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PAPER

Synthesis of MUC1 *Neo*glycopeptides using efficient microwave-enhanced chaotrope-assisted click chemistry†

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The first synthesis of click *neo*glycopeptide analogues of the biologically relevant MUC1 sequence is reported. In the process, microwave-enhanced chaotrope-assisted click reaction conditions that may be used on a routine basis for the synthesis of click peptide conjugates have been developed. The convergent route for the synthesis of *neo*glycopeptides using these reaction conditions enables the facile, rapid, and highly efficient preparation of focused *neo*glycopeptide libraries of defined chemical structure for biological evaluation.

Introduction

The membrane-bound tumor-associated MUC1 glycoproteins occur ubiquitously in almost all epithelial tissues of most organs, and are also excessively expressed on tumor tissue.[1] They are also involved in cell signalling events, and their aberrant expression has been found to aid the metastatic spread of tumor cells.[2] The extracellular domain of MUC1 contains a variable number of tandem repeat domains of 20 amino acids (HGVTSAPDTRPAPGSTAPPA), possessing five potential *O*-glycosylation sites on Thr and Ser.[3] The altered glycan patterns of MUC1 on tumour cells are caused by down-regulation of β6-*N*-acetylglucosaminyltransferase (β6GnT) and the concomitant up-regulation of α3-sialyltransferase (α3ST) resulting in shortened sialylated oligosaccharides (sialyl-T_N and T_N antigens) instead of the longer chains found to be expressed on normal cells.[4] Over-expression of these antigens on tumor cells renders them attractive candidates for selective cancer vaccines, and these have been studied by several research groups.[5]

Difficulties experienced in making the native GalNAcα1-*O*-Thr/Ser building blocks in the large quantities needed for Fmoc SPPS prompted us to explore more convergent methods to obtain MUC1-like glycopeptides. Previously, glycoconjugates containing a variety of unnatural linkages between the carbohydrate and aglycone moieties have been explored.[6] When carried out in a convergent manner this mimicry allows rapid and efficient access to a wide variety of *neo*glycopeptides that may retain the biological activity of the natural glycoproteins.

One of the most studied of these unnatural linkages is the triazole ring formed *via* 1,3-dipolar cycloaddition (click chemistry) of an organic azide with a terminal alkyne.[7] Rutjes *et al.*[8] made use of protected building blocks to synthesize a series of triazole-linked glycosyl amino acids and dipeptides. Danishefsky's group[9] and Walsh *et al.*[10] have independently reported the union of unprotected carbohydrates and peptides containing a limited range of side chains using click chemistry. Macmillan's group reported the synthesis of a glycosyl amino acid linked *via* a triazole linkage which was incorporated into solid-phase peptide synthesis.[11] Moroder *et al.*[12] have utilized click chemistry to synthesize semi-synthetic lipoproteins. Davis *et al.*[13] have also utilized click chemistry to demonstrate the diversity of post-translational chemical protein modification. Besides these examples, click chemistry is also widely used in other areas such as analytical and material sciences.[14,15] An excellent example of this was demonstrated by Ravoo *et al.* who used click chemistry to prepare carbohydrate microarrays by microcontact printing of carbohydrate alkyne conjugates onto azide self-assembled monolayers.[15] Recently we reported the synthesis of *neo*glycopeptides using one-pot native chemical ligation and click chemistry using unprotected sugar-azides and propargylated peptides.[16] Click analogues of anti-freeze glycopeptides have also been reported by our group with azido-alanine incorporated in the peptide chain; however, one limitation is that the azide moiety is susceptible to elimination during Fmoc deprotection cycles in SPPS.[17]

We therefore envisaged that the complementary click chemistry using unprotected sugar-azides and peptides containing one or more propargyl groups would facilitate rapid synthesis of a library of MUC1 *neo*glycopeptides with a variable number of GalNAcα1-*O*-Thr/Ser mimics for biological evaluation (Fig. 1). Surprisingly, click mimics of the biologically relevant MUC1 sequence have not been reported to date. Importantly, the chemistry reported herein is amenable to the synthesis of a focused library of *neo*glycopeptides of defined structures, thereby overcoming the problems of working with native glycoproteins that often exist as a variety of glycoforms.[18]

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† Electronic supplementary information (ESI) available: Details of synthesis of propargylated peptides, click reaction procedures, HPLC and ESI-MS profiles of click neoglycopeptides are provided. See DOI: 10.1039/c0ob01043j

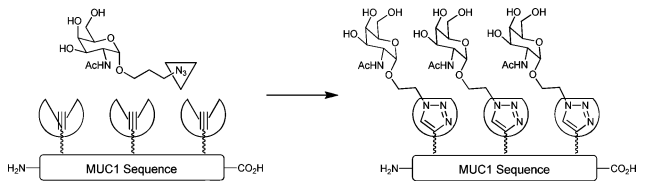
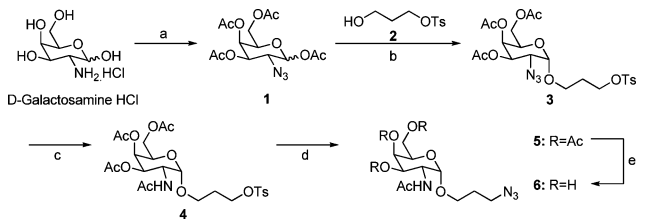


Fig. 1 Synthesis of click *neo*glycopeptides of MUC1 sequence.

Synthesis of sugar-azide and propargylated peptides

The key GalNAcα1-*O*-Thr/Ser *neo*glycosyl amino acid mimic **6** was designed to incorporate an *N*-acetyl group at C-2, and an α-*O*-glycosidic link at the C-1 anomeric position (Scheme 1). Azido-acetate **1** was formed from galactosamine hydrochloride *via* diazo-transfer then acetylation. Glycosylation of alcohol **2** with **1** afforded tosylate **3** as a 2 : 1 mixture of α : β anomers, which were separated to give the α-anomer in 41% yield. The α-anomer was subjected to reductive acetylation in 87% yield, then tosylate **4** underwent subsequent azide displacement and deacetylation to give the desired 3′-azido-1′-propyl-2-acetamido-2-deoxy-α-D-galactopyranoside **6** ready for reaction with the synthetic propargylated peptides.



Scheme 1 (a) i. Imidazole-1-sulfonyl azide hydrochloride, K_2CO_3, $CuSO_4 \cdot 5H_2O$, MeOH, 3 h, rt.; ii. pyridine/Ac_2O, DMAP, 12 h, rt., 83%; (b) $BF_3 \cdot OEt_2$, DCM, 48 h, 40 °C, 41% (α purified); (c) THF/Ac_2O/AcOH (3/2/1 (v/v/v)), powdered Zn, aq. $CuSO_4$ (sat.), 1 h, rt., 87%; (d) NaN_3, $^nBu_4N^+HSO_4^-$, H_2O/DCM, 16 h, rt., 80%; (e) 1 M NaOMe, 3 h, rt., 98%.

A series of propargylated peptide derivatives of the MUC1 sequence were synthesized on amino-functionalized polystyrene resin containing the acid labile hydroxymethylphenoxypropionic acid (HMPP) linker using microwave enhanced Fmoc SPPS (Scheme 2). Fmoc-L-propargylglycine[16] **7** was incorporated into the peptide chain using the conditions shown in Scheme 2, replacing Thr/Ser at selected positions in the MUC1 sequence. These replacements were chosen to aid the determination of which specific sites were important for incorporation of the GalNAc mimic.

Click *neo*glycopeptides

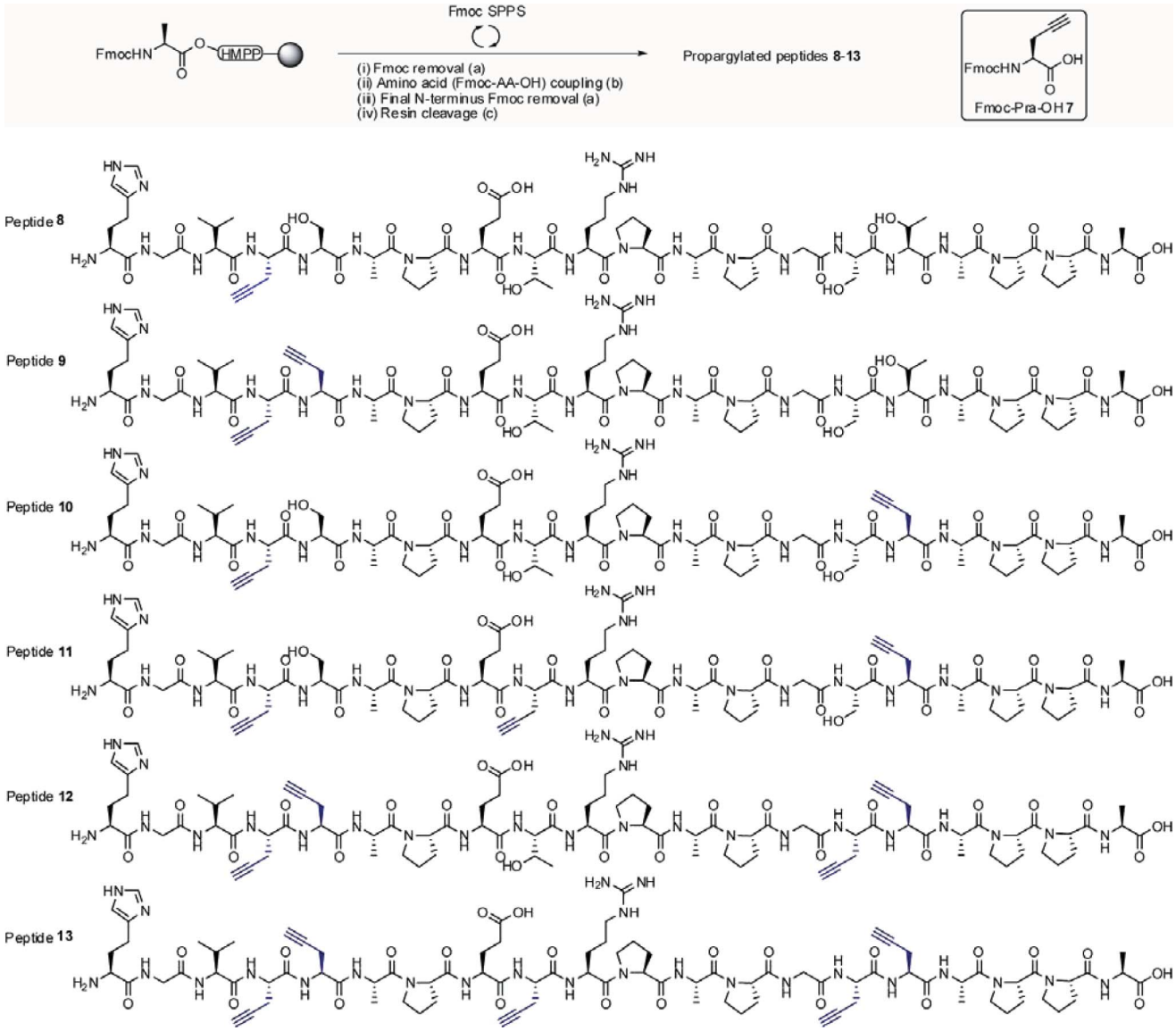
Initial attempts to carry out click reactions using catalytic amounts of $CuSO_4$ and sodium ascorbate (NaAsc) in aqueous phosphate buffer were unsuccessful, even at elevated temperatures using microwave irradiation. Our previous studies[16] of click reactions involving unprotected peptides and sugars highlighted the need for more than equimolar amounts of Cu(I) species per propargyl group, possibly due to chelation. The desired click product was only observed when a large excess of $CuSO_4$/NaAsc was used; however, significant formation of side products complicated subsequent purification (see the ESI†). Furthermore, large quantities of $CuSO_4$/NaAsc are undesirable in bioconjugation due to copper-mediated generation of reactive oxygen species.[19] Dehydroascorbate and other ascorbate byproducts can also react with lysine amino and arginine guanidino groups, resulting in covalent modification and aggregation of proteins.[20] Given the drawbacks, we therefore focused on a much milder and biologically friendly reductant, namely tris(carboxyethyl)phosphine (TCEP). TCEP is often used in bioconjugation chemistry and native chemical ligation reactions,[21] and its oxidised byproduct, TCEP oxide, is relatively inert. When aqueous $CuSO_4$ and TCEP were premixed before addition to the peptide solution, blue aggregates were observed suggesting incomplete reduction of $CuSO_4$ by TCEP. After exploring several different solvent systems, it was established that use of 6 M guanidine hydrochloride (GnHCl)/0.2 M Na_2HPO_4 solution helped to solubilise the blue aggregates facilitating complete reduction of the $CuSO_4$ almost immediately upon addition. In addition to its use as a common solvent for the native chemical ligation of peptides,[22] GnHCl is an important chaotrope used to minimize aggregation in proteins and to facilitate chemical modification.[23] In our study, use of GnHCl was critical to the success of the click chemistry. It appeared to accelerate the reduction of $CuSO_4$ by TCEP, and importantly the Cu(I) species formed seemed to be less susceptible to air oxidation. Furthermore, it is noteworthy that a broad peak was observed on the HPLC chromatograms (see Scheme 3A and the ESI†) where $CuSO_4$ and TCEP coexisted with GnHCl that was not present in aqueous buffer solutions lacking GnHCl. When the reaction mixture was blue (*i.e.* suggesting that the Cu(II) species was present) this broad peak was not observed by HPLC (see the ESI†) and the click reaction did not progress to completion.

Table 1 Summary of click reactions. All click reactions were carried out in 6 M GnHCl/0.2 M Na_2HPO_4 buffer at pH 7 with microwave irradiation (25 W)

Peptide (3mM)	$CuSO_4$ conc./mM	TCEP conc./M	Time taken[a]/h	Yield (%)[b]
8	20	20	1.0	95 (43)
9	40	40	2.5	95 (36)
10	40	40	4.0	95 (34)
11	60	60	4.5	95 (28)
12	80	80	3.5	95 (34)
13	100	100	4.0	90 (30)

[a] Time taken to completion observed by HPLC. [b] % Conversion from starting peptide to product observed by HPLC. Isolated yield after reverse phase HPLC chromatography and lyophilisation, depicted in parentheses.

Click reactions on all peptides **8–13** using 20 mM of both $CuSO_4$ and TCEP per 3 mM of propargyl moiety proceeded cleanly to afford the desired click peptides at both room temperature and 50 °C. In addition, microwave irradiation reduced the reaction times, often by 2–4 fold compared to the use of conventional heating, with slightly improved purities. Using microwave conditions, the key click reactions were successfully executed on all mono-, di-, tri-, tetra-, and penta-propargylated peptides in a matter of hours (Table 1). Scheme 3 highlights the success of our multiple, contiguous click reaction on a penta-propargylated peptide **13**. The reaction progression was monitored by the presence of multiple peaks in the HPLC chromatogram at t = 30 min that corresponded to mono-, di-, tri-, and tetra-clicked intermediates



Scheme 2 Synthesis of propargylated peptides. (a) 20% (v/v) piperidine in DMF, MW 25 W; (b) Fmoc-Aaa-OH (incl. **7**), HBTU, DIPEA in DMF, MW 25 W; (c) TFA/TIS/H_2O (38/1/1), 2.5 h.

with only a minor amount of starting peptide remaining (Scheme 3B). At t = 4 h, the reaction was essentially complete affording the desired penta-clicked *neo*glycopeptide **14** (Scheme 3C), which was isolated by solid phase extraction and then lyophilized. The crude product was formed in 90% yield, and this was further purified by reverse phase HPLC to give the *neo*glycopeptide in high purity and isolated yield (Table 1).

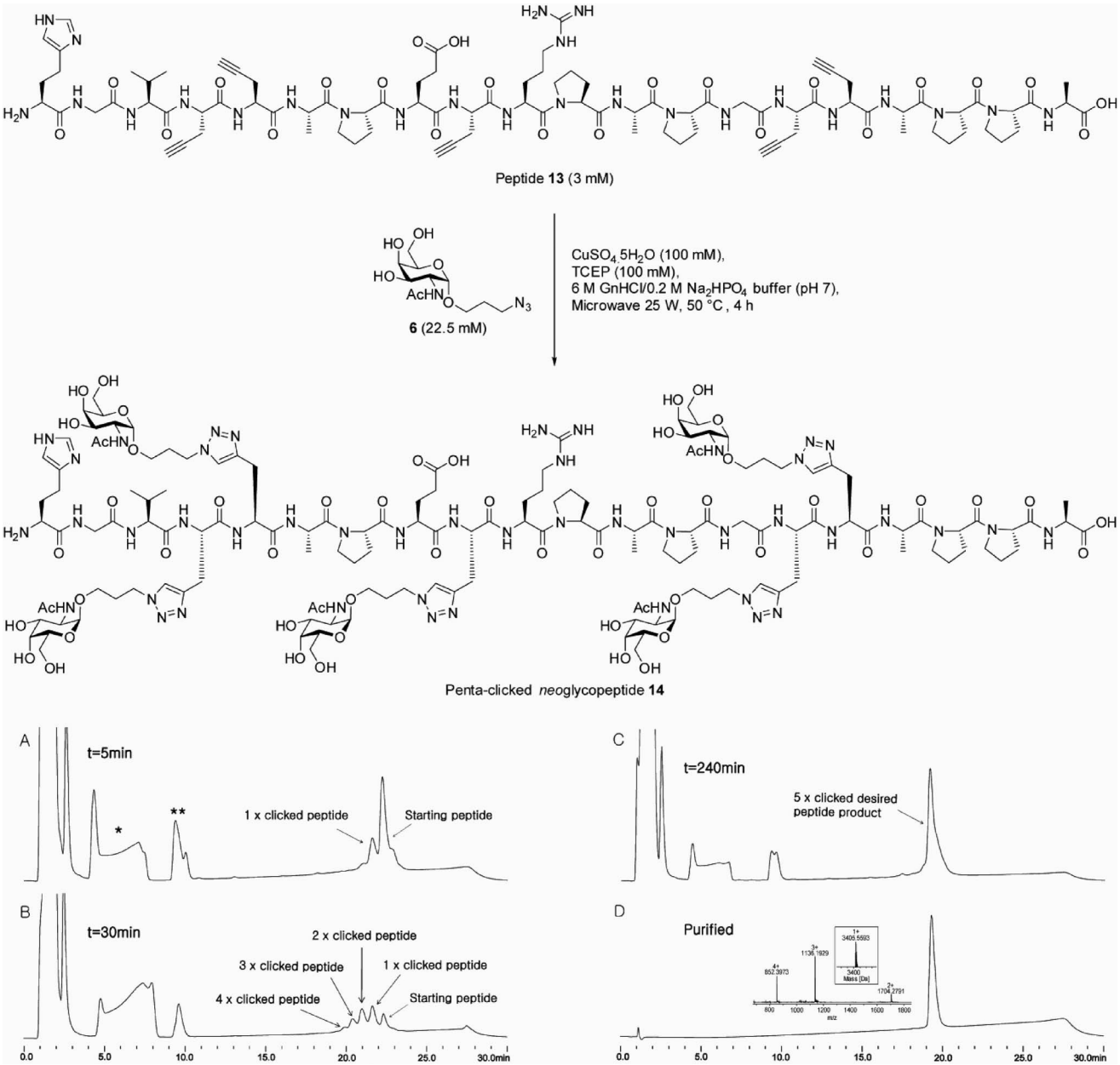
Conclusions

In conclusion, we have successfully synthesized the first click *neo*glycopeptide analogues of the biologically relevant MUC1 sequence. In the process, a new combination of click reaction conditions that may be used on a routine basis for the synthesis of click *neo*peptides using unprotected sugar-azides and peptides has been developed. This convergent route for the synthesis of *neo*glycopeptide analogues using click chemistry and the improved reaction conditions developed in this study enable the facile, rapid, and highly efficient preparation of focused *neo*glycopeptide libraries of defined chemical structure for biological evaluation. Access to *neo*glycopeptides of defined structures overcomes the inherent problems of working with native glycoproteins that are often complicated by their existence in a variety of glycoforms. Biological evaluation of the synthetic click *neo*glycopeptides prepared herein is in progress.

Experimental Section

General Information

All reagents were purchased as reagent grade and used without further purification. Analytical thin layer chromatography was performed using 0.2 mm plates of Kieselgel F_{254} (Merck) and compounds were visualised by ultra-violet fluorescence or by



Scheme 3 Synthesis of penta-clicked product **14** with reaction profile HPLC traces. A) t = 5 min. * = Broad peak observed when $CuSO_4$/TCEP was added to 6 M GnHCl/0.2 M Na_2HPO_4 solution. ** = GalNAcα1-propylazide **6** peak. B) t = 30 min. Multiple peaks eluting before the starting peptide peak correspond to mono-, di-, tri-, and tetra-clicked peptide intermediates. C) t = 240 min. Penta-clicked *neo*glycopeptide peak observed with essentially no starting/intermediate peptide peaks remaining. D) Purified penta-clicked *neo*glycopeptide by RP-HPLC.

staining with 4% sulfuric acid in ethanol or ethanolic ninhydrin solution (0.3% ninhydrin in ethanol + 1% v/v acetic acid), followed by heating the plate for a few minutes. Flash chromatography was performed using Kieselgel F_{254} S 0.063–0.1 mm (Riedel de Hahn) silica gel with indicated solvents. Infrared spectra were obtained using a Perkin Elmer Spectrum One Fourier Transform infrared spectrometer as a thin film between sodium chloride plates. Absorption maxima are expressed in wavenumbers (cm^{-1}) with the following abbreviations: s = strong, m = medium, w = weak, br = broad and v = varying. Optical rotations were determined at the sodium D line (589 nm) at 20 °C with a Perkin Elmer 341 polarimeter and are given in units of 10^{-1} deg cm^2 g^{-1}. Nuclear magnetic resonance (NMR) spectra were recorded as indicated on a Bruker AVANCE DRX300 (^{1}H, 300 MHz, ^{13}C, 75 MHz). Chemical shifts are reported in parts per million (ppm) relative to the tetramethylsilane signal recorded at δ_H 0.00 ppm in $CDCl_3/SiMe_4$ solvent or were referenced to the residual water signal at δ_H 4.79 ppm in D_2O solvent. The ^{13}C values were referenced to the residual chloroform signal at δ_C 77.0 ppm in $CDCl_3/SiMe_4$ solvent. ^{1}H NMR shift values are reported as chemical shift (δ_H), relative integral, multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet; br s, broad singlet; dd, doublet of doublets, ddd, doublet of doublets of doublets), coupling constant (J in Hz) and assignments. ^{13}C values are

reported as chemical shift (δ_C), degree of hybridisation and assignment.

Synthesis of 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α,β-D-galactopyranosyl acetate 1

Imidazole-1-sulfonyl azide hydrochloride[24] (2.52 g, 12.0 mmol) was added to D-galactosamine hydrochloride (2.15 g, 10.0 mmol), K_2CO_3 (3.73 g, 27.0 mmol), and $CuSO_4 \cdot 5H_2O$ (25.0 mg, 0.010 mmol) in MeOH (50 mL) and the mixture stirred at room temperature for 2 h. The mixture was concentrated and co-evaporated with PhMe (2 × 50 mL). The resulting residue was suspended in pyridine (40 mL) at 0 °C, then acetic anhydride (30 mL) was added and the reaction mixture stirred at room temperature for 16 h. The reaction mixture was diluted with EtOAc (200 mL), washed with 10% HCl(aq) (2 × 50 mL), sat. $NaHCO_3$ (50 mL), dried with $MgSO_4$ then concentrated. The crude product was purified by flash chromatography to afford the title compound **1** as a pale-yellow oil (3.08 g, 8.25 mmol, 83% (both α and β anomers (2 : 1 α : β) were obtained). ^{1}H NMR data was in agreement with that reported in the literature.[25]

Synthesis of 3′-*p*-toluenesulfonyl-1′-propyl-3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactopyranoside 3

3,4,6-Tri-*O*-acetyl-2-azido-2-deoxy-α,β-D-galactopyranosyl acetate 1 (0.80 g, 2.14 mmol) and 3′-hydroxy-1′-propyl *p*-toluenesulfonate 2 (0.74 g, 3.21 mmol) were dried together in the presence of P_2O_5 under high vacuum for 16 h prior to the reaction. The mixture was dissolved in dry CH_2Cl_2 (25 mL) and stirred under argon for 30 min at 0 °C. $BF_3 \cdot Et_2O$ (2.63 mL, 21.4 mmol) was added slowly and the reaction mixture was stirred at 40 °C for 48 h. The solution was cooled to room temperature then neutralized with sat. $NaHCO_3$. The product was extracted with CH_2Cl_2 (2 × 75 mL), washed with sat. $NaHCO_3$ (50 mL), brine (25 mL), dried with $MgSO_4$ then concentrated. The crude product was purified by flash chromatography to afford the title compound **3** as a colorless solid (0.48 g, 0.88 mmol, 41%): (Found: $[M+Na]^+$, 566.1422, $C_{22}H_{29}N_3NaO_{11}S$ requires 566.1415); mp 105–107 °C; $[\alpha]_D^{20}$ +94.9 (*c* 1.0 in $CHCl_3$); ν_{max} (film)/cm^{-1} 2964 (s, C═H alkane), 2110 (s, N_3), 1750, 1734 (s, C═O ester), 1465, 1404 (m, ArC═C), 1364, 1175 (s, O═S═O), 1229, 1128 (s, C–O), 1035 (s, C–N); δ_H (300 MHz, $CDCl_3$) 2.01–2.02 (2H, m, -CH_2-CH_2-OTs), 2.04, 2.05, 2.13 (3 × 3H, s, COCH_3), 2.45 (3H, s, PhCH_3), 3.51–3.56 (1H, m, -O–CH_2-CH_2-CH_2-OTs), 3.60 (1H, dd, *J* 11.0, 3.6, H-2), 3.78–3.85 (1H, m, -O–CH_2-CH_2-CH_2-OTs), 4.04–4.12 (4H, m, -CH_2-OTs, H-6$_{A,B}$), 4.17 (1H, t, *J* 6.0, H-5), 4.93 (1H, d, *J* 3.3, H-1), 5.25 (1H, dd, *J* 11.1, 3.3, H-3), 5.38 (1H, d, *J* 3.0, H-4), 7.37 (2H, d, *J* 7.8, Ar*H*), 7.80 (2H, d, *J* 8.1, Ar*H*); δ_C (75 MHz, $CDCl_3$) 20.4, 20.4, 20.5 (CO*C*H$_3$), 21.4 (Ph*C*H$_3$), 28.9 (-*C*H$_2$-CH_2-OTs), 57.2 (C-2), 61.6 (-*C*H$_2$-OTs), 64.0 (*O*-*C*H$_2$-CH_2-CH_2-OTs), 66.6 (C-5), 66.9 (C-6), 67.4 (C-4), 67.9 (C-3), 98.0 (C-1), 127.7 (Ar-CH), 129.8 (Ar-CH), 132.7 (quat., Ar–C), 144.8 (quat., Ar–C), 169.6, 169.9, 170.3 (quat., *C*OCH$_3$).

Synthesis of 3′-*p*-toluenesulfonyl-1′-propyl-3,4,6-tri-*O*-acetyl-2-acetamido-2-deoxy-α-D-galactopyranoside 4

3′-*p*-Toluenesulfonyl-1′-propyl-3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactopyranoside 3 (80.0 mg, 0.15 mmol) was dissolved in a mixture of THF/Ac_2O/AcOH (3/2/1 (v/v/v), 5 mL), then powdered zinc (100 mg, 1.53 mmol) was added. After addition of sat. $CuSO_4$ (200 μL) the reaction mixture was left to stir for 1 h at room temperature. The reaction mixture was then filtered through Celite®, diluted with CH_2Cl_2 and co-evaporated with PhMe (2 × 20 mL). The crude product was purified by flash chromatography to afford the title compound **4** as a yellow oil (71.0 mg, 0.13 mmol, 87%): (Found: MH^+, 560.1790, $C_{24}H_{34}NO_{12}S$ requires 560.1796); $[\alpha]_D^{20}$ +76.5 (*c* 1.0 in $CHCl_3$); ν_{max} (film)/cm^{-1} 2964 (s, C–H alkane), 1742 (s, C═O ester), 1661 (s, C═O amide), 1537 (m, C═O amide), 1436 (m, ArC═C), 1368, 1175 (s, O═S═O), 1229, 1132 (s, C–O), 1041 (s, C–N); δ_H (300 MHz, $CDCl_3$) 1.88–1.90 (2H, m, -CH_2-CH_2-OTs), 1.93, 1.95, 1.99, 2.11 (4 × 3H, s, COCH_3), 2.41 (3H, s, PhCH_3), 3.47–3.54 (1H, m, -O–CH_2-CH_2-CH_2-OTs), 3.72-3.79 (1H, m, -O–CH_2-CH_2-CH_2-OTs), 3.99–4.07 (4H, m, -CH_2-OTs, H-6$_{A,B}$), 4.20–4.27 (1H, m, H-5), 4.51–4.59 (1H, m, H-2), 4.81 (1H, d, *J* 3.3, H-1), 5.07 (1H, dd, *J* 11.4, 3.3, H-3), 5.30 (1H, d, *J* 3.0, H-4), 5.99 (1H, d, *J* 9.6, N*H*), 7.32 (2H, d, *J* 8.1, Ar*H*), 7.73 (2H, d, *J* 8.1, Ar*H*); δ_C (75 MHz, $CDCl_3$) 20.5, 20.6, 20.6, 21.5 (CO*C*H$_3$), 23.0 (Ph*C*H$_3$), 28.5 (-*C*H$_2$-CH_2-OTs), 47.4 (C-2), 61.8 (*O*-*C*H$_2$-CH_2-CH_2-OTs), 63.4 (-*C*H$_2$-OTs), 66.7 (C-5), 66.8 (C-6), 67.2 (C-4), 68.3 (C-3), 97.6 (C-1), 127.6 (Ar-CH), 129.9 (Ar-CH), 132.7 (quat., Ar–C), 145.0 (quat., Ar–C), 170.2, 170.3, 170.4, 170.7 (quat., *C*OCH$_3$).

Synthesis of 3′-azido-1′-propyl-3,4,6-tri-*O*-acetyl-2-acetamido-2-deoxy-α-D-galactopyranoside 5

Tetrabutylammonium hydrogen sulfate (137 mg, 0.40 mmol) was added to a solution of 3′-*p*-toluenesulfonyl-1′-propyl-3,4,6-tri-*O*-acetyl-2-acetamido-2-deoxy-α-D-galactopyranoside 4 (150 mg, 0.27 mmol) in CH_2Cl_2 (10 mL). NaN_3 (170 mg, 2.62 mmol) in H_2O (10 mL) was added and the reaction mixture was stirred vigorously for 16 h. The aqueous layer was extracted with CH_2Cl_2 (2 × 50 mL), and the combined organic layer was dried with $MgSO_4$ and concentrated. The crude product was purified by flash chromatography to afford the title compound **5** as a yellow oil (92.2 mg, 0.21 mmol, 80%): (Found: MH^+, 431.1757, $C_{17}H_{27}N_4O_9$ requires 431.1773); $[\alpha]_D^{20}$ +80.1 (*c* 1.0 in $CHCl_3$); ν_{max} (film)/cm^{-1} 2935 (s, C–H alkane), 2097 (s, N_3), 1743 (s, C═O ester), 1660 (s, C═O amide), 1535 (m, C═O amide), 1435 (m, ArC═C), 1219, 1131 (s, C–O), 1042 (s, C–N); δ_H (300 MHz, $CDCl_3$) 1.93 (2H, p, *J* 6.3, -CH_2-CH_2–N_3), 1.97, 1.99, 2.05, 2.16 (4 × 3H, s, COCH_3), 3.38–3.48 (2H, m, -CH_2-N_3), 3.51–3.58 (1H, m, -O–CH_2-CH_2-CH_2–N_3), 3.78–3.86 (1H, m, -O–CH_2-CH_2-CH_2–N_3), 4.06–4.19 (3H, m, H-5, H-6$_{A,B}$), 4.52–4.60 (1H, m, H-2), 4.91 (1H, d, *J* 3.6, H-1), 5.14 (1H, dd, *J* 11.4, 3.3, H-3), 5.38 (1H, d, *J* 3.0, H-4), 5.97 (1H, d, *J* 9.6, N*H*); δ_C (75 MHz, $CDCl_3$) 20.4, 20.4, 20.4, 22.9 (CO*C*H$_3$), 28.3 (-*C*H$_2$-CH_2–N_3), 47.5 (C-2), 48.2 (-*C*H$_2$–N_3), 61.8 (C-6), 65.1 (*O*-*C*H$_2$-CH_2-CH_2–N_3), 66.6 (C-5), 67.1 (C-4), 68.1 (C-3), 97.5 (C-1), 169.9, 170.0, 170.2, 170.6 (quat., *C*OCH$_3$).

Synthesis of 3′-azido-1′-propyl-2-acetamido-2-deoxy-α-D-galactopyranoside 6

3′-Azido-1′-propyl-3,4,6-tri-*O*-acetyl-2-acetamido-2-deoxy-α-D-galactopyranoside **5** (220 mg, 0.51 mmol) was stirred in 1 M NaOMe (5 mL, pH 11.5) for 3 h at room temperature. The reaction mixture was acidified with H^+ Dowex resin then filtered.

The filtrate was concentrated, diluted with Milli Q water (15 mL) and lyophilized to afford the pure title compound **6** as a colorless solid (152 mg, 0.50 mmol, 98%): (Found: $[M+Na]^+$, 327.1273, $C_{11}H_{20}N_4NaO_6$ requires 327.1275); mp 142–144 °C; $[\alpha]_D^{20}$ +160.7 (*c* 1.0 in H_2O); ν_{max} (film)/cm^{-1} 3297 (s, O–H), 2924 (s, C–H alkane), 2097 (s, N_3), 1616 (s, C═O amide), 1553 (m, C═O amide), 1448 (m, ArC═C), 1115, 1065, 1017 (s, alcohol C–O), 1037 (s, C–N); δ_H (300 MHz, D_2O) 1.96 (2H, p, -CH_2-CH_2–N_3), 2.11 (3H, s, COCH_3), 3.46–3.64 (2H, m, -CH_2-N_3, -O–CH_2-CH_2-CH_2–N_3), 3.79–3.91 (3H, m, -O–CH_2-CH_2-CH_2–N_3, H-$6_{A,B}$), 3.96–4.06 (3H, m, H-3, H-4, H-5), 4.22 (1H, dd, *J* 12.0, 3.0, H-2), 4.97 (1H, d, *J* 3.0, H-1); δ_C (75 MHz, D_2O) 22.0 (CO*C*H_3), 28.0 (-*C*H_2-CH_2–N_3), 48.2 (-*C*H_2–N_3), 50.0 (C-2), 61.3 (C-6), 65.0 (*O*-*C*H_2-CH_2-CH_2–N_3), 67.7 (C-5), 68.5 (C-4), 71.0 (C-3), 97.0 (C-1).

General click chemistry procedure

Peptide (3 mM) and $CuSO_4$/TCEP (20 mM per 3 mM of propargyl group) were mixed in a degassed solution of 6 M GnHCl/0.2 M Na_2HPO_4 buffer. After 30 min, 3′-azido-1′-propyl-2-acetamido-2-deoxy-α-D-galactopyranoside **6** (1.5 equiv. per propargyl group) was added and the reaction was carried out under argon with microwave irradiation (25 W). The reaction progress was monitored by analytical RP-HPLC, and upon completion of the reaction the crude product was isolated by solid phase extraction then lyophilized. The crude product was purified by semi-prep RP-HPLC to afford the desired clicked *neo*glycopeptide products.

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