

# Synthesis of Erythropoietins Site-Specifically Conjugated with Complex-Type *N*-Glycans

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The biological activity of the glycoprotein hormone erythropoietin (EPO) is dependent mainly on the structure of its *N*-linked glycans. We aimed to readily attach defined *N*-glycans to EPO through copper-catalyzed azide alkyne cycloaddition. EPO variants with an alkyne-bearing non-natural amino acid (Plk) at the *N*-glycosylation sites 24, 38, and 83 were obtained by amber suppression followed by protein purification and refolding. Click conjugation of the alkynyl EPOs with biantennary *N*-glycan azides provided biologically active site-specifically modified EPO glycoconjugates.

Human erythropoietin (EPO) is a glycoprotein that plays a key role in the growth of erythroid cells.<sup>[1]</sup> The  $\alpha$ -helical EPO consists of 165 amino acids and is stabilized by two disulfide bridges. EPO is *N*-glycosylated at Asn24, Asn38, and Asn83 and *O*-glycosylated at Ser126.<sup>[2]</sup> Recombinant human erythropoietin is one of the best-selling therapeutic proteins and is administered to treat anemia.<sup>[3]</sup> EPO for therapeutic purposes is mainly expressed in CHO cells, which leads to a heterogeneous glycosylation pattern dominated by sialylated bi-, tri-, and tetra-antennary *N*-glycans.<sup>[4]</sup> The expression of EPO in *Saccharomyces cerevisiae* leads to hypermannosylated *N*-glycans, which can be immunogenic.<sup>[5]</sup> The *N*-glycans found on EPO have only a marginal effect on the binding to the rEPO receptor, but determine the bioactivity of the protein in vivo.<sup>[6]</sup>

The glycan chains of EPO are highly heterogeneous;<sup>[7]</sup> this impedes a systematic elucidation of structure–function relationships for individual glycans of EPO. Herein, we show that, by combining bacterial amber suppression and click conjuga-

tion of semisynthetic *N*-glycan azides, homogeneous and biologically active EPO glycoconjugates can be obtained.

Recently, glycoengineering of the methylotrophic yeast *Pichia pastoris* enabled the expression and purification of EPO to a higher level of homogeneity,<sup>[8]</sup> but with a glycosylation pattern differing from that of humans.<sup>[9]</sup> The production of EPO with a more human-like glycosylation in glycoengineered insect cells<sup>[10]</sup> and asialo-EPO in plants<sup>[11]</sup> has been reported. However, none of the recombinant expression systems can provide homogeneously glycosylated EPO.

To address this problem, several chemical and enzymatic methods have been elaborated for the synthesis of uniform EPO.<sup>[12]</sup> Flitsch and co-workers established the expression of nonglycosylated EPO in *Escherichia coli* and introduced single cysteine residues at the natural glycosylation sites suitable for coupling to *N*-glycosylidoacetamides.<sup>[13]</sup>

Kent's group synthesized EPO by native chemical ligation (NCL); conjugating it with two synthetic polymers led to a prolonged half-life.<sup>[14]</sup> An EPO conjugated to two *N*-glycans was generated by protein semisynthesis.<sup>[15]</sup> However, NCL of a glycopeptide- $\alpha$ -thioester and a recombinantly expressed protein fragment can limit the choice of accessible glycosylation sites. The all-chemical synthesis of the EPO polypeptide with three uniform glycosylations at native sites was performed in the laboratories of Danishefsky.<sup>[16]</sup> Recently, Kajihara and co-workers succeeded in creating various EPO glycoforms covering all natural glycosylation sites by using Boc solid-phase synthesis and NCL.<sup>[17]</sup>

As an alternative approach to rapidly derivatize EPO with the desired oligosaccharides, we considered the recombinant expression of EPO site-specifically modified with the unnatural amino acid *N*<sup>6</sup>-(propargyloxycarbonyl)-L-lysine (Plk) suitable for subsequent modification with *N*-glycan-azides by click chemistry.<sup>[18]</sup> We aimed to combine the advantages of bacterial expression with the ease of click conjugation. This strategy should facilitate the divergent generation of stable site-specifically modified glycoconjugates that could serve as reference compounds for the establishment of structure–activity relationships for complex-type *N*-glycans on the EPO scaffold (Scheme 1).

Native EPO expressed in *E. coli* (EPO-0) is not glycosylated and is prone to unspecific aggregation during expression and refolding. To overcome this problem, we generated an EPO variant with five amino acid replacements (N24K, N38K, N83K, P121N, and P122S: EPO-1). The asparagine residues of the three glycosylation sites were mutated to lysine to increase the solubility of the protein at neutral pH by increasing the net

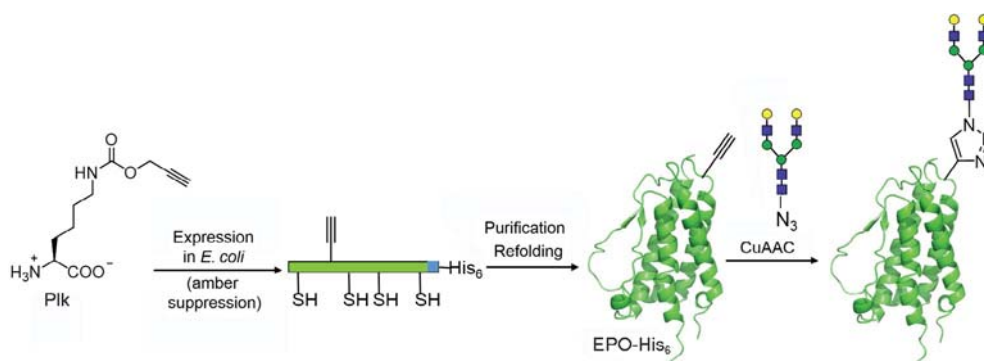
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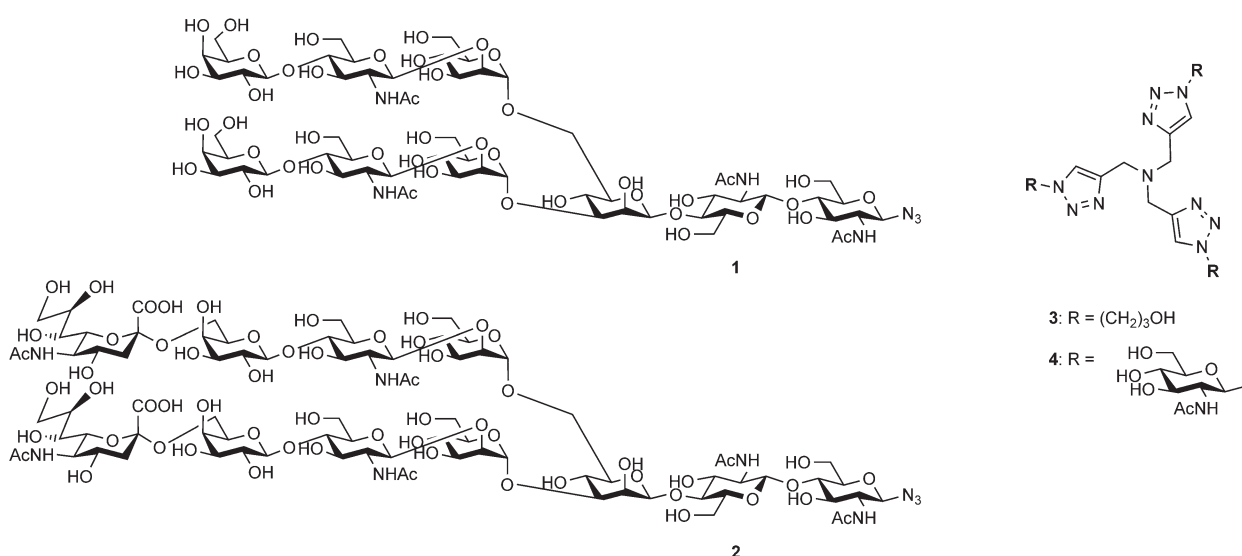


**Scheme 1.** A novel semisynthetic approach for the synthesis of EPO conjugated with defined and uniform *N*-glycans. The lysine derivative (Plk) bearing an alkyne function is selectively introduced into EPO at the natural glycosylation sites by using amber suppression. After purification and refolding of the recombinant EPO variants, defined glycan azides are attached to the sites by copper-catalyzed cycloaddition.

positive charge.<sup>[19]</sup> Pro121 and Pro122 were mutated to serine and asparagine, respectively, to eliminate non-native prolyl-*cis/trans* isomers that could lead to unspecific aggregation during refolding.<sup>[20]</sup> Due to a sequence conflict in the first published sequence of human EPO, the mutation G140R was also present.<sup>[21]</sup> The unnatural amino acid Plk serves as a pyrrolysine analogue as it has an alkyne group on its side chain for coupling to the synthetic glycan azides (Scheme 1). Plk can be incorporated by amber suppression, by exploiting the PylRS/tRNA<sup>Pyl</sup> orthogonal pair from *Methanosarcina barkeri*.<sup>[22]</sup> An amber stop codon was inserted by site-directed mutagenesis at the glycosylation site 24, 38, or 83 of EPO-1 as a reference protein. Whereas the EPO variants with Plk at position 24 (**A**) and 83 (**C**) were efficiently expressed in *E. coli* BL21(DE3) cells, the variant with Plk at position 38 (**B**) was initially not obtained, probably due to insufficient suppression efficiency. The problem was overcome by co-expression of the elongation factor tufB. Surprisingly, only tufB from *E. coli* (Figure S2 in the Supporting Information) improved the expression; the homologue from *M. barkeri* was not effective. EPO polypeptides with a C-termi-

nal His<sub>6</sub> tag were recovered from inclusion bodies by solubilization in 6 M guanidine chloride and purified by Ni-affinity chromatography. As recombinant human EPO fused to a His<sub>6</sub> tag is commercially available for research purposes, we reasoned that the additional tag does not disrupt the biological functionality of the protein. Refolding conditions were optimized (see the Supporting Information) to enable the correct formation of the two disulfide bridges. After oxidative refolding and purification by size-exclusion chromatography (SEC), the Plk-containing EPOs **A**, **B**, and **C** were obtained in yields of 0.5–1 mg per L of bacterial culture. The incorporation of Plk was slightly less efficient than in a previous expression of human EPO by using amber suppression with *p*-azidophenylalanine (pAzF), especially at position 38.<sup>[23]</sup>

With the EPO-Plk variants **A**, **B**, and **C** in hand, we investigated conjugation with the azido-functionalized biantennary *N*-glycans **1** and **2** by copper-catalyzed azide-alkyne cycloaddition (CuAAC; Scheme 2).<sup>[24]</sup> The biantennary nonasaccharide azide **1**<sup>[25]</sup> and the sialylated undecasaccharide azide **2** were synthesized from a glycopeptide isolated from egg yolk



**Scheme 2.** *N*-glycan azides and ligands used in this study.

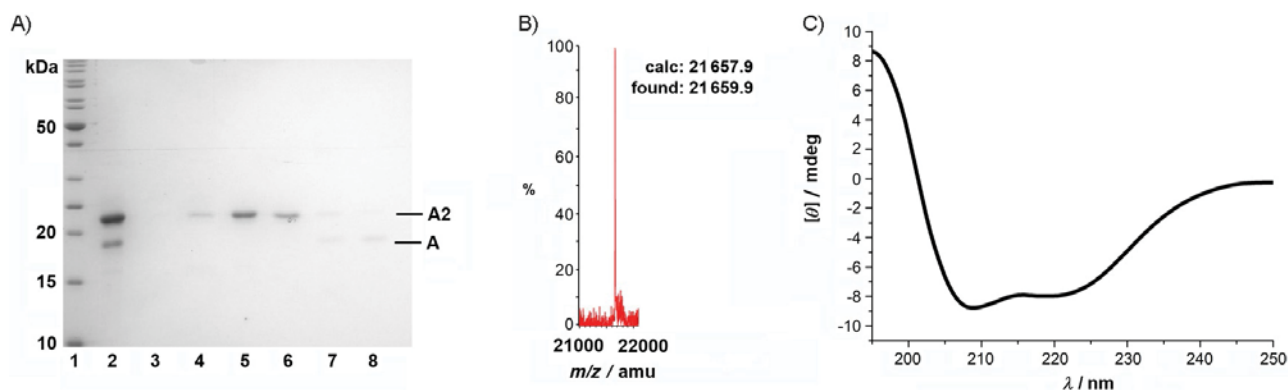
followed by PNGase F digestion and aqueous azidation of the hemiacetals under Shoda's conditions (Figure S1).<sup>[26]</sup>

We tested different conditions for the click reaction of EPOs **A**, **B**, and **C** and glycan azide **2** on an analytical scale (Figure S3). We found that the frequently used combination of  $\text{CuSO}_4$  and TCEP or ascorbic acid led to nearly complete precipitation of the nonglycosylated EPOs **A**, **B**, and **C** during the reaction (Figure S3). In order to additionally minimize the Fenton-type degradation of the *N*-glycan azide **2** during click conjugation,<sup>[27]</sup> we used  $(\text{CH}_3\text{CN})_4\text{Cu}(\text{I})\text{-PF}_6$  as a stable  $\text{Cu}^{\text{I}}$  source and the tris triazole **3** or **4** (Scheme 2) as complex ligand under exclusion of oxygen. The following conditions

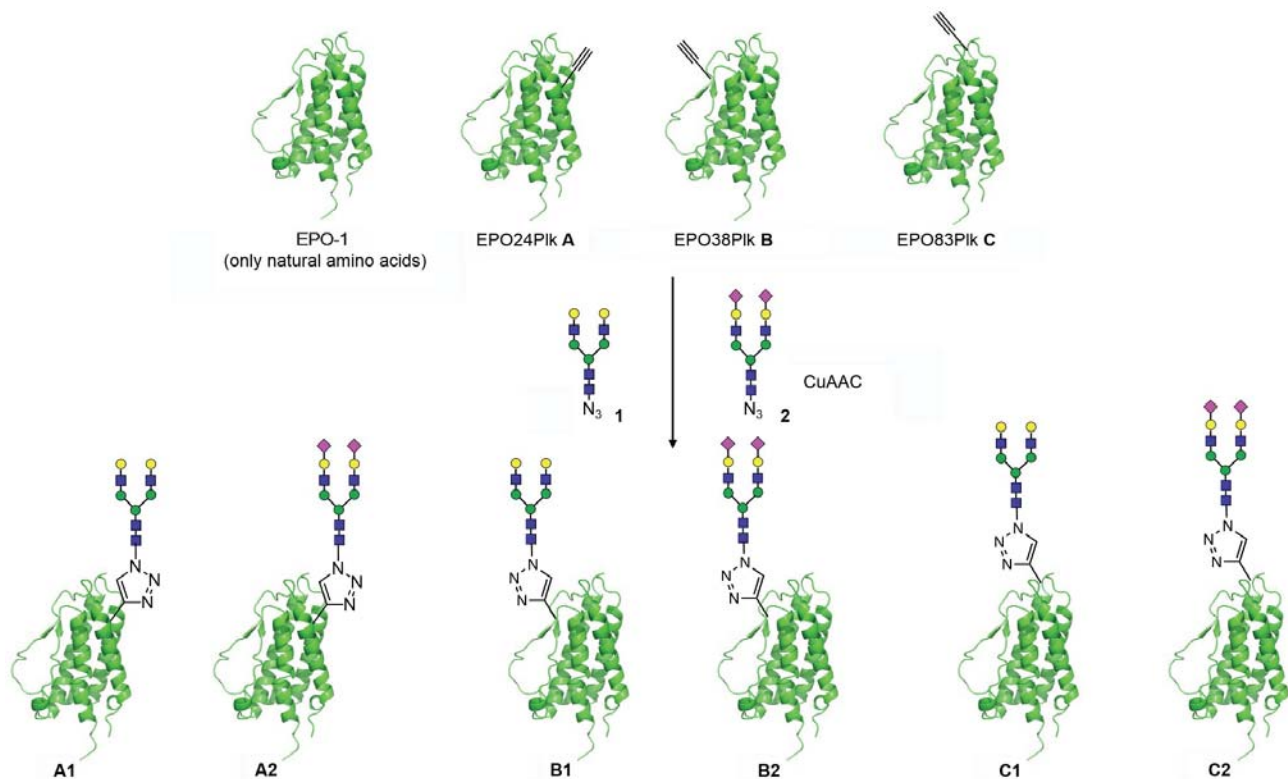
were found to be best:  $40\ \mu\text{M}$  EPO-PIk **A**, **B**, or **C**;  $400\ \mu\text{M}$  glycan azide **1** or **2**;  $4\ \text{mM}$  **3**;  $8\ \text{mM}$  **3** in  $20\ \text{mM}$  Tris-HCl (pH 7.5). Gratifyingly, the EPO conjugates could be purified by SEC. The mass spectra indicated that the glycans remained intact after CuAAC (Figures 1 and S4–S6).

The CD spectra of the EPO glycoconjugates obtained had a shape that is characteristic for EPO. The final amounts of the six purified EPO conjugates **A1–C2** were in the range 0.1–0.3 mg.

With the panel of systematically varied EPO glycoconjugates (Scheme 3) and the unmodified parent compounds at hand, we tested their biological activity in a cellular proliferation



**Figure 1.** A) SDS-PAGE: 1: protein marker, 2: crude CuAAC of EPO24PIk (**A**) and *N*-glycan azide **2**, 3–8 fractions from SEC (Superdex 75). B) Deconvoluted ESI-MS spectrum and C) Far-UV CD spectrum of **A2**.



**Scheme 3.** EPO glycoconjugates obtained by the semisynthetic approach. Defined synthetic glycans were site-specifically conjugated to the protein variants by CuAAC.

assay. At first, we determined the dose–response of EPO-dependent TF-1 cells at different EPO concentrations.<sup>[28]</sup> Cell proliferation was measured by detecting metabolic activity through the reduction of resazurin to the highly fluorescent resorufin.<sup>[29]</sup>

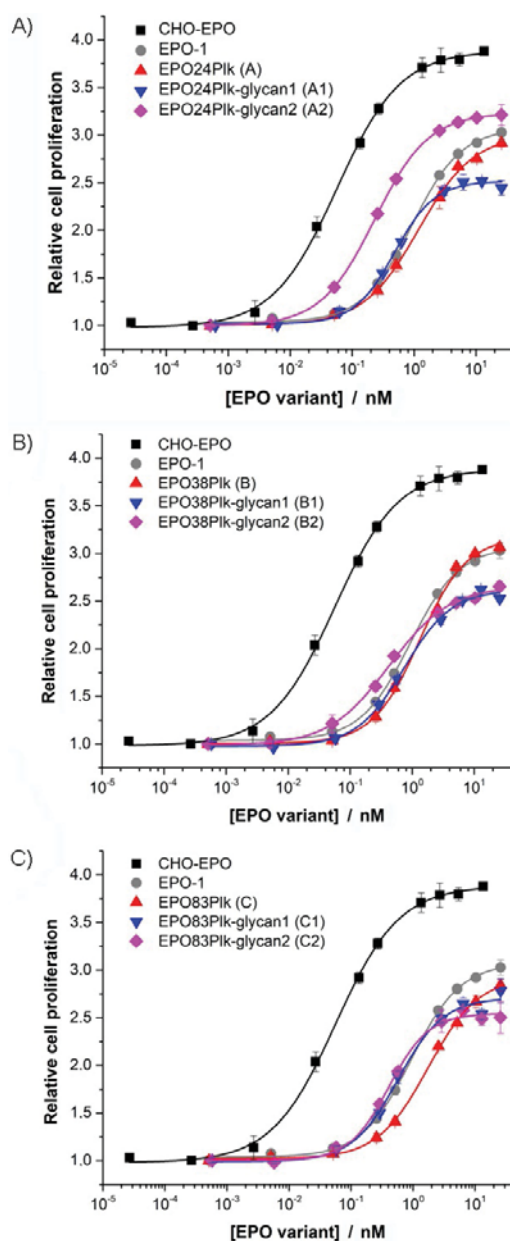
The EC<sub>50</sub> value for our reference protein EPO-1 measured in the TF-1 proliferation assay is in agreement with data reported in the literature both for bacterial recombinant EPO<sup>[30]</sup> and for an EPO obtained by chemical synthesis.<sup>[31]</sup> It is important to note that all EPO-Plk variants (A, B, C) displayed biological activity similar to that of EPO-1, thus indicating that a single Plk residue has little impact on EPO functionality. All six EPO glycoconjugates A1–C2 elicited a stronger proliferation activity on TF-1 cells than EPO-1. As expected, the efficiency of A1–C2 was lower than that of the fully glycosylated EPO from CHO cells (Figure 2, Table 1). Of all the conjugates, A2 (attachment of the sialylated glycan 2 at position 24) gave the best EC<sub>50</sub> value (0.232 nM) and the maximal efficiency (85%) relative to the CHO-EPO reference.

Attachment of glycan 1 at the three individual positions induced a 1.8- to 3.7-fold reduction in the EC<sub>50</sub> value in comparison to the nonglycosylated EPO-Plks A–C, whereas the sialylated glycan 2 promoted a three- to sevenfold reduction (Table 1). These results suggest that, at least to a certain extent, the terminal sialylation also plays a role in the cellular proliferation assay and not only in the biological activity of EPO in vivo.

It might be that the terminal sialic acids enhance the solubility of the protein and increase its stability at 37 °C, thereby enabling a longer lifetime in the medium. It has been previously reported that nonglycosylated EPO aggregates rapidly at physiological temperature and sticks to vessels due to hydrophobic patches.<sup>[32]</sup> Furthermore, the EC<sub>50</sub> values obtained for PEGylated EPO variants (140G)<sup>[23]</sup> cannot be easily compared to the results of this study, as EPO with the G140R mutation showed a sevenfold decrease in the bioactivity in the TF-1 cell assay.

For EPO with pAzF at position 24 or 38, conjugation of branched PEG750 and PEG2000 phosphites led to a three- or fivefold decrease in the EC<sub>50</sub> value of the corresponding conjugate.<sup>[23]</sup> In this respect, the effects of the conjugated glycans resemble those of the branched PEG chains. The introduction of pAzF at position 83 completely abolished the bioactivity of EPO, severely destabilizing the protein. In contrast, the incorporation of Plk at position 83 is tolerated by the protein scaffold and less disruptive for the biological activity of EPO (Table 1).

Subsequently, we assayed the most active synthetic EPO glycoconjugate (A2) in a cell-differentiation test. We performed a colony forming units (CFU) assay in mouse bone marrow cells.<sup>[33]</sup> This assay relies on the ability of hematopoietic progenitor cells in methylcellulose medium to proliferate and differentiate into burst-forming unit-erythroid (BFU-E) colonies in response to EPO stimulation. Although the assay is qualitative and does not produce classical dose–response curves, it directly tests the erythropoietic potency of EPO on the differentiation of hematopoietic progenitors into mature red cells; the addition of EPO glycoconjugate A2 to the methylcellulose



**Figure 2.** Concentration-dependent proliferation assay of TF-1 cells with EPO variants. Each experiment was performed in triplicate. The relative increase in cell number was plotted against EPO concentration. The data were fitted to a non-cooperative binding reaction with a single binding site (Hill coefficient = 1).

EPO variant	EC <sub>50</sub> [nM]	Max proliferation activity
CHO-EPO	0.056 ± 0.003	100
EPO-1	0.923 ± 0.047	79
EPO24Plk (A)	1.177 ± 0.098	75
EPO24Plk-glycan1 (A1)	0.483 ± 0.037	67
EPO24Plk-glycan2 (A2)	0.232 ± 0.001	85
EPO38Plk (B)	1.316 ± 0.128	82
EPO38Plk-glycan1 (B1)	0.742 ± 0.081	69
EPO38Plk-glycan2 (B2)	0.429 ± 0.038	71
EPO83Plk (C)	1.753 ± 0.106	78
EPO83Plk-glycan1 (C1)	0.570 ± 0.085	72
EPO83Plk-glycan2 (C2)	0.410 ± 0.032	67

medium triggered the formation of BFU-E colonies significantly, thus showing that it possesses erythropoietic activity (Figure S7).

Furthermore, the conjugated glycan chains had a beneficial effect on the stability of the EPO protein against unspecific aggregation. After repeated freeze-thaw cycles, we observed a pronounced precipitation of EPO24PIk (A); this phenomenon was nearly absent in the *N*-glycan-derivatives A1 and A2 (Figure S8).

In summary, we have expressed and refolded three EPO variants with alkyne groups at the natural glycosylation sites by using amber suppression. After click conjugation with synthetic *N*-glycan azides under mild conditions the biological activity of all six EPOs conjugated at a single site was increased.

The outlined approach is generally applicable to modifications of proteins by site-specific conjugation using glycan azides and can provide a platform to investigate the effect of glycan composition and positioning on protein function and stability in detail. By introducing an additional amber stop codon into the gene of the protein of interest, conjugates with multiple glycans should become accessible.

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## Conflict of Interest

The authors declare no conflict of interest.

**Keywords:** click chemistry · glycoproteins · protein engineering · protein modification · synthetic glycans

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