Peptide Linkage

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A PEGylated Photocleavable Auxiliary Mediates the Sequential Enzymatic Glycosylation and Native Chemical Ligation of Peptides**

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Abstract: *Research aimed at understanding the specific role of glycosylation patterns in protein function would greatly benefit from additional approaches allowing direct access to homogeneous glycoproteins. Herein the development and application of an efficient approach for the synthesis of complex homogenously glycosylated peptides based on a multifunctional photocleavable auxiliary is described. The presence of a PEG polymer within the auxiliary enables sequential enzymatic glycosylation and straightforward isolation in excellent yields. The auxiliary-modified peptides can be directly used in native chemical ligations with peptide thioesters easily obtained by direct hydrazinolysis of the respective glycosylated peptidyl resins and subsequent oxidation. The ligated glycopeptides can be smoothly deprotected by UV irradiation. We apply this approach to the preparation of variants of the epithelial tumor marker MUC1 carrying one or more Tn, T, or sialyl-T antigens.*

Posttranslational modifications (PTMs) are essential in determining the folding and activity of many proteins and their misregulation is often involved in the progression of severe diseases.[1] Despite many advances in our understanding of protein modifications with carbohydrates,[2] and the ability to generate glycosylated proteins by highly evolved chemical synthesis approaches[3] as well as by combinations of enzymatic and synthetic means,[4] insights into the specific roles of this class of PTMs in protein function remain a challenge due to their complexity and heterogeneity. We have recently developed an efficient method for the preparation of site-specifically O-glycosylated mucin 1 (MUC1) polypeptides,[5] in which a monodisperse PEG polymer at the N-terminus of the peptides allowed quantitative enzymatic glycosylation in solution and the recovery of the modified peptides by simple precipitation and spin column gel permeation chromatography (GPC), avoiding elaborate intermediate purifications. This method proved to be high-yielding and versatile, as it could be coupled with native chemical ligation (NCL), thereby giving access to longer site-specifically O-glycosylated peptides.

However, the proteolytic removal of the polymer required installation of a protease recognition site consisting of six amino acids, and a non-native cysteine residue had to be introduced to carry out NCL reactions. To overcome these limitations we now combined both functionalities (PEG attachment[6] and thiol group for NCL[7]) in a photocleavable auxiliary, based on the 1-nitrophenyl-2-sulfanylethyl scaffold previously described by the groups of Aimoto[8] and Dawson.[9] Upon photolytic removal a native glycine residue remains at the ligation site. The mild deprotection conditions together with the high occurrence of glycine in proteins have made this approach suitable for several applications in protein (semi)synthesis.[10] Herein we present the synthesis and application of a photocleavable auxiliary that allows attachment of the PEG polymer for efficient enzymatic glycosylation, provides the functional groups for NCL, and is cleanly removed by UV irradiation (Scheme 1).

The synthesis of the auxiliary (see Scheme S1 in the Supporting Information, also for the numbered compounds) was started using the procedure developed by Dawson and co-workers.[9] Starting from vanillin (**1**), the desired methyl butanoate, nitro, and methylene groups were efficiently introduced (**2**). The planned subsequent Sharpless aminohydroxylation[11] was not efficient, even under various conditions. Therefore, a stepwise strategy was adopted, starting with the Sharpless dihydroxylation[12] to obtain diol **3**, which was then converted into the corresponding amino alcohol **4** in four steps and 63% overall yield.[13] The Mitsunobu reaction,[14] followed by protecting-group exchange (acetyl to *tert*-butylsulfanyl),[15] gave the protected sulfanylaminoethyl group (**5**) essential for the NCL reaction. Catalytic ester–amide exchange[16] provided the attachment point for the PEG polymer and potentially for many other modifications (**6**). Final PEGylation of the free primary amine, removal of the Boc group, and purification gave the desired auxiliary **7** in 68% yield over the last two steps.

With the desired auxiliary in hand, we investigated the conditions for its incorporation into a MUC1 peptide through the replacement of a native N-terminal glycine residue (Scheme 1). The MUC1 tandem repeat peptide was synthesized by Fmoc-based solid-phase peptide synthesis (SPPS) and the N-terminus was modified with iodoacetic acid (analytical data in Figure S3) through HBTU-mediated coupling and then submitted to an S_N2 reaction with auxiliary

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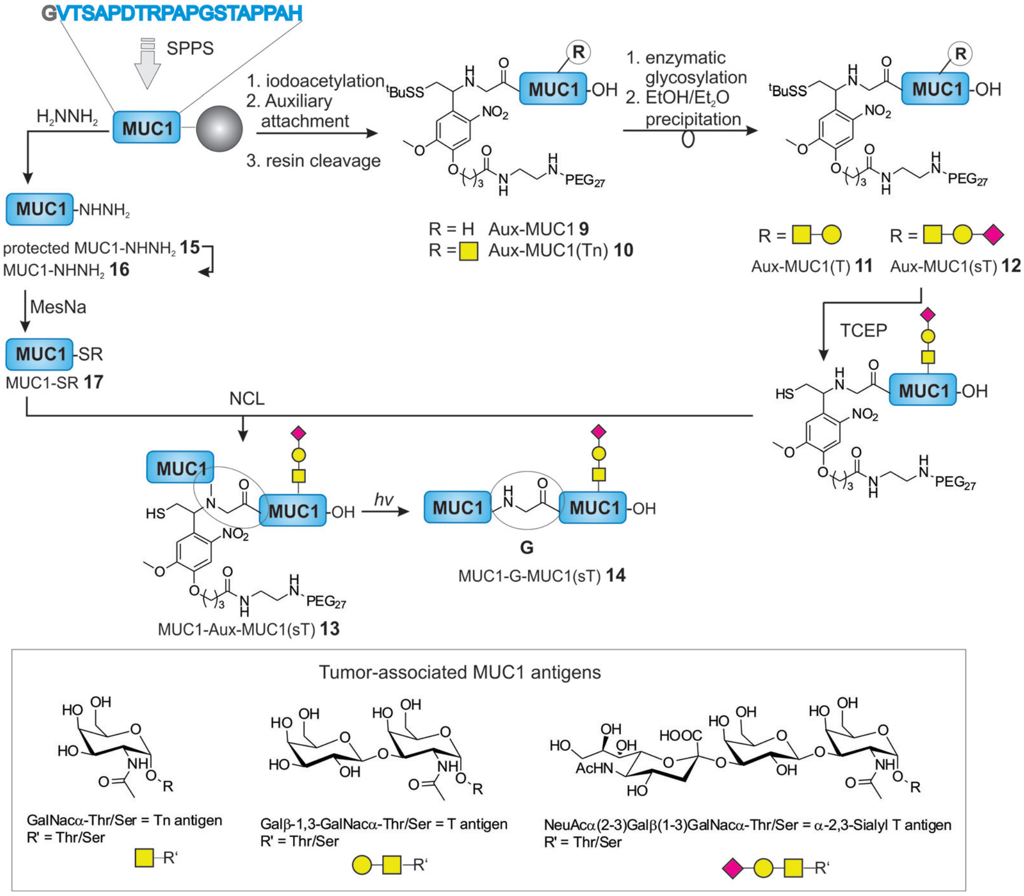

Scheme 1. The auxiliary-mediated chemoenzymatic glycosylation approach. The solid-phase peptide synthesis (SPPS) of a 20 mer MUC1 motif with a PEGylated auxiliary provides material for consecutive glycosylation and precipitation steps (right). The same peptide precursor can be converted into a peptide hydrazide and subsequently into a peptide thioester (left). Both peptides are used in ligation reactions to give extended glycosylated MUC1 repeats.

employed for site-selective generation of the Tn antigen.[5] Iodoacetylation, attachment of the auxiliary, cleavage from the resin, and subsequent hydrazinolysis of the acetyl protecting groups on the glycan gave conjugate **10** (see also Figure S8) in 4% overall yield (based on synthesis scale).

In a first attempt to prepare **10** the GalNAc deprotection was performed on resin by overnight incubation with 5% (v/v) hydrazine monohydrate in methanol. Surprisingly, this led not only to acetyl removal but also to complete release of the peptide from the Wang TentaGel resin. Further investigation demonstrated that hydrazine quantitatively cleaved the protected peptide from the resin by nucleophilic attack on the linker, forming a peptide hydrazide.[17,18] This initially appeared as a nuisance on route to **10**; however, since peptide hydrazides can be efficiently converted into the corresponding peptide thioesters,[19] we decided to take advantage of this intermediate to generate larger MUC1 segments by sequential NCL reactions with auxiliary and hydrazide-modified MUC1 peptides. To fully exploit the bifunctional MUC1 peptides we needed to prove that MUC1-NHNH$_2$ (**16**; Scheme 1) can be easily converted into the corresponding thioester **17** and that chemoenzymatic glycosylation works on Aux-MUC1(Tn)NHNH$_2$ (**19**; Figure 2). We knew from previous attempts that peptide thioesters are hydrolyzed under glycosylation conditions; therefore a peptide hydrazide could be an ideal masked peptide thioester sufficiently stable to allow enzymatic glycosylation and subsequent conversion into the desired glycosylated peptide thioester. Moreover, the same peptidyl resin provided access both to the peptide acid and to the peptide hydrazide.

To test conversion of MUC1-NHNH$_2$ (**16**) into the corresponding MUC1-thioester **17**, MUC1-peptidyl resin was incubated with 5% (v/v) hydrazine monohydrate in methanol overnight. Protected MUC1-NHNH$_2$ (**15**) was obtained in 85% yield and deprotection of the side chains in solution by treatment with a mixture of TFA/TIS/H_2O (92.5:5:2.5) gave the desired **16** (Figure S9). Treatment with

7. Unfortunately, all tested conditions gave poor conversion even after several days.

To improve this critical step, the synthesis of the MUC1 peptide was repeated with lower peptide loading on TentaGel resin, which has better swelling properties than the previously used Wang PS resin. To reduce any potential steric hindrance by the long PEG chain during the substitution reaction, auxiliary **8**, lacking the PEG moiety, was installed first and subsequently PEGylated on resin. The auxiliary synthesis was modified accordingly by introducing an Fmoc protecting group on the free primary amine (step m in Scheme S1). The S_N2 reaction of auxiliary **8** with the iodoacetylated MUC1 peptidyl (Wang TentaGel) resin now proceeded with very high conversion (Figure S5) and PEGylation on resin was quantitative. After cleavage and purification, the pure MUC1–auxiliary conjugate Aux-MUC1 (**9**; Scheme 1 and Figure S7) was obtained in 34% yield.

This strategy was applied for the preparation of an auxiliary-bearing MUC1 peptide including the Tn antigen (GalNAcα1-O-Thr) at position 14 (Aux-MUC1(Tn) (**10**)). The use of Fmoc-Thr(GalNAc-Ac$_3$)-OH ensured full control over the O-glycan attachment site. Alternatively *Drosophila* polypeptidyl GalNAc-transferase 1 (dGalNAcT1) can be

$NaNO_2$ followed by addition of MesNa and HPLC purification gave the MUC1-SR (**17**; Figure S10) in 14% overall yield.

Next, we demonstrated that the PEGylated auxiliary was indeed able to facilitate the fast sequential enzymatic glycosylation of the MUC1 peptides as previously described for N-terminally PEGylated MUC1.[5] Glycosylation of Aux-MUC1(Tn) (**10**) with human C1GalT1 gave Aux-MUC1(T) (**11**) with a single T antigen (Galβ1-3GalNAcα disaccharide attached to Thr14) in excellent conversion (Figure 1 and Figures S12 A,B). The incubation with a mixture of ethanol and diethyl ether at −80°C induced the precipitation of **11**, which was then collected in 95% yield by centrifugation (Figure 1 and Figure S12 B) and used in the next glycosylation step without further purification. Incubation of **11** with CMP-Neu5Ac in the presence of recombinant ST3Gal1 allowed efficient extension of the disaccharide to the Neu5Acα2-3Galβ1-3GalNAcα trisaccharide and gave Aux-MUC1(sT) (**12**) with the sialyl-T antigen in 90% yield (Figure 1 and Figure S12B). Using similar conditions for all glycosylation reactions but by increasing times of incubation with ethanol and ether at −80°C (6–12 h instead of 4 h), we were able to simplify the sequential glycosylation procedure by omitting the GPC spin column step. As a control we have also used MUC1(Tn) without the auxiliary in this glycosylation–precipitation procedure and only 27% yield of MUC1(T) was recovered, clearly demonstrating the advantages of PEGylation (Figure S12C).

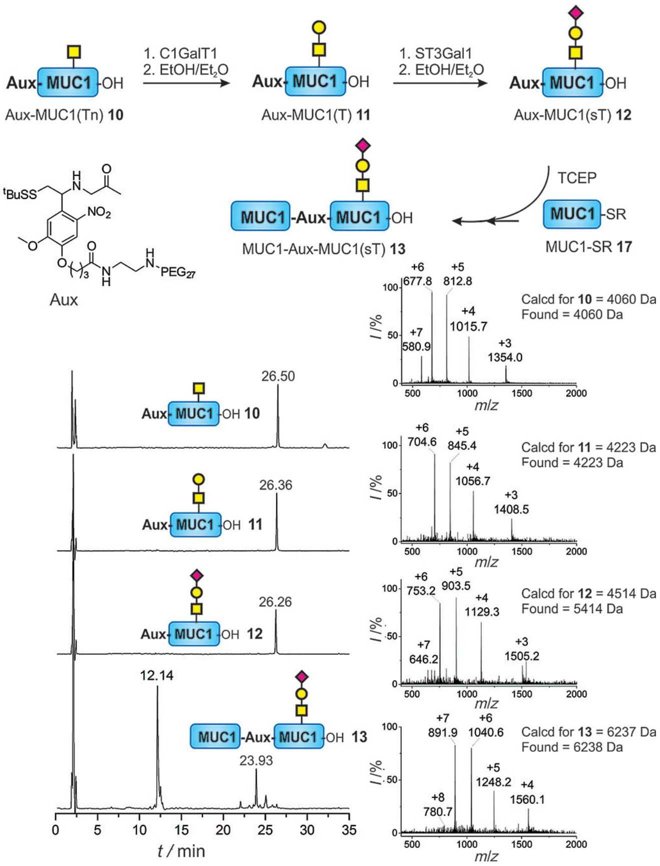

Figure 1. Top: Sequential enzymatic glycosylation of **10** gave **11** and **12** for NCL reactions with **17**. MUC1: Tandem repeat sequence of mucin 1 VTSAPDTRPAPGSTAPPAH; the O-glycans on the side chain of Thr14 are indicated in brackets. Tn: GalNacα, T: Galβ1-3GalNAcα, sT: Neu5Acα2-3Galβ1-3GalNacα. Bottom left: HPLC chromatograms after precipitation and of the NCL reaction after 24 h. Injection peak at 2 min; peak at 12.14 min: peptide thioester **17**. Bottom right: ESI mass spectra.

Next, the glycosylated peptide conjugates were linked to MUC1-SR (**17**) by NCL to demonstrate all advantages of the auxiliary. Non-glycosylated Aux-MUC1 (**9**) was used to establish optimal ligation conditions. The thiol group of the auxiliary was deprotected through incubation with TCEP at 24°C for 6 h before addition of **17**. Optimized NCL conditions for MUC1 peptides (65% conversion after two days) are NaPi buffer (pH 7.5) at 30°C with Aux-MUC1 (**9**) at 8 mM concentration and a 2.5-fold excess of **17** (Figure S15A). These conditions were applied for the synthesis of glycosylated MUC1-Aux-MUC1(Tn) (**18**), which was obtained from **17** and **10** in 78% conversion after 36 h of incubation at 30°C (Figure S15B).

After demonstrating the individual functions of the auxiliary in MUC1 peptides, we coupled glycosylation and NCL reaction by performing sequential enzymatic glycosylation of **10** to the sialylated core 1 containing conjugate **12**, which was recovered by precipitation and directly used in a ligation reaction with **17**. In this case consecutive additions of TCEP were needed to efficiently remove the *tert*-butylsulfanyl group from the auxiliary (Figure S15C). Addition of **17** and MesNa to the ligation mixture led to the desired product **13** in one day and with 70% conversion (Figure 1).

Finally, light-induced removal of the PEGylated auxiliary was demonstrated for non-glycosylated MUC1-Aux-MUC1 as well as for **13** and **18**. This was accomplished by UV irradiation of the crude ligation mixtures in water or in a water/acetonitrile mixture. In all cases no starting material was detectable after 30 min of irradiation with an UV-A lamp and simultaneously a new peak formed corresponding to the desired product, which was isolated by HPLC purification (Figures S16–S18).

As described above, peptide hydrazides are useful thioester precursors that remain unaffected in glycosylation reactions in which peptide thioesters quickly hydrolyze. Hydrazine-induced cleavage of Aux-MUC1(Tn) peptidyl resin gave protected Aux-MUC1(Tn)-$NHNH_2$ (**19**; Figure S11A), which, after acidic deprotection in solution, was successfully used in sequential glycosylation reactions giving the corresponding hydrazide Aux-MUC1(sT)-$NHNH_2$ (**21**) with yields similar to those found for **12** (Figure 2). Treatment with $NaNO_2$ followed by ascorbic acid (to suppress nitrosamine formation on the auxiliary) and MesNa gave access to the desired thioester Aux-MUC1(sT)-SR (**22**) in 63% conversion (see the Supporting Information).

To further explore the potential of this approach, the synthesis of fully unprotected conjugates comprising two glycosylated MUC1 peptides was accomplished (Figure 3). A MUC1 peptide carrying the Tn antigen at position Thr7 (**23**, instead of Thr14) was synthesized and converted into a peptide α-thioester. Hydrazine cleavage, deprotection, and

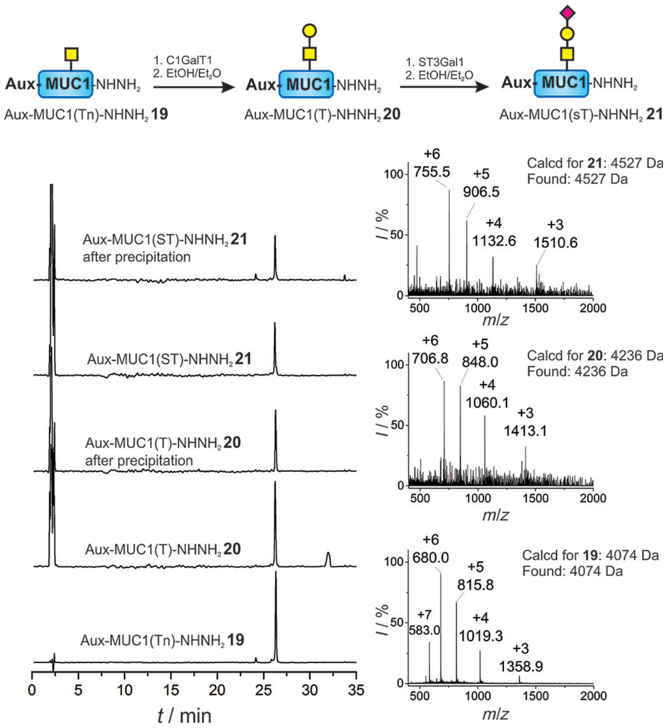

Figure 2. Sequential enzymatic glycosylation of **19**, a precursor for glycosylated peptide thioesters, and the corresponding ESI mass spectra.

lated peptides and its combination with NCL to obtain larger polypeptides with different but specific glycosylation patterns.

In summary, we have developed a PEGylated ligation auxiliary that efficiently supports the sequential quantitative enzymatic glycosylation of peptides in solution without the need for chromatographic purification and it can mediate NCL reactions. The auxiliary-modified (and glycosylated) peptides can be used in NCL reactions with other MUC1 tandem repeat peptides carrying a C-terminal thioester. All peptide α-thioesters used herein were obtained by hydrazinolysis followed by oxidation of the corresponding hydrazide. Subsequent NCL reactions carried out in this study give conversions of $>65\%$ and the auxiliary is cleanly removed from ligation products. Light-induced removal of the auxiliary leaves a glycine residue at the ligation site, limiting the use of this approach to glycine residues, which are fortunately quite abundant in proteins. The combination of the new PEGylated auxiliary with the synthesis of peptide α-thioesters by direct hydrazinolysis allows the preparation of site-selectively O-glycosylated peptide α-thioesters, opening the way to the synthesis of polypeptides comprising two or more MUC1 tandem repeats with different glycosylation patterns. Controlling thioester generation and deprotection of the thiol group within the auxiliary also allows the controlled extension of each building block in C- and N-terminal direction. We will

treatment with $NaNO_2$ and MesNa smoothly led to the desired MUC1-(Tn^7)-SR (**24**) in 43% yield (Figure S20). **24** was used in NCL with **10** to give **25** in 35% yield (Figure S21).

Compound **25** was efficiently used in the glycosylation–precipitation procedure leading to MUC1(T^7)-Aux-MUC1(T) (**26**; Figure 3 and Figure S22), consisting of two MUC1 tandem repeats with a T antigen at different positions. This much longer peptide with only one auxiliary was efficiently recovered by precipitation (80% recovery) under similar conditions as described above. Subsequently, **26** was submitted to UV irradiation at 365 nm for 6 min and the desired MUC1(T^7)-G-MUC1(T) **27** was obtained in 53% yield. MUC1(T^7)-Aux-MUC1(T) **26** was also used in a further glycosylation–precipitation step leading to sialylated MUC1(sT^7)-Aux-MUC1(sT) **28** (68% recovery, Figure 3 and Figure S23). UV irradiation cleanly removed the auxiliary and pure MUC1-(sT^7)-G-MUC1(sT) (**29**) was obtained in 12% yield after HPLC purification (Figure 3). These results demonstrate the power of this approach for the chemoenzymatic synthesis of glycosy-

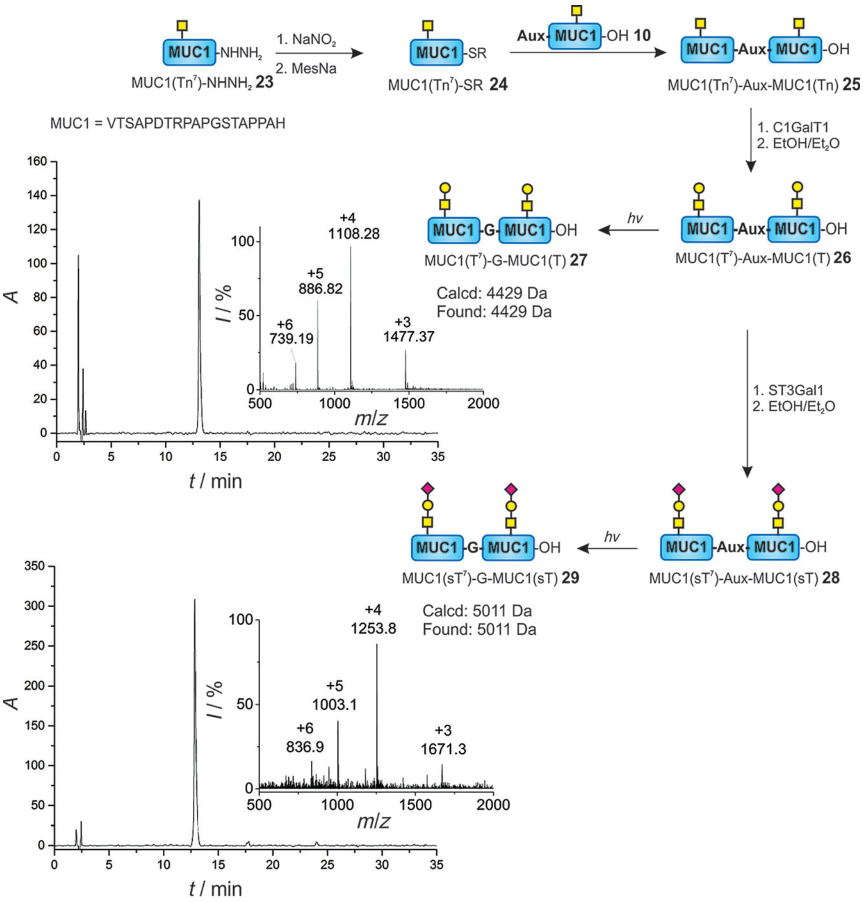

Figure 3. Synthesis of unprotected glycosylated peptides **27** and **29** by NCL, glycosylation, and UV irradiation, and their HPLC chromatograms and mass spectra.

use this approach to create a library of site-selectively O-glycosylated MUC1 variants with different glycosylation patterns for a detailed study of these patterns in MUC1 function.

This approach is not limited to the synthesis of glycosylated MUC1 analogues. It is applicable to the synthesis of many larger, posttranslationally modified proteins, only limited by the identification of a suitable glycine residue as well as the availability of chemistry or enzymes that introduce the desired PTMs.

Keywords: bioorganic chemistry · chemoenzymatic glycosylation · PEGylation · photocleavable ligation auxiliary · protein modifications

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