Supplementary Information

Efficient use of the Dmab Protecting Group: Applications for the Solid-Phase Synthesis of *N*-Linked Glycopeptides

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General procedures

^{1}H NMR spectra were recorded using a Bruker Avance DPX 400 at a frequency of 400.2 MHz. The spectra are reported as parts per million (ppm) downfield shift using the solvent peak as an internal reference. The data are reported as chemical shift (δ), multiplicity, relative integral, coupling constant (*J* Hz) and assignment where possible. Low resolution mass spectra were recorded on a Finnigan LCQ Deca ion trap mass spectrometer (ESI). High resolution mass spectra were recorded on a Bruker 7T Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FTICR)

Analytical reverse-phase HPLC was performed on a Waters System 2695 separations module with an Alliance series column heater at 30 °C and 2996 photodiode array detector. A Waters Sunfire 5 μm, 2.1 x 150 mm column was used at a flow rate of 0.2 mL min^{-1} using a mobile phase of 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B) and a linear gradient of 2-50% B over 30 min. The results were analysed with Waters Empower software. Preparative reverse-phase HPLC was performed using a Waters 600 Multisolvent Delivery System and Waters 500 pump with 2996 photodiode array detector or Waters 490E Programmable wavelength detector operating at 254 and 280 nm. A Waters Sunfire 5 μm, 19 x 150 mm column was used at a flow rate of 7 mL min^{-1} using a mobile phase of 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B) using a linear gradient of 2-50% B over 30 min.

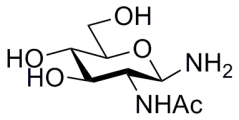
LC-MS was performed on a Thermo Separation Products: Spectra System consisting of P400 Pump and a UV6000LP Photodiode array detector on a Phenomenex Jupiter 5 μm, 2.1 x 150 mm column at a flow rate of 0.2 mL min^{-1} coupled to a Thermoquest Finnigan LCQ Deca mass spectrometer (ESI) operating in positive mode. Separations involved a mobile phase of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) using a linear gradient of 2-50% B over 30 min.

Analytical thin layer chromatography (TLC) was performed on commercially prepared silica plates (Merck Kieselgel 60 0.25 mm F254). Flash column chromatography was performed using 230-400 mesh Kieselgel 60 silica eluting with distilled solvents as described.

Commercial materials were used as received unless otherwise noted. Amino acids, coupling reagents and resins were obtained from Novabiochem. DCM and methanol were

distilled from calcium hydride. DMF was obtained as peptide synthesis grade from Auspep or Labscan.

2-acetamido-2-deoxy-β-D-glucopyranosylamine (5)[1]



N-acetyl-D-glucosamine (1.05 g, 4.74 mmol) was added to saturated aqueous ammonium bicarbonate (25 mL) and stirred for 4 days at 40 °C with periodic addition of ammonium bicarbonate to maintain saturation. The reaction was concentrated *in vacuo* until cessation of gas evolution, after which it was lyophilised to a constant weight. The crude product was purified by column chromatography (eluent: 1:1 v/v chloroform/methanol) to give an oily residue which was redissolved in water (15 mL) and lyophilised, to afford amino sugar **5** as a white solid (0.88 g, 84%). R_f [1:1 v/v chloroform/methanol] = 0.34; m.p. = 59-61 °C; ^{1}H NMR (400 MHz, CD_3OD); 4.04 (d, 1H, J = 8.8 Hz, H1), 3.87 (dd, 1H, J = 12.0, 2.0 Hz, H6), 3.67 (dd, 1H, J = 12.0, 5.6 Hz, H6'), 3.58 (dd, J = 10.0, 10.0 Hz, H3), 3.44 (dd, 1H, J = 10.0, 8.4 Hz, H2), 3.33 (dd, 1H, J = 10.0, 10.0 Hz, H4), 3.30-3.26 (m, 1H, H5), 2.03 (s, 3H, CH_3); MS (ESI) *m/z* 243.1 [$(M+Na)^+$, 100%]. These data were in agreement with those reported by Likhosherstov *et. al*.[1]

SPPS of peptides and glycopeptides

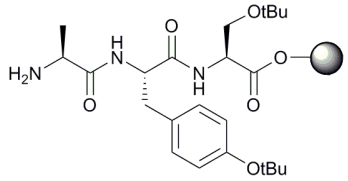
Solid-phase syntheses of peptides and glycopeptides were carried out in polypropylene syringes equipped with Teflon filters, purchased from Torviq.

Loading Fmoc-Ser(*t*Bu)-OH onto Wang resin

Wang resin LL (100-200 mesh) (0.45 g, 0.44 mmol g^{-1}, 200 μmol) was initially washed with DMF (5 x 5 mL), DCM (5 x 5 mL) and DMF (5 x 5 mL), then allowed to swell in DMF (5 mL) for 30 min. Fmoc-Ser(*t*Bu)-OH (0.38 g, 2.0 mmol) was dissolved in dry DCM (15 mL) and DMF (0.5 mL) was added to improve solubility. The solution was cooled to 0 °C before DIC (155 μL, 1.0 mmol) was added and the reaction stirred for 20 min before the solvent was removed *in vacuo*. The resulting symmetrical

anhydride was redissolved in DMF (5 mL) before adding directly to the resin. A solution of DMAP (2.5 mg, 20 μmol) in DMF (1 mL) was added and the resin shaken for 1 h before washing with DMF (5 x 5 mL), DCM (5 x 5 mL) and DMF (5 x 5 mL). The resin was then treated with Ac_2O/pyridine (1:9 v/v) for 10 min followed by washing with DMF (5 x 5 mL), DCM (5 x 5 mL) and DMF (5 x 5 mL). Treatment of the resin with piperidine/DMF (5 mL, 1:9 v/v) for 3 min (x 2) and measurement of the resulting fulvene-piperidine adduct at λ = 301 nm showed that amino acid loading onto the resin was quantitative. The resin was subsequently washed with DMF (10 x 5 mL) and DCM (10 x 5 mL) before drying *in vacuo*.

Synthesis of resin bound tripeptide 3 (25 μmol scale)



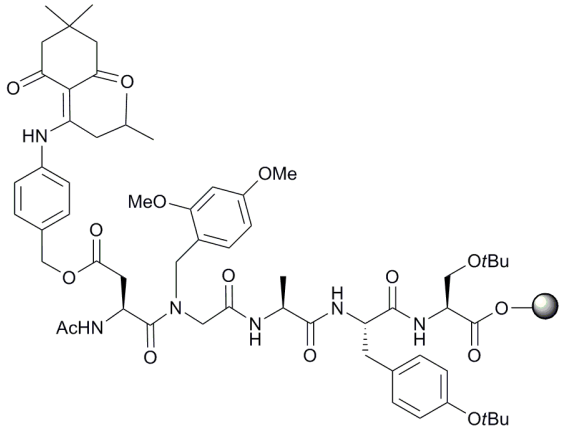
Amino Acid Coupling: A solution of Fmoc-Tyr(*t*Bu)-OH (46 mg, 100 μmol), PyBOP (52 mg, 100 μmol) and NMM (22 μL, 200 μmol) in DMF (1 mL) was added to the resin and shaken. After 1 h the resin was washed with DMF (5 x 3 mL), DCM (5 x 3 mL), and DMF (5 x 3 mL).

Capping: Ac_2O/pyridine (1:9 v/v) was added to the resin and shaken. After 3 min the resin was washed DMF (5 x 3 mL), DCM (5 x 3 mL), and DMF (5 x 3 mL).

Fmoc Deprotection: A solution of piperidine/DMF (5 mL, 1:9 v/v) was added to the resin and shaken for 3 min (x 2). The resin was subsequently washed with DMF (5 x 3 mL), DCM (5 x 3 mL), and DMF (5 x 3 mL). The efficiency of the previous amino acid coupling was determined by measurement of the resulting fulvene-piperidine adduct at λ = 301 nm.

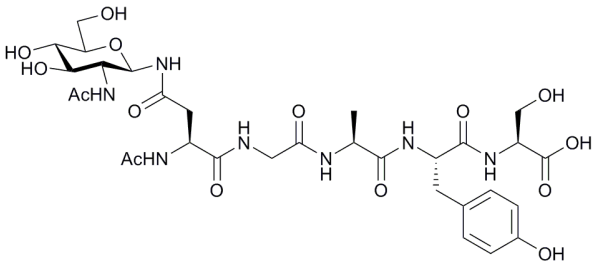
Fmoc-Ala-OH (31 mg, 100 μmol) was *coupled*, *capped* and *Fmoc-deprotected* in an identical fashion to that described above.

Synthesis of resin bound pentapeptide 1 (25 µmol scale)



Fmoc-(Dmb)Gly-OH (23 mg, 50 µmol) was coupled to resin bound tripeptide **3**, using PyBOP (26 mg, 50 µmol) and NMM (11 µL, 100 µmol) in DMF (0.5 mL) for 1 h. Following *capping* and *Fmoc-deprotection* as described above Fmoc-Asp(ODmab)-OH (33 mg, 50 µmol) was coupled to the peptide using HATU (19 mg, 50 µmol) and NMM (11 µL, 100 µmol) in DMF (0.5 mL) for 2 h. The peptide was then *Fmoc-deprotected* as described above and the loading determined to be quantitative. The *N*-terminus was then acetylated by treatment with Ac_2O/pyridine (5 mL, 1:9 v/v) for 5 minutes and the resin washed with DMF (5 x 3 mL), DCM (5 x 3 mL) and DMF (5 x 3 mL).

Synthesis of *N*-linked glycopeptide - Ac-Asn(β-GlcNAc)-Gly-Ala-Tyr-Ser-OH (6)



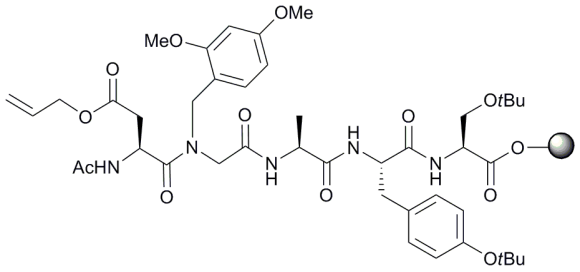
Solid-phase aspartylation via 1

Dmab deprotection: A solution of hydrazine monohydrate in DMF (2% v/v, 3 mL) was added to resin **1**, shaken for 3 minutes and the procedure repeated a further 4 times. Removal of the ivDde moiety was determined by measuring the UV absorbance at 290 nm until no further indazole adduct could be detected. Following ivDde removal, the resin was treated with a 5mM solution of NaOH in H_2O/methanol (3 mL, 1:1 v/v) for 3 hours. The resin was subsequently washed with methanol (5 x 3 mL), DMF (5 x 3 mL), DCM (5 x 3 mL) and DMF (5 x 3 mL).

Aspartylation: A solution of 2-acetamido-2-deoxy-β-D-glucopyranosylamine **5** (8.3 mg, 37.5 μmol), PyBOP (19.5 mg, 37.5 μmol) and NMM (8.3 μL, 75 μmol) in DMF (0.5 mL), was added to the resin and shaken for 16 h. The resin was subsequently washed with DMF (10 x 3 mL) then DCM (10 x 3 mL).

A mixture of TFA/thioanisole/triisopropylsilane/water (17:1:1:1 v/v/v/v) was added to the resin and shaken for 1.5 hrs. The resin was then washed with TFA (3 x 3 mL), and the combined cleavage and washing solutions were concentrated *in vacuo*. The resulting residue was dissolved in DMSO (1.5 mL), purified by preparative HPLC and lyophilised to afford the desired glycopeptide as a fluffy white solid (9.5 mg, 50%).

Synthesis of resin bound pentapeptide 2 (25 μmol scale)



Fmoc-(Dmb)Gly-OH (23 mg, 50 μmol) was coupled to resin bound tripeptide **3**, using PyBOP (26 mg, 50 μmol) and NMM (11 μL, 100 μmol) in DMF (0.5 mL) for 1 h. Following *capping* and *Fmoc-deprotection* as described above Fmoc-Asp(OAllyl)-OH (40 mg, 100 μmol) was coupled to the peptide using HATU (38 mg, 100 μmol) and NMM (22 μL, 200 μmol) in DMF (1 mL) for 1 h. The peptide was then *Fmoc-deprotected* as described above and the loading determined to be quantitative. The *N*-terminus was then acetylated by treatment with Ac_2O/pyridine (5 mL, 1:9 v/v) for 5 minutes and the resin washed with DMF (10 x 3 mL) then DCM (10 x 3 mL).

Solid-phase aspartylation via 2

Allyl deprotection: A solution of tetrakis(triphenylphosphine)palladium(0) (20 mg, 18 μmol) and phenylsilane (123 μL, 1 mmol) in dry DCM (1 mL) was added to resin **2** (25 μmol), shaken for 1 h and the procedure repeated. The resin was subsequently washed with DMF (10 x 3 mL), DCM (10 x 3 mL), and DMF (5 x 3 mL).

Aspartylation: A solution of 2-acetamido-2-deoxy-β-D-glucopyranosylamine **5** (8.3 mg, 37.5 μmol), PyBOP (19.5 mg, 37.5 μmol) and NMM (8.3 μL, 75 μmol) in DMF (0.5 mL), was added to the resin and shaken for 16 h. The resin was subsequently washed with DMF (10 x 3 mL) then DCM (10 x 3 mL).

A mixture of TFA/thioanisole/triisopropylsilane/water (17:1:1:1 v/v/v/v) was added to the resin and shaken for 1.5 hrs. The resin was then washed with TFA (3 x 3 mL), and the combined cleavage and washing solutions were concentrated *in vacuo*. The resulting residue was dissolved in DMSO (1.5 mL), purified by preparative HPLC and lyophilised to afford the desired glycopeptide as a fluffy white solid (17.2 mg, 91%).

Analytical HPLC: R_t = 11.0 min, LCMS: R_t = 11.3 min; MS (ESI) *m/z* 756.0 [$(M+H)^+$, 100%]; HRMS Calcd for $C_{31}H_{45}N_7O_{15}Na$: MNa^+, 778.2866 found MNa^+, 778.2867.

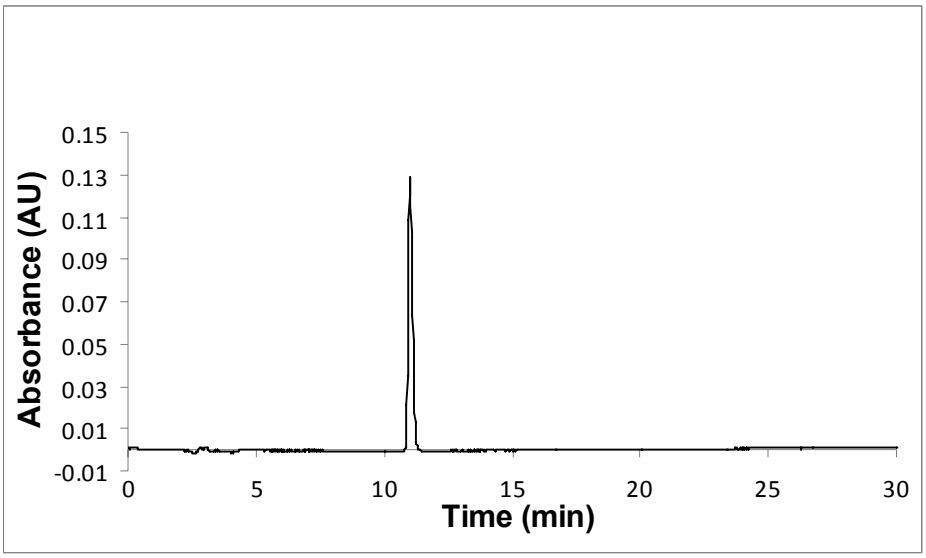


Figure S1. Analytical HPLC of glycopeptide **6**

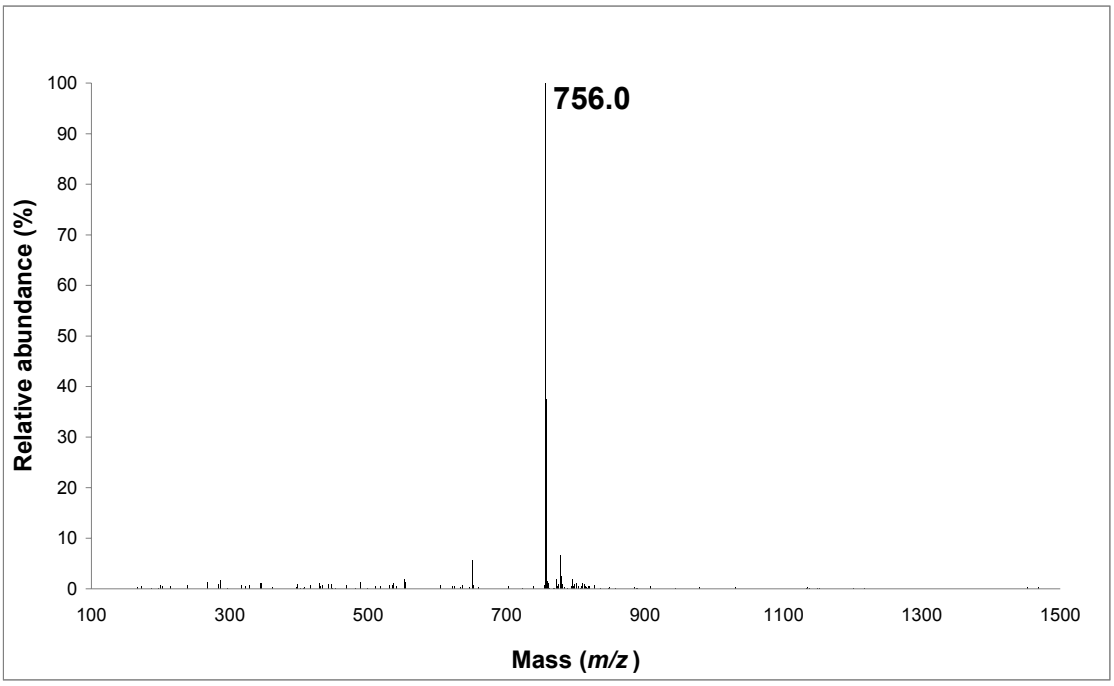
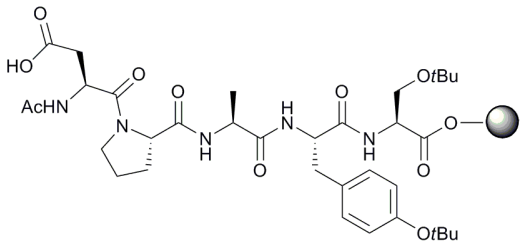


Figure S2. Electrospray mass specrtrum of glycopeptide **6**.

Synthesis of resin bound pentapeptide 7 (25 µmol scale)

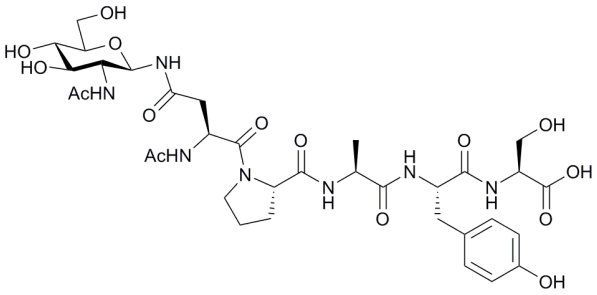


Fmoc-Pro-OH (34 mg, 100 µmol) was coupled to resin bound tripeptide **3**, using PyBOP (52 mg, 100 µmol) and NMM (22 µL, 200 µmol) in DMF (1 mL) for 1 h. Following *capping* and *Fmoc-deprotection* as described above Fmoc-Asp(ODmab)-OH (33 mg, 50 µmol) was coupled to the peptide using HATU (19 mg, 50 µmol) and NMM (11 µL, 100 µmol) in DMF (0.5 mL) for 2 h. The peptide was then *Fmoc-deprotected* as described above and the loading determined to be quantitative. The *N*-terminus was acetylated by treatment with Ac_2O/pyridine (5 mL, 1:9 v/v) for 5 minutes and the resin washed with DMF (5 x 3 mL), DCM (5 x 3 mL) and DMF (5 x 3 mL).

A solution of hydrazine monohydrate in DMF (2% v/v, 3 mL) was added to the resin, shaken for 3 minutes and the procedure repeated a further 4 times. Removal of the ivDde moiety was determined by measuring the UV absorbance at 290 nm until no further

indazole adduct could be detected. Following ivDde removal, the resin was treated with a 5mM solution of NaOH in H_2O/methanol (3 mL, 1:1 v/v). The resin was subsequently washed with methanol (5 x 3 mL), DMF (5 x 3 mL), DCM (5 x 3 mL) and DMF (5 x 3 mL).

Synthesis of *N*-linked glycopeptide Ac-Asn(β-GlcNAc)-Pro-Ala-Tyr-Ser-OH (9)



A solution of 2-acetamido-2-deoxy-β-D-glucopyranosylamine **5** (8.3 mg, 37.5 μmol), PyBOP (19.5 mg, 37.5 μmol) and NMM (8.3 μL, 75 μmol) in DMF (0.5 mL), was added to the resin-bound peptide **7** and shaken for 16 h. The resin was subsequently washed with DMF (10 x 3 mL) then DCM (10 x 3 mL).

A mixture of TFA/thioanisole/triisopropylsilane/water (17:1:1:1 v/v/v/v) was added to the resin and shaken for 1.5 hrs. The resin was then washed with TFA (3 x 3 mL), and the combined cleavage and washing solutions were concentrated *in vacuo*. The resulting residue was dissolved in DMSO (1.5 mL), purified by preparative HPLC and lyophilised to afford the desired glycopeptide **9** as a fluffy white solid (17.1 mg, 86%).

Analytical HPLC: R_t = 13.2 min; LCMS: R_t = 12.4 min, MS (ESI) *m/z* 796.1 [$(M+H)^+$, 100%]; HRMS Calcd for $C_{34}H_{49}N_7O_{15}Na$: MNa^+, 818.3179 found MNa^+, 818.3178.

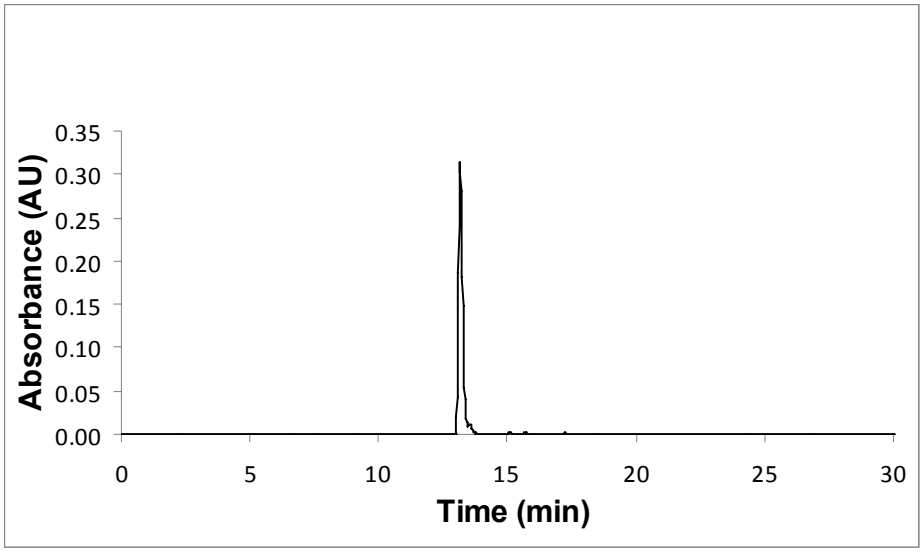


Figure S3. Analytical HPLC of glycopeptide **9.**

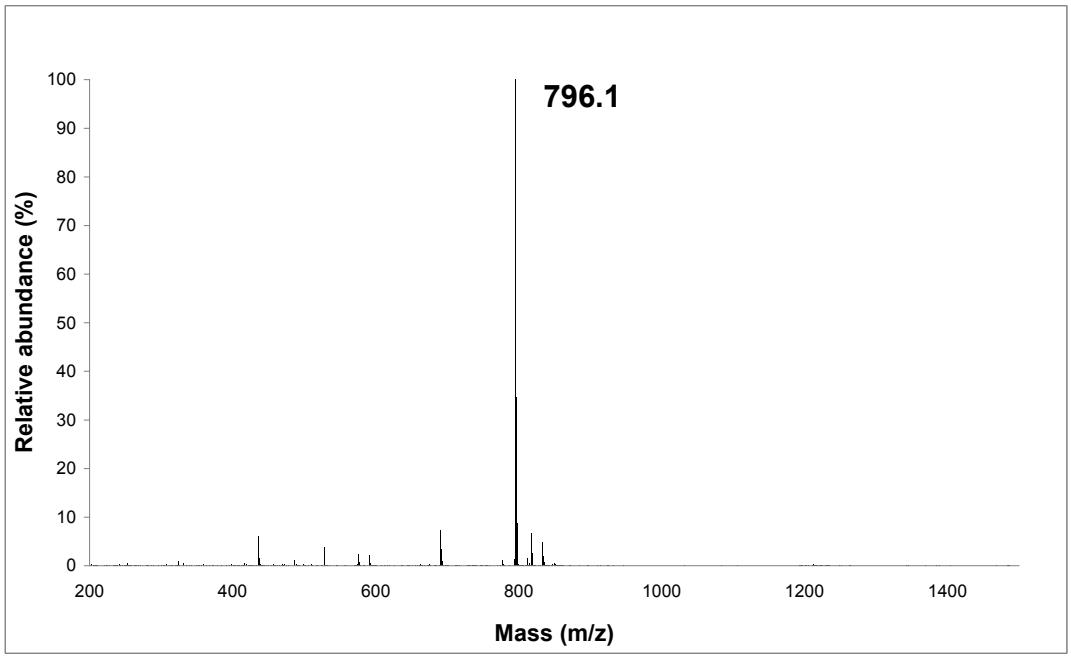
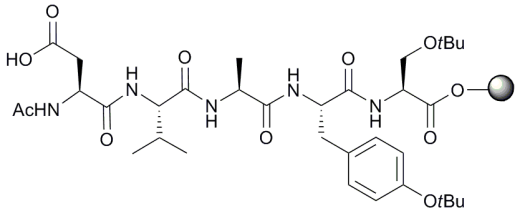


Figure S4. Electrospray mass spectrum of glycopeptide **9.**

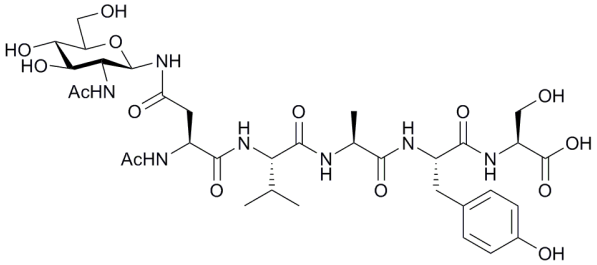
Synthesis of resin bound pentapeptide 8 (25 µmol scale)



Fmoc-Val-OH (34 mg, 100 µmol) was coupled to resin bound tripeptide **3**, using PyBOP (52 mg, 100 µmol) and NMM (22 µL, 200 µmol) in DMF (1 mL) for 1 h. Following *capping* and *Fmoc-deprotection* as described above Fmoc-Asp(ODmab)-OH (33 mg, 50 µmol) was coupled to the peptide using HATU (19 mg, 50 µmol) and NMM (11 µL, 100 µmol) in DMF (0.5 mL) for 2 h. The peptide was then *Fmoc-deprotected* as described above and the loading determined to be quantitative. The *N*-terminus was acetylated by treatment with Ac_2O/pyridine (5 mL, 1:9 v/v) for 5 minutes and the resin washed with DMF (5 x 3 mL), DCM (5 x 3 mL) and DMF (5 x 3 mL).

A solution of hydrazine monohydrate in DMF (2% v/v, 3 mL) was added to the resin, shaken for 3 minutes and the procedure repeated a further 4 times. Removal of the ivDde moiety was determined by measuring the UV absorbance at 290 nm until no further indazole adduct could be detected. Following ivDde removal, the resin was treated with a 5 mM solution of NaOH in H_2O/methanol (3 mL, 1:1 v/v) for 3 hours. The resin was subsequently washed with methanol (5 x 3 mL), DMF (5 x 3 mL), DCM (5 x 3 mL) and DMF (5 x 3 mL).

Synthesis of *N*-linked glycopeptide Ac-Asn(β-GlcNAc)-Val-Ala-Tyr-Ser-OH (10)



A solution of 2-acetamido-2-deoxy-β-D-glucopyranosylamine **5** (8.3 mg, 37.5 µmol), PyBOP (19.5 mg, 37.5 µmol) and NMM (8.3 µL, 75 µmol) in DMF (0.5 mL), was added to the resin-bound peptide **8** and shaken for 16 h. The resin was subsequently washed with DMF (10 x 3 mL) then DCM (10 x 3 mL).

A mixture of TFA/thioanisole/triisopropylsilane/water (17:1:1:1 v/v/v/v) was added to the resin and shaken for 1.5 hrs. The resin was then washed with TFA (3 x 3 mL), and the combined cleavage and washing solutions were concentrated *in vacuo*. The resulting residue was dissolved in DMSO (1.5 mL), purified by preparative HPLC and lyophilised to afford the desired glycopeptide **10** as a fluffy white solid (11.2 mg, 56%).

Analytical HPLC: R_t = 15.3 min, LCMS: R_t = 14.3 min; MS (ESI) *m/z* 798.1 [$(M+H)^+$, 100%]; HRMS Calcd for $C_{34}H_{51}N_7O_{15}Na$: MNa^+, 820.3354 found MNa^+, 820.3342.

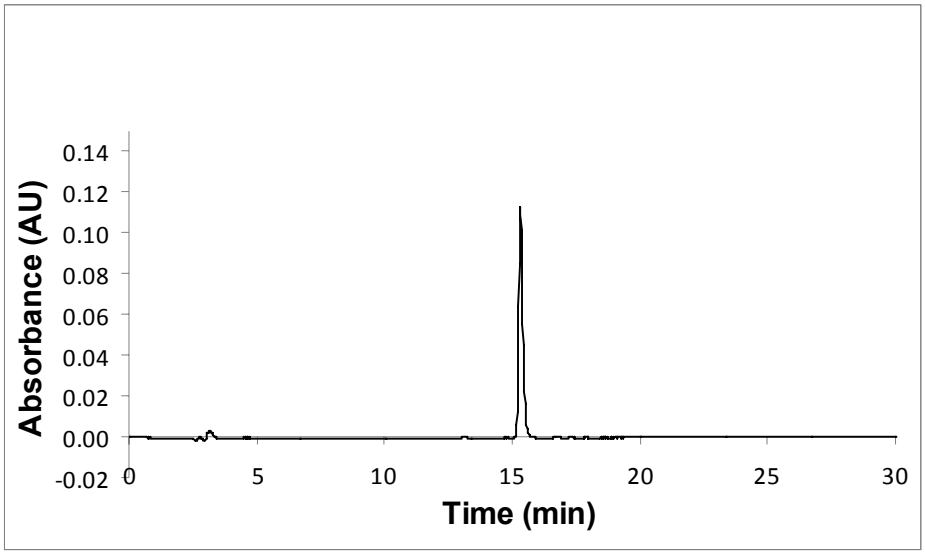


Figure S5. Analytical HPLC of glycopeptide **10**

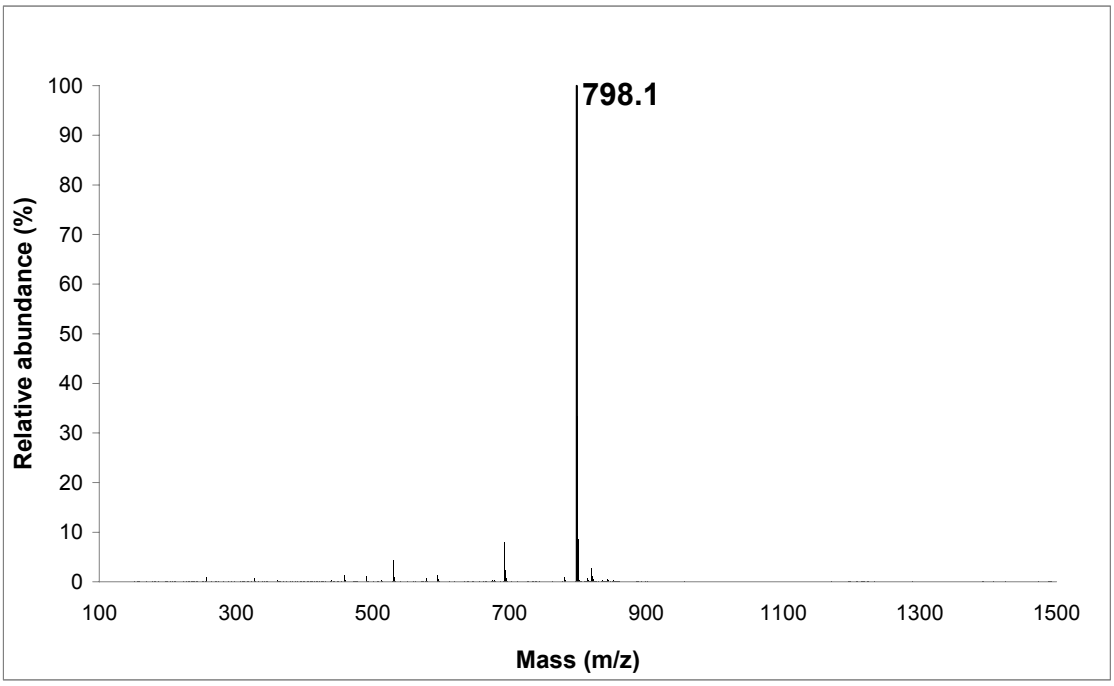
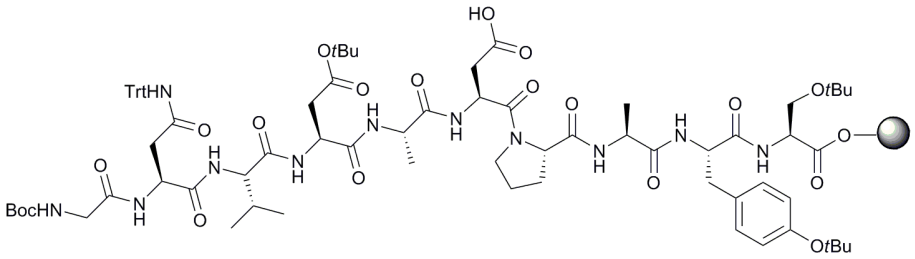


Figure S6. Electrospray mass spectrum of glycopeptide **10**.

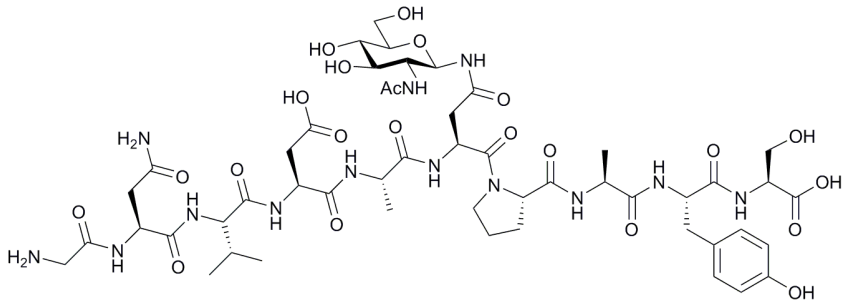
Synthesis of resin bound peptide 12 (12.5 µmol scale)



Fmoc-Pro-OH (17 mg, 50 µmol) was coupled to resin bound tripeptide **3** (12.5 µmol), using PyBOP (26 mg, 50 µmol) and NMM (11 µL, 100 µmol) in DMF (0.5 mL) for 1 h. Following *capping* and *Fmoc-deprotection* as described above Fmoc-Asp(ODmab)-OH (17 mg, 25 µmol) was coupled to the peptide using HATU (9 mg, 25 µmol) and NMM (6 µL, 50 µmol) in DMF (0.5 mL) for 2 h. The peptide was then *Fmoc-deprotected* as described above and the loading determined to be quantitative. Fmoc-Ala-OH, Fmoc-Asp(OtBu)-OH (50 µmol), Fmoc-Val-OH (50 µmol), Fmoc-Gln(Trt)-OH (50 µmol) and Boc-Gly-OH (50 µmol) were coupled to the peptide according to the standard Fmoc-SPPS procedure described above. A solution of hydrazine monohydrate in DMF (2% v/v, 3 mL) was added to the resin, shaken for 3 minutes and the procedure repeated a further 4 times. Removal of the ivDde moiety was determined by measuring the UV absorbance at

290 nm until no further indazole adduct could be detected. Following ivDde removal, the resin was treated with a 5 mM solution of NaOH in H_2O/methanol (3 mL, 1:1 v/v) for 3 hours. The resin was subsequently washed with methanol (5 x 3 mL), DMF (5 x 3 mL), DCM (5 x 3 mL) and DMF (5 x 3 mL).

Synthesis of *N*-linked glycopeptide NH_2-Gly-Asn-Val-Asp-Ala-Asn(β-GlcNAc)-Pro-Ala-Tyr-Ser-OH 11



A solution of 2-acetamido-2-deoxy-β-D-glucopyranosylamine **5** (4.2 mg, 18.8 μmol), PyBOP (9.8 mg, 18.8 μmol) and NMM (4.2 μL, 38 μmol) in DMF (0.5 mL), was added to resin-bound peptide **12** and shaken for 16 h. The resin was subsequently washed with DMF (10 x 3 mL) then DCM (10 x 3 mL).

A mixture of TFA/thioanisole/triisopropylsilane/water (17:1:1:1 v/v/v/v) was added to the resin and shaken for 1.5 hrs. The resin was then washed with TFA (3 x 3 mL), and the combined cleavage and washing solutions were concentrated *in vacuo*. The resulting residue was redissolved in water and purified by preparative HPLC and lyophilised to afford the desired glycopeptide **11** as a fluffy white solid (9.3 mg, 61%).

Analytical HPLC: R_t = 15.5 min, LCMS: R_t = 10.2 min; MS (ESI) *m/z* 1210.5 [$(M+H)^+$, 100%]; HRMS Calcd for $C_{50}H_{76}N_{13}O_{22}$: MH^+, 1210.5222 found MH^+, 1210.5248.

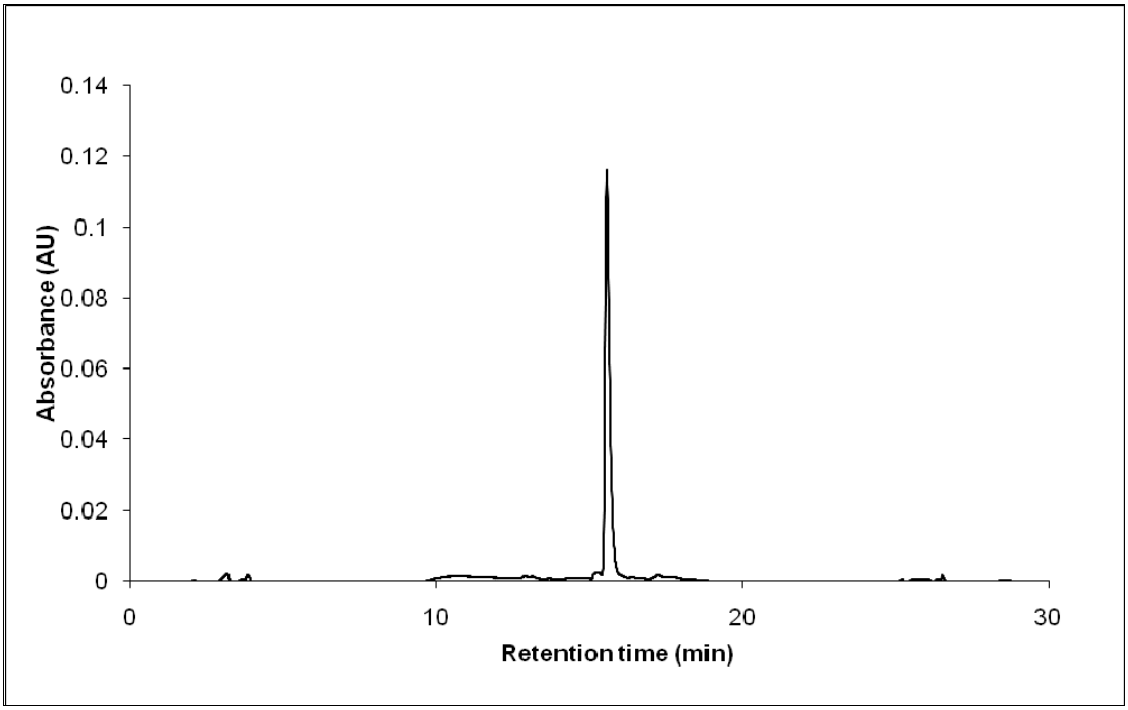


Figure S7. Analytical HPLC of glycopeptide **11**

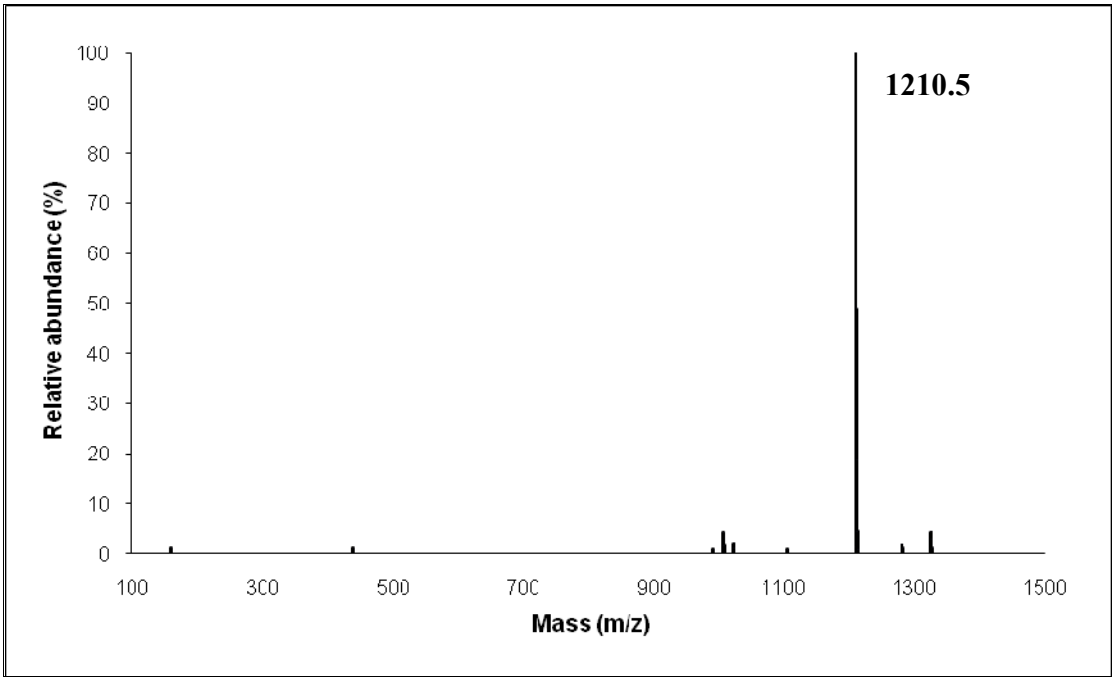
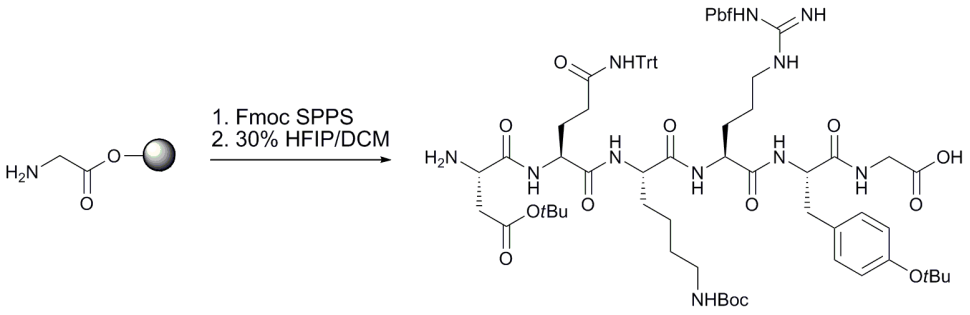


Figure S8. Electrospray mass spectrum of glycopeptide **11.**

Testing the stability of protecting groups in Fmoc strategy SPPS to the Dmab deprotection conditions (5mM NaOH)

A model hexapeptide was synthesised possessing a representative range of side chain protecting groups. The peptide was synthesised on 2-chlorotrityl resin which allowed for the peptide to be cleaved from the resin using the mild acid hexafluoroisopopanol (HFIP) leaving the side chain protecting groups intact (**13**, Scheme S1). Treatment of this peptide with 5mM NaOH in methanol and water for 12 hours (four times the period required for complete elimination of the aminobenzyl ester) followed by HPLC analysis showed that the protecting groups are stable to this low concentration of hydroxide (Figure S7). In addition, there was no detectable epimerisation, aspartimides or aspartimide related products formed under these conditions.



13

Scheme S1. Synthesis of model hexapeptide **13** on trityl-Cl resin

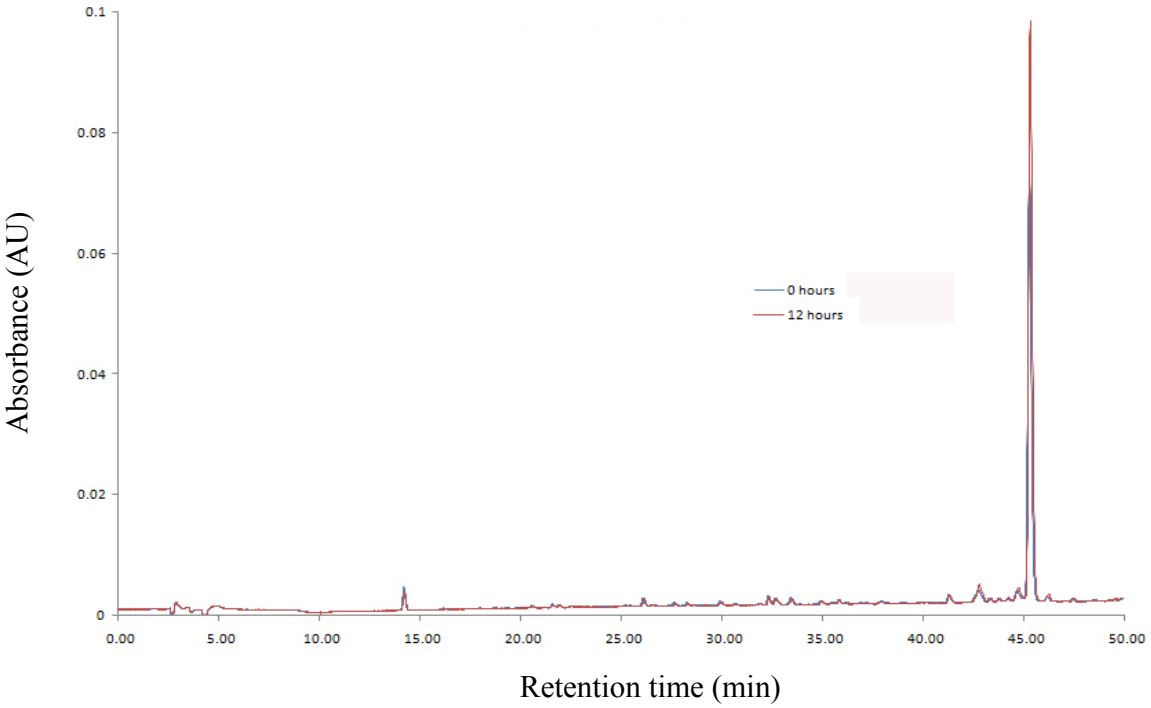
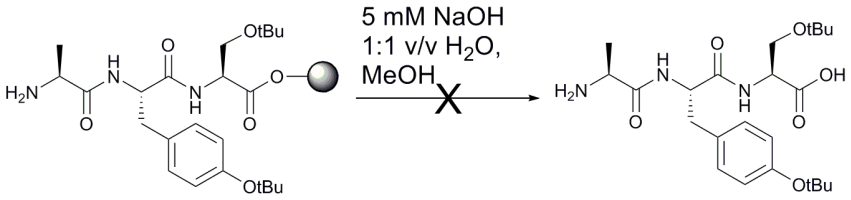


Figure S7: HPLC trace for aqueous NaOH treatment of the protected hexapeptide **13**.

Experimental:

The hexapeptide was synthesised from 2-chlorotrityl resin preloaded with glycine (50 µmol), using the standard Fmoc strategy procedure described above. Following its assembly, the resin was treated with a solution of 30% HFIP/DCM and shaken for 30 min. The resin was subsequently washed with DCM (3 x 2 mL), and the combined cleavage and washing solutions were concentrated *in vacuo* to afford the side chain protected hexapeptide (0.08 g, quant.), which was used without purification. The crude peptide (0.05 g) was added to a solution of 5 mM NaOH in MeOH/H_2O, (1:1 v/v, 6 mL) and analysed by analytical HPLC immediately and after 12 h. Analytical HPLC (2-75% B over 45 min): R_t = 45.4 min.

Testing the stability of the ester linkage to Wang resin under the Dmab deprotection conditions (5mM NaOH)



Scheme S2. Stability of ester linkage to Wang resin using 5 mM NaOH.

Experimental:

Resin bound peptide **3** was treated with a 5 mM solution of NaOH in H_2O/methanol (3 mL, 1:1 v/v) for 3 hours. This solution was then collected, concentrated *in vacuo* and analysed by LCMS. Tripeptide **14** was undetectable by UV (λ=280), HPLC or by mass spectrometry indicating that the ester linkage was stable to the Dmab deprotection conditions.

References:

1. L. M. Likhosherstov, O. S. Novikova, V. A. Derevitskaja and N. K. Kochetkov, *Carbohydrate Res.*, 1986, **146**, C1-C5.