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Investigation of acyl transfer auxiliary-assisted glycoconjugation for glycoprotein semi-synthesis†

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Homogeneous glycoprotein syntheses have become possible in the last decade due to advances in chemical ligation strategies, particularly Native Chemical Ligation (NCL). For native glycoproteins this still requires laborious and technically challenging syntheses of glycopeptide components, combined with multi-segment ligation reactions. Here we explore new reactions between sugar-linked acyl transfer auxiliaries and peptide thioesters. We show that native glycoproteins are difficult to produce using this approach but various related analogues are accessible. The results show that site-specific neoglycoconjugation is a viable route to simply glycosylated proteins, which may be extended using well-documented enzymatic processes.

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Introduction

Protein glycosylation remains an unsolved synthetic chemical problem.¹ During the biosynthesis of *N*-linked glycoproteins, specific asparagine (Asn) residues are singled out by the multi-enzyme complex oligosaccharyl transferase (OST) and the carboxamide group of the asparagine sidechain is destabilised to facilitate reaction, as a nucleophile, towards an oligosaccharide acceptor bearing a complex phospholipid leaving group.² The details concerning recognition and activation of specific Asn residues are beginning to be understood, where conserved OST amino acid residues mediate recognition and twisting of the target Asn amide bond.³

The carbohydrate moiety confers desirable properties upon the protein to which it is attached. These include prolonged longevity in the blood, which is particularly relevant to therapeutic proteins, reducing the dosing regimen and consequently the potential for intolerance and error, as well as protecting the protein backbone from proteolysis, and can target the protein to the appropriate site within the body.⁴ The *N*-glycans in the Fc domain of antibodies are also crucial for directing effector function.⁵

Despite over 100 years of carbohydrate chemistry, the bio-synthetic process has not been replicated using organic syn-

thesis. Formation of the *N*-glycopeptide bond typically involves amide bond formation between suitably protected aspartic acid and glycosylamine building blocks in a process introduced by Lansbury.⁶ Further advances have increased the efficiency of *N*-glycoside bond formation⁷ but *N*-glycoprotein chemical synthesis remains the endeavour of specialized synthetic groups that not only construct the short peptides decorated with complex sugars, but also combine those short peptides into larger bioactive proteins, using chemoselective coupling processes such as native chemical ligation (NCL).⁸ In an alternative strategy full length proteins of biological origin, and with *N*-acetylglucosamine (GlcNAc) pre-installed at a specific location as a result of pre-existing cellular glycosylation, are redesigned using *endo*-glycosidases.^{1,9} An important challenge is how to attach this first required GlcNAc residue in recombinant proteins, at a site of your choice?

Here we explore a new paradigm for semi-synthetic glycoprotein synthesis by uniting synthetic monosaccharide building blocks with fully recombinant protein, containing the unnatural amino acid “ThioD” (1),¹⁰ or “BnE” (2),¹¹ and their corresponding hydrazides (Scheme 1).

Results and discussion

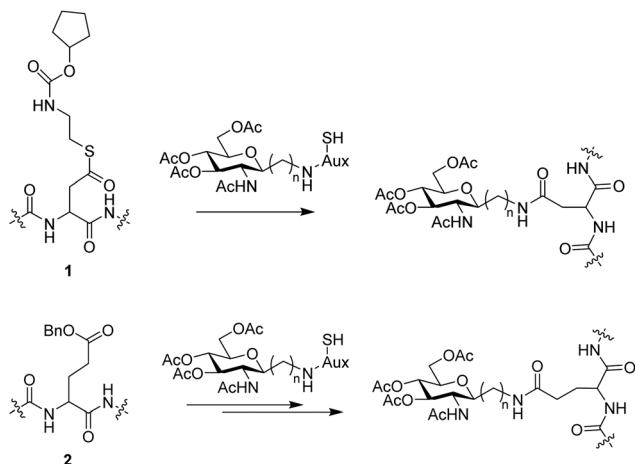
Garner *et al.* demonstrated that thioester containing amino acid ThioD could be introduced to recombinant proteins,¹⁰ and oligosaccharide structures were ligated to green fluorescent protein using cysteine terminated carbohydrates.¹² There is only one example, albeit in fully synthetic peptides, reporting NCL-type chemistry, employing “traceless” acyl transfer auxiliaries to unite carbohydrates with sidechain thioester containing peptides.¹³ In a related study, we examined the

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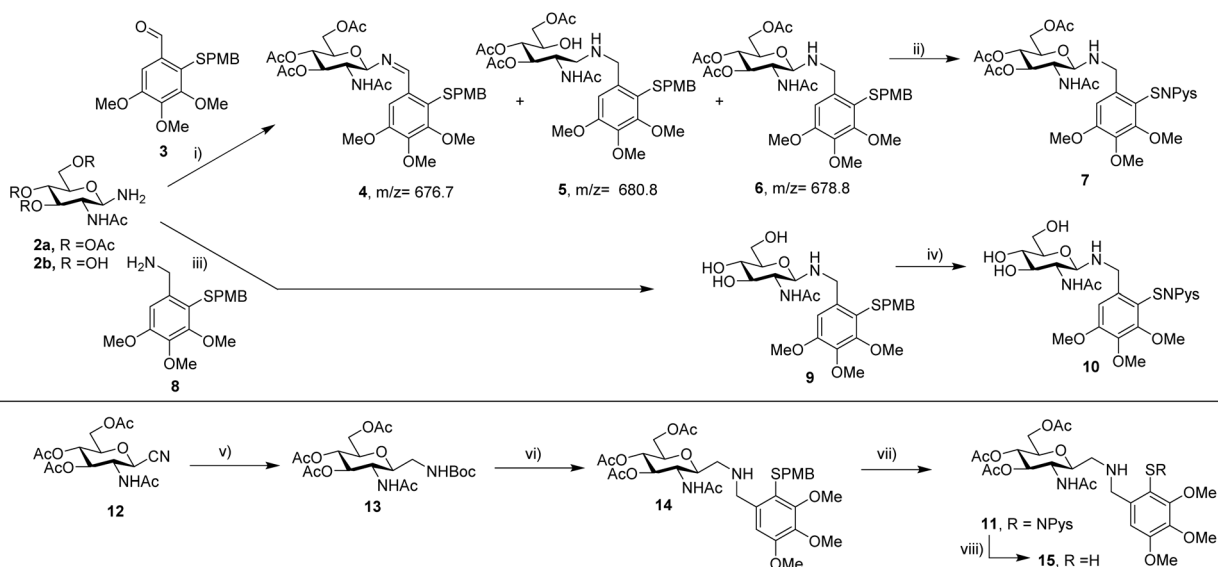


Scheme 1 Proposed auxiliary-mediated "neoglycoligation" from fully recombinant side-chain thioester (1) or benzyl ester (2) Aux = acyl transfer auxiliary, $n = 0$, or 1. Auxiliary-mediated ligation has the potential to introduce native *N*-glycopeptide linkages.

ability of an acyl transfer auxiliary to unite synthetic sugar derivatives, linked to an acyl transfer auxiliary, with synthetic peptide thioesters derived from the corresponding acyl hydrazides.¹⁴ Our reactions were successful, but only when employing an additional methylene spacer (a *C*-glycoside) in the carbohydrate building block. Currently, proteins adorned with sidechain hydrazides of aspartic acid are not directly accessible using genetic means, although can be obtained from the corresponding genetically encoded benzyl ester.^{11,15} We concluded that our failure to form native glycopeptides was

perhaps a result of incompatibility between the sugar-linked auxiliary and conditions required for hydrazide pre-activation.

This work encouraged us to prepare sugar-linked acyl transfer auxiliaries that may enable site-specific attachment of GlcNAc to fully recombinant proteins with pre-installed thioesters such as ThioD (Scheme 2). To this end, a reductive amination between glycosylamine **2a**¹⁶ and aldehyde **3**¹⁷ was investigated, but found to produce a mixture of products including imine **4** and over-reduced product **5**, with the desired product **6** forming in only 36% yield at best. This was not unexpected, considering the dearth of examples of reductive aminations featuring glycosylamines in the literature. However, once isolated, *p*-methoxybenzyl (PMB) cleavage, using 3-nitro-2-pyridylsulfenyl chloride (NPysCl) produced the mixed disulfide **7** in 41% yield. In an attempt to overcome the troublesome reductive amination, a transamination process was also investigated, using the fully unprotected glycosyl amine **2b**.¹⁸ A test transamination with benzylamine proceeded efficiently and, following purification was observed to be exclusively β -configured. Upon extension to benzylic amine **8**,¹⁹ the reaction was found to proceed similarly and **9** was isolated in 40% yield. Once more the *S*-PMB protecting group was exchanged to form the mixed disulfide. However **10** proved unstable to chromatography and was used without further purification. For comparison, our methylene extended building block **11** was also prepared. Whilst unnatural, it has precedent as a valuable glycopeptide analogue.²⁰ As expected, the synthesis of **11** was more straightforward. Glycosyl nitrile **12**²¹ was reduced by catalytic hydrogenation in the presence of Boc₂O allowing isolation of pure **13** in 82% yield.^{20a,22} Utilising **13** in place of **2a** simpli-



Scheme 2 Synthesis of sugar-linked auxiliaries. Reagents and conditions: (i) NaB(OAc)₃H, AcOH, DCM 45 min rt, 36%. (ii) NPysCl, TFE/DCM (9 : 1), 1.5 h, rt, 41%. (iii) pyridine, 50 °C, 18 h, 40%. (iv) NPysCl, TFE, DCM (9 : 1). (v) H₂, 10% Pd/C, EtOH/THF (5 : 3), Et₃N, Boc₂O, 18 h, rt, 82%. (vi) TFA, DCM, 2 h, rt, then Na(CN)BH₃, DCM, AcOH, 2 h, rt, 68%. (vii) NPysCl, TFE/DCM (9 : 1), 2.5 h, rt, product isolated as 2 : 1 mixture of **11** and symmetrical disulfide. (viii) 6 M Guanidine.HCl, 0.1 M Na phosphate; pH 7.0, 30 mM TCEP, 10 min, rt, 40%. NPysCl = 3-nitro-2-pyridinesulfonyl chloride. TFE = 2,2,2-trifluoroethanol. TCEP = tris-carboxyethylphosphine.



fied the reductive amination and subsequent steps, confirming that the hemi-aminal motif present within **2** was the source of synthetic complications. It is noteworthy that, en-route to **7**, **10** or **11**, the intermediates were not significantly exposed to aqueous conditions. The NPys unsymmetrical disulfide **11**, formed from treatment of **14** with NPysCl, existed as a 2:1 mixture of desired product and symmetrical disulfide, which complicated analysis. We found that **11** could be reduced with tris-carboxyethylphosphine (TCEP) and the thiol **15** was isolated by preparative HPLC, which, although adding an additional step and compromising yield, afforded a compound that was significantly simpler to characterise. In contrast, **7** or **10** were not stable to the same reducing conditions. Nevertheless, both were used in NCL reactions, assuming that their disulfides would be reduced under ligation conditions.

With building blocks in hand, we turned our attention to model ligation reactions using preformed peptide thioesters.

In these experiments (Fig. 1) C-terminal peptide thioester **16**, derived from β -interferon residues 76–83 (sequence: H-STGWNETIG-SCH₂CH₂SO₃H) bearing a natural glycosylation site (N80) was initially employed. The C-terminal thioester was first investigated to avoid complicating factors that may arise from additional reagents used for *in situ* thioester formation at the sidechain, and potentially problematic aspartimide formation. Otherwise we considered it an acceptable model for thioD containing peptides. **16** was incubated with sugar-linked auxiliaries **7**, **10** and **15**. Reactions progressed to consume **16** but, in our hands, and under various reaction conditions (varying pH, thiol additive, and temperature), only **15** furnished the expected product, with **17** being isolated as the major species. When using **7** or **10**, the only peptide-based material that could be identified in the reaction was **18**, derived from hydrolysis of the glycosyl amine followed by ligation to the unglycosylated auxiliary. LC-MS also showed two signals, (labelled 7*) corresponding to **7** (by mass) which may

have arisen from anomerisation. We reasoned that, since the expected product is a glycosyl amide and relatively stable, hydrolysis of **7**, followed by ligation of the free auxiliary was the likely source of the undesirable conjugate. We attempted to combat this by conducting the ligation in organic solvent (DMF, Et₃N)²³ but no reaction was observed under these conditions. This phenomenon was not unique to the trimethoxybenzyl (Tmb) class of acyl transfer auxiliary. We encountered similar difficulties in the preparation and ligation of “naturally-linked” conjugates to the 2-mercapto-2-phenethyl auxiliary developed by Seitz.²⁴ Again, the introduction of a methylene spacer to afford **19** (Fig. 2a) solved the problem of building block hydrolysis during ligation. **19** was prepared in a similar manner to **15**, employing reductive amination between **13** and aldehyde **20**.²⁵ **19** was obtained upon exposure of **21** to triethylsilane and TFA in DCM. To make progress towards protein glycosylation we further examined ligation reactions between **15** or **19** and free ThioD and reactions were complete within 24 h (see ESI†). Whilst encouraging, it proved difficult to isolate the conjugates to free ThioD and so ligation using **19** was also performed against peptide **16** (Fig. 2b). In this case the product **22** was easily isolated and the auxiliary removed to afford the methylene bridged *N*-glycopeptide analogue **23** in 74% yield. In a final model reaction **19** was ligated to peptide **24** (H-STGWNETIG-OH), exploring reaction at the N80 asparagine (bold and underlined) residue of β -interferon (Fig. 3a). Because the sidechain thioester is not stable to Fmoc-based SPPS, it was produced *in situ* from the corresponding hydrazide.¹⁴ In contrast to the reaction with **16**, the ligation product was clearly observed as an inseparable pair of epimers, which returned to a single species, **25**, following cleavage of the racemic auxiliary (see ESI†). Confident that **15** and **19** served as reliable building blocks we turned to recombinant protein modification although, due to the ease of synthesis and use of **19** relative to **15**, only his building block was progressed.

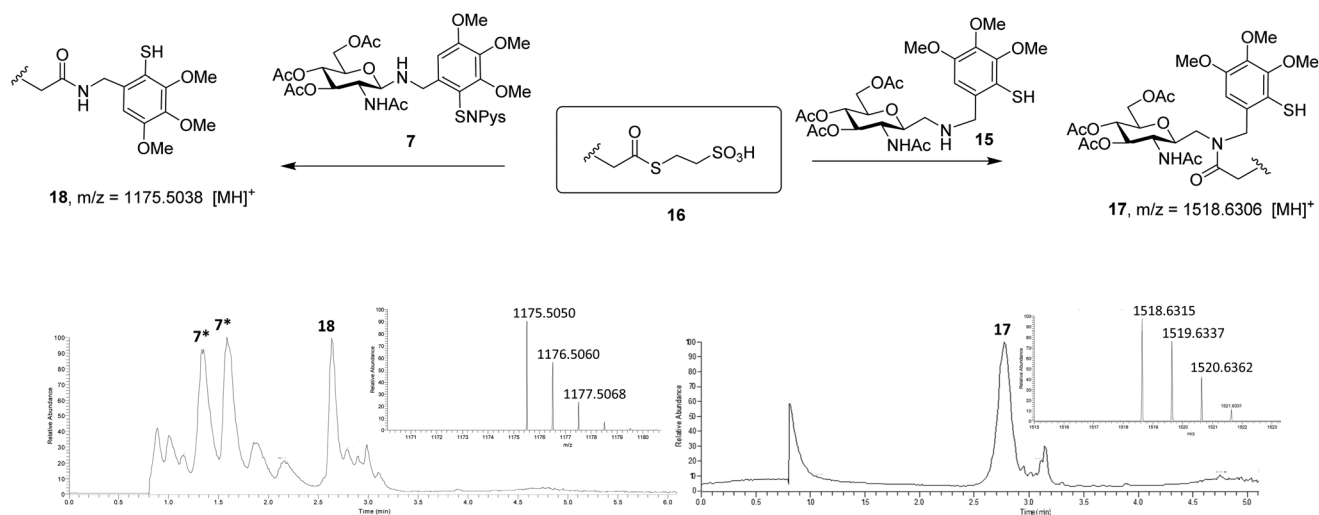


Fig. 1 Trial ligation reactions using naturally-linked **7** and methylene-extended **15**. Reaction conditions: **16** (1 mg ml⁻¹ final concentration), 0.1 M Na phosphate buffer; pH 7.0, 6.0 M guanidine.HCl, **7** or **15** (1.2 eq.), 0.2 M MPAA, 60 mM TCEP, rt, 72 h, 41% (**17**). MPAA = 4-mercaptophenyl acetic acid.



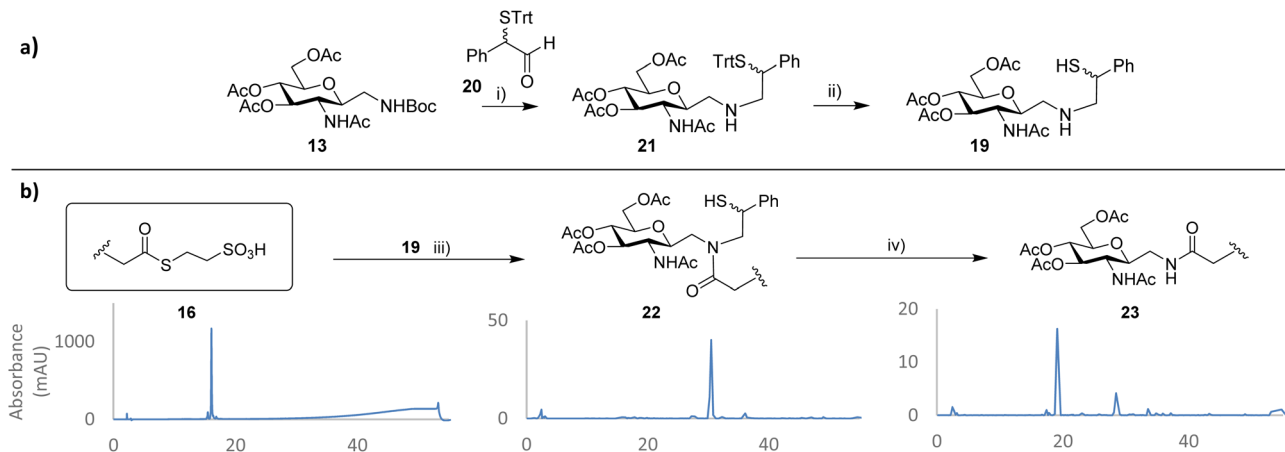


Fig. 2 (a) Synthesis of sugar-linked auxiliary **19**. Reagents and conditions (i) TFA, DCM, 3 h, rt, 96%, then **20**, NaB(OAc)₃H, DCM, 2% v/v acetic acid, rt, 4 h, 50% over 2 steps. (ii) TFA, Et₃SiH, DCM, rt, 2 h, 96%. (b) Ligation reaction between **19** and **16** with accompanying HPLC traces of isolated products. Reagents and conditions (iii) **19** (1.3 equiv.), 0.1 M Na phosphate; pH 7, 6 M guanidine.HCl, 0.2 M MPAA, 60 mM TCEP, 25 °C, 16 h, 34%. (iv) 0.5 M TCEP, 2 M morpholine (pH 8.5), rt, 5 h, 74%.^{24b}

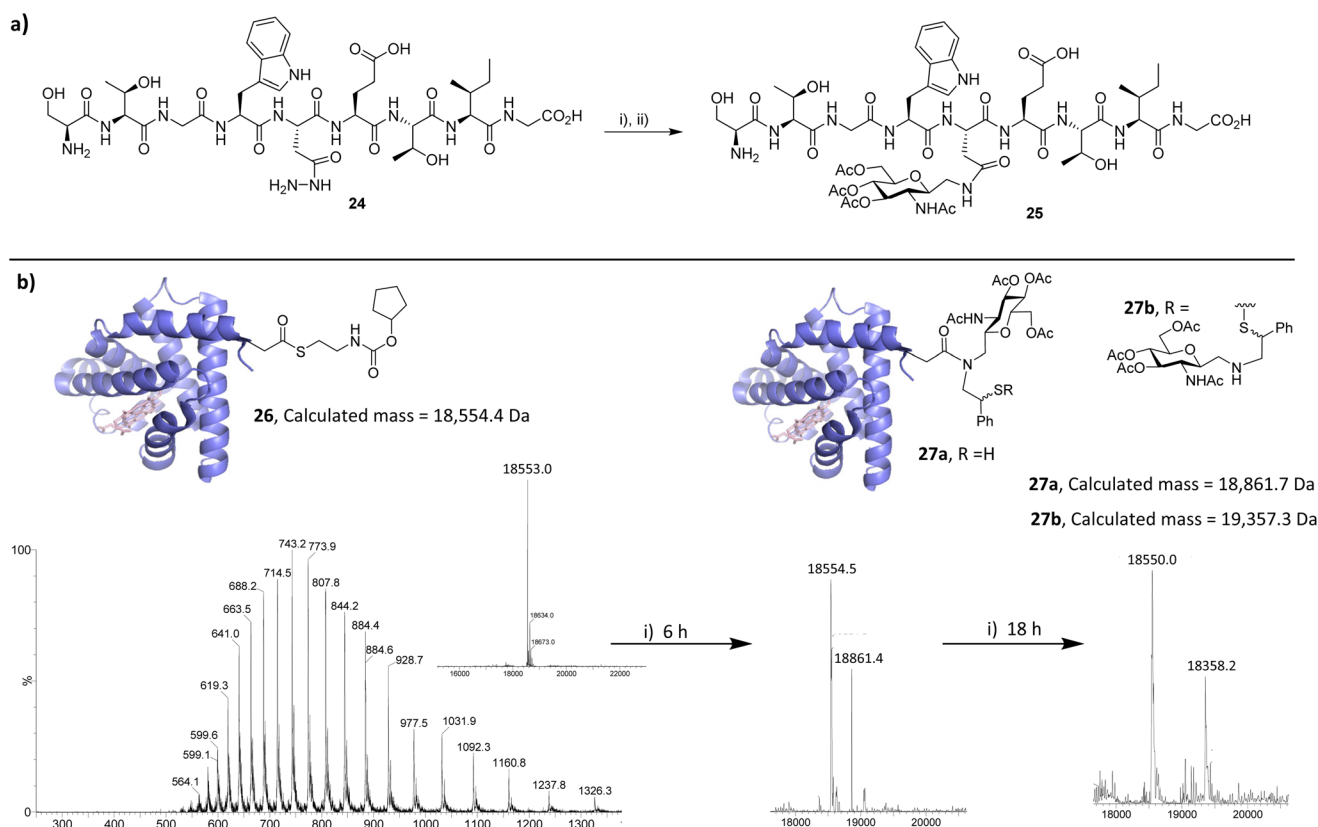


Fig. 3 (a) Ligation between **19** and model peptide **24**. Reagents and conditions: (i) 0.1 M Na phosphate (pH 3.0–4.0), 6.0 M guanidine.HCl, acetylacetone, 0.2 M MPAA, rt, 2 h, then **19** (1–2 eq.), 60 mM TCEP, pH 7.0, rt, 12 h, 34%. (ii) 0.5 M TCEP, 2 M morpholine (pH 8.5), rt, 16 h, 83%. (b) Attempted modification of myoglobin adorned with ThioD. Reagents and conditions: **19**, 50 mM Na phosphate; pH 7, 37 °C, 6 h. Reaction with 5–10 mg **19** initially showed modest conversion to the auxiliary-linked product that was converted to disulfide-linked product over a prolonged reaction time.

Sperm whale myoglobin, expressed from bacterial cells co-transformed with plasmid pMyo4TAG²⁶ and cognate ThioD tRNA synthetase (ThioDRS)¹⁰ was an available model system

and so was employed in proof of concept experiments. Using published procedures for protein expression we could isolate the protein containing ThioD (**26**) in yields of approximately



1 mg L⁻¹ and characterise it by SDS-PAGE and mass spectrometry (Fig. 3b). The presence of the thioester was confirmed by treatment of the protein, typically obtained at concentrations of 18.5 μM, with 2.5% v/v hydrazine hydrate, which showed complete conversion to the hydrazide within 2 h at room temperature (see ESI†). In contrast to reaction with hydrazine, myoglobin reacted only slowly with **19**. The reaction progressed to approximately 20% conversion after 6 h as judged by LC-MS despite employing a large excess of **19**, and heating to 37 °C. Heating to 37 °C for prolonged periods of time was generally unhelpful resulting in visible sample precipitation, loss of the of the protein's characteristic red/brown colour, and deterioration of the MS Signal. Encouragingly, only initial protein thioester **26**, product **27a**, and disulfide linked adduct between **27b** and **19**, were the major species observed in reactions by LC-MS. However, despite significant attempts to improve the reaction by (i) raising or lowering the reaction temperature (ii) conducting the reaction in 3 M guanidine hydrochloride, (iii) addition of MPAA, we were unable to improve the data. Unlike in our model systems there was no evidence of an MPAA thioester intermediate. We considered that the low conversion may be due to either the increased steric bulk of **19** and protein **29** relative to the model systems, or the oxidising nature of the model protein. Whilst **19** was significantly more stable towards oxidation relative to **15**, it still contains a benzylic thiol that is susceptible to oxidation. However, the oxidised dimeric form of **19** was not observed to accumulate significantly under the reaction conditions in the first 6 h and conducting reactions in the presence of 10 mM EDTA had a negligible effect on the outcome. Evidence of

expected by-products such as thioester hydrolysis or aspartimide formation were only observed after significantly longer reaction times (>6 h). Just as we were keen to investigate whether extending the glycoside by a methylene unit aided ligation of GlcNAc to model peptides, we additionally became interested in whether extending the protein sidechain from Asp to Glu might allow us to overcome the obstacle of low ThioD reactivity towards **19**. The corresponding "ThioE" is not available and so we prepared Glu thioesters from the benzyl ester (BnE) using the method recently reported by Xuan and co-workers.¹¹ From an inhouse PylRS mutant (N311A), we were able to recreate the BnE RNA synthetase (BnERS) by simple site directed mutagenesis of this template. We confirmed that the enzyme selectively introduced BnE (Fig. 4a) and once again expressed myoglobin containing the unnatural amino acid. Using an adaptation of the published procedure we could convert the BnE containing protein to the γ-glutamyl hydrazide (Fig. 4b). This procedure required exposing the protein to 30% v/v hydrazine hydrate and heating the sample at 37 °C for 2.5 h. Removal of excess hydrazine was accomplished by snap-freezing and lyophilisation of the reaction mixture. The presence of a viable protein hydrazide **28** was confirmed by reducing the pH of the sample to approximately pH 4 and incubating with 1 M GlcNAc at 40 °C for 4 h. In a reaction analogous to that recently reported at Asp β-hydrazides¹⁵ we observed near quantitative conversion to novel neoglycoprotein **29** (Fig. 4c). We did not test the longer-term stability of this linkage and neither confirm the β-stereochemistry at the anomeric linkage nor that the cyclic nature of sugar had been preserved, although preceding studies suggest both features are retained.²⁷

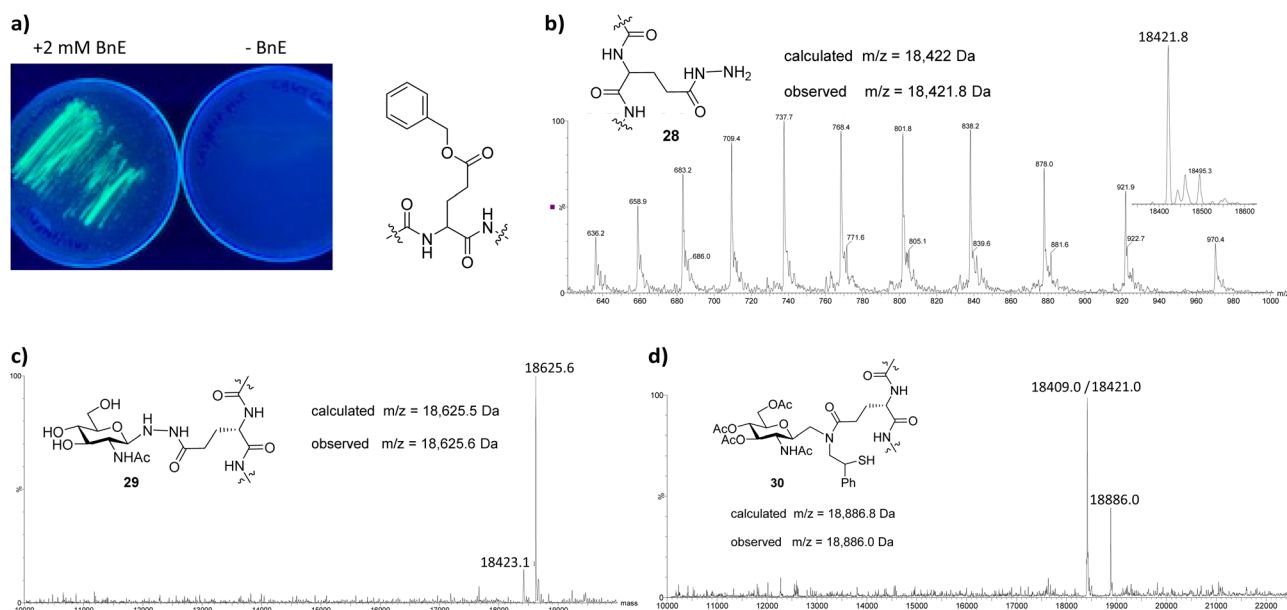


Fig. 4 (a) Selectivity of the γ-Benzyl glutamic acid pylRS mutant was confirmed by co-transformation with plasmid pREPpyIT for resistance to 50 μg ml⁻¹ chloramphenicol in the presence (left) and absence (right) of 2 mM BnE.²⁶ (b) Purified benzyl ester is converted to the γ-hydrazide by exposure to 30% v/v hydrazine hydrate at 37 °C for 2 h. (c) The presence of the hydrazide was confirmed by GlcNAc bioconjugation. Reagents and conditions: **28** (18.5 μM), 50 mM Na phosphate buffer; pH 4, 1 M GlcNAc, 40 °C, 4 h >80%. (d) Ligation between **28** and **19**. Reagents and conditions: 50 mM Na phosphate buffer; pH 4, 1 mM NaNO₂, 0 °C, 20 min, then **19**, 2.0 M guanidine.HCl 50 mM MPAA, 25 mM TCEP, pH 7, 25 °C, 4 h.



Confident that the hydrazide **28** should serve as a suitable thioester precursor we investigated ligation with **19** using both oxidative²⁸ and pyrazole forming²⁹ methods for hydrazide activation. In preliminary investigations, and in contrast to experiments with model peptide **24**, only oxidative activation of the hydrazide with NaNO₂ proved successful. However, as with ThioD containing protein **26**, only partial conversion to the desired neoglycoconjugate was observed after 3 h. A significant quantity of material had also failed to activate and/or hydrolysed during hydrazide activation and ligation, which is a disadvantage of not utilising a preformed thioester. However the results suggested that the nature of the amino acid itself (Asp vs. Glu) was not the only factor affecting the extent of protein modification. Since the reactions between protein samples and **19** gave rise to a mixture of starting material and product, no attempt was made to remove the auxiliary.

Conclusions

In summary we have explored a novel paradigm for glycoprotein synthesis which has the potential to reduce the synthetic burden required to assemble them. If site-specific glycosylation can be directed by the amino acid, and extended enzymatically after conjugation,^{9,30} then homogeneous semi-synthetic glycoproteins could be accessed without recourse to lengthy peptide synthesis-based processes. By utilizing novel unnatural C-glycosides (**15** or **19**), glycoside hydrolysis was abolished and ligation to amino acids and model peptides could be easily accomplished in reasonable yields. Whilst reaction of myoglobin thioester **26** with hydrazine hydrate was rapid at room temperature, reaction between **26** and **19** was less efficient. Investigation of ligation employing **19** and γ -glutamyl thioester **28** again showed modest conversion. It is likely that these reactions are compromised for different reasons. In the case of ThioD, the reactivity of the alkyl thioester appears low, whereas the need for pre-activation of **28** may increase hydrolysis and aspartimide formation. Reactions failed to reach completion within a time compatible with the fragile nature of the model protein myoglobin, which additionally displays a wide range of catalytic activities.³¹

Overall, we have demonstrated an important proof of concept, uniting sugar-linked auxiliaries, *via* an amide linkage, with recombinant protein sidechains. After cleavage of the auxiliary the glycoconjugate is extended by a single methylene unit when compared to a natural N-linked oligosaccharide. Unlike cysteine based “tag and modify” approaches³² the introduction of ThioD or the corresponding benzyl esters of aspartic and glutamic acids don't create the potential for disulfide scrambling and provide access to stable amide linked products. Owing to its more sustainable and less synthetically demanding components, as well as the potential to combine with developed enzymatic GlcNAc extension protocols,^{9,30} this approach may be developed into a useful glycoprotein and neoglycoprotein synthesis strategy.

Experimental details

General experimental details

Preparative reversed-phase high performance liquid chromatography (RP-HPLC) was performed using a Dionex Ultimate 3000 system equipped with a Phenomenex Jupiter Proteo 90A, C₁₂, 250 × 21.2 mm column. Separations involved a mobile phase of 0.1% TFA (v/v) in water (solvent A)/acetonitrile (solvent B) employing a 5–60% acetonitrile gradient over 60 min, and were monitored at wavelengths 230 nm, 254 nm, and 280 nm. Analytical reversed-phase high performance liquid chromatography (RP-HPLC) was performed using a Dionex Ultimate 3000 equipped with a Phenomenex SphereClone 5 μ ODS, C₁₈ 250 × 4.6 mm column. Separations used a mobile phase of 0.1% TFA (v/v) in water (solvent A)/acetonitrile (solvent B) employing a 5–95% acetonitrile gradient over 45 min, and were monitored at wavelengths 230 nm, 254 nm, and 280 nm. Analytical LC-MS was carried out on Waters uPLC/SQD-LC mass spectrometer instrument equipped with C₁₈, 2.1 × 50 mm column. Separations were conducted with a linear gradient of 5–95% acetonitrile containing 0.1% formic acid over 10 minutes using a flow rate of 0.2 ml min⁻¹. High resolution mass spectrometry was obtained from a Thermo Q-Exactive Vanquish Orbitrap mass spectrometer equipped with a liquid chromatography for separations. The liquid chromatography was equipped with Thermo Scientific Hypersil Gold C₄ 50 × 2.1 column and a linear gradient of 5–95% acetonitrile containing 0.1% Formic acid was used over 6 minutes.

General method for manual solid phase peptide synthesis

Manual peptide synthesis was carried out using standard Fmoc amino acids on NovaSyn TGT resin pre-loaded with Fmoc-Cys(Trt)-OH for the synthesis of **16** (loading = 0.19 mmol g⁻¹) and Fmoc-Gly-OH (loading = 0.21 mmol g⁻¹) for the synthesis of sidechain peptide hydrazide **24**. Syntheses were performed on 0.05 mmol scale. Deprotection of the N-terminal Fmoc protecting groups was achieved with 20% piperidine in DMF. The resin was then washed exhaustively with DMF and CH₂Cl₂. Fmoc-protected amino acids (5 equiv.) were pre-activated with HBTU/HOBt (5 equiv.) and DIPEA (10 equiv.) in DMF and then added to the reaction vessel for coupling. Each coupling reaction was conducted for 2 to 3 h. Subsequent couplings and deprotections led to the sequential assembly of the model peptide on resin. After chain assembly, deprotection of the peptide from the resin and of the side chain protecting groups was achieved with 4 mL of TFA:EDT: water (95:2.5:2.5) for 4 h. The resin was filtered off and to the filtrate added to diethyl ether (12 mL) which induced peptide precipitation. The mixture was centrifuged at 4000 rpm, 4 °C for 20 min. The ether layer was decanted and fresh Et₂O (12 mL) was added followed by further centrifugation. Ether was then decanted, and the peptide precipitate was dissolved in a minimum volume of water and purified by preparative reverse phase (RP) HPLC. Fractions containing product were identified by LC-MS and lyophilised to afford pure peptide.



Synthesis of model peptide thioester **16**

Peptide H-STGWNETIGC-OH was first constructed using the general method for manual peptide synthesis on Fmoc-Cys (Trt)-NovaSyn-TGT resin. After lyophilisation of the RP-HPLC fractions ($t_R = 28.5$ min), the peptide was isolated as a white fluffy solid (26 mg, 49%). ESI-HRMS: m/z calculated for $C_{44}H_{66}N_{12}O_{17}S$ $[M + H]^+$ 1067.4467, found $[M + H]$ 1067.4458.

This peptide (10 mg, 9.4 μmol) was dissolved in 0.1 M sodium phosphate buffer (pH 7.0) to a concentration of 1 mg mL^{-1} . To this solution was added 10% w/v MESNa (1.0 g, 6.1 mmol), 10% w/v hydrazine acetate (1.0 g, 10.9 mmol) and 0.5% w/v TCEP (0.05 g, 0.2 mmol). The reaction was mixed on an Eppendorf thermomixer (600 rpm) at 50 °C for 30 h. The crude mixture was purified by RP-HPLC ($t_R = 26.0$ min) and fractions containing product were lyophilised to afford a pure peptide hydrazide (8.0 mg, 87%) as a white solid. ESI-HRMS: m/z calculated for $C_{41}H_{63}N_{13}O_{15}$ $[M + H]^+$ 978.4645, found $[M + H]^+$ 978.4634.

Finally, the peptide C-terminal hydrazide (14 mg, 14.3 μmol) was dissolved in 7.0 mL of ligation buffer comprised of 0.1 M sodium phosphate buffer pH 3.0–4.0 and 6.0 M guanidine.HCl. The solution was cooled to –15 °C and NaNO_2 (0.7 mL of 0.2 M stock solution dissolved in deionised water) was then added dropwise and the reaction was stirred at –15 °C for 20 min.²⁸ The pH of the reaction was then adjusted to pH 7.0 by dropwise addition of 2 M NaOH. MESNa (2.3 g, 14 mmol) was dissolved in 6M guanidine.HCl (7.0 mL) and added to the reaction mixture followed by TCEP to final concentration of 30 mM. The reaction was stirred at room temperature for 2 h. The crude mixture was purified by RP-HPLC ($t_R = 27.5$ min) and fractions containing **16** were lyophilised to afford pure peptide thioester (9.0 mg, 60%) as a white solid. ESI-HRMS: m/z calculated for $C_{43}H_{66}N_{11}O_{18}S_2$ $[M + H]^+$ 1088.4029, found $[M + H]^+$ 1088.4015.

Ligations between **16** and **7**, **10**, or **15**

Thioester peptide **16** was dissolved in ligation buffer comprised of 0.1 M sodium phosphate buffer; pH 7.0 in 6.0 M guanidine.HCl to a concentration of 2 mg mL^{-1} . 0.5 mL of the solution was added to an Eppendorf tube. **7**, **10** or **15** (1.2 eq.) were dissolved in ligation buffer (0.5 mL) and then added, followed by MPAA to a final concentration of 0.2 M, and TCEP to a final concentration of 60 mM. Reactions were shaken in an Eppendorf thermomixer (750 rpm) at room temperature. LC-MS analysis of ligation reactions between **16** and **7** or **10** failed to identify any desired product and the by-products were not isolated. Successful reaction between **16** and **15** was scaled up 10-fold and the reaction was monitored for 72 h. The crude mixture was purified by preparative reverse-phase HPLC ($t_R = 39$ min) and lyophilised to yield **17** (5.7 mg, 40%). ESI-HRMS: m/z calculated for $C_{66}H_{96}N_{13}O_{26}S$ $[M + H]^+$ 1518.6310, found $[M + H]^+$ 1518.6316.

Ligation reaction between **16** and **19**, and removal of the auxiliary

16 (10 mg, 9.2 μmol) was dissolved in ligation buffer comprised of 0.1 M sodium phosphate buffer pH 7.0 in 6.0 M gua-

nidine.HCl to a concentration of 2 mg mL^{-1} . To this solution (5.0 ml) was added **19** (5.8 mg, 0.01 mmol, 1.2 eq.) dissolved in ligation buffer (5.0 mL) with MPAA (0.5 g, 3.0 mmol), 60 mM TCEP (260 mg, 1.04 mmol) and the reaction was stirred at room temperature. After 12 h, the product was purified from the reaction mixture by semi-preparative reverse-phase HPLC ($t_R = 39.0$ min) and lyophilised to yield **22** (4 mg, 34%). ESI-MS: m/z calculated for $C_{64}H_{91}N_{13}O_{23}S$ $[M + H]^+$ 1442.62, found $[M + H]^+$ 1443.73.

22 (1.8 mg, 1.25 μmol) was dissolved in 0.5 mL solution of 2 M morpholine in deionised water. TCEP (62.5 mg, 0.25 mmol) was added to the reaction and the pH was adjusted to pH 8.5 using 2 M NaOH. The mixture was placed in an Eppendorf thermomixer (750 rpm) and shaken at room temperature. After 5 h the reaction was complete and product isolated *via* preparative HPLC ($t_R = 31.0$ min). Lyophilization resulted in a white solid of **23** (1.2 mg, 74%). ESI-MS: m/z calculated for $C_{56}H_{84}N_{13}O_{23}$ $[M + H]^+$ 1306.58, found $[M + H]^+$ 1307.0.

Synthesis of model peptide **24**

IFN (76–83) with aspartic acid sidechain benzyl ester at position 80 was constructed using the general method for manual peptide synthesis on Fmoc-Gly-NovaSyn-TGT preloaded resin. Commercially available Fmoc-L-aspartic acid 4-benzyl ester was used for the coupling of Asp(OBzl)-OH at position 80. After lyophilisation of the semi-preparative reverse phase HPLC fractions ($t_R = 35.5$ min), peptide ester was isolated as a white fluffy solid (9.6 mg, 19%). The peptide was characterised by mass spectrometry. ESI-MS: m/z calculated for $C_{48}H_{67}N_{10}O_{17}$ $[M + H]^+$ 1056.48, found $[M + H]$ 1056.39.

To form the hydrazide 50–60% v/v hydrazine hydrate (200 μmol) was added to (8 mg, 7.59 μmol) of peptide benzyl ester dissolved in 8 mL of water. The mixture was stirred at room temperature for 1 h then purified by RP-HPLC ($t_R = 26.0$ min) and lyophilised to yield **24** (6.8 mg, 92%). ESI-HRMS: m/z calculated for $C_{41}H_{63}N_{12}O_{16}$ $[M + H]^+$ 979.4485, found $[M + H]$ 979.4484.

Ligation between peptide **24** and **19**

Peptide hydrazide was dissolved in ligation buffer comprised of 0.1 M sodium phosphate buffer pH 3.0–4.0 in 6.0 M guanidine.HCl to a concentration of 2 mg mL^{-1} (5.0 mL). Acetylacetone (125 mg, 1.25 mmol) was added from a 0.25 M stock solution along with 0.2 M MPAA (168 mg, 1.0 mmol) and the mixture was stirred at room temperature. After 2 h, **19** (1.2 eq.) and 60 mM TCEP (75 mg, 0.3 mmol) were added and the pH adjusted to pH 7.0 using 2 M NaOH. The reactions were stirred at room temperature and monitored by LC-MS. After 12 h the crude mixture was purified by preparative reverse phase (RP) HPLC ($t_R = 40.5$ min) and lyophilised to yield the product as a epimeric mixture (4 mg, 34%). ESI-MS: m/z calculated for $C_{64}H_{91}N_{12}O_{24}S$ $[M + H]^+$ 1443.56, found $[M + H]^+$ 1443.69. The product (4 mg, 2.77 μM) was dissolved in a solution of 0.5 M TCEP and 2 M morpholine to a concentration of 4 mg mL^{-1} . The pH was adjusted to pH 8.5 using 2 M NaOH



and the mixture was stirred at rt for 16 h. The crude mixture was purified by preparative reverse phase HPLC ($t_R = 31.0$ min) and lyophilised to yield **25** (3 mg, 83%). ESI-MS: m/z calculated for $C_{56}H_{83}N_{12}O_{24}$ $[M + H]^+$ 1307.56, found $[M + H]^+$ 1307.46.

Expression and purification of Sperm whale myoglobin containing ThioD or BnE

E. coli strain DH10B was co-transformed with pBK-ThioDRS (or pBK-BnERS) and pMyo4TAG-pyIT.²⁶ Successfully transformed clones were selected on LB-KT agar plates supplemented with kanamycin (25 $\mu\text{g mL}^{-1}$) and tetracycline (12.5 $\mu\text{g mL}^{-1}$). A single colony was picked and inoculated into LB-KT medium (10.0 mL) and grown overnight with shaking (250 rpm) at 37 °C. The culture was then diluted 100-fold into LB-KT medium (0.5 L) and grown at 37 °C. When the $OD_{600} = 0.2$, solid ThioD (or BnE) was directly added to the culture to a final concentration of 2 mM, and the culture was grown for another 30 min at 37 °C before induction with 0.2% w/v arabinose. The induced cells were grown for 6 h at 37 °C. The cells were pelleted by centrifugation at 8000 rpm for 10 min. The cells were resuspended in His-Bind buffer (50 mM Tris.HCl, 0.5 M NaCl, 5 mM imidazole; pH 8.0) and lysed with a sonic disruptor (10 \times 30s cycles) in the presence of protease inhibitor (0.01% PMSF). Insoluble protein and cell debris were removed by centrifugation at 10 000 rpm for 15 min. The protein was purified by Ni^{2+} affinity chromatography, eluting from the column in 50 mM Tris.HCl, 0.5 M NaCl, 250 mM imidazole; pH 8.0. Prior to analysis and ligation reactions, samples were buffer-exchanged into 50 mM sodium phosphate buffer (pH 8.0), and concentrated, using an Amicon Ultra-15 centrifugal filter unit (3000 MWCO) according to the manufacturers instructions. Yields were typically 0.9 mg L^{-1} after 6 h induction. After concentration to 1.0 mL, typical working protein concentrations of 18.5 μM were obtained. Expected mass for MbThioD (**26**) $M = 18554.1$ Da, found $M = 18553.0$ Da. Expected mass for Mb4BnE M^+ , 18487.2 Da, found $M = 18497.5$ Da. Note: The isolated protein containing BnE has a mass anomaly of +10 Da, presumably arising from a post translational modification, but all subsequent masses are correct relative to each other).

Ligation between **26** and **19**

In an Eppendorf tube **26** (0.35 mg, 18.9 nmol) dissolved in 50 mM sodium phosphate buffer pH 7.0 (0.5 mL) was added to excess **19** (2.5 mg, 50.3 μmol) and TCEP (0.25 mg, 1.0 μmol). The reaction mixture was shaken in an Eppendorf thermomixer for 18 h at 37 °C. The formation of **27a/27b** was monitored by LC-MS. After 18 h, the reaction was stopped as no further changes to the LC-MS spectra were observed. **27** was not isolated. ESI-MS: calculated mass for **27a** = 18861.7 Da, observed mass = 18861.4 Da.

Hydrazinolysis of Myo4BnE

Hydrazine hydrate 30% v/v was added to concentrated (18.5 μM) samples of Myo4BnE in 50 mM Na phosphate buffer (pH 8.0). The reaction was shaken in an Eppendorf thermo-

mixer (37 °C, 750 rpm), monitored by LC-MS. Conversion was judged to be complete within 3 h. Samples were then snap-frozen in liquid nitrogen and lyophilised to afford crude **28**. Expected mass $M = 18421.5$ Da, found $M = 18421.8$ Da.

Ligation between **28** and *N*-acetyl D-glucosamine

Lyophilised hydrazide was reconstituted in deionised water to the original concentration (18.5 μM) and the pH was lowered to pH 4 with 2 M HCl. The solution was added to a fresh Eppendorf tube containing solid GlcNAc such that the final concentration of GlcNAc was 1 M. The reaction was shaken in an Eppendorf thermomixer (40 °C, 750 rpm), monitored by LC-MS and conversion was judged to be >80% complete within 4 h. The ligated product was separated from excess GlcNAc by centrifugal filtration (10 kDa MWCO) against 50 mM sodium phosphate buffer; pH 8.0. Expected mass = 18625.0 Da, observed mass = 18625.6 Da.

Ligation between hydrazide **28** and **19**

Lyophilised hydrazide was reconstituted to its original concentration (18.5 μM) in deionised water and the pH was lowered to pH 4 using 2 M HCl. The protein sample was cooled in an ice/NaCl bath and freshly prepared 0.1 M NaNO_2 solution was added to final concentration of 1 mM. After 20 min at 0 °C **19** (2.5 mg, nmol, predissolved in 6 M guanidine hydrochloride such that the final guanidine.HCl concentration was 2.0 M) was added along with MPAA (50 mM final concentration) and TCEP (25 mM final concentration). The pH was then carefully adjusted to pH 7 with 10 M NaOH and the reaction was shaken in an Eppendorf thermomixer (25 °C, 750 rpm). The reaction was monitored by LC-MS and conversion was judged to be approximately 25% complete after 4 h.

Conflicts of interest

There are no conflicts to declare.

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