Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

A silyl ether-protected building block for *O*-GlcNAcylated peptide synthesis to enable one-pot acidic deprotection

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Contents

1. Materials and Methods

Materials. 9-Fluoroenylmethoxylcarbonyl (Fmoc)-*L*-amino acids, Fmoc-Ser-OH, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylamonium hexafluorophosphate (HCTU), and Fmoc N-hydroxysuccinimide ester were purchased from Iris Biotech GmbH (Marktredwitz, Germany). 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU) was purchased from BACHEM (Bubendorf, Switzerland). Indium(III) bromide ($InBr_3$), *β*-N-acetylglucosamine (*β*-Ac_4GlcNAc), dry pyridine, Triisopropylsilane (TIPS), 4-Dimethylaminopyridine (DMAP), Piperidine, *N,N'*-Diisopropylcarbodiimide (DIC), 1-Hydroxybenzotriazole (HOBt) hydrate, and *tert*-Butyldimethylsilyl trifluoromethanesulfonate (TBDMS triflate) were purchased from Sigma Aldrich (Steinheim, Germany). Rink amide resin, Acetic acid glacial (AcOH), *L*-Ser-OH, Allyl chloroformate, Sodium carbonate, Anhydrous sodium sulfate, Acetic anhydrate, Glucosamine, 4Å molecule sieves, Boron trifluoride diethyl etherate ($BF_3{\cdot}Et_2O$), and Sodium methoxide (30% solution in methanol) were purchased from Sigma Aldrich (Darmstadt, Germany). Sodium bicarbonate ($NaHCO_3$), Ethyl cyanohydroxyiminoacetate (Oxyma), and Trifluoroacetic acid (TFA) were purchased from Carl Roth (Karlsruhe, Germany). 1,2-Dichloroethane, and Triethylamine (Et_3N) were purchased from Acros organics (Geel, Belgium). Methanol (MeOH), Dichloromethane (CH_2Cl_2), *N,N*-Diisopropylethylamine (DIPEA), and Dimethylformamide (DMF) were purchased from Fisher scientific (Geel, Belgium). Ethyl acetate (EtOAc), 37% Hydrochloric acid (HCl), Toluene, and Acetonitrile (CH_3CN) were purchased from VWR Chemicals (Fontenay-sous-Bois, France). Dry methanol (MeOH) was purchased from Acros organics (Fair Lawn, USA). Benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) was purchased from Merck KGaA (Darmstadt, Germany). Sep-Pak® Vac 12cc (2g) C18 Cartridges was purchased from Waters (Massachusetts, U.S.A.).

General peptide synthesis. The peptides were synthesized by Fmoc/*t*Bu solid-phase methods. Standard Fmoc-chemistry was used throughout with a 5-fold molar excess of the Fmoc-protected amino acids in the presence of 5-fold HBTU and 10-fold DIPEA on a PTI peptide synthesizer. The peptides were cleaved from the solid support resin with TFA in the presence of TIPS and water as scavenger (ratio 95:2:3) for 2 h at room temperature. After filtration to remove the resin, the filtrate was concentrated under a stream of nitrogen, and the peptide

products were precipitated in ice-cold diethyl ether and washed three times. The peptides were then purified by reversed-phase high performance liquid chromatography (RP-HPLC) in water and acetonitrile containing 0.1% TFA. The final products were characterized by both RP-HPLC and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Mass spectrometry

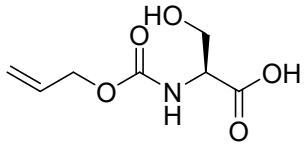
(High resolution) mass spectra (HRMS/MS) were measured on an Aquity system equipped with an ESI-MS Xevo® G2-XS QTof spectrometer (Waters Corporation, Massachusetts, USA) or an Aquity UPLC-MS system (Waters Corporation) (for compounds **3, s5**). The peptides **a-d** were measured with MALDI-time-of-flight (TOF)/TOF mass spectrometer (Bruker).

NMR

NMR spectra were recorded either with a Bruker AV III HD 300MHz spectrometer or a Bruker AV III 600MHz spectrometer (both Bruker Corporation, Billerica, Massachusetts USA) at ambient temperature. The chemical shifts for proton signals (^{1}H) are reported in ppm relative to the shift of tetramethylsilane.

2. Synthesis of Alloc-*L*-Ser(β-*O*-GlcNAc(TBDMS)$_3$)-OH s1

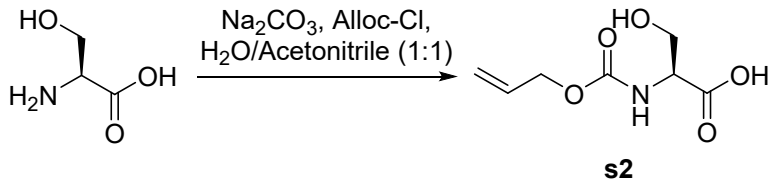
Alloc-*L*-Ser-OH s2



s2

L-Ser-OH (1 g, 9.5 mmol) and sodium carbonate (1g, 9.5 mmol) were dissolved in distilled water (10 ml). Allyl chloroformate (1 ml, 9.5 mmol) was dropwise added into the mixture followed by 5 ml acetonitrile. After 24 h, the acetonitrile was removed under vacuum and the mixture was acidified to pH 2 by concentrated HCl. Then, it was extracted with ethyl acetate and dried with anhydrous sodium sulfate. After filtration, the desired product, Alloc-*L*-Ser-OH **s2** (1.68 g, 8.9 mmol) in 93% yield without further purification. ^{1}H NMR (300 MHz, CHLOROFORM-d) δ ppm 3.83-3.95 (m, 1H), 4.02-4.12 (m, 1H), 4.32-4.50 (m, 1H), 4.58-4.60 (m, 2H), 5.17-5.39 (m, 2H), 5.82-5.99 (m, 1H), 6.33 (d, *J*=7.93 Hz, 1H), 7.34-7.65

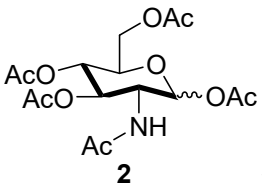
(m, 1H). ^{13}C NMR (300 MHz) δ ppm 60.47, 62.67, 66.24, 118.07, 132.31, 156.70, 173.61. ESI-MS Calculated $[M+H]^+$: 190.0710, Found $[M+H]^+$: 190.0717.



s2

Figure S1. Synthesis of Alloc-L-Ser-OH ***s2.***

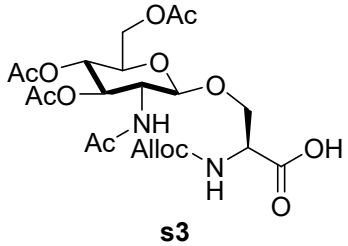
Acetylated glucosamine 2



2

D-Glucosamine (2 g, 6.7 mmol) was dissolved in pyridine (20 ml) with a catalytic amount of 4-dimethylaminopyridine (DMAP) (17 mg, 1.3 mmol). Then, acetic anhydrate (6.3 ml, 67 mmol) was added under ice bath and the mixture was stirred overnight at room temperature. The reaction was then poured into ice bath and stirred for 30 min. The solution was extracted with CH_2Cl_2 and dried with anhydrous sodium sulfate. The acetylated glucosamine **2** (2.58 g, 6.7 mmol) (α-anomer (*J*=3.6 Hz) and β-anomer (*J*=8.4 Hz) (20:1)) was obtained after evaporation in a quantitative yield. ^{1}H NMR (600 MHz, DMSO-d_6) δ ppm 1.81 (s, 3H), 1.95-2.04 (m, 9H), 2.19 (s, 3H), 4.01 (dd, *J*=12.51, 2.14 Hz, 1H), 4.12 (dt, *J*=10.38, 3.05 Hz, 1H), 4.19 (dd, *J*=12.51, 3.97 Hz, 1H), 4.23-4.32 (m, 1H), 5.00 (t, *J*=9.77 Hz, 1H), 5.18 (dd, *J*=10.99, 9.77 Hz, 1H), 5.72 (d, *J*=8.4 Hz, 1H, β-anomer), 5.92 (d, *J*=3.66 Hz, 1H, α-anomer), 8.05 (d, *J*=9.16 Hz, 1H). ^{13}C NMR (600 MHz) δ ppm 20.52, 20.58, 20.60, 20.72, 22.27, 51.95, 62.91, 69.73, 70.93, 71.74, 91.54, 170.73, 171.18, 171.91, 172.32, 173.71. ESI-MS Calculated $[M+Na]^+$: 412.1214, Found $[M+Na]^+$: 412.1227.

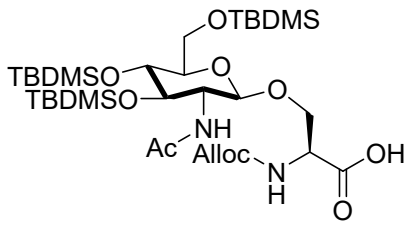
Alloc-*L*-Ser(β-*O*-GlcNAc(Ac)$_3$)-OH s3.



s3

Acetylated glucosamine **2** (2.86 g, 7.3 mmol) was added to a round bottom flask with 4 Å molecule sieves (2.1 g) dissolved in dry CH_2Cl_2 (10 ml) and stirred for 40 min under argon at r.t. Then, $BF_3{\cdot}Et_2O$ (46%, 2.74 ml, 10 mmol) was added dropwise into the mixture and stirred overnight. The Et_3N (0.9 ml, 6.4 mmol) was used to quench the reaction at 0 ℃ for 10 min, followed by the addition of Alloc-*L*-Ser-OH (1.5 g, 7.9 mmol) in CH_2Cl_2/acetonitrile (2:1, 15 ml). After reaction for 24 h and a second portion of fresh prepared oxazoline was added to the mixture and reacted for another 24 h. The reaction was quenched with water, extracted with CH_2Cl_2 and dried with anhydrous sodium sulfate. After filtration, the filtrate was concentrated. The crude product was purified by column chromatography on silica gel petroleum using CH_2Cl_2/MeOH (5:1) and reversed phase chromatography (RP-HPLC) to give the desired product, Alloc-*L*-Ser(β-*O*-GlcNAc(Ac)$_3$)-OH **s3** (610 mg, 1.2 mmol) in 15% yield over two steps. ^{1}H NMR (300 MHz, METHANOL-d4) δ ppm 1.94 (s, 3H), 1.99 (s, 3H), 2.02 (s, 3H), 3.76-3.99 (m, 3H), 4.08-4.20 (m, 2H), 4.24-4.36 (m, 1H), 4.39 (t, *J*=4.58 Hz, 1H), 4.54-4.61 (d, *J*=5.19 Hz, 2H), 4.69-4.78 (d, *J*=4.72 Hz, 1H), 5.00 (t, *J*=9.61 Hz, 1H), 5.16-5.26 (m, 2H), 5.35 (dd, *J*=17.24, 1.37 Hz, 1H), 5.80-6.10 (m, 1H). ^{13}C NMR (300 MHz) δ ppm 9.21, 20.55, 20.59, 20.66, 22.84, 55.26, 63.17, 66.76, 70.04, 70.09, 72.97, 73.97, 101.93, 117.82, 134.17, 158.18, 171.28, 171.80, 172.42, 172.96, 173.74. ESI-MS Calculated $[M+Na]^+$: 541.1646, Found $[M+Na]^+$: 541.1607.

Alloc-*L*-Ser(β-*O*-GlcNAc(TBDMS)$_3$)-OH s1.



s1

Alloc-*L*-Ser(β-*O*-GlcNAc(Ac)$_3$)-OH **s3** (50mg, 0.1 mmol) was dissolved in MeOH followed by the addition of sodium methoxide (0.4 M in MeOH, 1 ml).

The mixture was stirred for 30 min at r.t. The desired Alloc-*L*-Ser(β-*O*-GlcNAc)-OH **s4** was obtained under vacuum in a quantitative yield. Then, Alloc-*L*-Ser(β-*O*-GlcNAc)-OH **s4** and DMAP (0.3 equiv., 0.03 mmol) were dissolved with dry pyridine (5 ml) in a round bottom flask. Then, *tert*-Butyldimethylsilyl trifluoromethanesulfonate (TBDMS triflate) (16 equiv., 160 ul, 1.6 mmol) was added to the solution dropwise at ice bath. The mixture was reacted for overnight at r.t. Afterwards, the solvents was removed with toluene under vacuum. The crude mixture was purified by column chromatography on silica gel petroleum using MeOH/CH_2Cl_2 (2%-10%) to give the desired product, Alloc- L- Ser(β- *O*- GlcNAc(TBDMS)$_3$)-OH **s1** (73 mg, 0.1 mmol) in a quantitative conversion over two steps. The overall yield of compound s**1** is calculated based on the starting material **s4**. ^{1}H NMR (300 MHz, METHANOL-d4) δ ppm 0.08-0.20 (m, 16H), 0.87-0.97 (m, 25H), 2.03 (s, 3H), 3.56-3.69 (m, 1H), 3.72-3.90 (m, 5H), 3.99 (dd, *J*=10.22, 5.95 Hz, 1H), 4.04-4.13 (m, 1H), 4.15-4.21 (m, 1H), 4.56 (m, 4H), 5.21 (dd, *J*=10.53, 1.37 Hz, 1H), 5.33 (dd, *J*=17.24, 1.37 Hz, 1H), 5.87-6.04 (m, 1H). ^{13}C NMR (300 MHz) δ ppm 18.91, 18.96, 19.13, 23.26, 26.41, 26.44, 26.51, 56.34, 57.16, 64.46, 66.69, 70.91, 72.31, 75.70, 80.28, 101.97, 115.28, 117.90, 119.50, 123.71, 127.93, 134.09, 158.03, 162.99. 163.45. ESI-MS Calculated $[M+Na]^+$: 757.3943, Found $[M+Na]^+$: 757.3937.

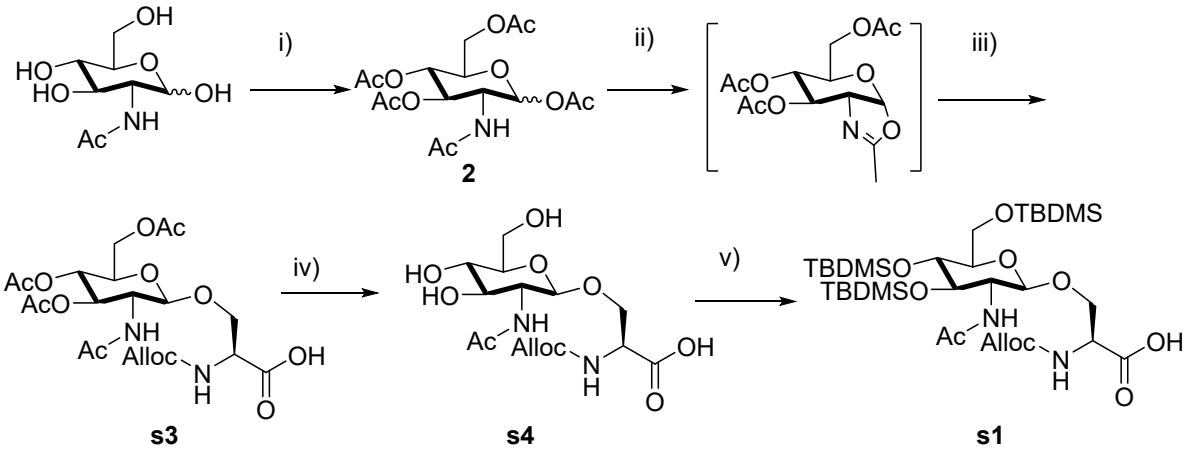


Figure S2. Synthesis of acid labile building block Alloc-L-Ser(β-O-GlcNAc(TBDMS)$_3$)-OH. i) Acetylic anhydride, DMAP in pyridine; ii) 4Å molecular sieves, $BF_3{\cdot}Et_2O$ in CH_2Cl_2, 0 °C, 12h; iii) Alloc-L-Ser-OH, 2 days; iv) 0.1 M MeONa in MeOH, 30 min; v) TBDMS-OTf, DMAP, dry pyridine.

3. Coupling of Alloc-*L*-Ser(β-*O*-GlcNAc(TBDMS)$_3$)-OH s1 *via* SPPS

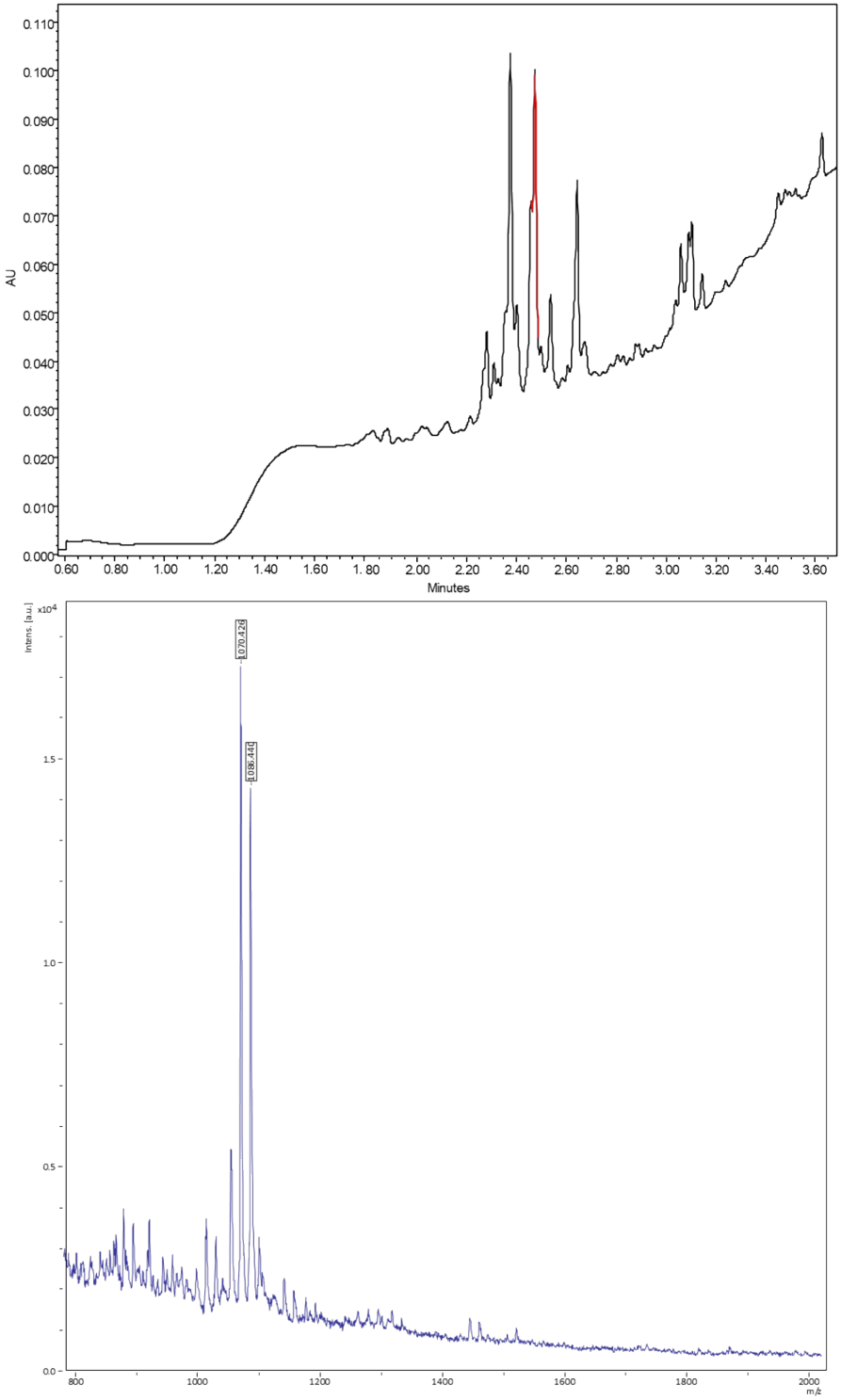
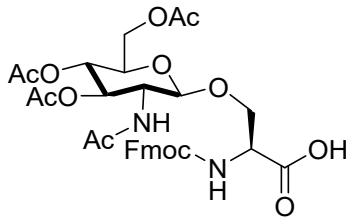


Figure S3 Attempted installation of Alloc-L-Ser(β-O-GlcNAc(TBDMS)$_3$)-OH to a short peptide (Alloc-L-Ser-(β-OGlcNAc(TBDMS)$_3$)-IAAC(tBu)-G-amide) via SPPS and deprotection with Pd(PPh$_3$)$_4$. The red highlighted peak and mass spectroscopy as the Alloc protected crude peptide before and after the deprotection treatment.

4. Synthesis of Fmoc-*L*-Ser(β-*O*-GlcNAc(TBDMS)$_3$)-OH 1

Fmoc-*L*-Ser(β-*O*-GlcNAc(Ac)$_3$)-OH 3

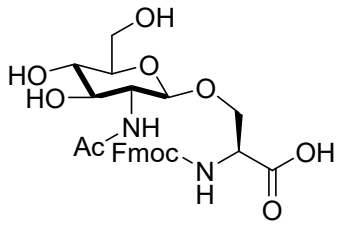


3

β-Ac$_4$GlcNAc (1.424g, 3.666mmol), Fmoc-Ser-OH (400mg, 1.222mmol), and $InBr_3$ (260mg, 0.7332mmol) were added into a round bottom flask containing a magnetic stir bar under open atmosphere. Mixture was then suspended in 1,2-dichloroethane (6.11ml). A reflux condenser was added and the round bottom flask was submerged in a silica oil bath set at 60°C at the beginning. A nitrogen balloon was added and the reaction was heated to reflux (84°C) for 16 hours under nitrogen protection. Once the reaction was no longer progressed as determined by TLC (7:2:1, EtOAc:MeOH:H_2O), the reaction was allowed to cool to r.t and concentrated under vacuum. The resulting black residue was rinsed with toluene several times under vacuum. The fluffy black solid was then re-dissolved in DCM and purified by flash column chromatography (0-5% MeOH in DCM, 0.1% AcOH, R_f = 0.5). Fractions containing the product were combined and concentrated under vacuum. The resulting acidic dark brown syrup was then further diluted with EtOAc and transferred into a separate funnel. Organic layer was made basic by adding aqueous saturated sodium bicarbonate. Aqueous layer was collected by gently mixing the two layers. The separated organic layer was made basic again and aqueous layer was collected afterwards. This procedure was repeated until no more products were detected in the organic layer as determined by TLC (7:2:1, EtOAc:MeOH:H_2O). The resulting basic aqueous fractions were combined and further washed with fresh EtOAc. The collected basic aqueous layer was then collected into a glass beaker and acidified gently with 6M HCl while stirring under r.t. The while solids were emerged gradually and were extracted from the aqueous by DCM. The organic layer was collected and dried by Magnesium sulfate. Following filtration, the organic fraction was concentrated in vacuum to achieve the desired product Fmoc-*L*-Ser(β-*O*-GlcNAc(Ac)$_3$)-OH **3** (642mg, 0.96mmol) in 80% yield. ^{1}H NMR (300 MHz, METHANOL-d4) δ ppm 1.86 (s, 3H), 1.97-2.09 (m, 9H), 3.53-3.64 (m, 1H), 3.81-3.84 (m, 2H), 3.92-4.01 (m, 1H), 4.10-4.19 (m, 2H), 4.24-4.34 (m, 2H), 4.35-4.50 (m, 3H), 4.73-4.76 (m, 1H), 4.97-5.06 (m, 2H), 5.19-5.31 (m, 1H), 7.27-7.46 (m, 5H), 7.67-7.70

(m, 2H), 7.78-7.82 (m, 2H). ^{13}C NMR (300 MHz) δ ppm 20.55, 20.59, 20.64, 20.86, 55.33, 55.50, 63.16, 68.13, 69.95, 70.06, 73.00, 73.90, 101.85, 120.95, 126.22, 128.22, 128.82, 142.55, 145.16, 158.37, 171.25, 171.83, 172.39, 172.89, 173.74. The obtained data were in accordance to the data provided by previous report[1,2]. UPLC-MS Calculated $[M+H]^+$: 657.23, Found $[M+H]^+$: 657.32.

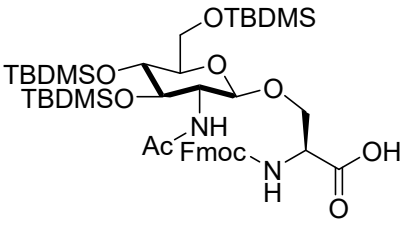
Fmoc-*L*-Ser(*β*-*O*-GlcNAc)-OH 5



5

Fmoc-*L*-Ser(β-*O*-GlcNAc(Ac)$_3$)-OH **3** (642mg, 0.96mmol) was dissolved in dry MeOH (3ml) followed by the equal volume of sodium methoxide (30% solution in MeOH, 3ml, 16mmol). The mixture was stirred for 30 min, acidified with 1 N HCl to pH 2. The desired unprotected Ser(β-GlcNAc)-OH **4** was achieved *via* concentrated in vacuum in a quantitative yield. Afterwards, Ser(β-GlcNAc)-OH **4** was redissolved in water (3 ml) and carefully adjusted pH to 8-9 by triethylamine and 1 N HCl. Then, Fmoc *N*-hydroxysuccinimide ester (1 equiv., 0.96 mmol) dissolved in 3 ml acetonitrile was added to react with **4** for 2 h at r.t. The reaction was quenched by 1 N HCl and extracted with ethyl acetate. In order to avoid triethylamine salts, the concentrated crude products was subjected to RP-HPLC to obtain pure Fmoc-*L*-Ser(*β*-*O*-GlcNAc)-OH **5** (93.1mg, 0.29mmol) in 30% yield over two steps. ^{1}H NMR (300 MHz, METHANOL-d4) δ ppm 1.96 (s, 3H), 3.43-3.53 (m, 1H), 3.58-3.75 (m, 2H), 3.85-3.96 (m, 2H), 4.19 (dd, *J*=10.68, 5.19 Hz, 1H), 4.23-4.30 (m, 1H), 4.33-4.53 (m, 4H), 7.29-7.46 (m, 4H), 7.71 (d, *J*=7.32 Hz, 2H), 7.83 (d, *J*=7.32 Hz, 2H). ^{13}C NMR (300 MHz) δ ppm 23.07, 62.65, 68.14, 69.14, 69.80, 71.91, 75.83, 78.12, 102.06, 102.65, 120.93, 126.30, 128.23, 128.82, 142.58, 142.26, 158.53, 174.08, 179.55. ESI-MS Calculated $[M+Na]^+$: 553.1793, Found $[M+Na]^+$: 553.1792.

Fmoc-*L*-Ser(β-*O*-GlcNAc(TBDMS)$_3$)-OH 1



1

Fmoc-*L*-Ser(β-*O*-GlcNAc)-OH **5** (33mg, 0.103mmol) with DMAP (0.3 equiv., 0.031mmol) were dissolved in dry pyridine (5 ml) in a round bottom flask. Then, *tert*-Butyldimethylsilyl trifluoromethanesulfonate (TBDMS triflate) (16 equiv., 1.648mmol) was added to the solution dropwise at ice bath. The mixture was reacted for overnight at r.t. Afterwards, the solvents was removed with toluene under vacuum for several times. The crude mixture was purified by solid phase extraction (10%-0% H_2O, 90-100% CH_3CN) to give the desired product, Fmoc-*L*-Ser(β-*O*-GlcNAc(TBDMS)$_3$)-OH **1** (37.8mg, 0.049mmol) in 48% yield. ^{1}H NMR (300 MHz, METHANOL-d4) δ ppm 0.07-0.19 (m, 18H), 0.86-0.99 (m, 28H), 1.96 (s, 3H), 3.61-3.74 (m, 2H), 3.76-3.96 (m, 5H), 4.05 (d, *J*=10.07, 7.02 Hz, 1H), 4.14-4.28 (m, 2H), 4.34-4.45 (m, 3H), 4.58-4.69 (m, 1H), 7.30-7.37 (m, 2H), 7.38-7.46 (m, 2H), 7.69 (d, *J*=7.32 Hz, 2H), 7.81 (d, *J*=7.32 Hz, 2H). ^{13}C NMR (300 MHz) δ ppm 14.47, 20.87, 25.76, 27.67, 28.72, 31.12, 43.91, 55.15, 55.66, 56.43, 61.55, 64.92, 72.55, 80.69, 114.86, 129.63, 130.68, 131.11, 131.20, 132.36, 134.67, 157.75, 159.99, 174.53. UPLC-MS (Intact molecule): Calculated $[M+K]^+$: 911.41, Found $[M+K]^+$: 911.63. ESI-MS (Minus one acid-labile TBDMS group): Calculated $[M-H]^-$: 758.3587, Found $[M-H]^-$: 758.3587.

5. Coupling of Fmoc-*L*-Ser(β-*O*-GlcNAc(TBDMS)$_3$)-OH 1 to short peptides *via* SPPS

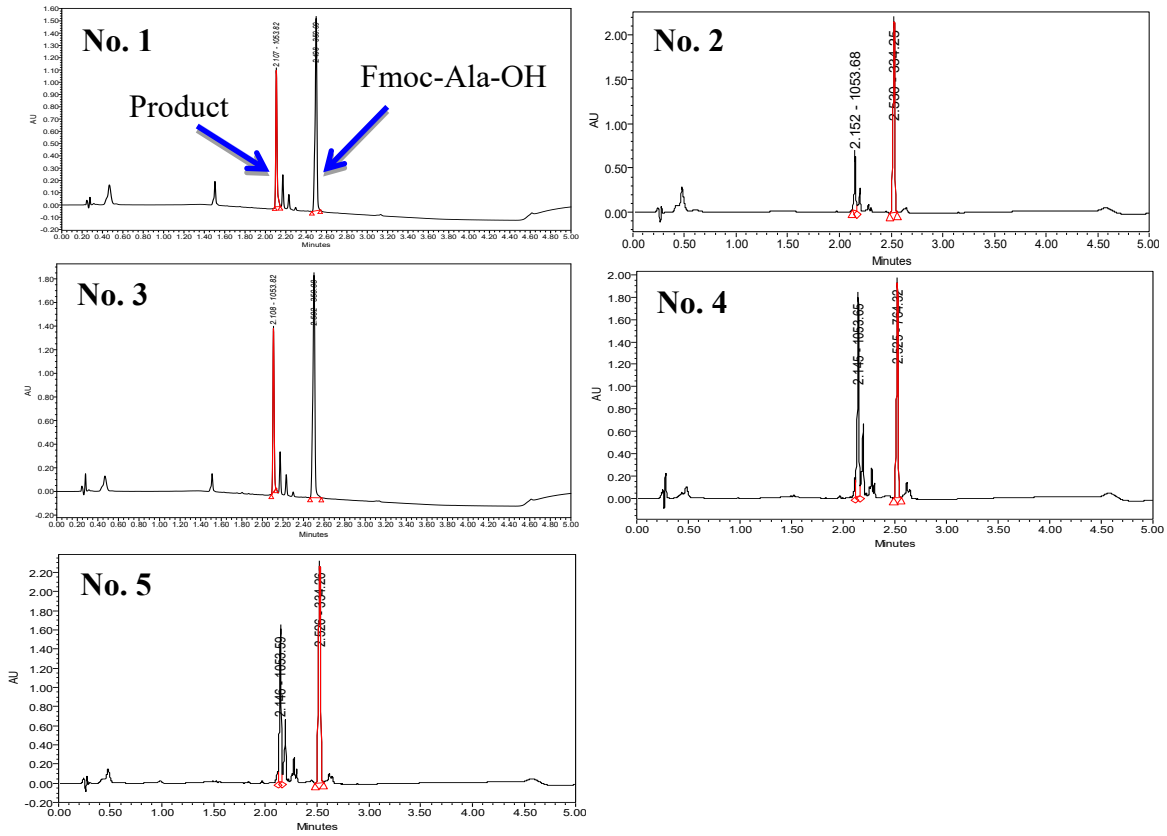


Figure S4 The UPLC-MS/MS profiles of the optimisation for the coupling of Fmoc-L-Ser(β-O-GlcNAc(TBDMS)$_3$)-OH ***1*** *(6 equiv.) on short peptide (g)SLQPGK-NH$_2$. The immobilized peptide was incubated with building block* ***1*** *for one hour at room temperature in DMF, before they were cleaved off the resin according to the general protocol for the peptide synthesis. The 1.65 mM Fmoc-Ala-OH (arrow pointed) was subjected as reference peak for the normalisation of the coupling efficiency. The product (arrow pointed), Fmoc-(g)SLQPGK-NH$_2$, was confirmed by UPLC-MS and calculated relatively of the reference peak area.*

6. MS/HPLC characterisation of FSPPSS(g)SLQPGK-NH_2 and VT(g)SGVKPSG-NH_2

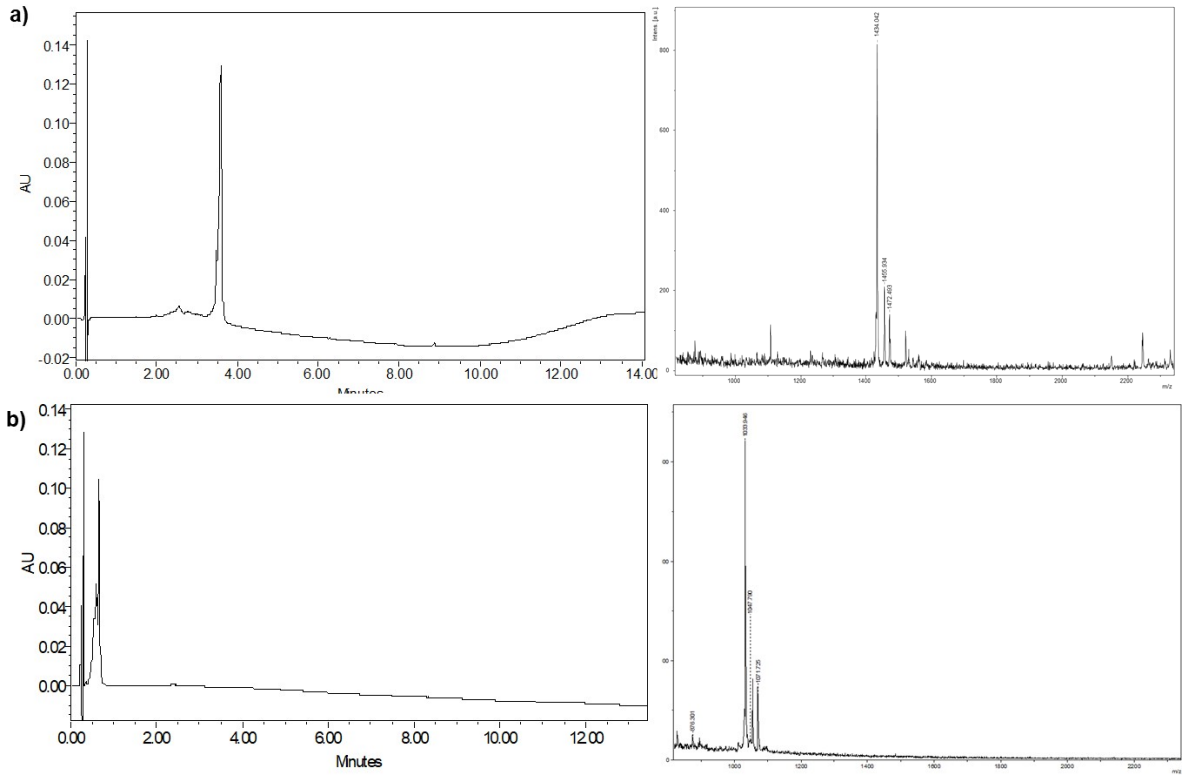
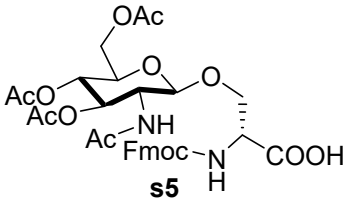


Figure S5 Characterisation of the synthetic FSPPSS(g)SLQPGK-NH_2 (a) and VT(g)SGVKPSG-NH_2 (b), which carry the native glycosylation sites. a), MALDI-TOF MS $[M+H]^+$ Found mass 1434.042 Da, calculated mass 1433.821 Da; b), MALDI-TOF MS $[M+H]^+$ Found mass 1033.946 Da, calculated mass 1033.250 Da.

7. Synthesis of Fmoc-D-Ser(β-*O*-GlcNAc(Ac)$_3$)-OH s5



s5

Acetylated glucosamine **2** (1g, 2.6 mmol) was added to a round bottom flask with 4 Å molecule sieves (1.2 g) dissolved in dry CH_2Cl_2 (5 ml) and stirred for 40 min under argon at r.t. Then, $BF_3{\cdot}Et_2O$ (46%, 1.05 ml, 3.9 mmol) was added dropwise into the mixture and stirred overnight. The Et_3N (0.32 ml, 2.2 mmol) was used to quench the reaction at 0 ℃ for 10 min, followed by the addition of Fmoc-D-Ser-OH (0.5 g, 1.5 mmol) in CH_2Cl_2/acetonitrile (2:1, 5 ml). After reaction for 24 h and a second portion of fresh prepared oxazoline was added to the mixture and reacted for another 24 h. The reaction was quenched with water, extracted with CH_2Cl_2 and dried with anhydrous sodium sulfate. After filtration, the filtrate was concentrated. The crude product was purified by column chromatography on silica gel petroleum using CH_2Cl_2/MeOH (5:1) and reversed phase chromatography (RP-HPLC) to give the desired product, Fmoc-D-Ser(β-*O*-GlcNAc(Ac)$_3$)-OH **s5** (80mg, 0.2 mmol) in 13% yield over two steps. ^{1}H NMR (300 MHz, METHANOL-d4) δ ppm 1.87-2.08 (m, 12H), 3.67-3.79 (m, 1H), 3.89 (t, *J*=9.46 Hz, 1H), 4.00-4.15 (m, 3H), 4.19-4.34 (m, 2H), 4.40-4.45 (M, 3H), 4.59 (d, *J*=8.24 Hz, 1H), 4.95-5.05 (m, 2H), 5.22 (t, *J*=9.92 Hz, 1H), 7.28-7.51 (m, 4H), 7.71 (d, *J*=7.02 Hz, 2H), 7.82 (d, *J*=7.02 Hz, 2H). ^{13}C NMR (300 MHz) δ ppm 20.55, 20.60, 22.80, 55.24, 55.61, 63.25, 68.01, 70.12, 71.44, 72.86, 73.71, 102.75, 120.93, 126.25, 128.22, 128.79, 128.84, 142.58, 145.20, 145.31, 158.45, 171.25, 171.81, 172.36, 172.78, 173.93. The obtained data were in accordance to the data provided by our previous report[3]. UPLC-MS Calculated $[M+Na]^+$: 679.21, Found $[M+Na]^+$: 679.22.

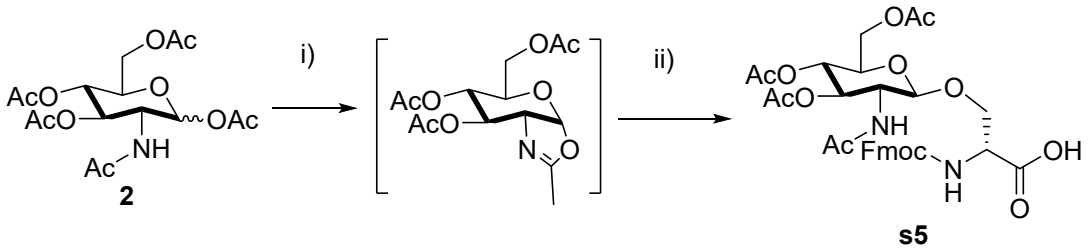


Figure S6 Fmoc-D-Ser(β-O-GlcNAc(Ac)$_3$)-OH. i) 4Å molecular sieves, $BF_3{\cdot}Et_2O$ in CH_2Cl_2, 0 °C, 12h; ii) Fmoc-D-Ser-OH, 2 days.

8. Epimerisation analysis of Fmoc-*L*-Ser(β-*O*-GlcNAc(TBDMS)$_3$)-OH 1 *via* SPPS

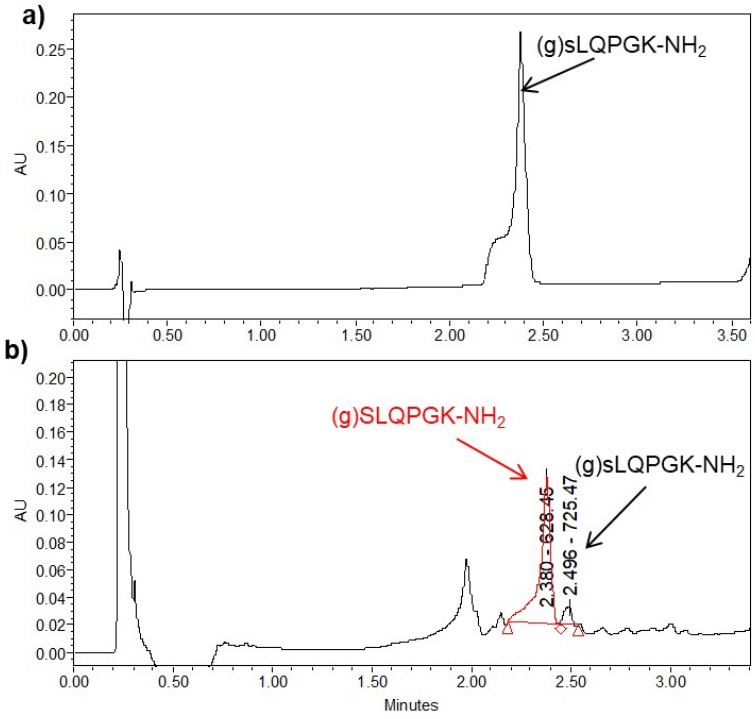


Figure S7 Epimerisation analysis of Fmoc-L-Ser(β-O-GlcNAc(TBDMS)$_3$)-OH. a) The purified isomeric peptide (g)SLQPGK-NH$_2$; b) The analysis of the coupled (g)SLQPGK-NH$_2$ with the acid labile building block, Fmoc-L-Ser(β-O-GlcNAc(TBDMS)$_3$)-OH.

9. MS and HPLC characterisation of *O*-GlcNAcylated peptides

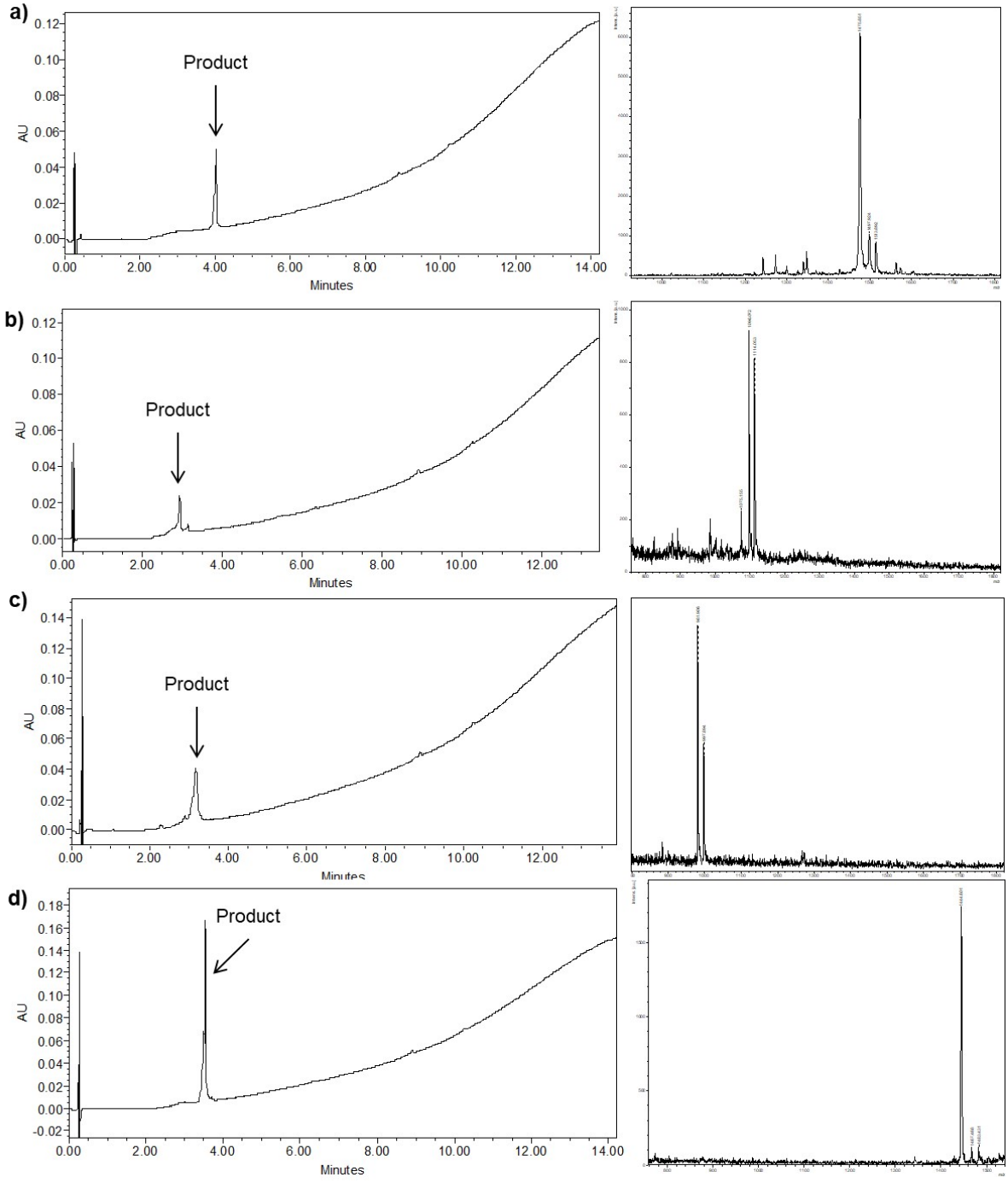


Figure S8 Characterisation of the synthetic peptides ***a-d,*** *which carry native glycosylation sites. a) Ac-FSPPSS(g)SLQPGK-NH_2, MALDI-TOF MS $[M+H]^+$ Found mass 1475.851 Da, calculated mass 1475.820 Da; b) Ac-VT(g)SGVKPSG-NH_2, MALDI-TOF MS $[M+H]^+$ Found mass 1075.156 Da, calculated mass 1075.250 Da; c) Ac-L(g)SPPASSG-NH_2, MALDI-TOF MS $[M+Na]^+$ Found mass 981.906 Da, calculated mass 982.090 Da; d) Ac-LNRTS(g)SDSALH-NH_2, MALDI-TOF MS $[M+H]^+$ Found mass 1444.601 Da, calculated mass 1444.690 Da.*

Reference

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2. S. Schwagerus, O. Reimann, C. Despres, C. Smet-Nocca and C. P. R. Hackenberger, *J. Pept. Sci.*, 2016, **22**, 327-333.
3. Y. Zhang, S. M. Muthana, D. Farnsworth, O. Ludek, K. Adams, J. J. Barchi and J. C. Gildersleeve, *J. Am. Chem. Soc.*, 2012, **134**, 6316-6325.