyl *C*-glycosides by an SH-free cysteine derivative propaedeutic to our ultimate goal consisting of peptide and protein glycosylation.^[20] The use of a *C*-glycoside as a reaction partner ensured the formation of a robust anomeric linkage in the target glycosyl amino acid. Thus, we set out to investigate the coupling between the peracetylated allyl α -*C*-galactoside (**1a**)^[21] and *N*-Fmoc cysteine (**2**)^[22] with the intent to prepare the *C*-linked galactosyl amino acid **3a** (Table 1). This glycosyl amino acid appeared to be suitable in *N*-Fmoc-based peptide synthesis owing to the set of orthogonal protective groups. Given the successful thermally induced coupling of cysteine **2** with different alkenes reported by Waldmann and co-workers^[23] we carried out the reaction of **1a** and **2** in refluxing dichloroethane (DCE) and in the presence of AIBN as the radical initiator. Surprisingly, reactions with different thiol/ene ratios (entries 1–3) showed both very low conversion of the reagents and low yields^[24] of isolated amino acid **3a**. Thus, we decided to perform experiments under photochemical conditions. We were pleased to find that the reaction of **1a** with excess **2** (3 equiv) occurred readily at room temperature by irradiation at λ_{max} 365 nm under argon and in the presence (10 mol %) of 2,2-dimethoxy-2-phenyl-acetophenone (DPAP) as the sensitizer. A nearly total conversion of **1a** was observed while the galactosyl cysteine **3a** was isolated in a very good yield (run 4).^[24] It is noteworthy that under these photochemical conditions the stereochemical integrity of both reagents **1a** and **2** was preserved as only **3a** and no other diastereoisomers were formed as judged by NMR analysis of the crude reaction mixture. The only side product that was observed was the cystine derivative arising from homocoupling of cysteine **2**. The excess of cysteine **2** turned out to be a key element in this approach because reactions with alkene/thiol in 1:1 and 3:1 ratio (runs 5 and 6) gave poor results. Very important was also the high concentration of the thiol (0.5 M) because reactions at low concentration (0.05 M) were much less efficient (runs 7 and 8). Also, the amount of sensitizer was key to reaction efficiency as in a control experiment the use of

Table 1. Optimization of **1a/2** coupling.^[a]

Entry	Condi- tions	1a/2	Solvent (c [M])	<i>t</i> [h]	Conv. [%] ^[b] Yield [%] ^[c]
1 ^[d]	Δ	3:1	DCE (0.25)	14	20
2 ^[d]	Δ	1:1	DCE (0.25)	14	< 5
3 ^[d]	Δ	1:3	DCE (0.25)	14	25
4	<i>h</i> <i>ν</i>	1:3	DMF (0.5)	1	15
5	<i>h</i> <i>ν</i>	1:1	DMF (0.5)	1	> 95
6	<i>h</i> <i>ν</i>	3:1	DMF (0.5)	1	78
7	<i>h</i> <i>ν</i>	1:3	DMF (0.05)	1	60
8	<i>h</i> <i>ν</i>	1:3	DCM (0.05)	1	40
9 ^[e]	<i>h</i> <i>ν</i>	1:3	DMF (0.5)	1	35
					21
					70
					52
					60
					48
					75
					56

[a] Photoinduced reactions were carried out with a household UVA lamp apparatus at λ_{max} 365 nm (see the Supporting Information) in the presence of 10 mol % DPAP. DPAP = 2,2-dimethoxy-2-phenyl-acetophenone; DCE = dichloroethane; DCM = dichloromethane; DMF = dimethyl formamide. [b] Estimated by ¹H NMR analysis of crude reaction mixtures. [c] Isolated yield of *t*Bu ester derivative of **3a** (see the Supporting Information). [d] Reactions were performed in refluxing solvent and using AIBN as initiator. [e] Reaction performed with 5 mol % of DPAP.

5 mol % of DPAP resulted in a low conversion of **1a** (75 %, entry 9).

In order to overcome the troublesome operation required for product isolation due to the presence of cysteine and cystine^[24] and evaluate the efficiency of the reaction on the basis of isolated product yield, the photoinduced reaction of allyl α -*C*-galactoside **1a** with *N*-Fmoc cysteine *tert*-butyl ester (**4**)^[25] was performed under the optimized conditions of run 4 in Table 1. From this reaction, the galactosyl amino ester **5a** was readily isolated by chromatography in excellent yield (92 %) (Table 2). Comparable high yields of coupling products **5b** and **5c** were obtained from the reactions of **4** with allyl β -*C*-galactoside **1b** and allyl α -*C*-mannoside **1c**. Hence, a route was paved to orthogonally protected glycosyl cysteine derivatives which could serve in glycopeptide synthesis via co-translational approach. We have also considered the isolation of the glycosyl amino ester **5a** by removing the residual cysteine **4** with a thiol-sequestering reagent such as (4-maleidobutylamidomethyl)polystyrene resin. This method appeared to work quite well although **5a** remained contaminated by small amount of cystine and the products derived from the decomposition of photoinitiator DPAP.

Table 2. Preparation of glycosyl cysteines **5a–c** and glutathione glycoconjugate **7**.^[a]

alkene + thiol (3 equiv) $\xrightarrow[\text{DMF (0.5 M), 1 h, } h\nu \text{ 365 nm}]{\text{DPAP (10 mol\%)}}$ sulfide			
Entry	Alkene	Thiol	Sulfide
1			
2			
3			
4			

[a] See the Supporting Information for experimental details (thiol concentration: 0.54 M). [b] Reaction performed in DMF/nH₂O 1:2 (thiol concentration: 0.27 M).

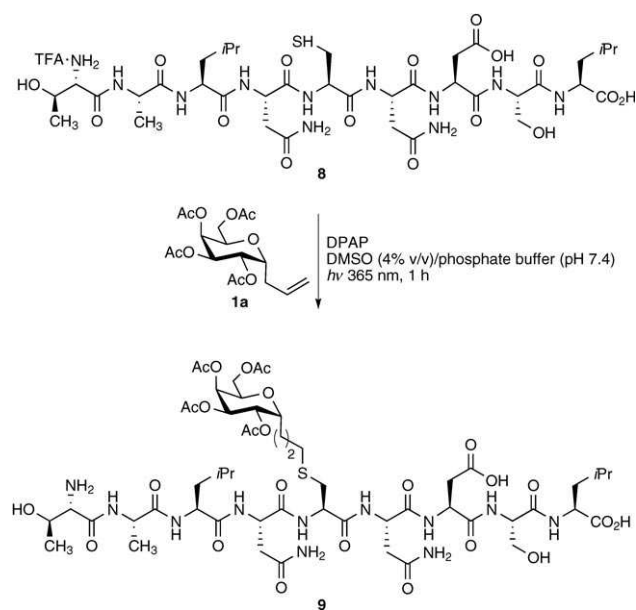
Hence **5a** was isolated in 95% yield and with a purity of 80% as determined by ¹H NMR analysis. It has to be pointed out, however, that this method of glycosyl cysteine isolation can be useful in library preparation for biological testing.

The glycosylation of cysteine containing peptide via TEC was the next step in our program. To this aim the commercially available tripeptide glutathione **6** (γ-L-Glu-L-Cys-Gly, GSH) was considered a suitable substrate for testing the viability of this approach. Thus, the photoinduced coupling of allyl α-C-galactoside **1a** with excess GSH **6** was carried out under the standard optimized conditions of run 4 in Table 1. The choice of a suitable reaction medium required some experimentation because **6** was insoluble in DMF and irradiation of a heterogeneous mixture of **1a** and **6** in this solvent gave the target glycosylated tripeptide **7** in only 15% yield. We found that a mixture of DMF/H₂O in 1:2 ratio was a suitable reaction medium. In this solvent the reaction of **1a** and **6** (Table 2, entry 4) furnished the coupling product **7** in 81% isolated yield by chromatography with Sephadex LH20.

Next we considered the photoinduced reaction of the allyl α-C-galactoside **1a** with the synthetic nonapeptide TALNCNDSL **8**^[26] featuring a single cysteine residue as well (Scheme 2). Besides expanding the scope of the methodology, we believed this experiment to be crucial for the

optimization of a different set of reaction conditions which were compatible with the planned protein glycosylation.

Hence, main requisites of this study were the use of non-denaturing reaction media, that is, aqueous solutions at physiological pH eventually diluted with salts for protein stabilization, and full conversion of the native peptide/protein for a simple and rapid purification process of the resulting glycoconjugate. Accordingly, after some experimentation, the coupling reaction of **1a** with **8** was optimized by adding a DPAP (80 mol %)^[27] containing solution of **1a** (30 equiv) in DMSO (0.12 M, 4% final volume content) to a 0.16 mM solution of **8** in 20 mM phosphate buffer (pH 7.4), and maintaining irradiation at λ_{max} 365 nm for 1 h under argon. The complete conversion of **8** into the corresponding glycosylated peptide **9**



Scheme 2. Preparation of glycopeptide **9**. DMSO = dimethyl sulfoxide.

was evaluated by LC-MS and MALDI-TOF MS analyses of the crude reaction mixture (Figure 1). The selective attachment of **1a** to the cysteine sulfhydryl group of **8** was also confirmed by ESI-QTOF MS/MS analysis (see Supporting Information). It has to be pointed out, however, that reac-

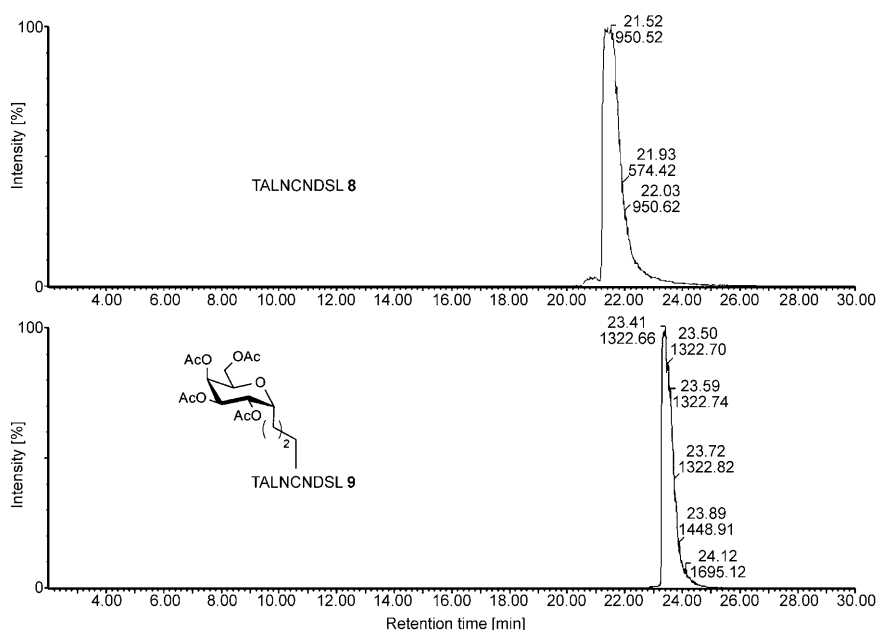


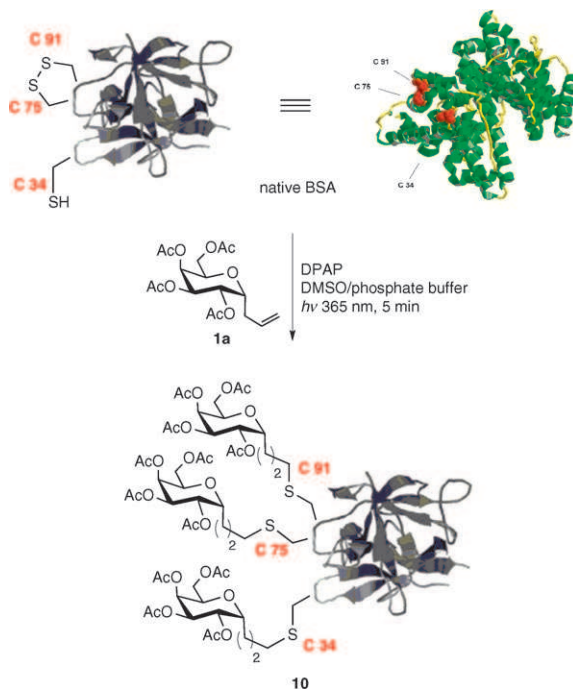
Figure 1. LC-MS chromatograms of peptide **8** (m/z 950.52) and glycopeptide **9** (m/z 1322.73).

tions performed at higher nonapeptide concentrations resulted in a partial loss of selectivity toward the cysteine residue (LC-MS analysis).

As anticipated, the ultimate goal of this study comprised the glycosylation of a cysteine residue embedded in a protein. To this aim we selected the 66 kDa globular bovine serum albumin (BSA) that is known to contain one free cysteine residue at position 34.^[28] Kunz and co-workers reported the photoinduced coupling of olefin-modified BSA with synthetic thiol-terminated glycopeptide antigens by irradiation with a mercury vapor lamp at $\lambda_{\max}$ 254 nm (70 W).^[17] As we observed (MALDI-TOF analysis) that under these conditions no glycosylation of BSA took place,^[29] we decided to trigger the coupling of BSA with the sugar alkene **1a** with a longer wavelength light at 365 nm which is known to cause minimum photodamage to the living cells and organisms.^[30] Much to our delight, irradiation of BSA and **1a** at this wavelength under conditions optimized for peptide **8** (DMSO/pH 7.4 phosphate buffer) afforded the BSA glycoconjugate **10** after 1 h in which three galactoside residues were introduced (Scheme 3). The quantitative formation of **10** was verified by MALDI-TOF analysis, which revealed peaks at masses 66652 and 67601 amu for native BSA and glycosylated BSA **10**, respectively, a shift corresponding to the addition of three molecules of galactoside **1a** per protein (Figure 2).^[31]

Tryptic digestion of **10** and ESI-QTOF MS/MS analysis of the resulting peptide fragments unequivocally demonstrated that BSA was glycosylated at cysteine residues 34 and 75 (see Supporting Information). Unfortunately, the third glycosylation site remained undetermined by this method. However, the analysis indicated that coupling of **1a** had occurred not only with SH-free cysteine 34 but also with sulfhydryl groups formed by cleavage of 75↔91 disulfide

bridge. Very likely Cys-91 resulted to be buried within the interior of protein after BSA glycosylation and thus not detectable by standard tryptic digestion.^[32] From a mechanistic point of view, it can be speculated that tryptophan residues present in BSA were responsible for the observed hyperglycosylation. It has been reported that prolonged near-UV irradiation of goat α -lactalbumin^[33] or hen egg white lysozyme^[34] induces tryptophan residue excitation and this is followed by electron transfer to some nearby cystine S–S bonds with consequential cleavage into thiol radical and thiolate anion. The same mechanism may be



Scheme 3. Preparation of BSA glycoconjugate **10**.

operative in the BSA irradiation, thus providing an extra number of thiol radicals centered on the former cysteine residues. Indeed, when irradiation was prolonged to 2 h the MALDI-TOF analysis indicated that seven glycoside residues were introduced (see Supporting Information). Hence, identification of a reaction window for selective Cys-34 glycosylation was next addressed by considering shorter irradiation times (45, 30, 15, 5, 3, 2, 1, 0.5 min) and/or aerobic conditions. Unfortunately, this goal could not be achieved

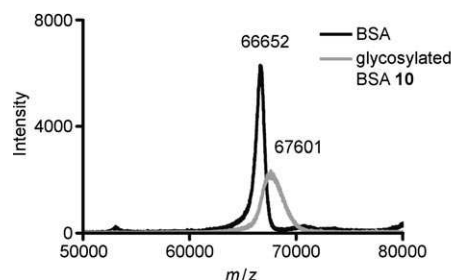


Figure 2. MALDI-TOF analysis of native BSA (black trace) and BSA glycoconjugate **10** (gray trace).

by these experiments. However, we demonstrated that BSA glycoconjugate **10** could be synthesized in a reproducible manner even under aerobic conditions in five minutes only. By contrast, no glycosylation took place in experiments performed with shorter irradiation times than five minutes as intact BSA was detected by MALDI-TOF analysis. Moreover, in order to exclude the potential addition of the sensitizer DPAP to the protein, a control experiment was carried out by irradiation of BSA in the presence of DPAP for 5 min. The MALDI-TOF spectrum of the recovered BSA proved to be identical to that of a non-irradiated sample; tryptic digestion followed by ESI-QTOF MS/MS analysis also confirmed that no modification occurred at Cys-34 residue.

In conclusion, we have demonstrated that the photoinduced thiol–ene coupling (TEC) is an efficient ligation method for the site-selective glycosylation of *N*- and *C*-protected cysteine derivatives and cysteine containing peptides such as glutathione and a synthetic nonapeptide. Moreover, post-translational glycosylation of bovine serum albumin (BSA) displaying the free Cys-34 has been deeply investigated. The use of simple carbohydrate substrates (sugar alkenes) and proteins, which could be employed in their native form without any chemical modification/activation, appeared the great advantage of this photoinduced ligation strategy over other existing methods for protein glycosylation. However, in addition to Cys-34, glycosylation of SH groups arising from 75↔91 disulfide bridge cleavage was observed. Very likely, disulfide bond cleavage was due to an electron transfer process from photoexcited tryptophan residues. Although photodegradation of proteins by UV irradiation is widely documented,^[34] the reported study on thiol–ene coupling of BSA constitutes the first example, to the best of our knowledge, of site-selective protein modification (hyperglycosylation) induced by a photocleavage reaction. As very recently pointed out by Davis et al.,^[35] this novel strategy of protein modification may offer new opportunities in biotechnology and proteomics. The potential of thiol–ene coupling in these areas is currently investigated in our laboratories.

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Keywords: alkynes • glycoproteins • glycosylation • protein modification • thiols

- [1] J. Marth, B. Lowe, A. Varki in *Essentials of Glycobiology* (Eds.: A. Varki, R. D. Cummings, J. D. Esko, H. H. Freeze, G. W. Hart, J. Marth), Cold Spring Harbor Laboratory, New York, **1999**, Chapters 8; J. Marth, B. Lowe, A. Varki in *Essentials of Glycobiology* (Eds.: A. Varki, R. D. Cummings, J. D. Esko, H. H. Freeze, G. W. Hart, J. Marth), Cold Spring Harbor Laboratory, New York, **1999**, Chapters 17; b) P. Sears, C.-H. Wong, *Cell. Mol. Life Sci.* **1998**, *54*, 223–252; c) H. J. Gabius, S. Andre, H. Kaltner, H. C. Siebert, *Biochim. Biophys. Acta* **2002**, *1572*, 165–177.
- [2] R. Apweiler, H. Hermjakob, N. Sharon, *Biochim. Biophys. Acta* **1999**, *1473*, 4–8.
- [3] Review: O. Seitz, *ChemBioChem* **2000**, *1*, 214–246.
- [4] a) A. Varki, *Glycobiology* **1993**, *3*, 97–130; b) R. A. Dwek, *Chem. Rev.* **1996**, *96*, 683–720; c) C. R. Bertozzi, L. L. Kiessling, *Science* **2001**, *291*, 2357–2364; d) P. M. Rubb, T. Elliott, P. Cresswell, I. A. Wilson, R. A. Dwek, *Science* **2001**, *291*, 2370–2376.
- [5] a) S. Kornfeld, *J. Clin. Invest.* **1998**, *101*, 1293–1295; b) R. G. Spiro, *Glycobiology* **2002**, *12*, 43R–56R.
- [6] Reviews: a) H. C. Hang, C. R. Bertozzi, *Acc. Chem. Res.* **2001**, *34*, 727–736; b) M. J. Grogan, M. R. Pratt, L. A. Marcaurelle, C. R. Bertozzi, *Annu. Rev. Biochem.* **2002**, *71*, 593–634; c) B. G. Davis, *Chem. Rev.* **2002**, *102*, 579–601; d) F. Peri, F. Nicotra, *Chem. Commun.* **2004**, 623–627; e) C.-H. Wong, *J. Org. Chem.* **2005**, *70*, 4219–4225; f) L. Liu, C. S. Bennett, C.-H. Wong, *Chem. Commun.* **2006**, 21–33; g) C. Haase, O. Seitz, *Top. Curr. Chem.* **2006**, *267*, 1–36; h) D. P. Gamblin, E. M. Scanlan, B. G. Davis, *Chem. Rev.* **2009**, *109*, 131–163. Selected articles: i) H. Lin, C. T. Walsh, *J. Am. Chem. Soc.* **2004**, *126*, 13998–14003; j) J. M. Langenhan, B. R. Griffith, J. S. Thorson, *J. Nat. Prod.* **2005**, *68*, 1696–1711; k) Q. Wan, J. Chen, G. Chen, S. J. Danishefsky, *J. Org. Chem.* **2006**, *71*, 8244–8249; l) R. J. Payne, S. Ficht, W. A. Greenberg, C.-H. Wong, *Angew. Chem.* **2008**, *120*, 4483–4487; *Angew. Chem. Int. Ed.* **2008**, *47*, 4411–4415; m) N. Yamamoto, Y. Tanabe, R. Okamoto, P. E. Dawson, Y. Kajihara, *J. Am. Chem. Soc.* **2008**, *130*, 501–510; o) R. Okamoto, S. Souma, Y. Kajihara, *J. Org. Chem.* **2008**, *73*, 3460–3466.
- [7] J. Wang, S. M. Schiller, P. G. Schultz, *Angew. Chem.* **2007**, *119*, 6973–6975; *Angew. Chem. Int. Ed.* **2007**, *46*, 6849–6851.
- [8] G. J. L. Bernardes, J. M. Calker, J. C. Errey, B. G. Davis, *J. Am. Chem. Soc.* **2008**, *130*, 5052–5053.
- [9] G. J. L. Bernardes, E. J. Grayson, S. Thompson, J. M. Chalker, J. C. Errey, F. El Oualid, T. D. W. Claridge, B. G. Davis, *Angew. Chem.* **2008**, *120*, 2276–2279; *Angew. Chem. Int. Ed.* **2008**, *47*, 2244–2247.
- [10] D. P. Gamblin, P. Garnier, S. J. Ward, N. J. Oldham, A. J. Fairbanks, B. G. Davis, *Org. Biomol. Chem.* **2003**, *1*, 3642–3644.
- [11] a) S. I. van Kasteren, H. B. Kramer, H. H. Jensen, S. J. Campbell, J. Kirkpatrick, N. J. Oldham, D. C. Anthony, B. G. Davis, *Nature* **2007**, *446*, 1105–1109; b) D. P. Gamblin, S. I. van Kasteren, J. M. Chalker, B. G. Davis, *FEBS J.* **2008**, *275*, 1949–1959.
- [12] For reviews, see: a) B. G. Davis, *Pure Appl. Chem.* **2009**, *81*, 285–298; b) C. P. Hackenberger, R. D. Schwarzer, *Angew. Chem.* **2008**, *120*, 10182–10228; *Angew. Chem. Int. Ed.* **2008**, *47*, 10030–10074. For a recent article, see: c) T. Kurpiers, H. D. Mootz, *Angew. Chem.* **2009**, *121*, 1757–1760; *Angew. Chem. Int. Ed.* **2009**, *48*, 1729–1731.
- [13] Y. A. Lin, J. M. Chalker, B. G. Davis, *ChemBioChem* **2009**, *10*, 959–969.
- [14] K. Griesbaum, *Angew. Chem.* **1970**, *82*, 276–290; *Angew. Chem. Int. Ed. Engl.* **1970**, *9*, 273–287.

- [15] For a brief commentary on the potential of this reaction in synthesis, see: A. Dondoni, *Angew. Chem.* **2008**, *120*, 9133–9135; *Angew. Chem. Int. Ed.* **2008**, *47*, 8995–8997.
- [16] M. Gómez-García, J. M. Benito, D. Rodríguez-Lucena, J.-X. Yu, K. Chmurski, C. Ortiz Mellet, R. Gutiérrez Gallego, A. Maestre, J. Defaye, J. M. García Fernández, *J. Am. Chem. Soc.* **2005**, *127*, 7970–7971.
- [17] S. Wittrock, T. Becker, H. Kunz, *Angew. Chem.* **2007**, *119*, 5319–5323; *Angew. Chem. Int. Ed.* **2007**, *46*, 5226–5230.
- [18] M. Fiore, A. Marra, A. Dondoni, *J. Org. Chem.* **2009**, *74*, 4422–4425. For further applications of TEC in bioorganic chemistry, see references therein.
- [19] P. Jonkheijm, D. Weinrich, M. KLhn, H. Engelkamp, P. C. M. Christensen, J. Kuhlmann, J. C. Maan, D. Nesse, H. Schroeder, R. Wacker, R. Breinbauer, C. M. Niemeyer, H. Waldmann, *Angew. Chem.* **2008**, *120*, 4493–4496; *Angew. Chem. Int. Ed.* **2008**, *47*, 4421–4424.
- [20] The thiol–ene reaction between *N*-allyl glycosides and cysteamine was reported by Klaffke and co-workers: D. Ramos, P. Rollin, W. Klaffke, *J. Org. Chem.* **2001**, *66*, 2948–2956.
- [21] Prepared by debenzoylation with $\text{BCl}_3\cdot\text{OEt}_2$ of the corresponding tetra-*O*-benzyl derivative and subsequent acetylation with Ac_2O /pyridine (J. Xie, M. Ménand, J.-M. Valéry, *Carbohydr. Res.* **2005**, *340*, 481–487).
- [22] M. Lumbierres, J. M. Palomo, G. Kragol, S. Roehers, O. Müller, H. Waldmann, *Chem. Eur. J.* **2005**, *11*, 7405–7415.
- [23] G. Triola, L. Brunsveld, H. Waldmann, *J. Org. Chem.* **2008**, *73*, 3646–3649.
- [24] Yields of isolated products were much lower than expected from the observed conversion of the reagents because of the difficult separation of **3a** by chromatography (silica gel, 95:5 $\text{CH}_2\text{Cl}_2/\text{MeOH}$) from unreacted cysteine **2** and cystine that formed as side-product. The product **3a** was characterized as *tert*-butyl ester obtained by treatment with *tert*-butyl trichloroacetimidate (2 equiv) in CH_2Cl_2 at room temperature.
- [25] M. F. M. Mustapa, R. Harris, N. Bulic-Subanovic, S. L. Elliott, S. Bregant, M. F. A. Groussier, J. Mould, D. Schultz, N. A. L. Chubb, P. R. J. Gaffney, P. C. Driscoll, A. B. Tabor, *J. Org. Chem.* **2003**, *68*, 8185–8192.
- [26] This compound was kindly provided as trifluoroacetic salt by UF-Peptides s.r.l. (University of Ferrara).
- [27] Surprisingly, the use of lower amounts of DPAP (50 and 10 mol%) were not sufficient for achieving a complete glycosylation of this peptide.
- [28] J. Janatova, J. K. Fuller, M. J. Hunter, *J. Biol. Chem.* **1968**, *243*, 3612–3622.
- [29] Control experiments were performed by irradiating ($\lambda_{\text{max}}=254\text{ nm}$, 70 W) solutions of BSA and sugar **1a** in DMSO–phosphate buffer (pH 7.4) for 3–6 h either in the absence or presence of 4,4'-azobis(4-cyanovaleic acid) as the sensitizer.
- [30] a) W. Song, Y. Wang, J. Qu, Q. Lin, *J. Am. Chem. Soc.* **2008**, *130*, 9654–9655; b) Y. Wang, W. J. Hu, W. Song, R. K. Lim, Q. Lin, *Org. Lett.* **2008**, *10*, 3725–3728, and references therein.
- [31] Indeed, the detected mass shift was lower than the value calculated for the addition of three sugar units ($3\times 372\text{ uma}$). This result is explained by the presence of a portion of **10** containing two galactose units only. As commonly observed for partially oxidized commercial batches of BSA, the BSA subjected to functionalization with **1a** contained only a fraction of available free sulfhydryl groups at Cys34 (ca. 45% from previous studies: a) P. De, M. Li, S. R. Gondi, S. B. Sumerlin, *J. Am. Chem. Soc.* **2008**, *130*, 11288–11289; b) B. Le Droumaguet, K. Velonia, *Angew. Chem.* **2008**, *120*, 6359–6362; *Angew. Chem. Int. Ed.* **2008**, *47*, 6263–6266).
- [32] Unfortunately, pre-treatment of glycosylated BSA **10** with dithiothreitol (DTT) for protein disulfide bonds reduction followed by excess iodoacetamide (IAA) sulfhydryl groups derivatization produced tryptic fragments, which displayed the acetamido group at cysteine residues 34, 75, and, very likely, at Cys-91 in place of the expected galactoside moiety (see the Supporting Information). This outcome is tentatively explained by the formation of intermediate *S*-acetamido sulfonium derivatives of glycosylated cysteine peptide fragments followed by a β -elimination process, which should result in the formation of the allyl galactoside **1a** and *S*-acetamido peptide fragments (see Scheme S1). The occurrence of this undesired side reaction that makes the DTT/IAA analysis method unsuitable for the characterization of glycosylated BSA **10** was also demonstrated by using the less complex glycopeptide **9** as the substrate (Supporting Information). For a deeper discussion on the β -elimination reaction of dialkylated sulfonium derivatives of Cys and the synthetic potential of related oxidative elimination of Cys for protein modification, see ref. [8].
- [33] a) A. Vanhooren, B. Devreese, K. Vanhee, J. Van Beeumen, I. Hanssens, *Biochemistry* **2002**, *41*, 11035–11143; b) E. A. Permyakov, S. E. Permyakov, G. Y. Deikus, L. A. Morozova-Roche, V. M. Grishchenko, L. P. Kalinichenko, V. N. Uversky, *Proteins Struct. Funct. Bioinf.* **2003**, *51*, 498–503.
- [34] L.-Z. Wu, Y.-B. Sheng, J.-B. Xie, W. Wang, *J. Mol. Struct.* **2008**, *882*, 101–106.
- [35] N. Floyd, N. J. Oldham, C. Eyles, M. Brouard, B. G. Davis, Abstracts of Papers, 237th ACS National Meeting, Salt Lake City, **2009**.

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