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Synthesis of a GlcNAcylated arginine building block for the solid-phase synthesis of death domain glycopeptide fragments

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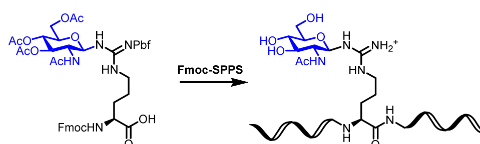

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Synthesis of a GlcNAcylated arginine building block for the solid phase synthesis of death domain glycopeptide fragments

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ABSTRACT

Herein we describe the synthesis of glycopeptide fragments from the death domains of TRADD and FADD bearing the recently discovered *Nω*-GlcNAc-β-arginine post-translational modification. TRADD and FADD glycopeptides were accessed through the use of a suitably protected synthetic glycosylamino acid 'cassette' that could be directly incorporated into conventional solid phase peptide synthesis (SPPS) protocols.

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1. Introduction

Protein glycosylation is a ubiquitous post-translational modification, thought to occur on more than 50% of human proteins.[1] In eukaryotic organisms, glycoproteins mediate diverse functions, including cell-cell communication, signal transduction and pathogenesis.[2] There exist two major classes of protein glycosylation, known as *N*- and *O*-linked glycosylation, which are classified according to the type of linkage to the amino acid side chain within the polypeptide backbone. *O*-linked glycans encompass a diverse range of structures which are attached through the hydroxyl group of serine, threonine, hydroxyproline, hydroxylysine or tyrosine.[3,4] In contrast, *N*-linked glycans exhibit limited diversity and are generally characterized by the presence of a common $Man_3GlcNAc_2$ pentasaccharide core, with the reducing terminal *N*-acetylglucosamine β-linked to the nitrogen atom of the asparagine amide side chain located within an Asn-Xaa-Ser/Thr consensus sequence.[4,5] Other forms of Asn-*N*-linked protein glycosylation are known but are exceedingly rare.[6-9]

In recent years, novel examples of *N*-glycosylation have been identified in plants,[10-12] bacteria[13-15] and humans,[16,17] whereby the sugar is linked to the ω-nitrogen of the guanidine moiety within the arginine (Arg) side chain. For instance, *N*-rhamnosyl-Arg was recently discovered as a modification in the protein elongation factor P expressed by pathogenic bacteria, such as *Pseudomonas aeruginosa, Shewanella oneidensis* and *Neisseria meningitidis*.[13-15] The presence of this modification was shown to be critical for each protein to prevent ribosome stalling during the translation of proline-proline motifs. The anomeric centre of the rhamnose motif was recently shown to have α-configuration through NMR analysis of the native EF-P glycoprotein[18] and through NMR and mass spectroscopic comparison of synthetic α- and β-linked glycopeptides with native and chemoenzymatically synthesized EF-P fragments.[19]

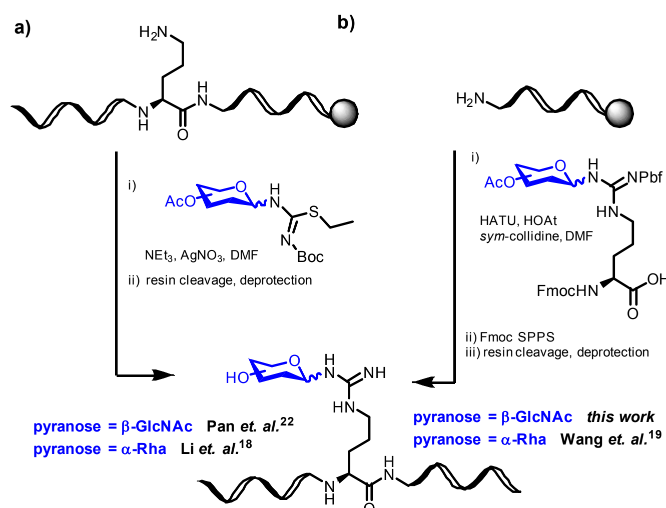


Figure 1. Complementary approaches for the preparation of native Arg-*N*ω-glycosylated peptides. (a) solid phase guanidinylation approach, (b) cassette strategy with preformed glycosylated Arg building blocks.

A further modification of Arg, β-GlcNAcylation has also been discovered in various human proteins bearing a death domain, including TRADD, FADD, TNFR1 and the kinase RIPK1.[16,17,20] In this case the post-translational modification was shown to be introduced by the glycosyltransferase activity of NIeB, a type III secretion system effector protein produced by attaching/effacing enteropathogenic strains of *Escherichia coli*. Interestingly, it was shown that GlcNAcylation of the Arg residue could functionally block the assembly of death-inducing signaling complex, effectively preventing *E.coli*-infected cells from undergoing apoptosis and necroptosis.[16,17] The importance of this protein modification elicited by *E. coli* to avoid regulatory clearance within mammalian cells suggests that this process might serve as a future adjuvant antimicrobial target.[21]

Recently Hu and co-workers reported synthetic routes to β-GlcNAcylated and α-rhamnosylated Arg-containing glycopeptides employing Ag-promoted on-resin guanidinylation of fully elongated and resin-bound peptides with an unprotected ornithine residue (Figure 1a).[18,22] These methods enabled the generation of peptides to which antibodies selective to the glycosylated Arg modifications could be raised.[18,22] Although the on-resin glycosylation strategy provided a direct and divergent route to glycosylated Arg glycopeptides, it is possible that the efficiency of this strategy could vary depending on the size and sequence of the peptide substrate. On-resin glycosylation of peptides requires the site of functionalisation to be solvent-accessible, which becomes less likely as the peptide length and number of β-sheet inducing amino acids increases. As a complementary approach, we recently prepared α- and β-linked rhamnosylated peptides through the so called 'cassette' strategy *via* the synthesis of *N*α-Fmoc and side chain-protected α- and β-linked rhamnosyl Arg building blocks and subsequent incorporation into target glycopeptides through Fmoc-SPPS (Figure 1b).[19] Our rationale for this approach was that the introduction of a suitable glycosylamino acid 'cassette' could serve as an alternative method for the introduction of the glycosylarginine moiety and would streamline access to glycopeptide libraries without the need to perform ornithine deprotection and guanidinylation on each resin-bound substrate. The successful implementation of this cassette strategy for introduction of the rhamnosylated Arg moiety prompted us to extend the methodology for the synthesis of biologically-relevant GlcNAcylated Arg glycopeptides that we report herein.

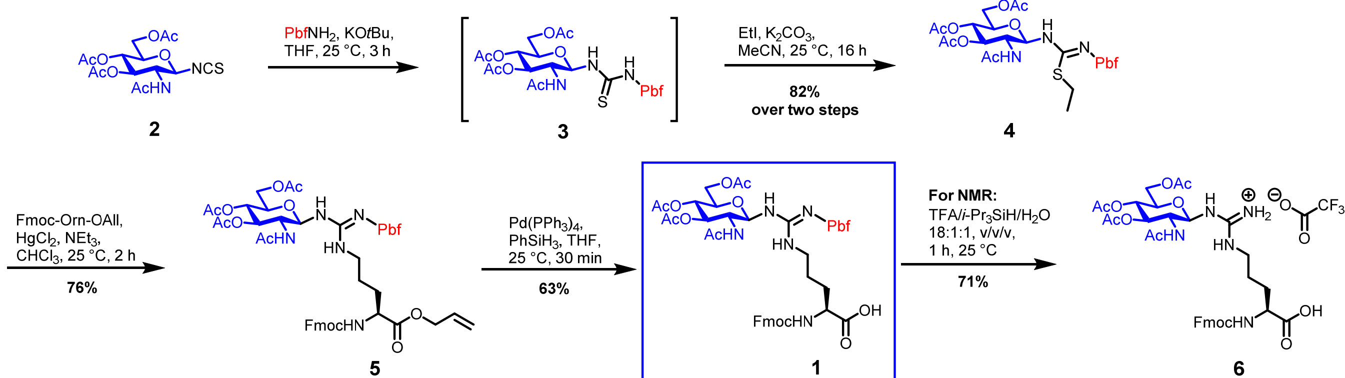
The synthesis of an effective glycosylamino acid building block for introduction of the *Nω*-GlcNAc-β-Arg moiety is dependent on appropriate protection of the Arg side chain, which would otherwise intramolecularly attack the α-carboxylic active ester during glycosylamino acid coupling, leading to an unproductive lactam derivative. Previous attempts by us (unpublished results), and the Hu group,[22] to implement the 'cassette' strategy have revealed that GlcNAcylated and rhamnosylated Arg building blocks bearing mono-*N*ω'-Boc and mono-*N*ω'-trifluoroacetyl[23] protecting groups couple very slowly to resin-bound peptides and are still prone to intramolecular lactamization during coupling. To address this limitation, and improve the viability of the glycosylamino acid building block approach for the synthesis of Arg-*N*-linked glycopeptides, we reported the synthesis of Arg-*Nω*-rhamnosylated glycosylamino acid building blocks bearing the electron withdrawing *N*ω'-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) side chain protecting group, which is commonly used for the protection of the standard guanidine side chain of Arg during Fmoc-SPPS.[24] Arg-*N*ω-rhamnosylated glycosylamino acids with the side chain Pbf protection were demonstrated to couple efficiently to resin-bound peptides and did not undergo unproductive lactamization. Herein, we report the efficient synthesis of an analogous glycosylamino acid bulding block **1**, for the introduction of the GlcNAc-β-Arg modification, and demonstrate its effective use for the synthesis of biologically relevant death domain-derived glycopeptides through Fmoc-SPPS.

2. Results and Discussion

Synthesis of GlcNAcylated Arg building block **1** began from glycosyl β-isothiocyanate **2**, which was prepared in two steps according to established methods (Scheme 1).[22] The isothiocyanate was subsequently treated with deprotonated Pbf-NH_2, which could be generated by pre-mixing Pbf-NH_2 with KO*t*Bu.[25] The resulting Pbf-thiourea **3** was not isolated, but immediately alkylated with EtI to afford the ethyl isothiourea **4** in 82% yield over the two steps. The ethyl isothiourea then underwent a Hg-promoted guanidinylation with Fmoc-Orn-OAll to form the allyl ester **5** in good yield. From here allyl deprotection, catalyzed by $Pd(PPh_3)_4$, afforded the desired building block **1** in 63% yield (39% overall yield over the 4 steps from **2**).

As the ^{1}H NMR spectrum of glycosylamino acids **1** and **5** exhibited significant broadening of the pyranose signals on account of a tautomeric or rotameric effect, the Pbf group of **1** was removed for characterisation purposes to yield free guanidine **6**, which could be more clearly interrogated by NMR spectroscopy. The extraction of a large anomeric coupling constant ($J_{1,2}$ = 9 Hz) confirmed the β-stereochemistry of the anomeric linkage.

Having successfully synthesized and characterized the desired glycosylamino acid building block **1**, we directed focus towards the synthesis of two target GlcNAcylated Arg glycopeptides through Fmoc SPPS (Scheme 2). We chose to synthesize glycopeptides **7** and **8** representing fragments of both human TRADD(232-239) and mouse FADD(115-125) death domains, each encompassing a central GlcNAcylated Arg residue. After loading the two C-terminal amino acids separately onto Chemmatrix® Trtyl-OH resin,[26] each resin-bound amino acid was elongated towards the glycosylation site using DIC/Oxyma coupling conditions to afford resin-bound peptides **9** and **10**. Each of the resin-bound peptides were then treated with a mixture of GlcNAcylated Arg cassette **1** (2.2 equiv.), 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-



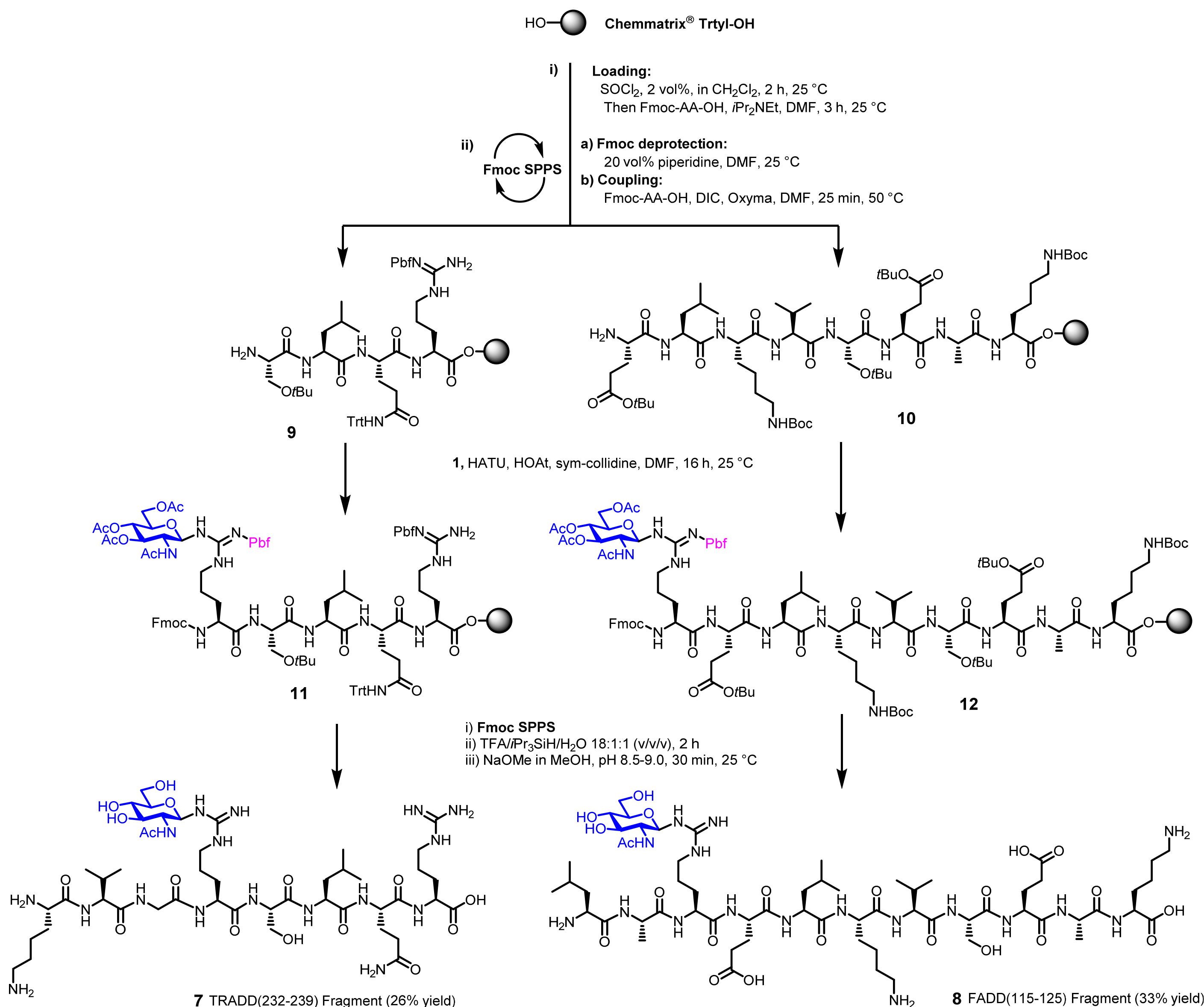
Scheme 1. Synthesis of GlcNAcylated Arg glycosylamino acid 'cassette' **1**

b]pyridinium 3-oxide hexafluorophosphate (HATU, 2.0 equiv.), 1-Hydroxy-7-azabenzotriazole (HOAt, 3.0 equiv.) and *sym*-collidine (2.4 equiv.).[27] These conditions facilitated complete incorporation of the glycosylamino acid as judged by analytical test cleave with TFA:*i*-Pr_3SiH:H_2O (18:1:1 *v/v/v*) followed by UPLC-MS analysis. The resulting two resin-bound glycopeptides **11** and **12** were further elongated under DIC/oxyma coupling conditions to afford the target resin-bound glycopeptides. From here, the resin-bound glycopeptides were treated with an acidic cocktail (TFA:*i*Pr_3SiH,H_2O; 18:1:1 v/v/v) to deprotect the peptide side chains and liberate the peptide from the resin. Finally, Zemplén deacetylation[28] was used to deprotect the *O*-acetate protecting groups on the GlcNAc moiety, to afford the desired TRADD(232-239) and FADD(115-125) glycopeptides **7** and **8** in 26% and 33% yield, respectively after reverse-phase HPLC purification (yields based on the Fmoc-loading of the C-terminal amino acid residue immobilized to resin).

A curious feature of α-*N*-rhamnosylated Arg glycopeptides is the tendency for anomerization in organic solvent under basic conditions, such as during deacetylation of the carbohydrate protecting groups with NaOMe/MeOH and hydrazine/DMF.[19] To ensure that this did not occur during the synthesis of *N*-GlcNAcylated TRADD(232-239) and FADD(115-125) peptides, both glycopeptides were characterized by a combination of ^1H NMR spectroscopy, and 2D COSY and HSQC. In both cases, the magnitude of the anomeric coupling constant ($J_{1,2}$ = 9.7 Hz) confirmed that the β-stereochemistry was retained during SPPS and deacetylation (see Supporting Information for NMR data). The results are consistent with the observation that the synthetic β-*N*-rhamnosylated counterpart, which also contains an equatorial anomeric linkage, was resistant to base catalysed anomerization.[19] These results support the notion that equatorial Arg-*N*-glycosidic linkages are more thermodynamically stable than their axial counterparts.

3. Conclusions

In summary, we have developed a new method for the synthesis of biologically-relevant glycopeptides containing the recently discovered GlcNAc-β-*N*-Arg linkage. Key to this methodology was the synthesis of a suitably protected GlcNAcylated Arg 'cassette' bearing side chain Pbf protection that could be synthesised in excellent overall yield in a short synthetic sequence. The building block was successfully incorporated into death domain fragments of TRADD(232-239) and FADD(115-125) peptides bearing the β-GlcNAcylated modification of Arg. The current methodology should find application in the study of proteins bearing this modification within death domains, and for the synthesis and interrogation of newly discovered proteins that contain the unusual GlcNAc-β-Arg post-translational modification.



Scheme 2. Synthesis of GlcNAcylated Arg-containing TRADD(232-239) and FADD(115-125) glycopeptides.

4. Experimental

4.1 General experimental procedures

All reactions were carried out under an argon atmosphere. Commercially available chemicals were obtained from Sigma-Aldrich, Merck, AK Scientifc Inc. or GL Biochem. All commercial materials were used as received without further purification unless otherwise noted. Flash chromatography was performed on silica gel (0.040 – 0.060 mm) manufactured by Grace. Solid-phase peptide synthesis (SPPS) was carried out in polypropylene syringes equipped with Teflon filters, purchased from Torviq. Dichloromethane was purchased from Merck. Peptide synthesis grade DMF was purchased from Labscan. Chemmatrix® Trtyl-OH resin was purchased from PCAS Biomatrix Inc.

All NMR spectra were recorded at 300 K using a Bruker Avance DRX400 or DRX500 spectrometer. Chemical shifts are reported in parts per million (ppm) and are referenced to solvent residue signals. Proton assignments were made with the assistance of COSY spectra. For 2D HSQC spectra contours, black represents positive cross peaks and red represents negative cross peaks.

Infrared (IR) absorption spectra were recorded on a Bruker ALPHA Spectrometer with Attenuated Total Reflection (ATR) capability using OPUS 6.5 software. Compounds were deposited as films on the ATR plate *via* solid compression. Optical rotations were recorded at ambient temperature (293K) on a Perkin–Elmer 341 polarimeter at 589 nm (sodium D line) with a cell path length of 1 dm, and the concentrations are reported in g/100 mL.

All HPLC separations were performed using a buffer consisting of 0.1% TFA in H_2O (solvent A) and 0.1% TFA in MeCN (solvent B). Preparative reverse-phase HPLC was performed on a Waters 2535 quaternary gradient module, equipped with a Waters 2489 UV detector operating at 214 nm using Waters Empower 3 software. Separations of building block **1** and small molecule intermediates were performed on a Waters XBridge OBD C18 50 mm x 150 mm preparative column at a flow rate of 38 mL/min. Separations of glycopeptides **7** and **8** were performed on a Waters XBridge OBD C18 30 mm x 150 mm preparative column at a flow rate of 7 mL/min. Analytical HPLC was performed on a Waters e2695 separations module equipped with a Waters 2489 UV/Vis detector operating at 214 nm. The separations were achieved on a Waters Atlantis T3 column using a gradient of 0% B for 1 min, followed by 0% B to 30% B over 15 min with a flow rate of 0.4 mL/min.

Reaction monitoring by UPLC-MS was performed on a Shimadzu NexeraX2 UPLC equipped with a SPD-M30A diode array detector and a LCMS-2020 low resolution ESI mass spectrometer operating in positive ion mode. Samples were analyzed with an Acquity UPLC® BEH C18 1.7 µm, 2.1 x 50 mm column using eluents of MeCN (0.1% formic acid) and H_2O (0.1% formic acid) and gradients as specified. High Resolution ESI+ mass spectra were obtained on a Bruker Apex Qe 7T Fourier Transform Ion cyclotron resonance mass spectrometer equipped with a ESI/MALDI (Nd:YAG) dual source. Low Resolution MALDI-TOF mass spectra was recorded on a Bruker Autoflex™ Speed MALDI-TOF mass spectrometer operating in reflectron mode.

4.2 Synthesis of glycosylamino acid building block

Glycosyl isothiocyanate **2** was synthesized according to the method described by Pan *et al.*[22]

4.2.1: 1-(2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl)-3-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl)-2-ethylisothiourea (**4**)

A solution of Pbf-NH_2 (1.4 g, 5.2 mmol) and KO*t*Bu (0.60 g, 5.0 mmol) in anhydrous THF (20 mL) was stirred under an argon atmosphere for 1 h. A solution of GlcNAc isothiocyanate **2** (2.0 g, 5.2 mmol) in THF (10 mL) was added dropwise to the reaction mixture. After 3 h, the solution was neutralized with Amberlite® IR-120 ion exchange resin. After filtration, the filtrate was concentrated under reduced pressure. The crude mixture was dissolved in anhydrous MeCN (20 mL) under an argon atmosphere. Anhydrous K_2CO_3 (3.4 g, 25 mmol) was added into the reaction followed by EtI (800 µL, 10 mmol). The mixture was stirred at 25 °C for 16 h. After the reaction was complete (monitored by UPLC-MS), the mixture was concentrated and purified by HPLC (20% to 80% B over 20 min) yielding the desired isothiourea **4** (2.9 g, 4.2 mmol, 82%) as a white foam. $[\alpha]_D$ 2.0 (c 0.4, methanol). **IR:** 3282 (N-H), 1743 (C=O), 1370 (S=O), 1156 (S=O) cm^{-1}. **^{1}H NMR** (400 MHz, d_6-acetone): δ 7.40 (br, NH), 5.37 (t_{apt}, 1H, $J_{2,3}$ = $J_{3,4}$ = 10.0 Hz, H-3), 5.27 (d, 1H, $J_{1,2}$ = 9.8 Hz, H-1), 5.02 (t_{apt}, 1H, $J_{3,4}$ = $J_{4,5}$ = 9.7 Hz, H-4), 4.26 (dd, 1H, $J_{5,6a}$ = 5.0 Hz, $J_{6a,6b}$ = 12.3 Hz, H-6_a), 4.15 (t_{apt}, 1H, $J_{1,2}$ = $J_{2,3}$ = 10.0 Hz, H-2), 4.07 (dd, 1H, $J_{5,6b}$ = 2.3 Hz, $J_{6a,6b}$ = 12.3 Hz, H-6_b), 3.92 (ddd, 1H, $J_{4,5}$ = 10.0 Hz, $J_{5,6a}$ = 5.2 Hz, $J_{5,6b}$ = 2.3 Hz, H-5), 3.04 (s, 2H, Ar-C$\underline{H}_2$), 2.95 (q, 2H, J = 7.5 Hz, S-C$\underline{H}_2$-CH_3), 2.57 (s, 3H, Ar-C$\underline{H}_3$), 2.51 (s, 3H, Ar-C$\underline{H}_3$), 2.08 (s, 3H, Ar-C$\underline{H}_3$), 2.02 (s, 3H, Ac), 2.01 (s, 3H, Ac), 1.99 (s, 3H, Ac), 1.91 (s, 3H, Ac), 1.47 (s, 6H, C-(C$\underline{H}_3$)$_2$), 1.20 (t, 3H, J = 7.3 Hz, S-CH_2-C$\underline{H}_3$) ppm. **^{13}C NMR** (100 MHz, d_6-acetone): δ 169.8 , 169.2, 159.0, 138.7, 133.0, 124.9, 117.0, 86.6, 81.9, 73.1, 72.4, 68.8, 61.8, 52.8, 42.5, 27.8, 25.4, 22.0, 19.8, 19.7, 19.7, 18.5, 17.3, 13.5, 11.5 ppm. **HRMS**: (+ESI) Calcd. for $C_{30}H_{44}N_3O_{11}S_2$ $[M + H]^+$ m/z, 686.2414; found: 686.2412.

4.2.2: *N*α-[(9H-Fluoren-9-yl)-methoxycarbonyl]-*N*ω-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-*N*ω'-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl)-L-arginine allyl ester (**5**)

To a solution of isothiourea **4** (0.37 g, 0.54 mmol), Fmoc-Orn-OAll (0.39 g, 1.0 mmol) and NEt_3 (280 µL, 2.0 mmol) in chloroform (10 mL) was added $HgCl_2$ (0.16 g, 0.6 mmol). The mixture was stirred at 25 °C for 2 h. After completion (monitored by TLC), the mixture was filtered through a pad of Celite®. The filtrate was concentrated and purified by HPLC to yield the desired GlcNAcylated Arg allyl ester **5** (410 mg, 0.39 mmol, 72%). $[\alpha]_D$ -11 (c 0.98, methanol). **IR:** 3315 (N-H), 1741 (C=O), 1367(S=O), 1156 (S=O) cm^{-1}. **^{1}H NMR** (500 MHz, d_6-acetone): δ 7.85 (d, 2H, J = 7.5 Hz, Ar-H), 7.70 (dd_{apt}, 2H, J = 7.3 Hz, J = 2.5 Hz, Ar-H), 7.40 (t_{apt}, 2H, J = 7.4 Hz, Ar-H), 7.31 (t_{apt}, 2H, J = 7.4 Hz, Ar-H), 6.97 (br, 1H, Fmoc-NH), 6.00-5.80 (m, 1H, O-CH_2-C$\underline{H}$=CH_2), 5.34 (dq, 1H, J_1 = 17.3 Hz, J_2 = 1.4 Hz, O-CH_2-CH=C$\underline{H}_2$), 5.29-5.24 (br, 2H, 2 x GlcNAc-H), 5.18 (dq, 1H, J_1 = 10.5 Hz, J_2 = 1.4 Hz, O-CH_2-CH=C$\underline{H}_2$), 5.00 (t, 1H, J = 9.7 Hz, GlcNAc-H), 4.60 (d, 2H, J = 4.5 Hz, O-C$\underline{H}_2$-CH=CH_2), 4.50-4.30 (m, 2H, J = 7.0 Hz, FmocCH-C$\underline{H}_2$-O-CO), 4.30-4.10 (m, 3H, FmocC$\underline{H}$-CH_2-O-CO, Arg-Hα, GlcNAc-H6_a), 4.10-3.97 (br, 3H, 2 x GlcNAc-H, GlcNAc-H6_b), 3.40-3.10 (m, 2H, Arg-Hδ), 2.97 (s, 2H, Ar-C$\underline{H}_2$), 2.57 (s, 3H, Ar-C$\underline{H}_3$), 2.49 (s, 3H, Ar-C$\underline{H}_3$), 2.05 (s, 3H, Ar-C$\underline{H}_3$), 1.96 (s_{apt}, 6H, 2 x Ac), 1.95 (s, 3H, Ac), 1.88 (s_{apt}, 4H, Ac, Arg-Hβ_a), 1.85-1.75 (m, 1H, Arg-Hβ_b), 1.75-1.60 (m, 2H, Arg-Hγ), 1.42 (s, 6H, C-(C$\underline{H}_3$)$_2$) ppm. **^{13}C NMR** (100 MHz, d_6-acetone): 171.9, 170.0, 170.0, 169.3, 158.4, 156.4, 156.4, 154.5, 154.5, 144.3, 144.2, 141.4, 138.2, 134.3, 132.6, 132.2, 127.8, 127.2, 127.2, 125.4, 125.4, 124.7, 120.1, 117.6, 116.8, 86.3, 81.4, 72.9, 72.7, 68.9, 66.4, 65.3, 62.0, 54.1,

54.1, 53.1, 47.3, 42.8, 40.8, 28.0, 25.8, 22.2, 22.2, 19.9, 19.9, 19.9, 18.8, 17.5, 11.8 ppm. **HRMS**: (+ESI) Calcd. for $C_{51}H_{64}N_5O_{15}S$ $[M + H]^+$ m/z, 1018.4114, found: 1018.4118.

4.2.3: *N*α-[(9*H*-Fluoren-9-yl)-methoxycarbonyl]-*N*ω-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-*N*ω'-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-glucopyranos-1-yl)-L-arginine (**1**)

The allyl ester **5** (200 mg, 0.19 mmol) was dissolved in anhydrous THF (10 mL). $Pd(PPh_3)_4$ (230 mg, 0.20 mmol) was added to the solution followed by phenylsilane (0.25 mL, 2.0 mmol). The mixture was stirred at 25 °C for 30 min. After the reaction was complete (monitored by TLC), the solution was concentrated under reduced pressure. The mixture was then filtered through a pad of silica with 1% AcOH in ethyl acetate as the eluent. The combined filtrate was decolorized over activated charcoal, concentrated and purified through reverse-phase HPLC to yield the desired β-GlcNAcylated arginine building block **1** (123 mg, 0.13 mmol, 66%) as a white solid following lyophilization. $[\alpha]_D$ -20 (c 0.19, methanol). **IR:** 3359 (N-H, O-H), 1727 (C=O), 1365 (S=O), 1132 (S=O) cm^{-1}. **^{1}H NMR** (500 MHz, d_6-acetone): δ 7.87 (d, 2H, J = 7.5 Hz, Ar-H), 7.74 (d, 2H, J = 7.5 Hz, Ar-H), 7.42 (t_{apt}, 2H, J = 7.5 Hz, Ar-H), 7.34 (t_{apt}, 2H, J = 7.6 Hz, Ar-H), 5.40-5.20 (m, 2H, 2 x GlcNAc-H), 5.01, (t_{apt}, 1H, J = 8.7 Hz, GlcNAc-H), 4.42-4.32 (m, 2H, FmocCH-C<u>H</u>$_2$-O-CO), 4.27-3.70 (m, 6H, FmocC<u>H</u>-CH_2-O-CO, Arg-Hα, 4 x GlcNAc-H), 3.30-3.20 (m, 2H, Arg-Hδ), 3.00 (s, 2H, Ar-C<u>H</u>$_2$), 2.60 (s, 3H, Ar-C<u>H</u>$_3$), 2.52 (s, 3H, Ar-C<u>H</u>$_3$), 2.15-1.85 (m, 16H, Ar-C<u>H</u>$_3$ 4 x Ac, Arg-$H\beta_a$), 1.85-1.75 (m, 1H, Arg-$H\beta_b$), 1.74-1.64 (m, 2H, Arg-Hγ), 1.44 (s, 6H, C-(C<u>H</u>$_3$)$_2$) ppm. **^{13}C NMR** (125 MHz, d_6-acetone): 173.9, 170.8, 170.1, 159.2, 157.2, 155.3, 145.1, 145.0, 142.1, 138.9, 135.1, 133.0, 128.5, 128.0, 128.0, 126.2, 125.4, 120.8, 117.6, 87.1, 82.0, 73.7, 73.5, 69.7, 67.2, 62.8, 54.4, 48.1, 43.6, 41.7, 29.6, 28.7, 26.5, 22.9, 20.7, 20.64, 20.62, 19.5, 18.3, 12.5 ppm. **HRMS**: (+ESI) Calcd. for $C_{48}H_{60}N_5O_{15}S$ $[M + H]^+$ m/z, 978.3801, found: 978.3812

4.2.4: *N*α-[(9*H*-fluoren-9-yl)-methoxycarbonyl]-*N*ω-(2-acetoamino-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-glucopyranos-1-yl)-L-arginine (**6**)

Fmoc-Arg(β-GlcNAc,Pbf)-OH **1** (40 mg, 0.04 mmol) was suspended in TFA/*i*-Pr_3SiH/H_2O (18:1:1, v/v/v, 10 ml) at 25 °C. The mixture was stirred vigorously for 60 min before concentrating under reduced pressure. The residue was purified by RP-HPLC to yield Fmoc-Arg(β-GlcNAc)-OH **6** as the trifluoroacetate salt (25.0 mg, 0.028 mmol, 71%) as white solid after lyophilization. $[\alpha]_D$ -14 (c 0.40, methanol). **IR:** 3360 (N-H), 3216 (O-H), 1674 (C=O) cm^{-1}. **^{1}H NMR** (400 MHz, CD_3OD): δ 7.82 (d, 2H, J = 7.5 Hz, Ar-H), 7.68 (t_{apt}, 2H, J = 7.5 Hz, Ar-H), 7.42 (t_{apt}, 2H, J = 7.5 Hz, Ar-H), 7.32 (t_{apt}, 2H, J = 7.3 Hz, Ar-H), 5.28 (t_{apt}, 1H, $J_{2,3} = J_{3,4}$ = 9.9 Hz, H-3), 5.06 (t_{apt}, 1H, $J_{3,4} = J_{4,5}$ = 9.7 Hz, H-4), 5.03 (d, 1H, $J_{1,2}$ = 9.4 Hz, H-1), 4.45-4.37 (m, 2H, FmocCH-C<u>H</u>$_2$-O-CO), 4.32 (dd, 1H, $J_{5,6a}$ = 4.0 Hz, $J_{6a,6b}$ = 12.5 Hz, H-6_a), 4.30-4.25 (m, 1H, FmocC<u>H</u>-CH_2-O-CO), 4.22-4.16 (m, 1H, Arg-Hα), 4.15 (dd, 1H, $J_{5,6b}$ = 1.0 Hz, $J_{6a,6b}$ = 12.5 Hz, H-6_b), 4.03 (br, 1H, H-2), 3.96 (ddd, 1H, $J_{4,5}$ = 10.0 Hz, $J_{5,6a}$ = 4.0 Hz, $J_{5,6b}$ = 1.0 Hz, H-5), 3.40-3.20 (m, 2H, Arg-Hδ), 2.01-2.00 (m, 9H, 3 x Ac), 1.96-1.90 (m, 4H, Ac, Arg-$H\beta_a$), 1.80-1.60 (m, 3H, Arg-$H\beta_b$ and 2 x Arg-Hγ). **^{13}C NMR** (100 MHz, CD_3OD): 173.9, 172.9, 170.8, 170.3, 169.8, 157.4, 156.3, 143.9, 143.7, 141.2, 127.4, 126.8, 124.8, 124.8, 119.5, 72.8, 72.4, 68.1, 66.6, 61.5, 53.3, 52.9, 40.9, 28.5, 24.8, 21.3, 19.2, 19.1, 19.1 ppm. **HRMS**: (+ESI) Calcd. for $C_{35}H_{44}N_5O_{12}$ $[M + H]^+$ m/z, 726.2981, found: 726.2978.

4.3 General procedures for glycopeptide synthesis

4.3.1: Preloading of Chemmatrix® Trtyl-OH Resin

Chemmatrix® Trtyl-OH resin (loading capacity 0.3 mmol/g, 0.2 g) was swelled in dichloromethane (6 mL) for 30 min. A solution of 2% $SOCl_2$ in dichloromethane (6 mL) was then added and the resin was shaken for 2 h. After filtration, the resin was washed with dichloromethane (5 x 3 mL) and DMF (5 x 3 mL). Then a solution of Fmoc-AA-OH (1 mmol) and *i*Pr_2NEt (2 mmol) in dichloromethane (3 mL) was added into the resin and the mixture was agitated for 3 h. The resin was again washed with DMF (5 x 3 mL), dichloromethane (5 x 3 mL) and DMF (5 x 3 mL).

4.3.2: Fmoc deprotection

The resin was treated with piperidine (20% v/v) in DMF (5 mL, 2 x 5 min, 25 °C) and then washed with DMF (5 x 3 mL), dichloromethane (5 x 3 mL) and DMF (5 x 3 mL).

4.3.3: Coupling of standard amino acids

A solution of Fmoc-AA-OH (4 equiv.), *N,N'*-diisopropylcarbodiimide (DIC, 4 equiv.), and Oxyma (4 equiv.) in DMF (3 mL) was added to the resin and the mixture was agitated at 50 °C for 25 min. After filtration, the resin was washed with DMF (5 x 3 mL), dichloromethane (5 x 3 mL) and DMF (5 x 3 mL).

4.3.4: Coupling of glycosylated arginine building block **1**

A solution of Fmoc-Arg(β-GlcNAc,Pbf)-OH **1** (2.1 equiv.), HATU (2.0 equiv.), HOAt (3.0 equiv.) and *sym*-collidine (2.2 equiv.) in DMF (3 mL) was added to the resin and the mixture was agitated at 25 °C for 18 h. After filtration, the resin was washed with DMF (5 x 3 mL), dichloromethane (5 x 3 mL) and DMF (5 x 3 mL).

4.3.5: Capping

A solution of Ac_2O/pyridine (1:9, v/v, 3 mL), was added to the resin. The mixture was agitated at 25 °C for 5 min. After filtration, the resin was washed with DMF (5 x 3 mL), dichloromethane (5 x 3 mL) and DMF (5 x 3 mL).

4.3.6: Resin cleavage and deprotection

After washing thoroughly with dichloromethane (10 x 5 mL), the resin was suspended in TFA/*i*-Pr_3SiH/H_2O (18:1:1,v/v/v) for 2 h. After filtration, the filtrate was concentrated by blowing with a gentle flow of nitrogen gas. The residue was then re-dissolved in MeOH (5 mL). NaOMe (0.5 M in MeOH, 1 mL) was added into the solution to give a final pH of 8-9. The resulting solution was stirred at 25 °C for 2 h. After completion (reaction monitoring by UPLC-MS), the reaction was quenched with formic acid (100 µL). The resulting mixture was concentrated and purified with RP-HPLC.

4.4 Synthesis of glycopeptides **7** and **8**

4.4.1: TRADD glycopeptide (**7**)

The synthesis of TRADD peptide fragment peptide **7** was conducted on a 25 µmol scale based on a Fmoc-loading assay after loading the first amino acid. After HPLC purification and lyophilization, the peptide was obtained as a white powder (9.9 mg, 6.5 µmol, 26%). **^{1}H NMR** (400 MHz, CD_3OD): δ 4.70 (d, 1H, $J_{H1,H2}$ = 9.7 Hz, GlcNAc-H1), 4.50–4.30 (m, 5H, 2 x Arg-Hα, Ser-Hα, Leu-Hα, Gln-Hα), 4.25 (d, 1H, Val-Hα), 4.10-3.76 (m 6H, 2 x Gly-Hα, 2 x Ser-Hβ, GlcNAc-H2, GlcNAc-$H6_a$), 3.72 (dd, 1H, $J_{H5,H6b}$ = 5.7 Hz, $J_{H6a,H6b}$ = 11.8 Hz, GlcNAc-$H6_b$), 3.57 (t_{apt}, 1H, $J_{H2,H3} = J_{H3,H4}$ = 9.7 Hz, GlcNAc-H3), 3.50-3.40 (m, 1H, GlcNAc-H5), 3.37 (t_{apt}, $J_{H3,H4} = J_{H4,H5}$ = 9.0 Hz, GlcNAc-H4),

3.30-3.20 (m, 4H, 4 x Arg-Hδ), 2.98 (t, 2H, 2 x Lys-Hε), 2.37 (t, 2H, 2 x Gln-Hγ), 2.20-1.40 (m, 23H, Ac, 2 x Lys-Hβ, 2 x Lys-Hγ, 2 x Lys-Hδ, 4 x Arg-Hβ, 4 x Arg-Hγ, Val-Hβ, 2 x Leu-Hβ, Leu-Hγ, 2 x Gln-Hβ), 1.03 (d, 6H, J = 6.2 Hz, 6 x Val-Hγ), 0.98 (d, 3H, J = 6.2 Hz, 3 x Leu-Hδ), 0.94 (d, 3H, J = 6.1 Hz, 3 x Leu-Hδ). **HRMS**: (+ESI) Calcd. for $C_{47}H_{88}N_{17}O_{16}$ $[M + H]^+$ m/z 1146.6587, Found: 1146.6600. **LRMS:** (MALDI-TOF) m/z, $[M + H]^+$, Calcd: 1146.66, Found: 1146.67.

4.4.2 FADD glycopeptide **8**

The synthesis of FADD peptide fragment peptide **14** was conducted on a 25 μmol scale based on a Fmoc-loading assay after loading the first amino acid. After HPLC purification and lyophilization, the peptide was obtained as a white powder (13.9 mg, 7.5 μmol, 30%). **^{1}H NMR** (400 MHz, CD_3OD): δ 4.67 (d, 1H, $J_{H1,H2}$ = 9.7 Hz, GlcNAc-H1), 4.50–4.20 (m, 9H, Arg-Hα, Ser-Hα, 2 x Leu-Hα, 2 x Glu-Hα, 2 x Ala-Hα, Lys6-Hα), 4.11 (d, 1H, Val-Hα), 3.95-3.85 (m, 3H, Ser-H$β_a$, Lys11- Hα, GlcNAc-H6_a), 3.85-3.75 (m, 2H, Ser-H$β_a$, GlcNAc-H2), 3.70 (dd, 1H, $J_{H5,H6b}$ = 5.8 Hz, $J_{H6a,H6b}$ = 11.9 Hz, GlcNAc-H6_b), 3.54 (t_{apt}, 1H, $J_{H2,H3}$ = $J_{H3,H4}$ = 9.5 Hz, GlcNAc-H3), 3.50-3.40 (m, 1H, GlcNAc-H5), 3.34 (t_{apt}, $J_{H3,H4}$ = $J_{H4,H5}$ = 9.1 Hz, GlcNAc-H4), 3.25-3.15 (m, 2H, 2 x Arg-Hδ), 3.00-2.90 (m, 4H, 4 x Lys-Hε), 2.50-2.40 (m, 4H, 4 x Glu-Hγ), 2.22-1.34 (m, 36H, Ac, 4 x Lys-Hβ, 4 x Lys-Hγ, 4 x Lys-Hδ, 2 x Arg-Hβ, 2 x Arg-Hγ, Val-Hβ, 4 x Leu-Hβ, 2 x Leu-Hγ, 4 x Glu-Hβ, 6 x Ala-Hβ), 1.10-0.80 (m, 18H, 6 x Val-Hγ, 12 x Leu-Hδ. **HRMS**: (+ESI) Calcd. for $C_{62}H_{113}N_{17}O_{22}$ $[M + 2H]^{2+}$ m/z, 723.9118, Found: 723.9118. **LRMS:** (MALDI-TOF) m/z, $[M + H]^+$, Calcd: 1446.81, Found: 1446.79.

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