A novel neoglycopeptide linkage compatible with native chemical ligation†

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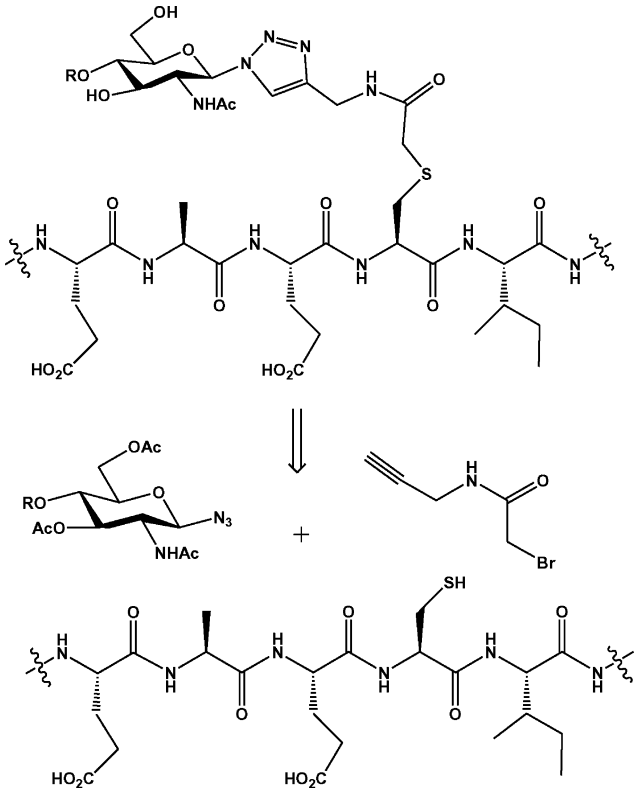
The straightforward synthesis of a novel class of neoglycopeptide and its fusion with a larger peptide thioester using sequential chemoselective ligations is described.

The synthesis of glycosylated proteins is a formidable challenge and it is fair to say that synthetic glycoproteins have had modest impact, thus far, on the study of glycobiology when compared to genetic methods. This is largely due to the well documented difficulties associated with synthesis of oligosaccharides and glycopeptides such as the requirement for extensive protection and deprotection strategies and the stereoselective formation of glycosidic linkages. We, and others, have pursued alternative methodologies for the assembly of modified peptides and proteins.[1] Using the click "ligation"[2] for bioconjugation was of particular interest due to its reliability and compatibility with the aqueous milieu.[3] We had previously shown that glycosyl iodoacetamides can react with the sulfhydryl groups of cysteine on solid-phase to afford glycopeptide mimetics and that this methodology was compatible with useful protein assembly methods such as native chemical ligation (NCL).[4] We were keen to investigate whether the coupling of glycosyl azides with peptides displaying acetylene dipolarophiles may also be useful for this purpose since the glycosyl azides are more stable and can be prepared in fewer steps than the corresponding glycosyl iodoacetamides (Scheme 1).

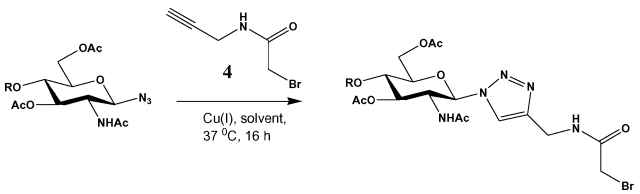
Initially we prepared the peracetylated glycopyranosylazides of *N*-acetylglucosamine (**1**) *N*-acetyllactosamine (**2**) and chitobiose (**3**) which are all constituents of the *N*-linked class of glycoproteins. We investigated conditions for their union with the heterobifunctional adaptor 2-bromoacetyl propargylamide (**4**)[5] and the reaction of these saccharides with **4** proceeded smoothly under conditions reported in the recent literature,[5,6] the 1,4-addition product being favoured by the presence of a Cu(I) catalyst (Table 1). The crude products did not require purification by column chromatography.

Pleased with the facility with which such glycoconjugates could be prepared we next aimed to expose the bromoacetamides to conditions typically encountered in peptide chemistry and native chemical ligation. We were particularly interested in the potential to modify the thiol groups of cysteine residues. Additionally, to demonstrate the versatility of the approach, we explored the possibility of reacting thiol groups directly with the bromoacetamide products **5–7** on solid phase *and* the reaction of cysteine thiols with **4** such that click chemistry could subsequently be investigated in solution or on solid-phase with peptides displaying acetylenes (Scheme 2). As anticipated, **4** and **5** reacted cleanly with benzyl mercaptan forming model thioethers **8** and **9** in 84% and 75% yield respectively. **8** also reacted cleanly with peracetylated 2-acetamido-2-deoxy-D-glucopyranosyl azide, affording **9** in 91%



Scheme 1 Construction of novel neoglycopeptides.

Table 1 Synthesis of neoglycopeptide precursor bromoacetamides



Saccharide	Catalyst	Product	Yield (%)[c]
1, R = Ac	Cu(I)I (5 eq.)[a]	**5**	100
1, R = Ac	$Cu(II)SO_4$ (0.1 eq.)[b]	**5**	97
2, R = $(OAc)_4$-β-Gal-	$Cu(II)SO_4$ (0.1 eq.)	**6**	91
3, R = $(OAc)_3$ β-GlcNAc-	$Cu(II)SO_4$ (0.1 eq.)	**7**	87

[a] Methanol as solvent. [b] Active copper species generated in the presence of 1.1 equivalent sodium ascorbate in 9 : 1 : 1 $CHCl_3$–EtOH–H_2O as solvent. [c] Isolated yield.

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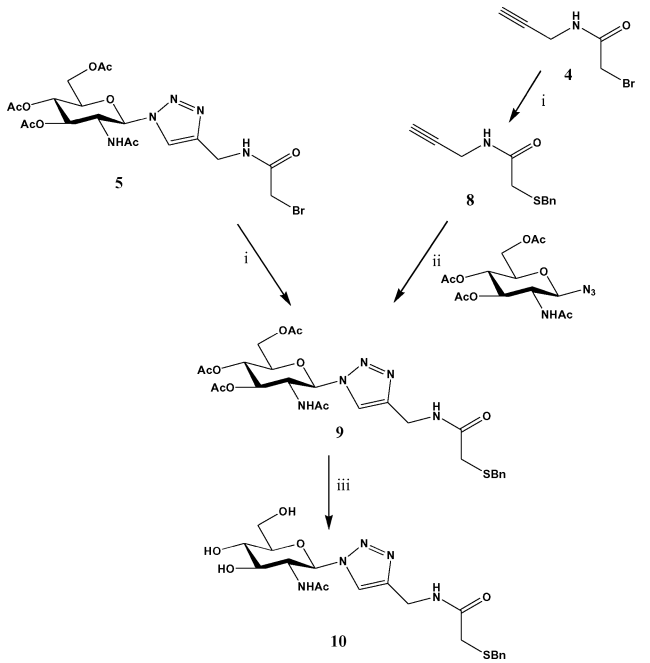
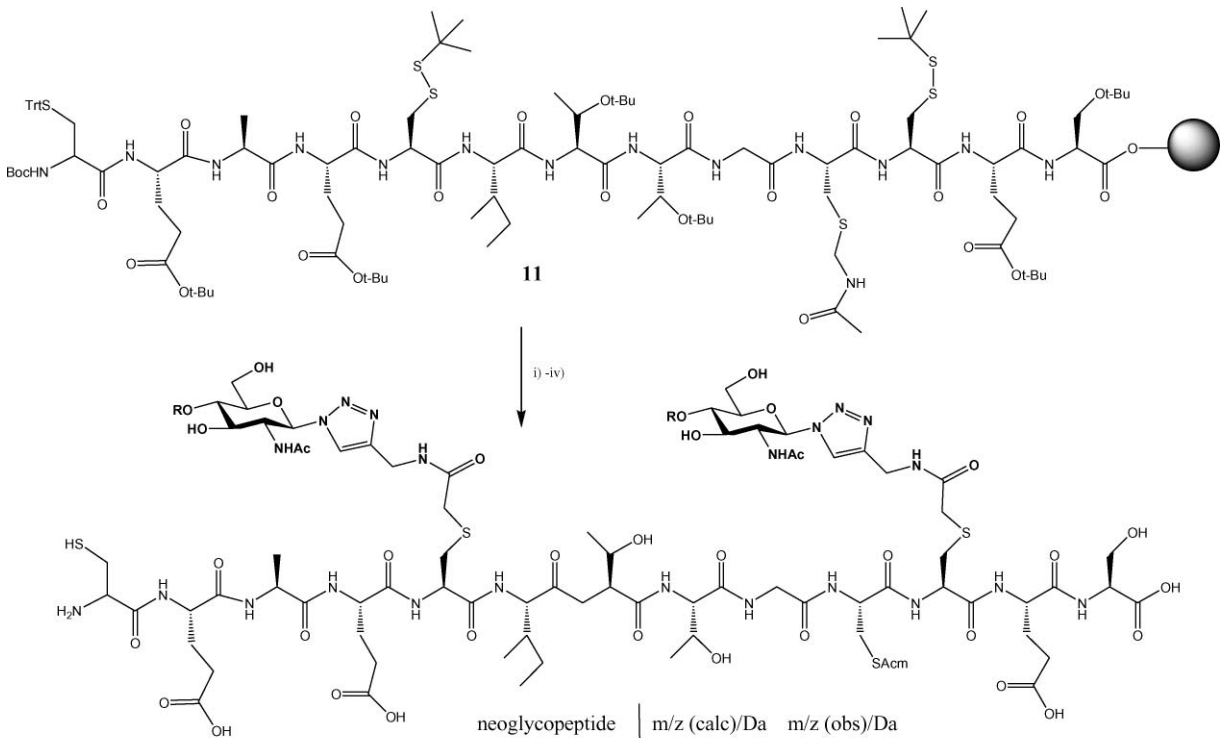


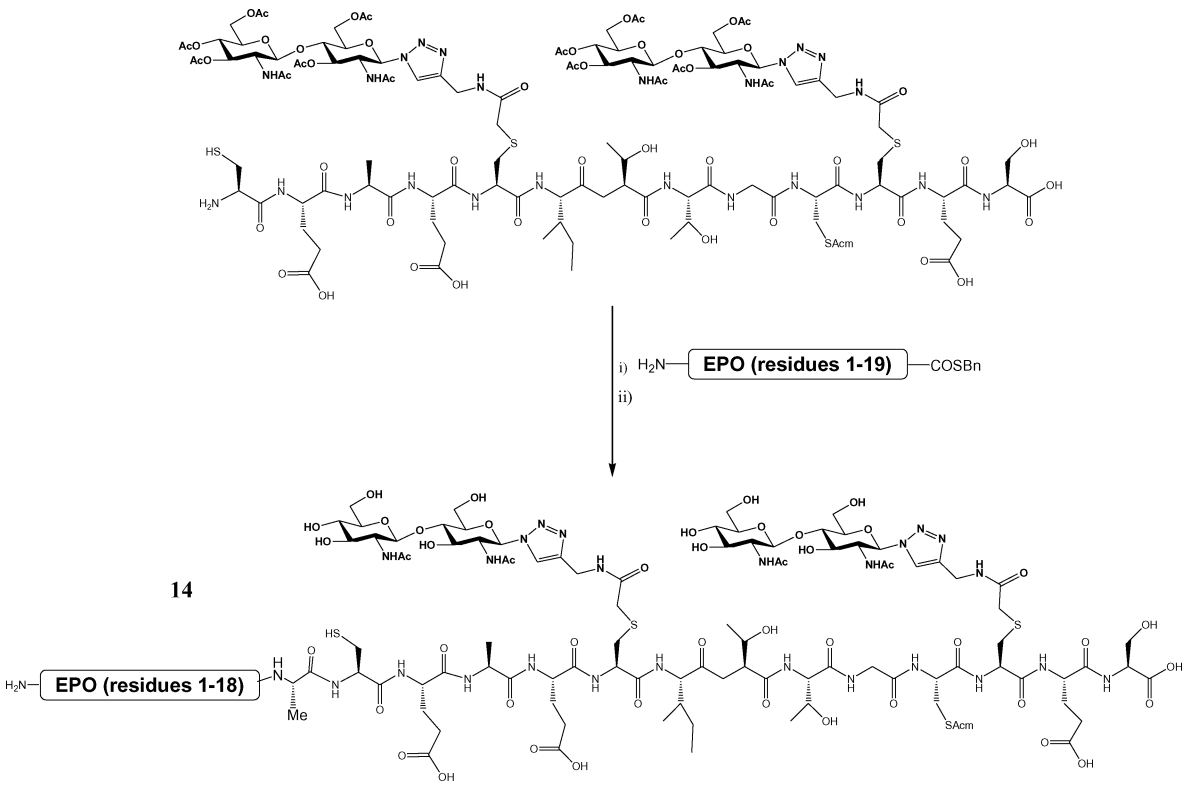
Scheme 2 Model reactions with benzylmercaptan demonstrate that the order in which the reactions take place appears unimportant. *Reagents and conditions*: i) BnSH, Et_3N, DMF, 16 h, 84% and 75% for **8** and **9** respectively ii) sodium ascorbate (1.1 eq.), Cu(II)SO_4·5H_2O (0.1 eq.), $CHCl_3$, EtOH, H_2O (9 : 1 : 1), 37 °C, 16 h, 91%, iii) 2% v/v hydrazine monohydrate EtOH, 72 h, 66%.[7]

yield. To establish whether the products might be stable to the usual acidic peptide cleavage conditions **9** was subjected to 95% aqueous TFA for 3 h. NMR analysis of the crude material after evaporation showed no decomposition had taken place. Finally the acetyl esters were cleanly removed upon exposure to 2% v/v hydrazine hydrate in EtOH for 72 h and the fully deprotected compound **10** was obtained.[7] Encouraged by the preliminary results we assembled a peptide fragment (**11**), similar in sequence to human erythropoietin (residues 21–32), plus an N-terminal cysteine residue, and furnished with two disulfide bond protected cysteine residues at pre-determined positions (Scheme 3). The peptide was assembled using standard protocols for Fmoc solid-phase peptide synthesis and in an automated fashion. The cysteine residues were deprotected on solid-phase by exposure to 10% w/v dithiothreitol (DTT) containing 2.5% v/v DIPEA to expose the thiol functional groups.[4] *N*-Acetylglucosamine and the disaccharide chitobiose were then incorporated by exposure of the resin to bromoacetamides **5** or **7**, employing three equivalents **5** or **7** per thiol in each reaction. After 16 h reaction at room temperature, cleavage of a small resin sample indicated that the reaction was complete as the starting material was not observed. After cleavage from the solid support by treatment of the resin with 95% TFA, 2.5% ethanedithiol and 2 5% H_2O for four hours the crude products were purified by semi-preparative HPLC, lyophilized, and treated with 2% v/v aqueous hydrazine hydrate containing 5% w/v DTT to obtain the fully deprotected products **12** and **13** in quantitative yield (determined by HPLC). Bromoacetamide **6** and acetylenic bromoacetamide **4** could also be incorporated into synthetic peptides in an identical fashion.



neoglycopeptide	m/z (calc)/Da	m/z (obs)/Da
12, R = H	2102.2	2103.4
13, R = β-GlcNAc	2508.6	2509.2

Scheme 3 *Reagents and conditions*: i) 10% w/v DTT, 2.5% DIPEA, DMF, 16 h, ii) **5** or **7** (3 eq. per thiol), 2.5% v/v Et_3N, DMF, 16 h, iii) 95% TFA, 2.5% ethanedithiol, 2.5% H_2O, 4 h, iv) 2% v/v aqueous hydrazine monohydrate, 1 h.



Scheme 4 The neoglycopeptides are compatible with native chemical ligation reactions.[7] *Reagents and conditions*: i) 6 M guanidine HCl, 1% w/v MESNA, 300 mM Na phosphate buffer (pH 8.0), 10 mM TCEP, ii) 2% aqueous H_2N-NH_2.

Fragment **13** was then coupled to a peptide thioester in a native chemical ligation reaction.[8] The construction of the peptide thioester, corresponding to human erythropoietin residues 1–19, and its release from the solid support were monitored using the dual-linker approach recently described by Unverzagt and co-workers.[9] In the ligation reaction equimolar quantities of each peptide were combined in 0.25 ml of 6 M guanidine hydrochloride containing 300 mM sodium phosphate buffer; pH 8.0, 1% w/v mercaptoethanesulfonic acid (MESNA) and 10 mM tris-carboxyethylphosphine (TCEP) for 36 h with shaking at room temperature (Scheme 4).[7] After this time the reaction mixture was purified by directly loading it onto a semi-preparative HPLC column. The ligated product (**14**) was the only species observed by HPLC.[7]

In summary we have developed a novel class of neoglycopeptide that is compatible with modification of cysteine mutant proteins, with synthetic peptides, and native chemical ligation. Furthermore the fusion of glycosyl azides with peptides displaying acetylenes may be of particular interest since NCL has been shown to fail when large oligosaccharide appendages are located proximal to the ligation site.[10] Therefore, if acetylenes can be installed so as to "encode" for glycosylation then bulky saccharide motifs may be installed after ligation using click chemistry in solution. We have already observed that the reaction between peptides displaying acetylenes derived from **4** and glycosylazides proceeds on solid-support.[7] Although the linkage between the carbohydrate and peptide moiety is unnatural, difficulties associated with the synthesis of native glycopeptides and glycoproteins dictates that new modes of presentation of carbohydrates (or other appendages) should be explored concomitantly.[1] Such neoglycoconjugates may improve the pharmacokinetic profile of therapeutic glycoproteins. The fact that the neoglycopeptides described are simple to prepare, homogeneous, and are also compatible with native chemical ligation, may render them attractive building blocks for neoglycoprotein assembly.

Acknowledgements

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