Automated Glycopeptide Assembly by Combined Solid-Phase Peptide and Oligosaccharide Synthesis

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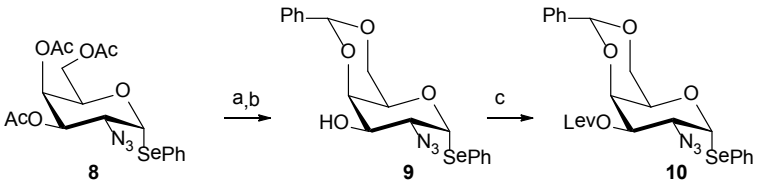
Supporting Information-Table of Contents

1. General Information

Solvents and reagents were used as supplied without any further purification. For use on the synthesizer, solvents were dried over molecular sieves until the water content was <50 ppm. NMR spectra were recorded on a Varian 400-MR (400 MHz) or 600 (600 MHz) spectrometer (Agilent). IR spectra were recorded on a Spectrum 100 FTIR spectrophotometer (Perkin–Elmer). Optical rotations were measured with a UniPol L 1000 polarimeter (Schmidt & Haensch, Berlin, Germany), with concentrations expressed in g per 100 mL. High resolution mass spectra were obtained with a 6210 ESI-TOF mass spectrometer (Agilent) with ES ionization (small organics), or an Amazon ETD ion trap mass spectrometer (Bruker). MALDI-TOF MS were obtained with an Autoflex Speed mass spectrometer (Bruker) with α-cyano-4-hydroxycinnamic acid. Analytical HPLC analysis was performed on Agilent 1200 series coupled to quadrupole-ESI LC/MS 6130 using Phenomenex Luna C_5 250 x 4.60 mm x 5 µm column. The same analytical gradient program was used for all runs, 50% B to 95% B in 25 min in a flow rate of 1 mL/min (A; TDW 0.1% formic acid, B; ACN 0.1% formic acid). Crude was purified on a preparative Agilent 1200 series HPLC using semi-preparative phenomenex Luna C_5 250 x 10 mm x 5 µm column. Gradient semi preparative system was used for all runs, 50% B to 95% B in 25 min in a flow rate of 5 mL/min (A; TDW 0.1% TFA, B; ACN 0.1% TFA).

2. Building Block and Linker Synthesis

2.1 Phenyl 2-Azido-4,6-O-benzylidene-2-deoxy-3-O-levulinoyl-1-seleno-D-galactopyranoside 10.



Scheme S1. Synthesis of Phenyl 2-Azido-4,6-*O*-benzylidene-2-deoxy-3-*O*-levulinoyl-1-seleno-D-galactopyranoside **10**. Conditions and reagents: a. NaOMe; b. benzaldehyde dimethylacetal, p-TsOH; c. 4-Oxopentanoic acid, DIC, DMAP

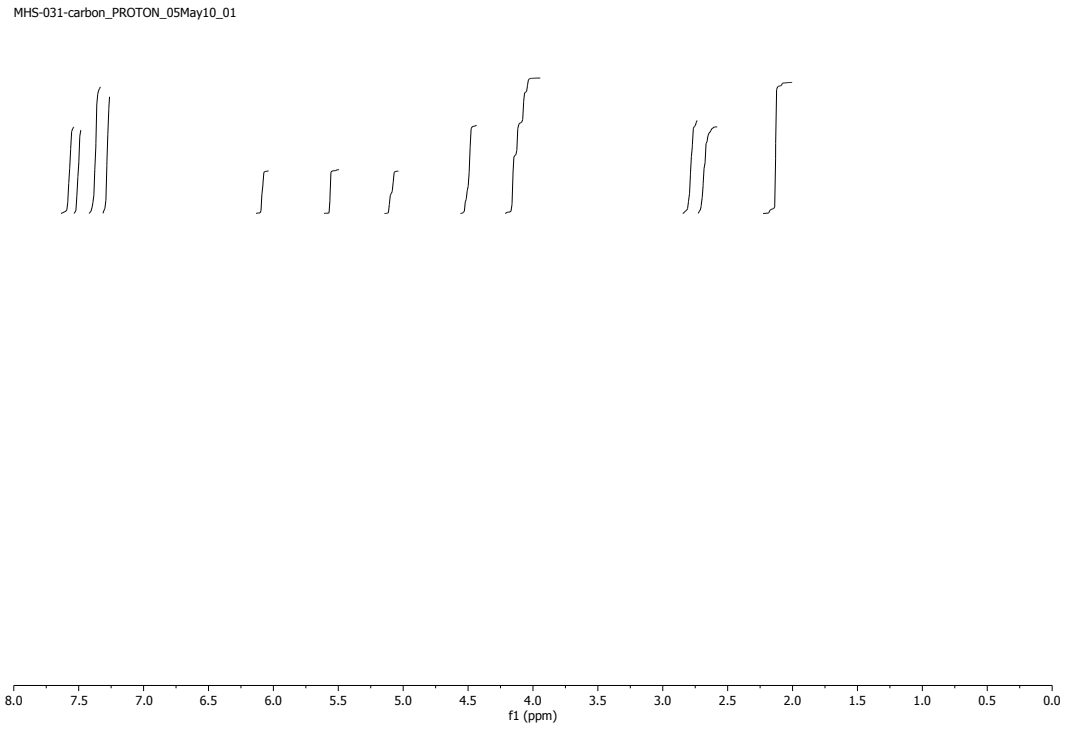
To a solution of **8**[1] in MeOH (100 mL) was added NaOMe (4.4 g, 9.3 mmol) and stirred for 4 hours at room temperature until no trace of starting material remained on TLC. The excess NaOMe was neutralized by the addition of Amberlite IR-120 (H^+ form) and solution was filtered and evaporated. The crude residue was co-evaporated twice with toluene and dissolved in acetonitrile. To this solution under Ar was added benzaldehyde dimethylacetal (4.2 mL, 28 mmol) and p-TsOH (0.2 g, 1 mmol) and the solution stirred at

room temperature overnight. The next morning, the reaction was quenched with Et_3N (2 mL) evaporated and purified by column chromatography over silica gel starting with cyclohexane:EtOAc 9:1 and ramped to 3:1 to elute compound **9**.

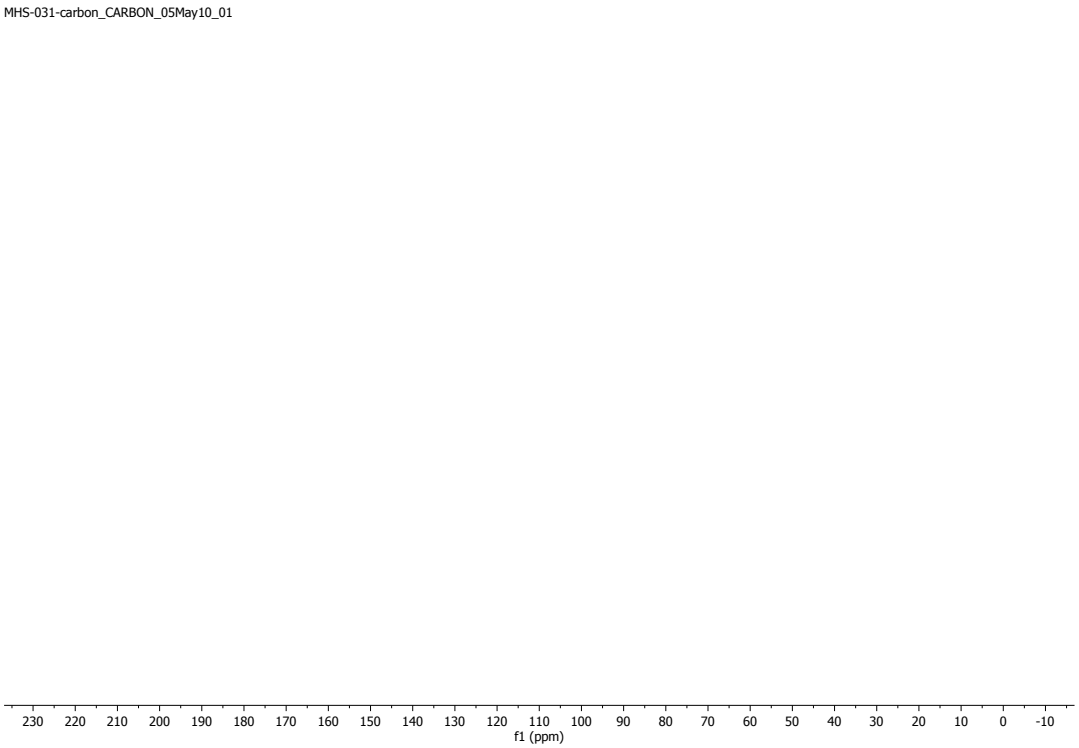
To a solution of **9** (1.7 g, 4 mmol) in CH_2Cl_2 cooled to on an ice bath under Ar was added 4-dimethylaminopyridine (4.8 g, 40 mmol), *N*,*N*'-diisopropylcarbodiimide (DIC) (6.8 mL, 40 mmol), and levulinic acid (4.5 mL, 44 mmol). The mixture was warmed to room temperature and left overnight under argon. The next morning, the crude residue was concentrated and purified by flash chromatography starting from 1:19 (EtOAc:cyclohexane) to 1:6 (EtOAc:cyclohexane) to elute compound **10**. Collected fractions were combined evaporated and left to dry in high vacuum. Impure fractions were crystallized from CH_2Cl_2/hexane to give pure compound **10** as yellow oil over three steps (1.8 g, 80%):

$[\alpha]^{20}_D$=+211.9 IR (neat) 2923, 2112; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 7.63 – 7.54 (m, 2H), 7.51 – 7.46 (m, 2H), 7.40 – 7.33 (m, 3H), 7.31 – 7.27 (m, 3H), 6.09 (d, *J*=5.3, 1H), 5.56 (s, 1H), 5.09 (dd, *J*=10.8, 3.4, 1H), 4.59 – 4.43 (m, 2H), 4.23 – 3.98 (m, 3H), 2.78 (dd, *J*=4.1, 3.3, 2H), 2.71 – 2.61 (m, 2H), 2.13 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm) = 205.93, 171.99, 137.33, 133.95, 129.20, 129.12, 128.19, 127.83, 126.15, 100.85, 84.89, 72.91, 72.42, 68.96, 64.78, 58.45, 37.81, 29.72, 28.09; ESI-HRMS. Calc for $[M+Na]^+$ $C_{24}H_{25}N_3NaO_6Se$ 554.0801, found 554.0804

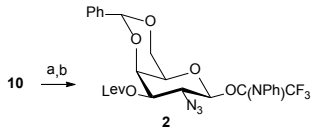
^{1}H NMR of 10 (400 MHz, $CDCl_3$)



^{13}C NMR of 10 (101 MHz, $CDCl_3$)



2.2 Azido-4,6-O-benzylidene-2-deoxy-3-O-levulinoyl-1-O-trifuoro-N-phenyl-imidate-D-galactopyranoside 2.

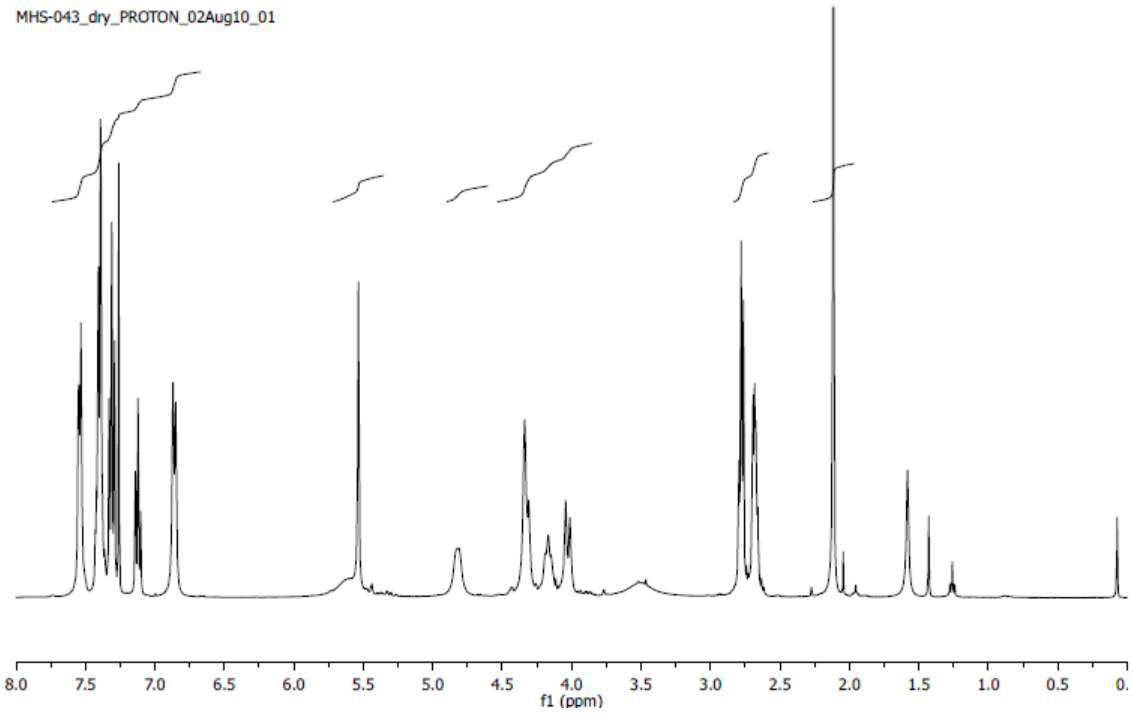


Scheme S2. Synthesis of 2-Azido-4,6-*O*-benzylidene-2-deoxy-3-*O*-levulinoyl-1-*O*-trifuoro-*N*-phenyl-imidate-D-galactopyranoside **2**. Reagents and conditions: a. NIS, acetone/H_2O; b. $CF_3C(NPh)Cl$, Cs_2CO_3

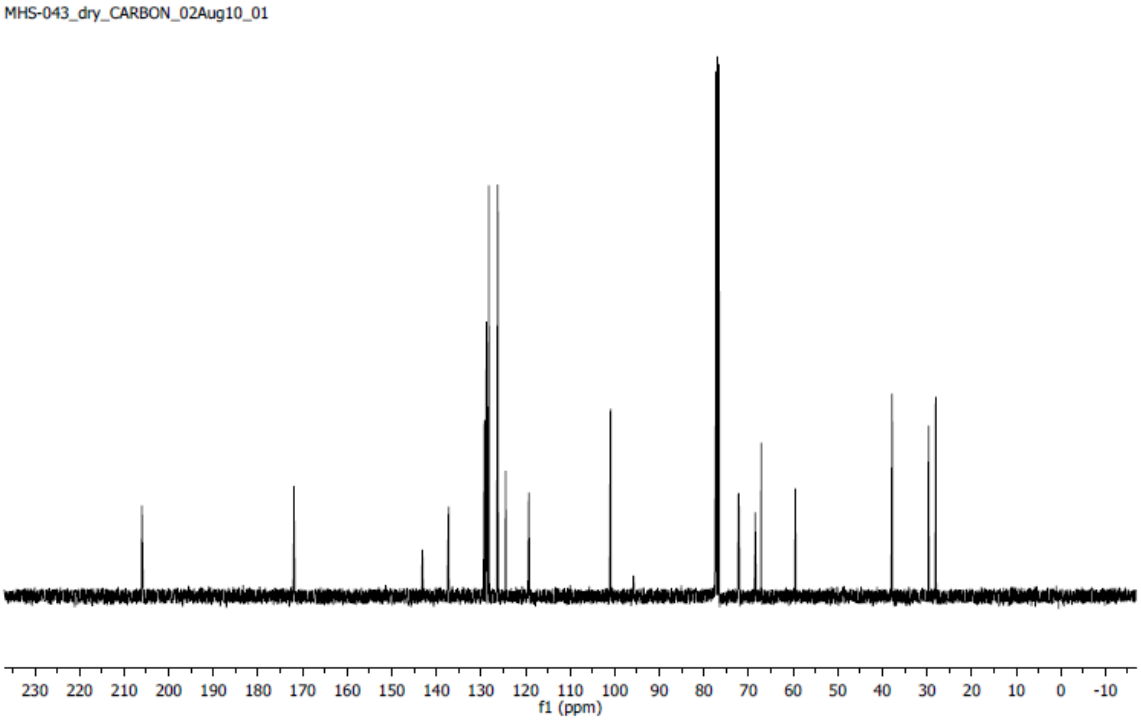
To a solution of **10** (0.7 g, 1.8 mmol) dissolved in a mixture of acetone/water (10 mL) was added NIS (0.8 g, 3.6 mmol) and the solution stirred at room temperature for 4 hours. The mixture was then diluted with CH_2Cl_2 (50 mL), and a mixture of sat. aq. $Na_2S_2O_3$ (15 mL) and sat. aq. $NaHCO_3$ (15 mL) was added and stirred for 30 min. The organic phase was separated, and the aqueous phase was extracted two more times with DCM. The combined organic phases were dried over $MgSO_4$, evaporated in vacuum, and co-evaporated twice with toluene. The crude oil was the dissolved in anhydrous CH_2Cl_2 (5 mL), cooled on an ice bath under Ar. To this solution was added 2,2,2-trifluoro-*N*-phenylacetimidoyl chloride (1.1 g, 5.3 mmol) and cesium carbonate (1.1 g, 2.6 mmol)

and the solution was left to warm slowly to room temperature. After ~2 h TLC indicated that the starting material was consumed at which point the reaction mixture was filtered through a pad of celite, washing with CH_2Cl_2. After evaporation of the solvent, the crude reside was purified over silica gel starting with 9:1 hexanes:EtOAc and ramping slowly to 3:1 (elution started in 6:1 and completed with 3:1) to afford compound **2** as a yellow foam over two steps (0.9 g, 89%): $[\alpha]^{20}_D$=+62.2; IR (neat) 2108,1754, 1725; ^{1}H NMR (400 MHz, CDCl3) δ (ppm) 7.47 (dd, *J* = 7.5, 2.0 Hz, 2H), 7.39 – 7.28 (m, 2H), 7.28 – 7.21 (m, 2H), 7.05 (m,, 1H), 6.79 (d, *J* = 7.7 Hz, 2H), 5.46 (s, 1H), 4.74 (bs, 1H), 4.26 (m, 2H), 4.14 – 4.05 (m, 1H), 3.96 (d, *J* = 12.1 Hz, 1H), 2.70 (m, 2H), 2.65 – 2.58 (m, 2H), 2.05 (s, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 205.99, 171.97, 137.24, 129.21, 128.73, 128.24, 126.23, 124.46, 119.26, 109.99, 100.95, 72.24, 67.15, 59.52, 37.82, 29.65, 28.03; ESI-HRMS. Calc for $[M+Na]^+$ $C_{26}H_{25}F_3N_4NaO_7$ 585.1568, found 585.1570

^{1}H NMR of 2 (400 MHz, $CDCl_3$)

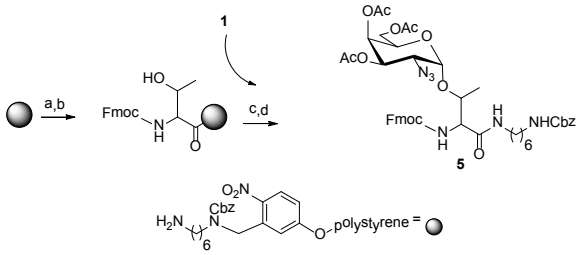


^{13}C NMR of 2 (101 MHz, $CDCl_3$)



3. Automated Synthesis of Glycopeptides

3.1 Automated Synthesis of Tn Antigen 5



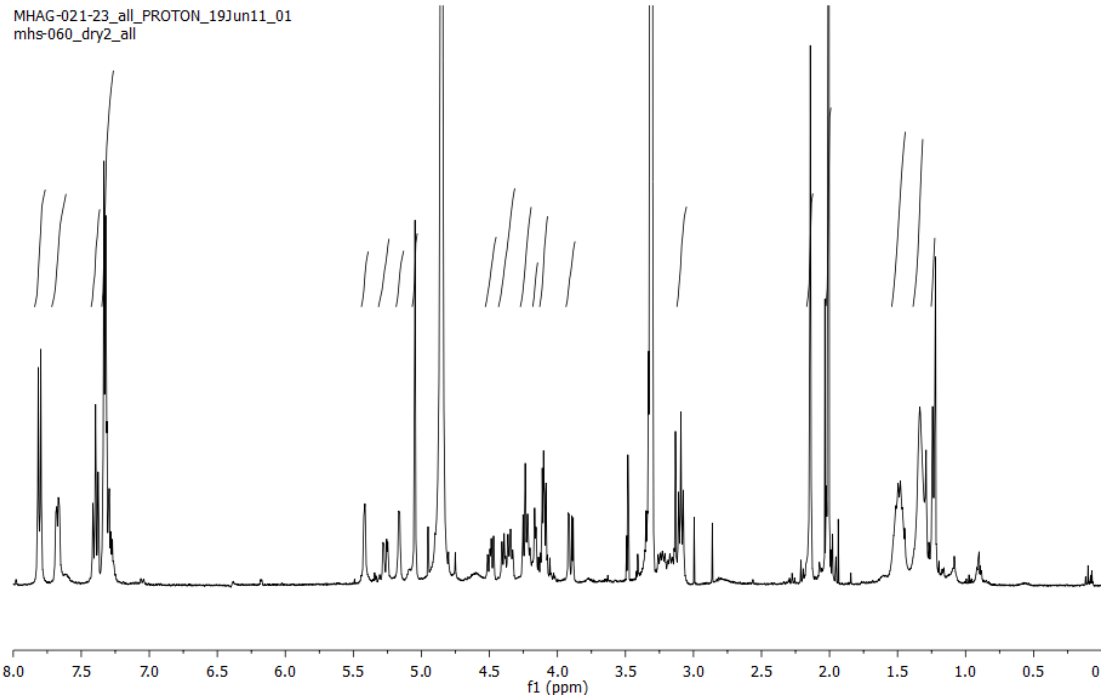
Scheme S3. Automated synthesis of Tn antigen **5**. Reagents and conditions: a. Fmoc-Thr(O*t*Bu)-OH, DIPEA, HBTU; b. TFA; c. TMSOTf, CH_2Cl_2/dioxane; d. *hv* 365nm.

Trifluoro-*N*-phenyl imidate **1**, was dried by repeated co-evaporation from toluene and left under high vacuum overnight. All solutions for the synthesis were prepared as described in the module appendix.

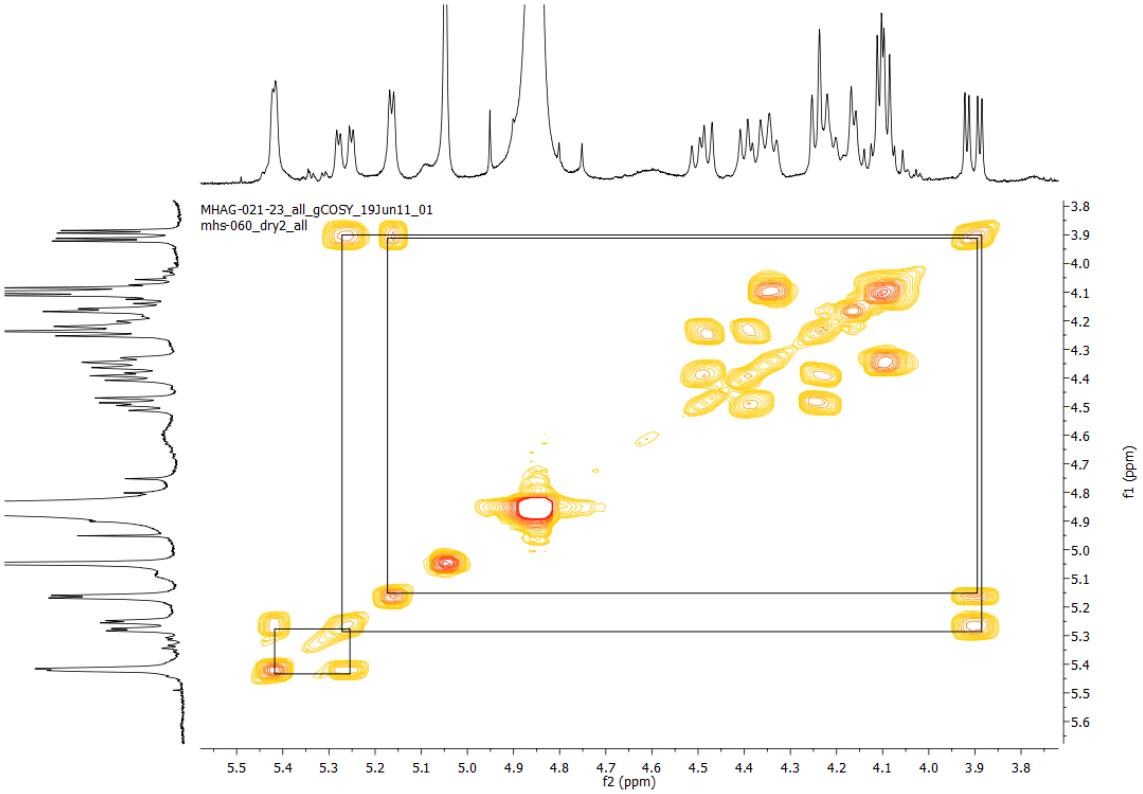
Linker-functionalized resin (loading 0.2 mmol/g, 125 mg, 25 μmol) was placed in the synthesizer and peptide synthesis was performed using Fmoc-Thr(O*t*-Bu)-OH and HBTU as described in module A. The *t*-BU protecting group was removed by treatment with

TMSOTf solution (module C) to afford a free hydroxyl group that was glycosylated with **1** and a catalytic amount of TMSOTf (module D) to give the resin bound glycopeptide. Glycopeptide **5** was the removed from the resin using a previously reported procedure.[2] The crude mixture was purified using RP-HPLC to give pure glycopeptide **5** as a yellow oil (3 mg, 13%): ^{1}H NMR (400 MHz, CD_3OD) δ (ppm) 7.81 (d, J = 7.5 Hz, 2H), 7.67 (d, J = 5.1 Hz, 2H), 7.40 (t, J = 7.5 Hz, 2H), 7.30 (ddd, J = 9.9, 8.0, 5.1 Hz, 7H), 5.42 (s, 1H), 5.26 (dd, J = 11.0, 3.2 Hz, 1H), 5.16 (d, J = 3.4 Hz, 1H), 5.05 (s, 2H), 4.49 (dd, J = 10.6, 6.8 Hz, 1H), 4.42 – 4.31 (m, 2H), 4.24 (t, J = 6.6 Hz, 2H), 4.16 (m, 1H), 4.10 (dd, J = 6.4, 4.3 Hz, 2H), 3.90 (dd, J = 11.1, 3.7 Hz, 1H), 3.09 (t, J = 6.9 Hz, 2H), 2.14 (s, 3H), 2.01 (s, 6H), 1.49 (m, 4H), 1.33 (m, 4H), 1.23 (d, J = 6.3 Hz, 3H); ESI MS: m/z calcd for $[M+Na]^+$ $C_{45}H_{54}N_6NaO_{13}$ 909.3, Found 909.2.

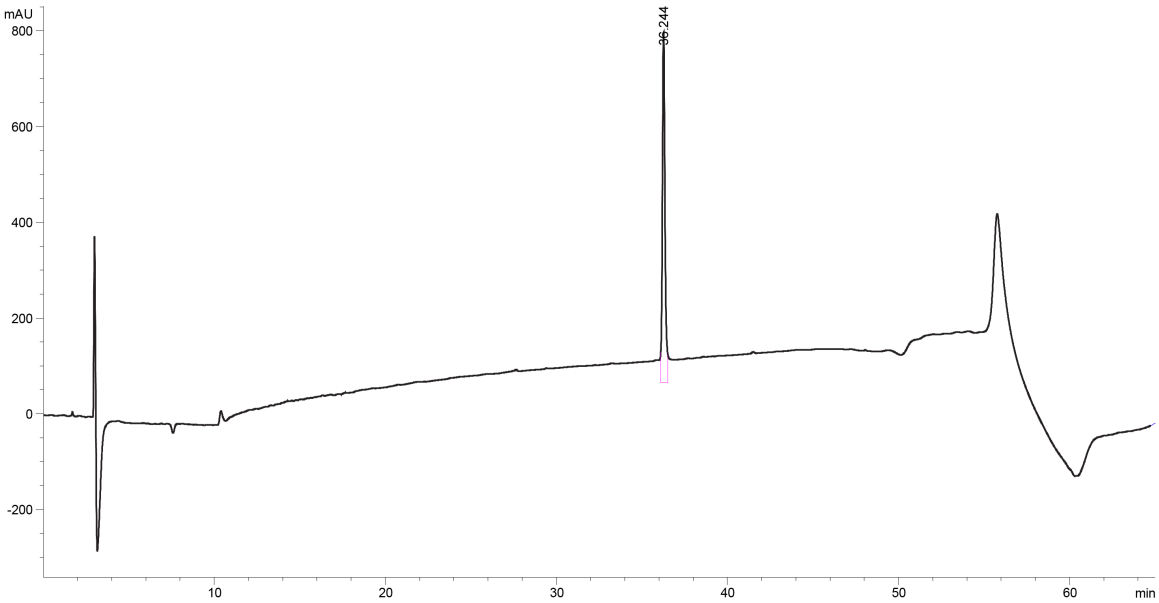
^{1}H NMR of Glycopeptide 5 (400 MHz, CD_3OD)



COSY NMR of Glycopeptide 5 (400 MHz, CD_3OD)

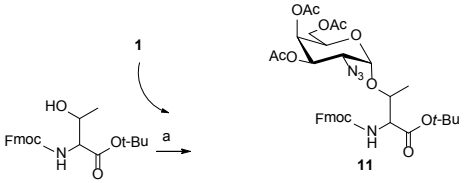


RP-HPLC Trace of 5



HPLC was performed using a Phenomenex C5 Luna column and a linear gradient of 5% to 95% ACN in TDW over 45 min (recorded at 220 nm). Glycopeptide **5** elutes at 36.2 min

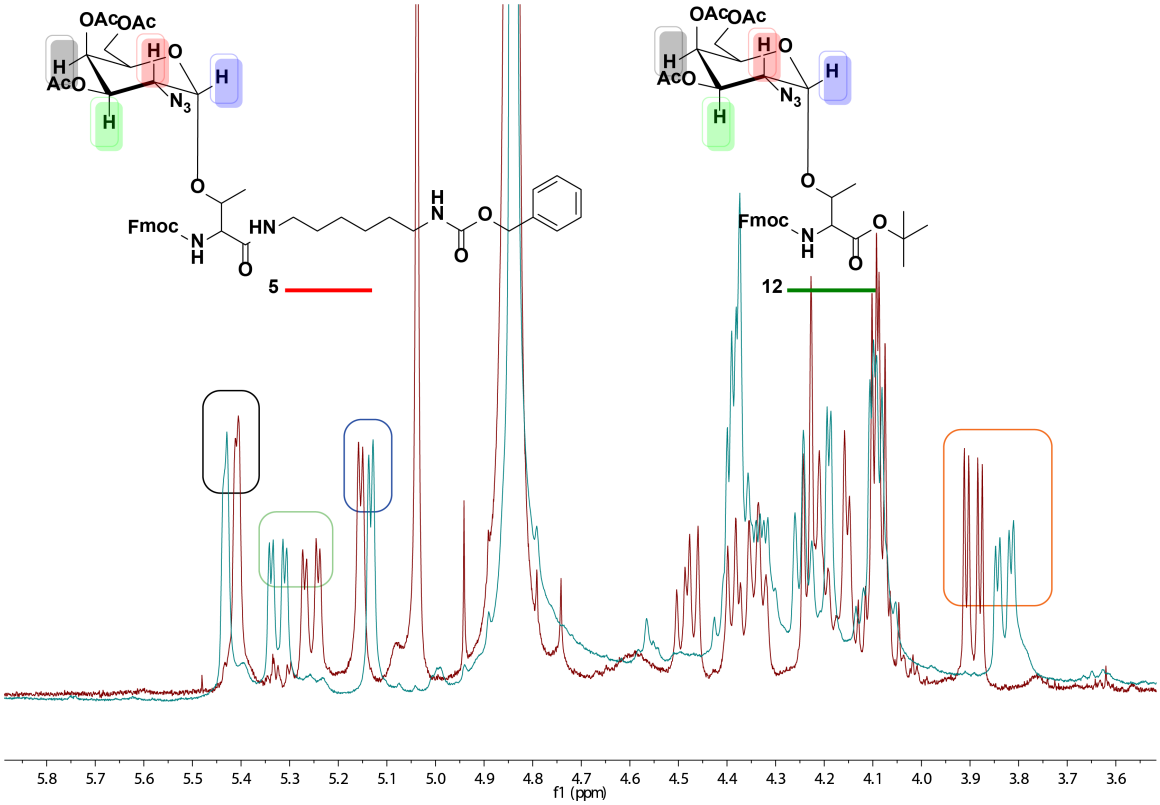
3.2 Synthesis of Tn antigen model 11



Scheme S4. Reagents and conditions: a. TMSOTf, CH_2Cl_2/ether -20 ˚C, 15 min.

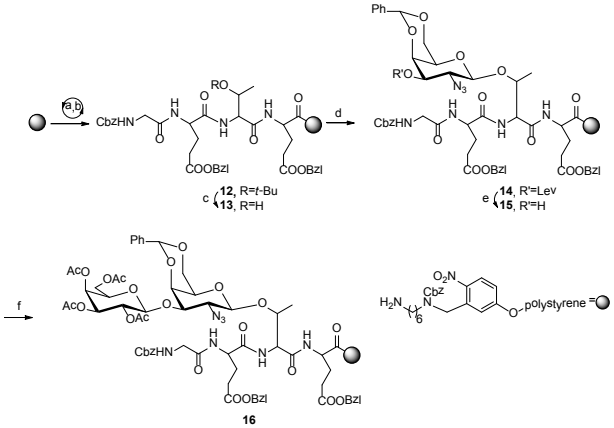
Tn antigen analog **11**,was synthesized following a previously described procedure.[3] The sugar patterns of **5** and **11** in the ^{1}H-NMR were compared. The similarities in the sugar patterns indicated that the desired stereoisomer was synthesized.

Overlay of ^{1}H NMRs of 5 and 11



The ^{1}H NMR of the two synthetic Tn antigen analogs **5** and **11** were compared. The sugar pattern represented by the different colors was similar in both cases confirming that the antigens are indeed identical.

3.3 Automated Synthesis of Glycopeptide 6

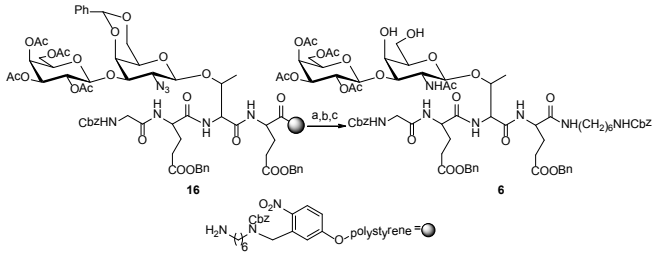


Scheme S5. Synthesis of glycopeptide **6.** Reagents and conditions. a. Amino acid, HBTU, DIPEA; b. Piperidine; c. TMSOTf (0.3 equiv); d. **2**, TMSOTf (0.2 equiv); e. hydrazine hydrate; f. **3**, NIS, TfOH.

Prior to automated synthesis, compounds **2** and **3**[4] were dried by repeated co-evaporation from toluene and left under high vacuum overnight. All solutions were prepared as described in the module appendix.

Linker-functionalized resin (loading 0.2 mmol/g, 125 mg, 25 μmol) was placed in the synthesizer and the peptide was elongated on a photocleavable linker repeating the amino acid coupling (module A) and Fmoc deprotection (module B) to give the fully protected tetrapeptide **12**. The protected peptide was treated eight times with TMSOTf solution (module C) to remove the *t*-Bu protecting group and afford **13**. The free hydroxyl group was subsequently glycosylated using **2** and TMSOTf as described in the imidate module to afford resin-bound glycopeptide **14**. The levulinate group was removed from **14** using hydrazine hydrate solution (module E) was and the free hydroxyl group glycosylated with **3** and NIS/TfOH (Module F) to provide resin-bound compound **16**.

To confirm the completion of each reaction, small amounts of resin were irradiated after each step to provide a sufficient amount of material for HPLC/MS analysis. HPLC traces of some of these crucial steps are presented in the next section.

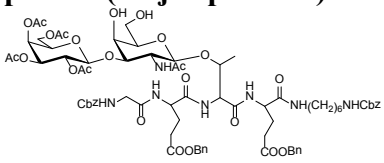


Scheme S6. Reagents and conditions: a. AcSH; b. TFA; c. *hv* 365nm.

After completion of the automated synthesis of **16**, the resin was transferred to a fritted syringe and incubated with AcSH for 48 h using previously reported methods.[5] After the resin was thoroughly washed, trifluoroacetic acid (TFA) solution (20% in CH_2Cl_2) was added at room temperature. After 30 min the resin was washed with CH_2Cl_2/MeOH and dried under vacuum.

The entire resin was exposed to UV irradiation in a flow reactor as previously described.[2] The collected crude solution was evaporated, dissolved in 1:1 ACN/H_2O, and purified using semi-preparative HPLC. The combined fractions were lyophilized to afford glycopeptide **6** as a yellow oil (2.2 mg, 5.7%) over ten automated steps.

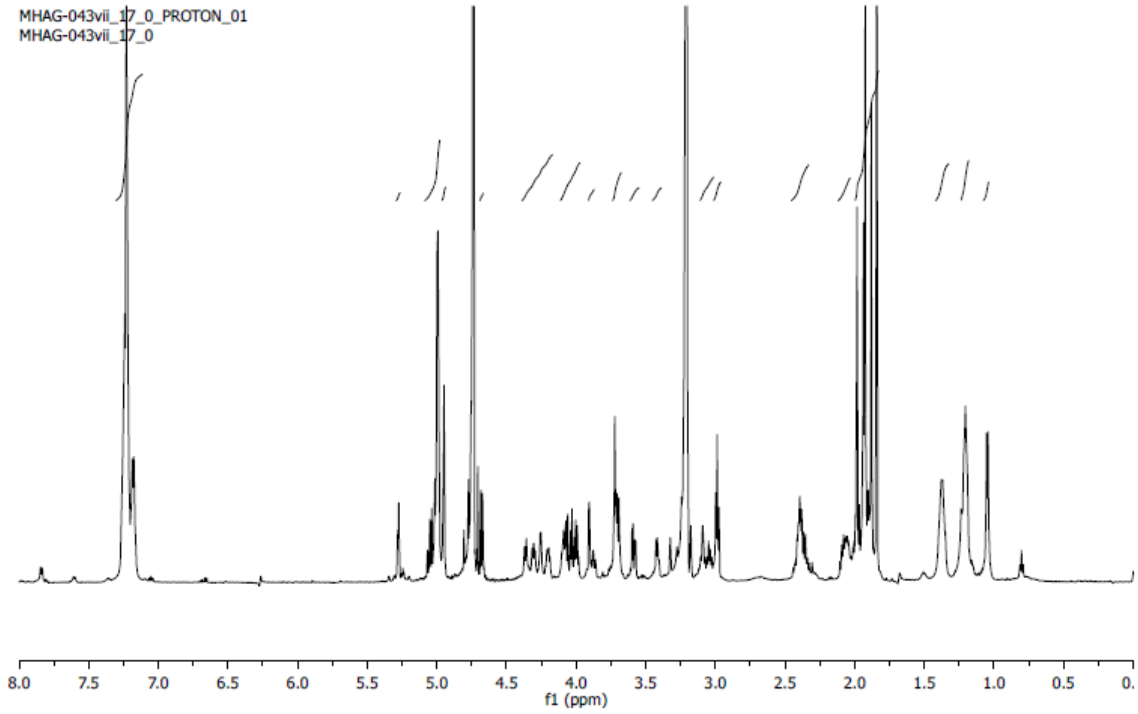
Characterization of Glycopeptide 6 (major product)



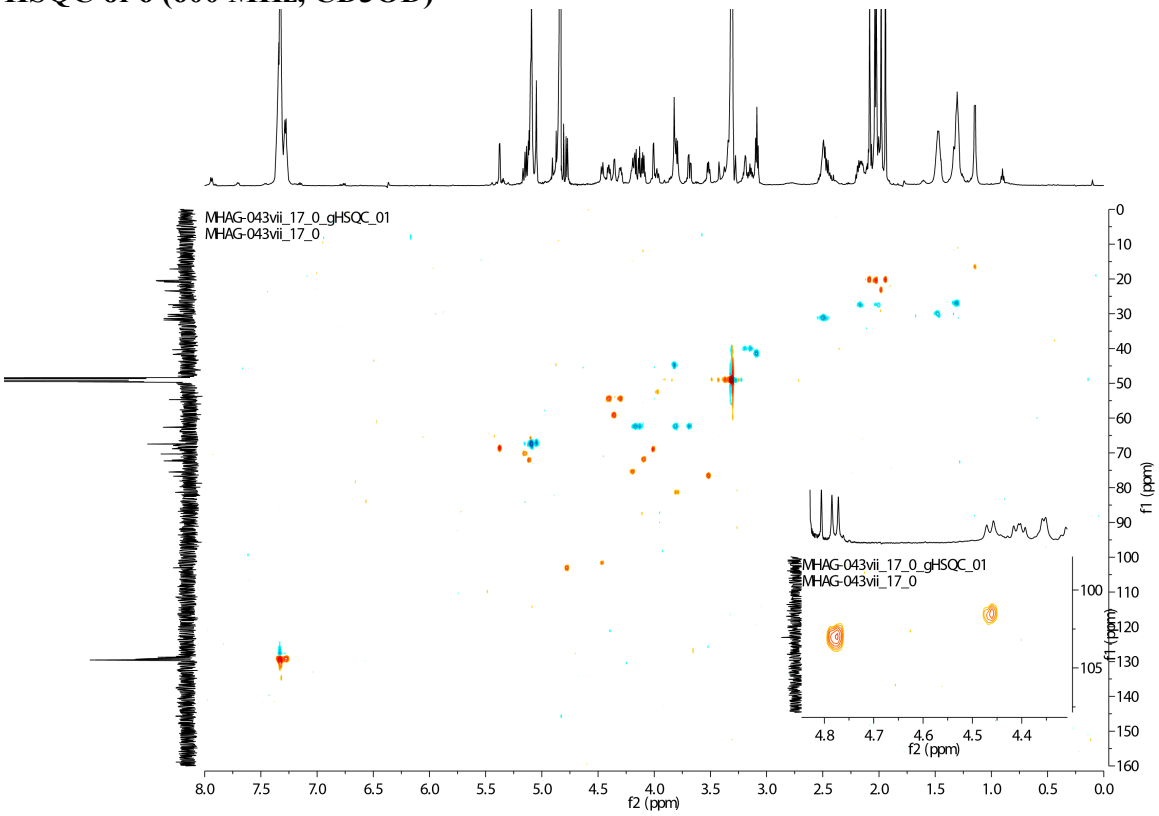
6

Analytical Data for compound **6**: ^{1}H NMR (600 MHz, CD_3OD) δ (ppm) 7.31 (m, 20H), 5.38 (d, J = 3.3 Hz, 1H), 5.19 – 5.02 (m, 10H), 4.78 (d, J = 7.6 Hz, 1H), 4.46 (d, J = 8.2 Hz, 1H), 4.43 – 4.37 (m, 1H), 4.36 (d, J = 3.9 Hz, 1H), 4.34 – 4.27 (m, 1H), 4.23 – 4.05 (m, 4H), 4.03 – 3.93 (m, 2H), 3.86 – 3.78 (m, 5H), 3.74 – 3.62 (m, 1H), 3.57-3.48 (m, 1H), 3.25 – 3.13 (m, 1H), 3.09 (t, J = 7.0 Hz, 2H), 2.60 – 2.37 (m, 4H), 2.24 – 1.88 (m, 19H), 1.49 – 1.44 (m, 4H), 1.35 – 1.27 (m, 4H), 1.15 (d, J = 6.2 Hz, 3H); ^{13}C NMR (151 MHz, CD_3OD) δ (ppm) 174.56, 173.20, 171.86, 171.36, 166.16, 129.54, 129.49, 129.45, 129.33, 129.23, 129.19, 129.14, 129.03, 128.94, 128.91, 128.75, 102.80, 101.32, 81.36, 76.70, 75.51, 72.31, 72.06, 70.32, 69.05, 68.81, 67.90, 67.46, 67.28, 66.34, 62.25, 65.22, 59.04 54.68, 54.71, 44.65, 41.71, 40.35, 31.80, 31.37, 30.75, 30.19, 28.16, 27.69, 27.63, 27.46, 27.40, 23.40, 20.88, 20.62, 20.47, 20.45, 17.15; ESI HR-MS: m/z calc for $[M+Na]^+$ $C_{74}H_{95}N_7NaO_{27}$ 1536.6169, found 1536.6155.

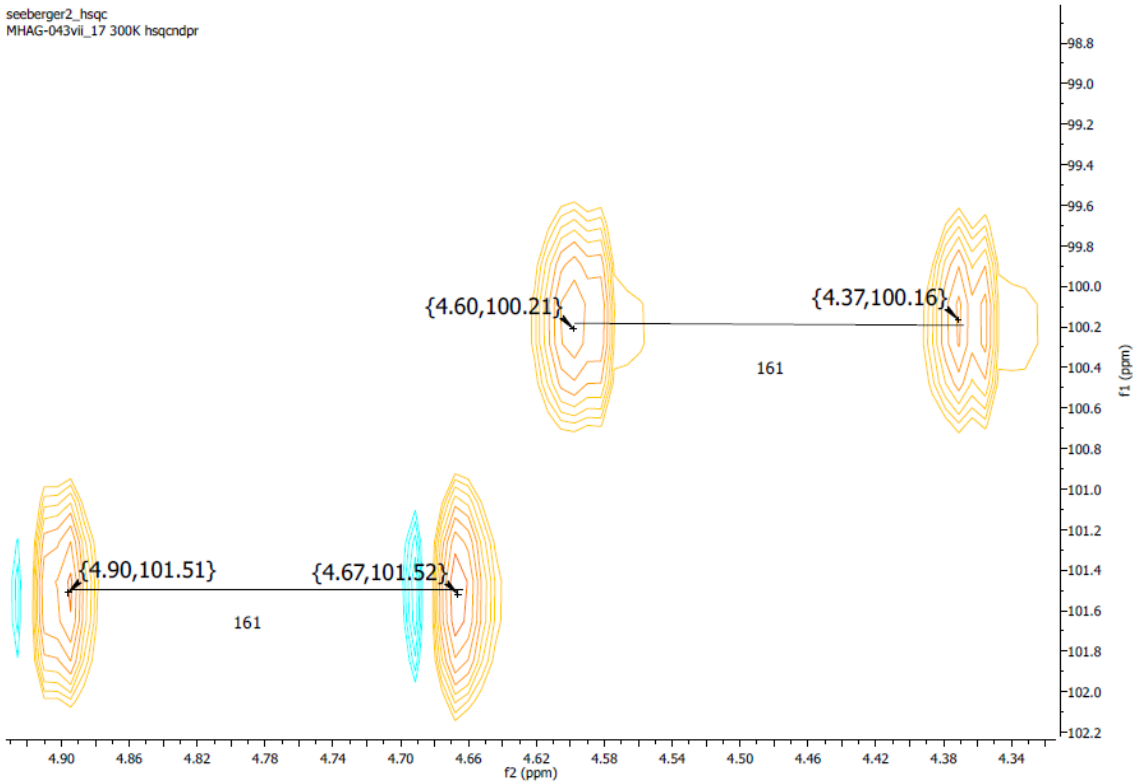
^{1}H NMR of 6 (600 MHz, CD_3OD)



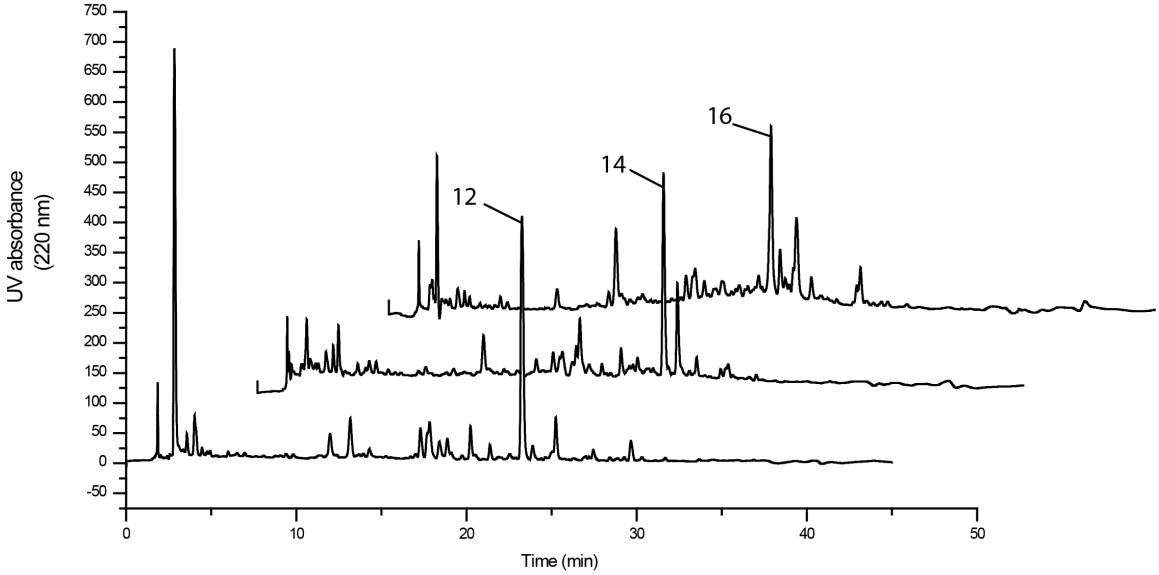
HSQC of 6 (600 MHz, CD3OD)



Coupled HSQC NMR of 6, Anomeric Region Zoom (700 MHz, CD_3OD)

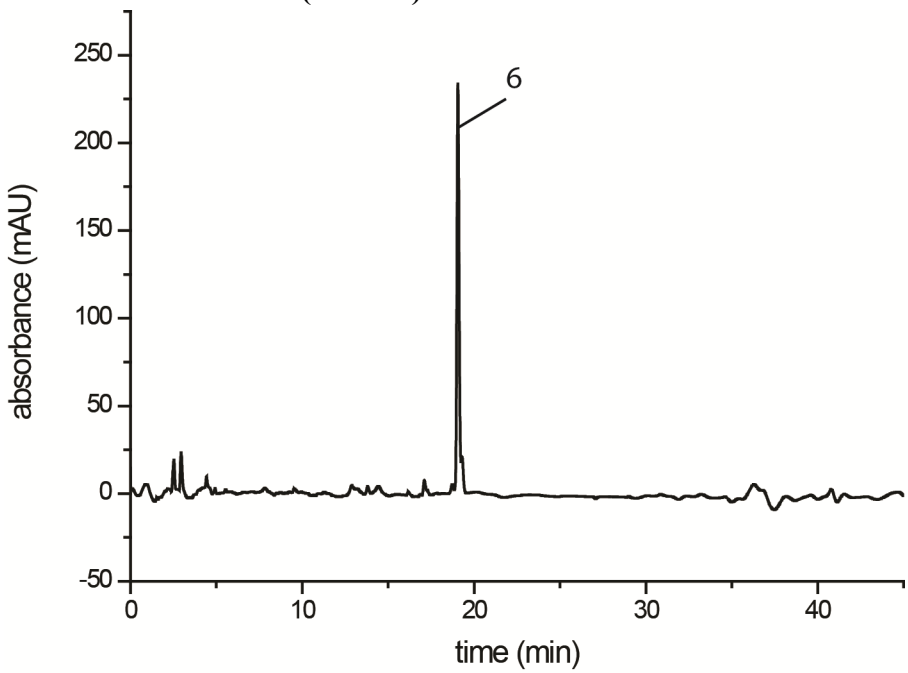


RP-HPLC Trace of Intermediates



HPLC was performed using a Phenomenex C5 Luna column and a linear gradient of 50% to 95% ACN in TDW over 45 min (recorded at 220 nm). The retention time indicates the advance in the synthesis as was confirmed also by coupled mass spectrometry analysis. Peptide **12** elutes at 18.7 min while glycopeptides **14** And **16** elutes in 23.85 and 22.45 min, respectively.

RP-HPLC Trace of 6 (220 nm).



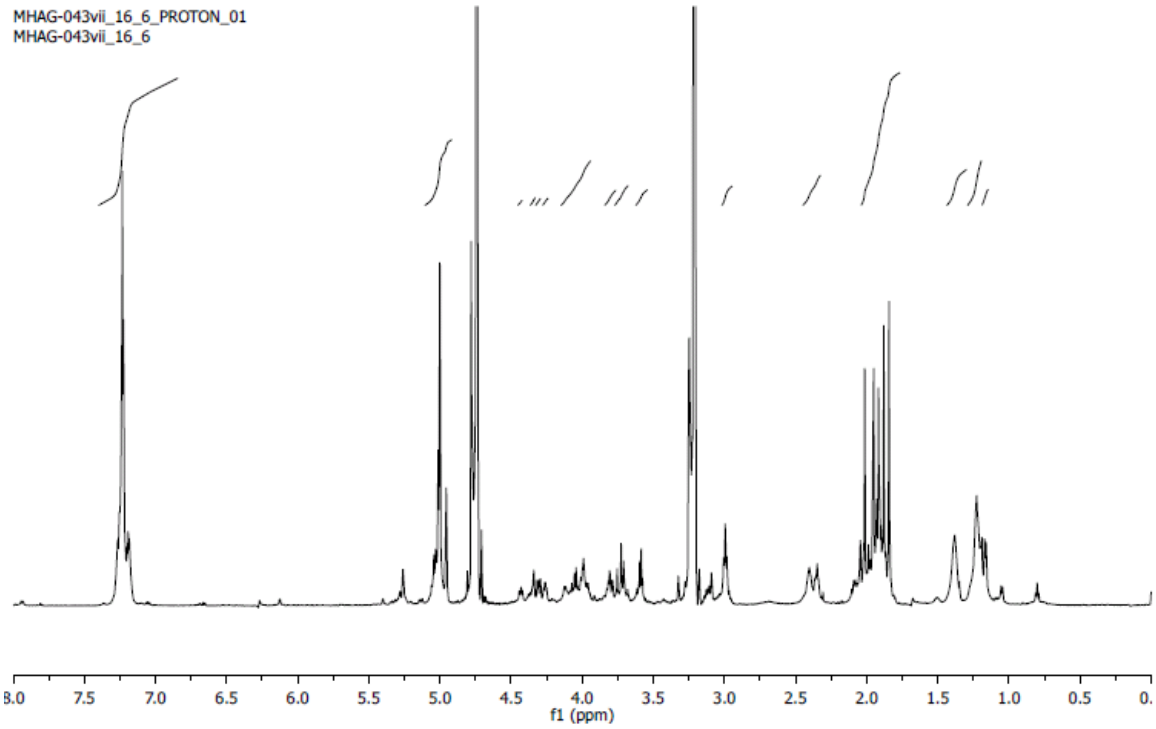
HPLC was performed using a Phenomenex C5 Luna column and a linear gradient of 50% to 95% ACN in TDW over 45 min (recorded at 220 nm). Glycopeptide **6** elutes at 18.66 min.

Characterization of Glycopeptide 12 (minor product)

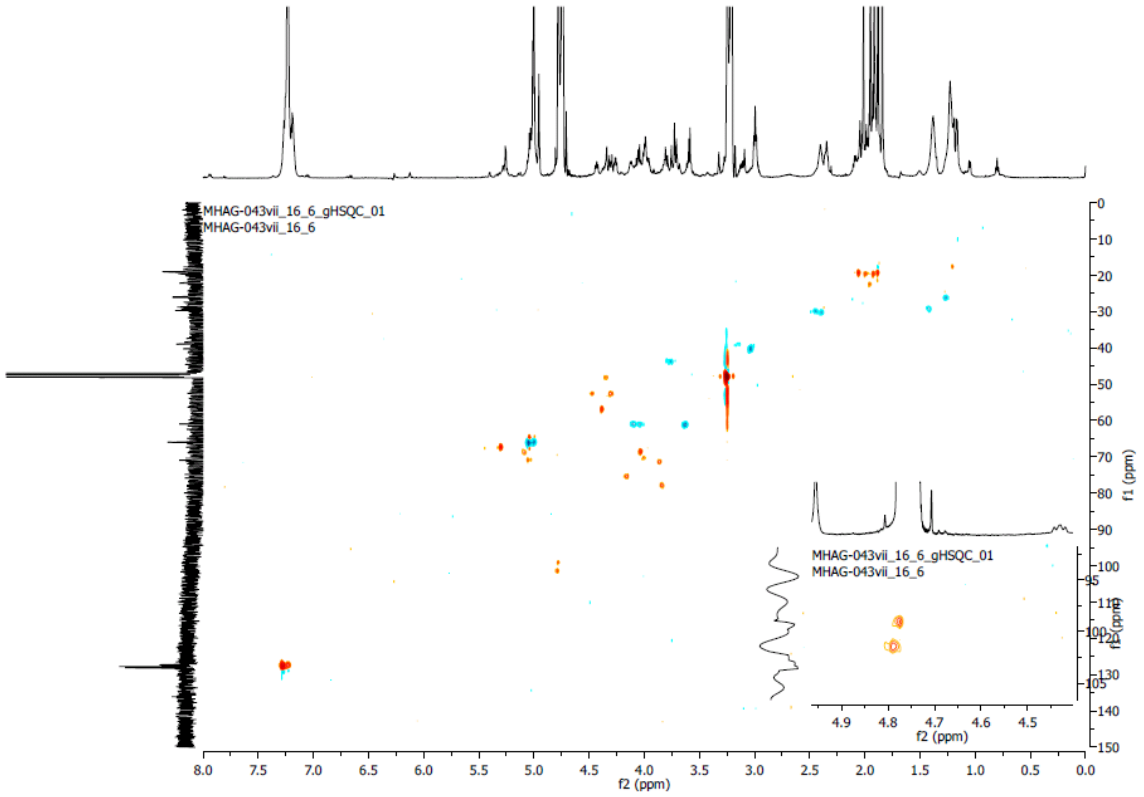
A minor side product with the same molecular weight as the desired product was also isolated and characterized. Using coupled HSQC, it was determined that this product contained a Gal-β(1→3)GalNAc-α(1-peptide) linkage.

Analysis of compound **6'** (minor product): Yield 2.2 mg, 6%; ^{1}H NMR (600 MHz, CD_3CD) δ (ppm) 7.38 – 7.26 (m, 19H), 5.36 (d, *J* = 3.1 Hz, 1H), 5.18 – 5.03 (m, 12H), 4.55 – 4.51 (m, 1H), 4.47 – 4.44 (m, 1H), 4.43 – 4.39 (m, 1H), 4.38 – 4.35 (m, 1H), 4.26 – 4.04 (m, 7H), 3.96 – 3.76 (m, 5H), 3.72 – 3.64 (m, 2H), 3.19 (dd, *J* = 2.0, 1.1 Hz, 1H), 3.12 – 3.07 (m, 3H), 2.53 – 2.48 (m, 2H), 2.47 – 2.44 (m, 2H), 2.26 – 1.81 (m, 30H), 1.54 – 1.42 (m, 6H), 1.38 – 1.26 (m, 12H); ESI HR-MS: m/z calc for $[M+Na]^+$ $C_{74}H_{95}N_7NaO_{27}$ 1536.6169 found 1536.6168

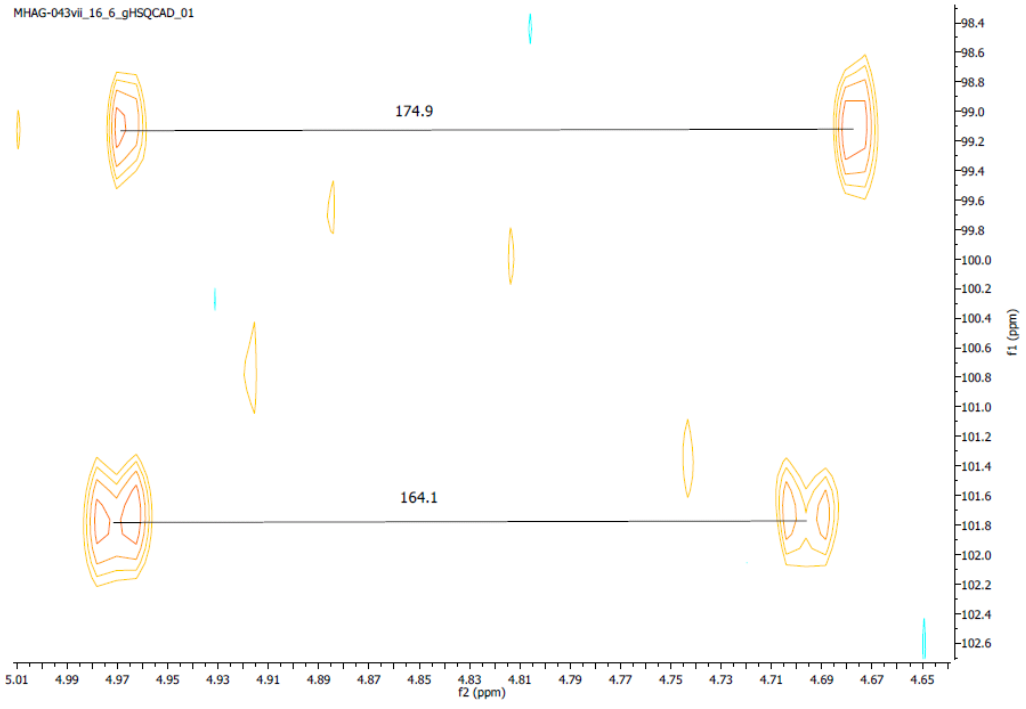
^{1}H NMR of 12 (600 MHz, CD_3CD)



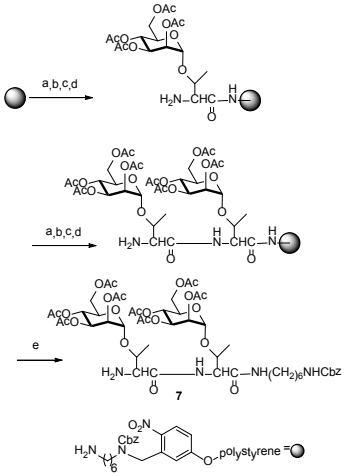
HSQC NMR of 12 (600 MHz, CD_3CD solution)



Coupled HSQC NMR of 12 Anomeric Region Zoom (700 MHz, CD_3OD)



3.4 Automated Synthesis of Glycopeptide 7

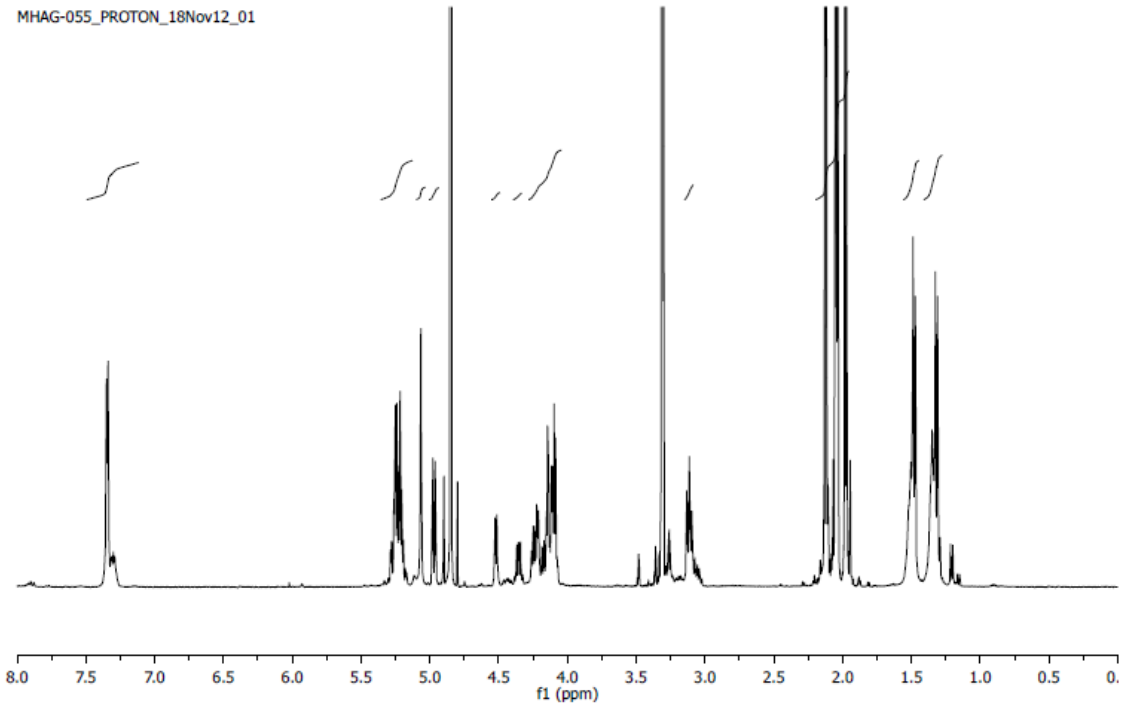


Scheme S7. Reagents and conditions: a. Fmoc-Thr(OTrt)-OH, HBTU, DIPEA; b. TMSOTf, (0.2 equiv) -30 ˚C; c. **4**, NIS,TfOH -40→-20 ˚C; d. Piperidine; e. *hv* 365nm.

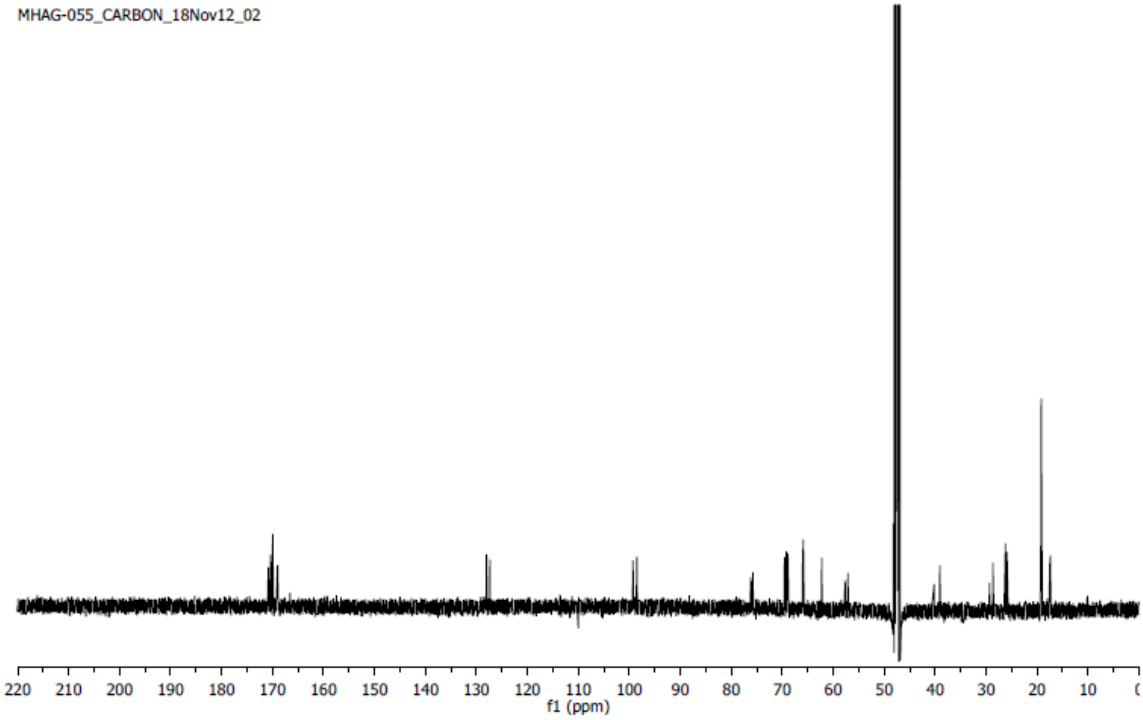
Prior to the automated synthesis, compound **4** was dried by repeated co-evaporation with toluene and left under high vacuum overnight. All other solutions were prepared as described in the module appendix.

Linker-functionalized resin (loading 0.2 mmol/g, 125 mg, 25 μmole) was placed in the synthesizer and peptide coupling using Fmoc-Thr(OTrt)-OH and HBTU performed (module A). The trityl group was cleaved with a catalytic amount of TMSOTf at -30 °C (module G). The free hydroxyl group was then glycosylated using **4** and NIS/TfOH (module E). The Fmoc protecting group was removed using a solution of piperidine (module B) and all steps were repeated one more time. Cleavage from the resin using UV irradiation yielded the desired diglycopeptide in over 80% purity. Purification of the crude residue afforded compound 7 as colorless oil over eight automated steps (3.1 mg, 14%) ^{1}H NMR (400 MHz, CD_3OD) δ (ppm) 7.34 (d, J = 4.4 Hz, 5H), 5.30 – 5.16 (m, 6H), 5.07 (s, 2H), 4.98 (d, J = 1.6 Hz, 1H), 4.96 (d, J = 1.7 Hz, 1H), 4.52 (d, J = 5.0 Hz, 1H), 4.35 (dd, J = 6.5, 4.3 Hz, 1H), 4.28 – 4.07 (m, 8H), 3.12 (dd, J = 10.2, 4.3 Hz, 2H), 2.13 (s, 3H), 2.12 (s, 3H), 2.05 (s, 3H), 2.04 (s, 6H), 2.03 (s, 3H), 1.98 (s, 3H), 1.97 (s, 3H), 1.49 (t, J = 6.5 Hz, 7H), 1.38 – 1.29 (m, 7H); ^{13}C NMR (101 MHz, CD_3OD) δ (ppm) 172.26, 172.22, 171.99, 171.83, 171.42, 171.32, 170.46, 168.00, 129.47, 128.96, 128.79, 100.74, 99.97, 77.63, 77.20, 70.99, 70.67, 70.59, 70.48, 70.44, 70.35, 67.45, 67.32, 63.71, 63.69, 59.13, 58.59, 41.66, 40.54, 30.80, 30.13, 27.66, 27.37, 20.72, 20.69, 20.64, 20.62, 20.59, 18.94, 18.83; ESI HR-MS: m/z calc for $[M+H]^+$ $C_{50}H_{73}N_4O_{24}$ 1113.4610, found 1113.4533.

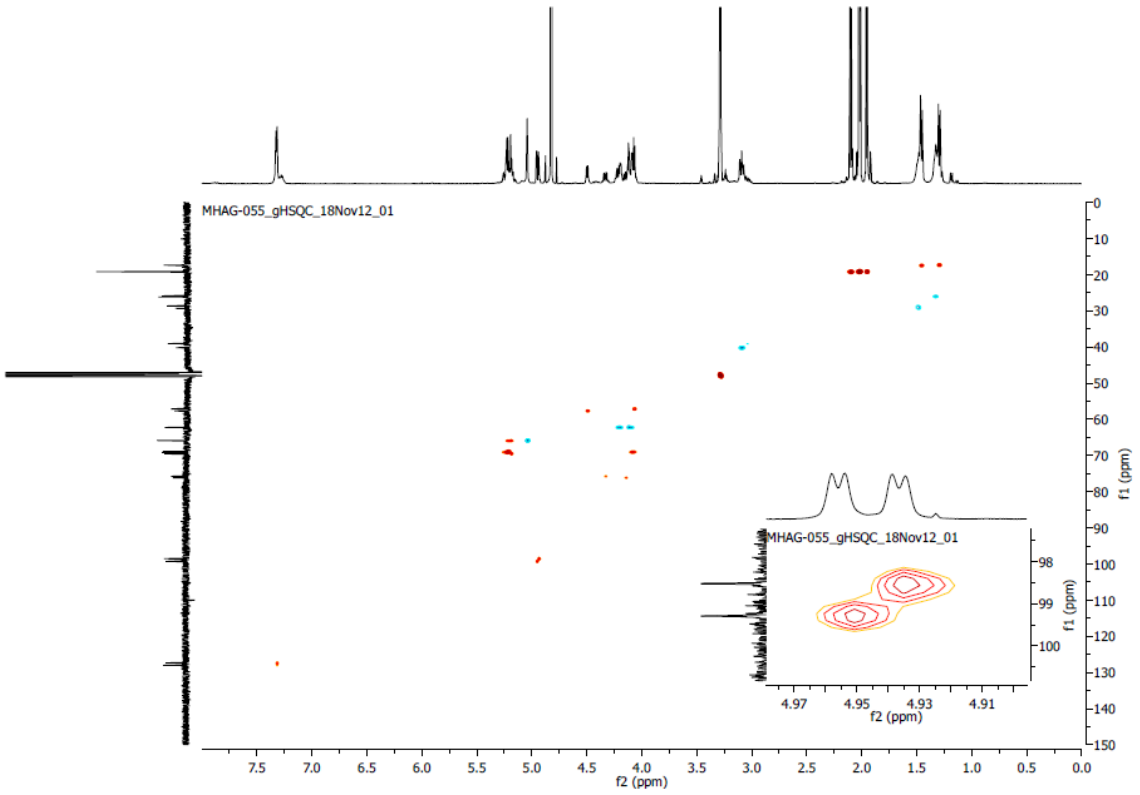
^{1}H of Glycopeptide 7 (400 MHz, CD_3OD)



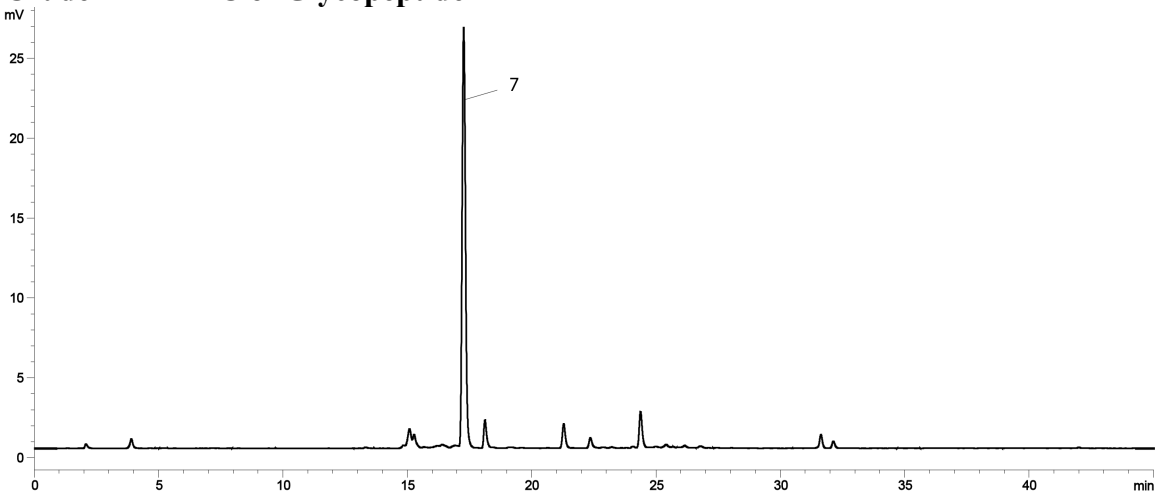
Carbon NMR of Glycopeptide 7 (101 MHz, CD_3OD)



HSQC NMR of Glycopeptide 7 (400 MHz, CD_3OD)



Crude RP-HPLC of Glycopeptide 7



HPLC was performed using Phenomenex C5 Luna column and a linear gradient of 50% to 95% ACN in TDW over 45 min (recorded with ELSD). Glycopeptide **7** elutes at 17.27 min.

4. Appendix: Synthesizer Modules and Conditions

4.1 Module A: Amino Acid Coupling Module

Solution preparation:

- Amino acid 0.25 mmol in DMF (2 mL) (0.125M)
- HBTU: 1.8 g (5 mmol) in DMF (40 mL) (0.125M)
- DIPEA: 1.7 mL (10 mmol) in DMF (40 mL) (0. 25M)

Action	Cycles	Solvent	Reagent 1 (mmol)	Reagent 2 (mmol)	Reagent 3 (mmol)	Temp ˚C	Time min
Wash	6	DMF	------	------	------	------	------
Coupling	2	DMF	amino acids (0.125)	HBTU (0.125)	DIPEA (0.25)	25	15
Wash	6	DMF	------	------	------	------	------

4.2 Module B: Fmoc Deprotection Module

Solution preparation:

- Piperidine (10 mL) in DMF (40 mL)

Action	Cycles	Solvent	Reagent 1	Temp ˚C	Time min
Wash	6	DMF	------	------	------
Deprotection	3	DMF	piperidine 20%	25	5
Wash	6	DMF	------	------	------

4.3 Module C: t-Bu Removal Module

Solution preparation:

- TMSOTf (364 µL, 2 mmol) in CH_2Cl_2 (20 mL) and dioxane (20 mL).

Action	Cycles	Solvent	Reagent 1 (mmol)	Temp ˚C	Time min
Wash	6	THF	------	------	------
Wash	6	CH_2Cl_2	------	------	------
Deprotection	8	CH_2Cl_2	TMSOTf (0.0375)	20	20
Wash	6	CH_2Cl_2	------	------	------

4.4 Module D: Imidate Double Coupling module

Solution preparation:

- TMSOTf (364 µl, 2 mmol) in CH_2Cl_2 (20 mL) and dioxane (20 mL).
- BB (0.25 mmol) in CH_2Cl_2 (750 µL) and dioxane (750 µL)

Action	Cycles	Solvent	Reagent 1 (mmol)	Reagent 2 (mmol)	Temp 1 ˚C	Time min
Wash	6	THF	------	------	------	------
Wash	6	CH_2Cl_2	------	------	------	------
Glycosylation	2	CH_2Cl_2/ dioxane	BB (0.125)	TMSOTf (0.025)	-10	1 h
Wash	6	CH_2Cl_2	------	------	------	------

4.5 Module E: Levulinoate Ester Removal Module

Solution preparation:

- hydrazine monohydrate (680 µL, 0.56 M) in pyridine (15 mL) and HOAc (10 mL).

Action	Cycles	Solvent	Reagent 1 (mmol)	Temp ˚C	Time min
Wash	6	CH_2Cl_2	------	------	------
Deprotection	3	pyridine/ AcOH	Hydrazine (0.5)	25	30 min
Wash	6	acetone/ CH_2Cl_2	------	------	------
Wash	6	CH_2Cl_2	------	------	------
Wash	6	Methanol	------	------	------
Wash	6	CH_2Cl_2	------	------	------

4.6 Module F: Thioglycoside Triple Coupling Module

Solution preparation:

- *N*-Iodosuccinimide (1.48 g, 6.66 mmol) and TfOH (60 µL, 0.66 mmol) in CH_2Cl_2 (20 mL) and dioxane (20 mL).
- BB 0.25 mmol in CH_2Cl_2 (3 mL)

Action	Cycles	Solvent	Reagent 1 (mmol)	Reagent 2 (mmol)	Reagent 3 (mmol)	Temp I ˚C	Time I min	Temp II ˚C	Time II min
Wash	6	THF	------	------	------	------	------	------	------
Wash	6	CH_2Cl_2	------	------	------	------	------	------	------
Glycosylation	2	CH_2Cl_2	BB (0.125)	NIS (0.125)	TfOH (0.05)	-40	5	-10	40
Wash	6	CH_2Cl_2	------	------	------	------	------	------	------

4.7 Module G: Trt Removal Module

Solution preparation:

- TMSOTf (364 µL, 2 mmol) in CH_2Cl_2 (20 mL) and dioxane (20 mL).

Action	Cycles	Solvent	Reagent 1 (mmol)	Temp ˚C	Time min
Wash	6	THF	------	------	------
Wash	6	CH_2Cl_2	------	------	------
Deprotection	3	CH_2Cl_2	TMSOTf (0.0375)	-30	5
Wash	6	CH_2Cl_2	------	------	------

5. Modified Synthesizer Port Settings

A schematic representation of each rotary valve unit is presented to describes the modifications made on the existing unit to enable glycopeptide synthesis.[6]

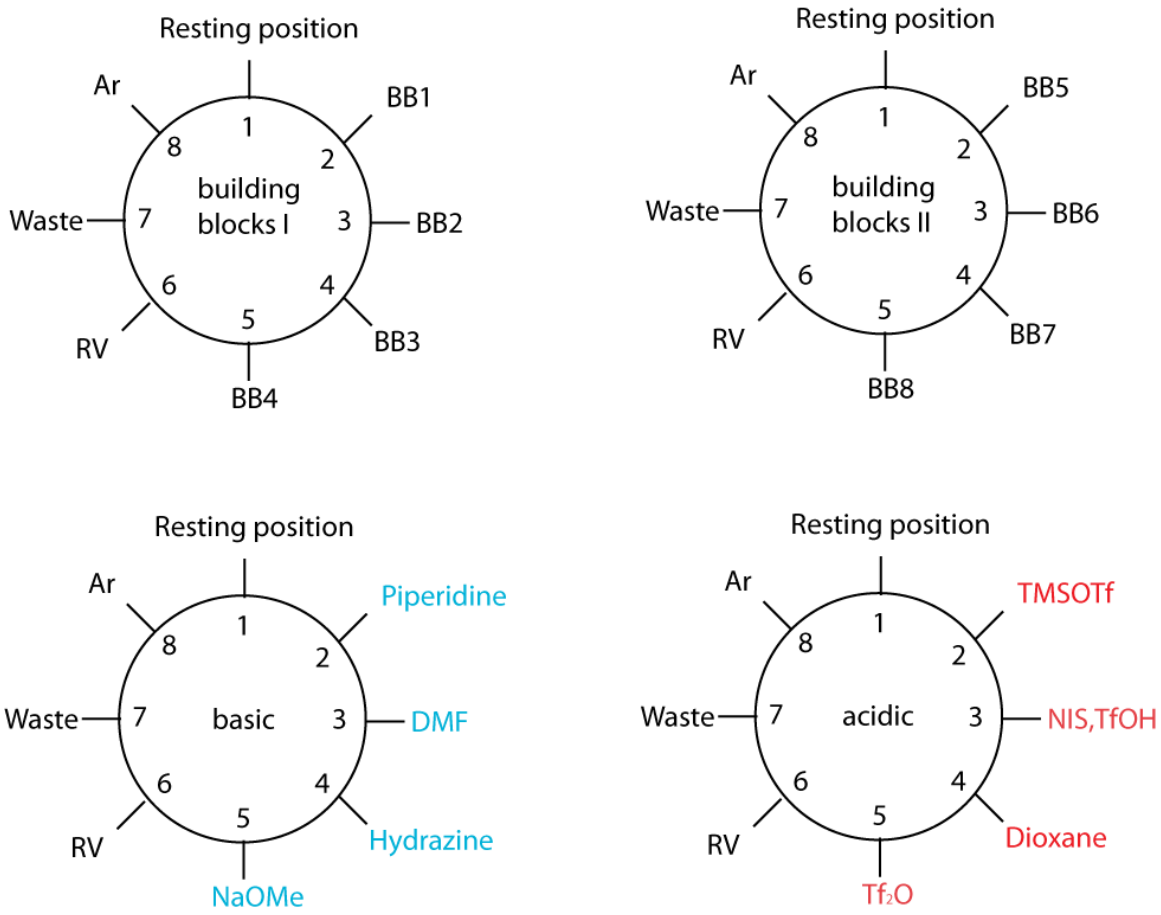


Figure 1. Synthesizer port settings for oligosaccharide synthesis.

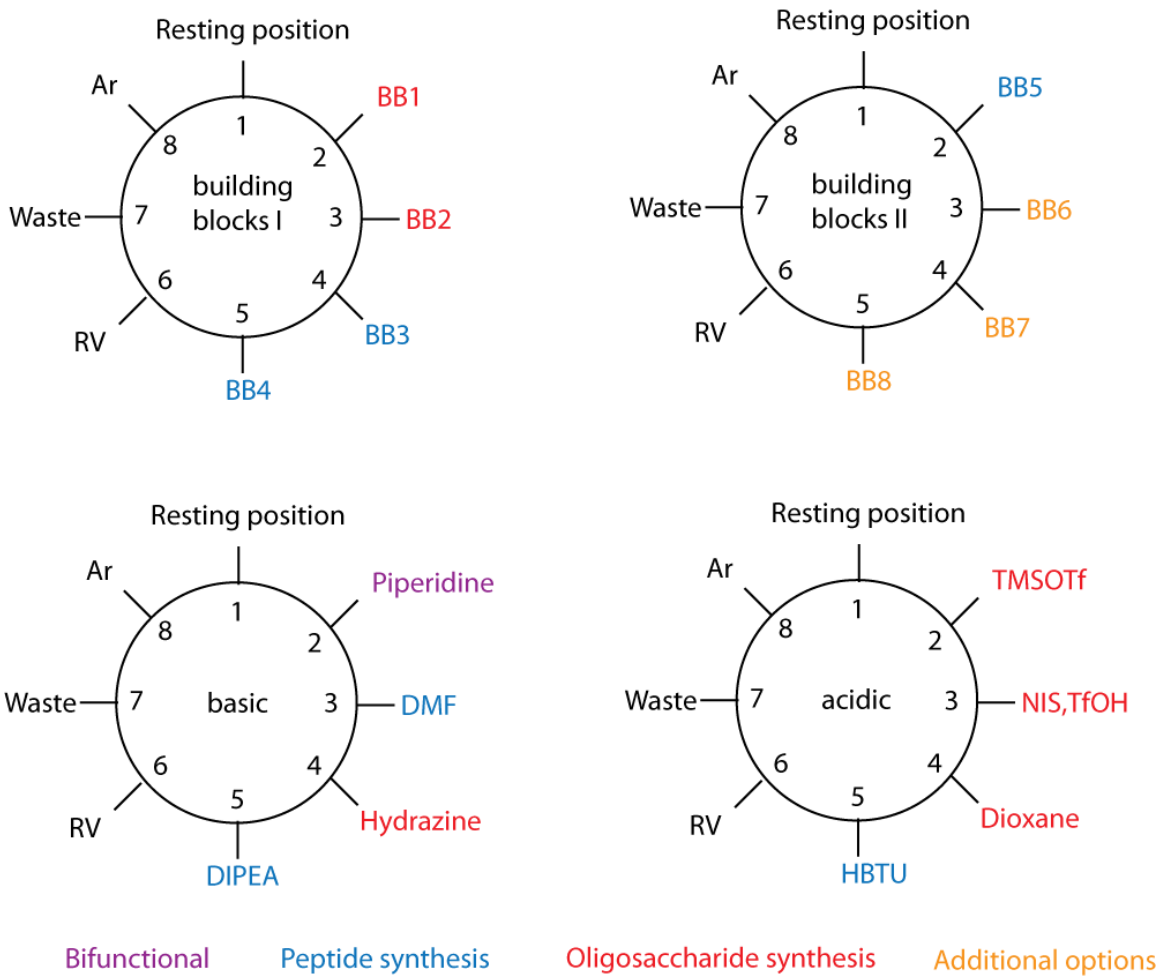
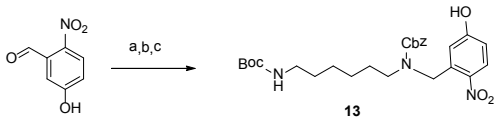


Figure 2. Synthesizer port settings for glycopeptide synthesis.

6. Linker Preparation and Solid Support Functionalization *

6.1 benzyl (6-((tert-butoxycarbonyl)amino)hexyl)(5-hydroxy-2-nitrobenzyl)carbamate 13.



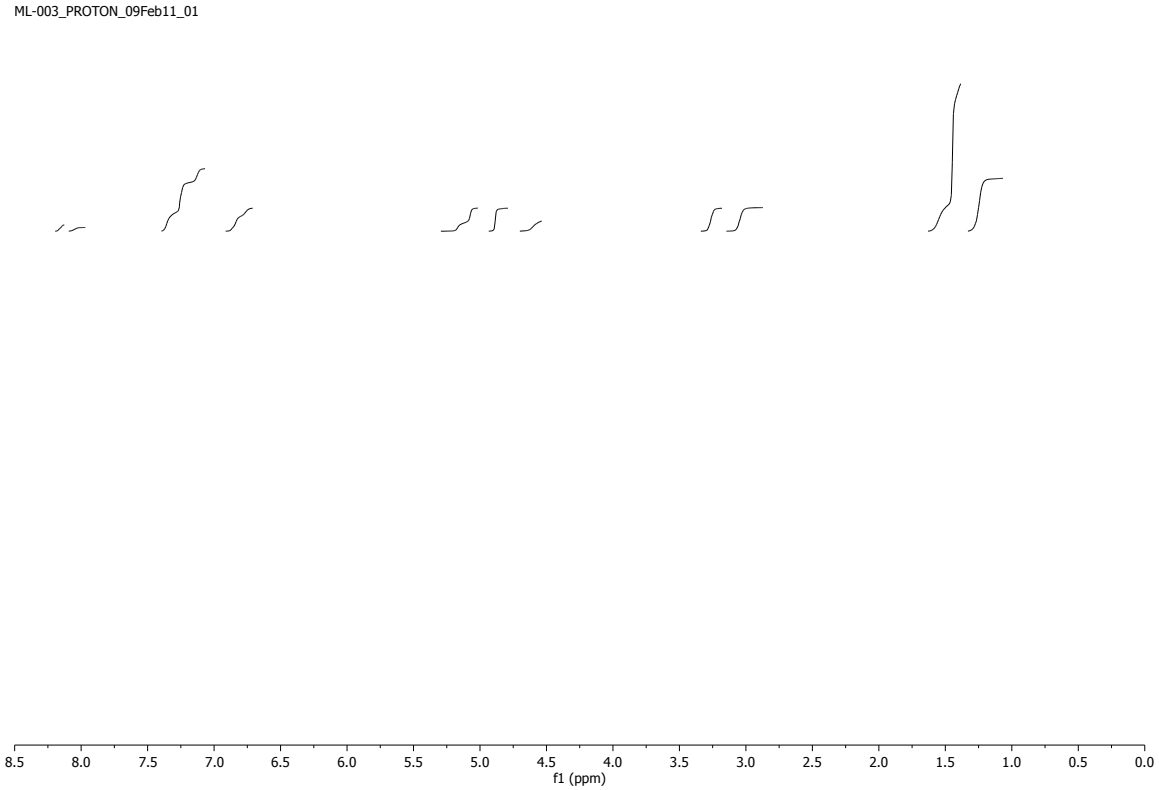
Scheme S2. Synthesis of linker **13**. Reagents and conditions: a. *N*-Boc-1,6-hexanediamine, toluene; b. $NaBH_4$; c. benzyl chloroformate, Et_3N, K_2CO_3

A solution of 5-hydroxy-2-nitrobenzaldehyde (2.260 g, 10.46 mmol) and *N*-Boc-1,6-hexanediamine (1.747 g, 10.46 mmol) in anhydrous toluene (70 mL) was stirred and heated at 120°C with Dean-Stark apparatus until the theoretical amount of released water

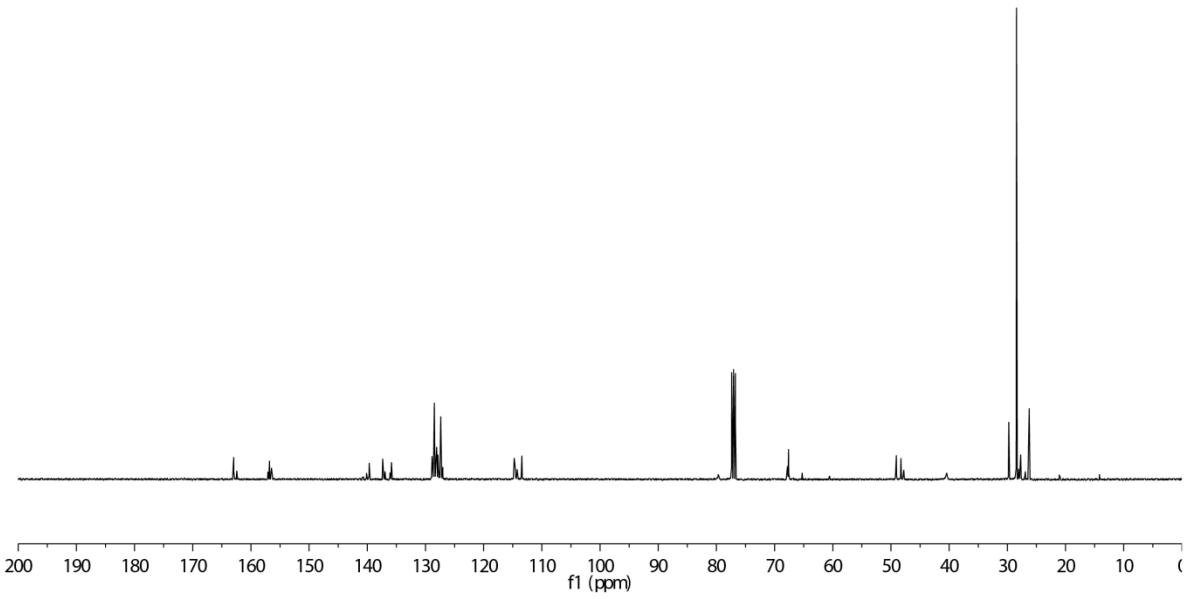
was reached. The solvent was then evaporated to furnish black foam. The imine was dissolved in methanol (80 mL) and sodium borohydride was slowly added (0.395 g, 10.47 mmol, 1eq) under bubbler control. After 30 minutes, acetone was slowly added (20 mL) and the solvent were evaporated to furnish a golden foam. To a solution of the amine in methanol (80 mL) was added Et_3N (3.2 mL, 23.03 mmol, 2.2 eq) and benzyl chlorofromate (3.0 mL, 20.94 mmol, 2.0 eq). After 1 h, to the stirred mixture was added potassium carbonate (4.33 g, 31.41 mmol, 3 eq) and stirred for another hour. The solution was filtered through celite and the solvents were evaporated. The crude product was dissolved in CH_2Cl_2 and washed successively with HCl (1M) and water. The organic layer was dried over $MgSO_4$, filtered and the solvent was evaporated. The residue was purified by flash chromatography starting from 8:2 (cyclohexane:EtOAc) and ramped to 7:3 (cyclohexane:EtOAc) to elute compound **13**. Collected fractions were evaporated and left to dry in high vacuum to yield linker **13** as light yellow foam (4.2 g, 80% over three steps): ^{1}H-NMR (400 MHz, $CDCl_3$): mixture of rotamers δ 8.15 (d, J = 8.8 Hz), 8.02 (d, J = 8.7 Hz), 7.35-7.11 (m, 5H), 6.87-6.72 (m, 2H), 5.15 (s, 1H), 5.06 (s, 1H), 4.88 (m, 2H), 4.69-4.67 (broad s, 1H), 3.24 (t, J = 6.8 Hz, 2H), 3.03 (t, J = 6.8 Hz, 2H), 1.57 (broad m, 2H), 1.44 (m, 11H), 1.28-1.23 (m, 4H). ^{13}C-NMR (100 MHz, $CDCl_3$): Mixture of rotamers δ 162.9, 162.4, 157.0, 156.8, 156.4, 140.1, 139.6, 137.3, 136.9, 136.1, 135.8, 128.8, 128.5, 128.5, 128.4, 128.2, 128.0, 127.8, 127.6, 127.3, 127.0, 114.7, 114.5, 114.2, 113.4, 67.7, 67.5, 65.2, 49.0, 48.2, 47.7, 40.4, 40.4, 40.3, 29.7, 28.4, 27.7, 26.2. MS ESI+-HRMS *m/z* $[M+H]^+$ calc for $C_{26}H_{36}N_3O7$ 502.2553 found 502.2561

*Similar procedure is reported in the accepted (under revision) manuscript by Hurevich *et. al.* in Organic Letters and is incorporated here to ensure all data is present.

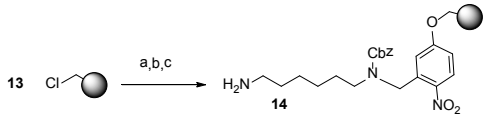
^{1}H NMR of 13 (400 MHz, $CDCl_3$)



^{13}C NMR of 13 (101 MHz, $CDCl_3$)



6.2 Solid Support Functionalization.



Scheme S2. Synthesis of linker **14**. Reagents and conditions: a. **13**, Cs_2CO_3, TBAI, DMF; b. CsOAc, DMF**;** c. TFA, DCM

Merrfiled resin (1 g, 0.53 mmol/g, 0.53 mmol) in DMF (30 mL) was added a solution of **13** (0.319 g, 0.636 mmol, 1.2 eq) in CH_2Cl_2 (5 mL) followed by Cs_2CO_3 (0.259 g, 0.795 mmol, 1.5 eq) and tetrabutylammonium iodide (TBAI) (0.294 g, 0.795 mmol, 1.5 eq). The solution was allowed to stir at 60°C and 200 mbar on the rotavap overnight. Resin was filtered and washed successively with DMF/Water (1/1), DMF, MeOH, CH_2Cl_2, MeOH, CH_2Cl_2 and then allowed to swell in CH_2Cl_2 for 1h. The swollen resin was placed in a flask with DMF (60 mL) and CsOAc (0.203 g, 1.06 mmol, 2 eq) was added. The solution was allowed to stir at 60°C and 200 mbar on the rotary evaporator overnight. The resin was washed successively with DMF/water (1/1), DMF, MeOH, CH_2Cl_2, MeOH, CH_2Cl_2, the solvent was drained and the resin was dried under vacuum.
Resin was swelled in CH_2Cl_2 for 30 min and was treated twice with a solution 10% TFA/ CH_2Cl_2 for 30 min. resin was washed with CH_2Cl_2, MeOH and dried under vacuum overnight to give solid support **14**.
Loading Determination of solid support **14**:
Dry resin **14** (100 mg, theoretical loading: 0.53 mmol/g, 0.053 mmol) was placed in a syringe (5 mL) equipped with a frit. DMF (3 mL) was added to swell the resin for 1 h. The DMF was drained and a solution of FmocCl (79 mg, 0.2 mmol) and DIPEA (0.07 mL, 0.4 mmol) in DMF (1 mL) was added to the resin. The reaction mixture was shaken for 12 h, the solution was drained and the resin was washed with DMF, CH_2Cl_2, MeOH and dried under vacuum. Loading determination was performed using standard Fmoc quantification[7] to give a typical loading of 0.21 mmol/g.

7. References

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