SUPPORTING INFORMATION

Efficient Synthesis and Enzymatic Extension of an *N*-GlcNAz Asparagine Building Block

Mikkel Haarslev Schröder Marqvorsen*[a], Sivasinthujah Paramasivam[b], Ward Doelman[a], Antony John Fairbanks[b,c] and Sander Izaak van Kasteren[a]

[a] *Leiden Institute of Chemistry (LIC), Division of Bio-Organic Chemistry, Einsteinweg 55, Leiden, Netherlands*

[b] *Department of Chemistry, University of Canterbury, Private Bag 4800, Christchurch, 8140, New Zealand*

[c] *Biomolecular Interaction Centre, University of Canterbury, Private Bag 4800, Christchurch, 8140, New Zealand*

Experimental

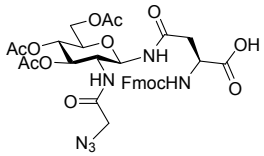
General

All commercially available reagents and solvents were used without further purification. Column chromatography was performed using Merck silica gel 60 (0.040-0.063 mm). High resolution mass spectra were recorded by direct injection (2 μL of a 2 μM solution in H_2O/MeCN 1:1 and 0.1% formic acid) on a mass spectrometer (Thermo Fisher Exactive HF Orbitrap) equipped with an electrospray ion source in positive mode. The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). ^{1}H and ^{13}C NMR spectra were recorded using a Brüker AV-300 (300/75 MHz), AV-400 (400/101 MHz) or AV-500 (500/126 MHz). Recorded data was interpreted and analysed using MestReNova 12 software. Chemical shifts are reported in ppm (δ) in reference to the residual solvent peak.

The WT Endo-M and N175Q Endo M mutant enzymes were purchased from Tokyo Chemical Industries Ltd (TCI). WT Endo A was expressed as previously described.[1] Units of enzyme activity are defined as follows: one unit of WT Endo M catalyses the release of 1.0 μmol of Fmoc-Asn(GlcNAc)-OH from Fmoc-Asn[$(NeuAcGalGlcNAcMan)_2ManGlcNAc_2$]-OH per minute at pH 6.0 at 37 ^{o}C; one unit of N175Q Endo M converts 1.0 μmol of pNP-GlcNAc to $(NeuAcGalGlcNAcMan)_2$ManGlcNAc-GlcNAc-pNP per minute at 30 ^{o}C at pH 7.0. Reverse phase high performance liquid chromatography (RP-HPLC) was performed on a Dionex P680 HPLC instrument with a Phenomenex Jupiter 5μ C18 300A column (5.0 μm, 4.6 × 250 mm) at 40 °C. The column was eluted with a linear gradient of 20-100 % MeCN containing 0.1% TFA at a flow rate of 1 mL/min for 30 mins. RP-HPLC analysis of glycosylation products was performed at appropriate reaction time intervals (0 min, 15 min, 30 min, 1 h, 2 h, 4 h and 6 h), using a 2 μL aliquot of the reaction mixture containing 0.2 % of 48 μL aqueous trifluoroacetic acid (TFA) solution. The yield of the reaction was determined by integration of product and acceptor peaks.

Synthesis

N^{γ}-[3,4,6-tri-*O*-Acetyl-2-azidoacetamido-2-deoxy-β-D-glucopyranosyl]-N^{α}-fluorenylmethoxycarbonyl-L-asparagine (2)

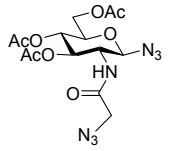


3,4,6-tri-*O*-acetyl-2-azidoacetamido-2-deoxy-β-D-glucopyranosyl azide (**3**) (2.09 g, 5 mmol) was dissolved in THF (45 mL) and cooled to 0 °C in an ice bath. To the cooled solution, PMe_3 (5 mL, 1 M in THF, 1 eq) was added dropwise and, after bubbling had subsided, the ice bath was removed and the reaction was stirred for an additional 5 minutes at room temperature. A crude ^{1}H NMR spectrum was consistent with formation of the expected 3,4,6-tri-*O*-acetyl-2-azidoacetamido-2-deoxy-β-D-glucopyranosyl trimethylphosphinimide (**7**)[2]; (300 MHz, THF-d8): δ_H (ppm) 9.63 (1H, br s, NH), 5.32 (1H, dd, $J_{2,3}$ 10.4 Hz, $J_{3,4}$ 9.2 Hz, H3), 4.95 (1H, t, *J* 9.7 Hz, H4), 4.93 (1H, dd, $J_{1,2}$ 8.8 Hz, $J_{H1,P}$ 28.9 Hz, H1), 4.17 (1H, dd, $J_{5,6a}$ 5.2 Hz, J_{gem} 11.9 Hz, H6a), 4.07 (1H, dd, $J_{5,6b}$ 2.6 Hz, J_{gem} 11.9 Hz, H6b), 3.81 (1H, d, J_{gem} 15.0 Hz, C$\underline{H}$HN$_3$), 3.79-3.68 (1H, m, H5), 3.69-3.48 (2H, m, CH$\underline{H}$N$_3$, H2), 2.00, 1.94, 1.90 (3H, s, C(O)C$\underline{H}_3$), 1.53 (9H, d, $J_{H,P}$ 13.0 Hz, N=P(C$\underline{H}_3$)$_3$). Water (900 µL, 50 mmol, 10 eq) was added to the crude phosphinimide and the reaction was stirred at room temperature for 2 hours and then reduced to a dark oil *in vacuo*. A crude ^{1}H NMR spectrum indicated the formation of the anomerically reduced compound, 3,4,6-tri-*O*-acetyl-2-azidoacetamido-2-deoxy-β-D-glucopyranosyl amine (**8**); (300 MHz, DMSO-d6) δ_H (ppm) 8.19 (1H, d, *J* 9.1 Hz, NH), 5.09 (1H, dd, $J_{2,3}$ 10.4 Hz, $J_{3,4}$ 9.4 Hz, H3), 4.78 (1H, t, *J* 9.7 Hz, H4), 4.33-4.16 (1H, m, H1), 4.12 (1H, dd, J_{gem} 12.1 Hz, $J_{5,6a}$ 4.9 Hz, H6a), 3.97 (1H, dd, J_{gem} 12.2 Hz, $J_{5,6b}$ 2.4 Hz, H6b), 3.76 (2H, s, CH_2N_3), 3.75-3.68 (1H, m, H5), 3.65 (1H, dt, $J_{NH,2}$=$J_{1,2}$ 10.8 Hz, $J_{2,3}$ 10.8 Hz, H2), 2.01, 1,96, 1.91 (3H, s, C(O)C$\underline{H}_3$). The crude glycosyl amine was dissolved in DMSO (2.5 mL). To this solution, *N*-Fmoc-aspartic anhydride **9**[3] (1.35 g, 4 mmol, 0.8 eq) was added and the reaction was stirred overnight at room temperature. The solution was diluted with 2.5 mL of methanol and added dropwise to a centrifuge tube containing 25 mL of methanol cooled in an ice bath. After 30 minutes, a precipitate had formed which was recovered by centrifugation followed by decantation. The precipitate was washed with a small amount of ice-cold methanol and dried *in vacuo* to afford the desired N^{γ}-[3,4,6-tri-*O*-Acetyl-2-azidoacetamido-2-deoxy-β-D-glucopyranosyl]-N^{α}-fluorenylmethoxycarbonyl-L-asparagine (**2**) of acceptable quality for SPPS (2.07 g, 2.86 mmol, 72%). This compound could be further recrystallized from ethanol (100 mL) to afford analytically pure **2** (1.13 g, 1.56 mmol, 39%) if desired.

$[\alpha]_D^{25°C}$ = -4.1 (c = 1.0 in DMSO); IR (Neat): ν = 2103 cm^{-1}, 1741 cm^{-1}, 1693 cm^{-1}, 1663 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d6): δ_H (ppm) 12.68 (1H, bs, COOH), 8.70 (1H, d, $J_{1,NH}$ 9.3 Hz, N$^{\gamma}$H), 8.23 (1H, d, $J_{2,NH}$ 9.2 Hz, N$\underline{H}$C(O)CH$_2$N$_3$), 7.89 (1H, d, *J* 7.5 Hz, Fmoc-Ar), 7.71 (1H, dd, *J* 7.4 Hz, *J* 3.6 Hz, Fmoc-Ar), 7.56 (1H, d, *J* 8.5 Hz, N$^{\alpha}\underline{H}$), 7.42 (2H, dt, *J* 1.1 Hz, *J* 7.4 Hz, Fmoc-Ar), 7.33 (2H, tt, *J* 1.5 Hz, *J* 7.4 Hz, Fmoc-Ar), 5.24 (1H, t, *J* 9.6, H1), 5.14 (1H, t, *J* 9.9, H3), 4.85 (1H, t, *J* 9.7, H4), 4.39 (1H, ddd, $J_{NH,Asn-CH}$ 8.5 Hz, $J_{Asn-CH,Asn-CH\underline{H}}$ 7.2 Hz, $J_{Asn-CH,Asn-C\underline{H}H}$ 5.8 Hz, Asn-CH), 4.31-4.16 (4H, m, H6a, Fmoc-CH, Fmoc-CH$_2$), 3.99-3.89 (2H, m, H4, H6b), 3.86 (1H, ddd, $J_{4,5}$ 10.1 Hz, $J_{5,6a}$ 4.2 Hz, $J_{5,6b}$ 2.4 Hz, H5), 3.79 (1H, d, J_{gem} 15.8 Hz,

C<u>H</u>HN$_3$), 3.69 (1H, d, J_{gem} 15.8 Hz, CH<u>H</u>N$_3$), 2.67 (1H, dd, J_{gem} 16.2 Hz, J_{vic} 5.7 Hz, Asn-C<u>H</u>H), 2.49 (1H, dd, J_{gem} 16.2 Hz, J_{vic} 7.3 Hz, Asn-CH<u>H</u>), 2.00, 1.97, 1.91 (3H, s, C(O)C<u>H</u>$_3$); ^{13}C-APT (125.8 MHz, DMSO-d6): δ_C (ppm) 173.0 (<u>C</u>OOH), 170.1, 169.9, 169.6, 169.4, 167.6 (3x<u>C</u>(O)CH$_3$, NH<u>C</u>(O)CH$_2$N$_3$, NHC(O)-Asn), 155.9 (NHC(O)-Fmoc), 143.87, 143.80, 140.76, 140.74 (Fmoc-Ar)[4], 127.7, 127.1, 125.3, 120.2 (Fmoc-Ar), 77.8 (C1), 73.3 (C3), 72.4 (C5), 68.3 (C4), 65.8 (CH$_2$-Fmoc), 61.8 (C6), 52.3 (C2), 50.8 (<u>C</u>H$_2$N$_3$), 50.0 (CH-Asn), 46.6 (CH-Fmoc), 36.9 (CH$_2$-Asn), 20.6, 20.43, 20.40 (OC(O)<u>C</u>H$_3$); HRMS (ESI$^+$) Calcd. For $C_{33}H_{36}N_6O_{13}$ (M+H$^+$) 725.2413. Found 725.2409.

3,4,6-tri-*O*-acetyl-2-azidoacetamido-2-deoxy-β-D-glucopyranosyl azide (3)

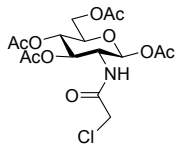


1,3,4,6-tetra-*O*-acetyl-2-chloroacetamido-2-deoxy-β-D-glucopyranose (**6**) (12.1 mmol, 4.83 g) was dissolved in DCM (46 mL). TiCl$_4$ (14.5 mL, 1.0 M in toluene, 1.2 eq) was added and the reaction was heated at reflux overnight. The reaction mixture was cooled to room temperature and diluted with DCM. The organic layer was washed successively with saturated NaHCO$_3$ (aq) and brine, and then dried over MgSO$_4$ (s), filtered and concentrated. The crude compound was filtered through a silica plug (1/1 ethyl acetate/pentane) to remove titanium salts to afford the intermediate 3,4,6-tri-*O*-acetyl-2-chloroacetamido-2-deoxy-α-D-glucopyranosyl chloride (**S1**) (3.88 g, 9.69 mmol) which was used without further purification; ^{1}H NMR (300 MHz, CDCl$_3$): δ_H (ppm) 6.96 (1H, d, $J_{2,NH}$ 8.5 Hz, NH), 6.23 (1H, d, $J_{1,2}$ 3.7 Hz, H1), 5.41 (1H, t, $J_{2,3}$ = $J_{3,4}$ 9.7 Hz, H3), 5.24 (1H, t, $J_{3,4}$ = $J_{4,5}$ 9.7 Hz, H4), 4.52 (1H, ddd, $J_{2,3}$ 10.7 Hz, $J_{2,NH}$ 8.5 Hz, $J_{1,2}$ 3.7 Hz, H2), 4.38 – 4.26 (2H, m, H5 + H6a), 4.20 – 4.11 (m, 1H, H6b), 4.07 (d, J_{gem} 14.9 Hz, C(O)C<u>H</u>HCl), 4.01 (d, J_{gem} 15.0 Hz, OC(O)CH<u>H</u>Cl), 2.11, 2.07, 2.06 (s, 3H, C(O)C<u>H</u>$_3$); ^{13}C-APT (75 MHz, CDCl$_3$): δ_C (ppm) 171.1, 170.5, 169.2, 166.6 (C=O), 92.8 (C1), 70.9 (C5), 69.7 (C3), 66.9 (C4), 61.1 (C6), 53.8 (C2), 42.1 (NHCO<u>C</u>H$_2$Cl), 20.63, 20.56, 20.5 (OC(O)<u>C</u>H$_3$). The crude dichloride was dissolved in dry DMF (20 mL) and NaN$_3$ (29.1 mmol, 1.89 g, 3 eq) was added. The reaction mixture was heated to 60 °C for 2 h, after which TLC (1/1 ethyl acetate/pentane) confirmed conversion into a single product. The reaction mixture was allowed to cool to room temperature before dilution with saturated NaHCO$_3$ (aq). This mixture was extracted with DCM, and the organic layer was washed further with NaHCO$_3$ (aq). The organic layer was dried over MgSO$_4$ (s), filtered and concentrated. Silica gel column chromatography (40% → 50% ethyl acetate/pentane) yielded the desired compound 3,4,6-tri-*O*-acetyl-2-azidoacetamido-2-deoxy-β-D-glucopyranosyl azide (**3**) (3.13 g, 7.58 mmol, 63%) as a pale yellow solid.

$[\alpha]_D^{25°C}$ = -26.4 (c = 1.0 in CHCl$_3$); IR (Thin film): ν = 2114 cm^{-1}, 1747 cm^{-1}, 1671 cm^{-1}; ^{1}H NMR (400 MHz, CDCl$_3$): δ_H (ppm) 7.01 (1H, d, $J_{2,NH}$ 9.0 Hz, NH), 5.39 (1H, t, $J_{2,3}$ = $J_{3,4}$ 9.6 Hz, H3), 5.12 (1H, t, $J_{3,4}$ = $J_{4,5}$ 9.7 Hz, H4), 4.95 (1H, d, $J_{1,2}$ = 9.2 Hz, H1), 4.29 (1H, dd, J_{gem} 12.4 Hz, $J_{5,6a}$ 5.0 Hz, H6a), 4.19 (1H, dd, J_{gem} 12.4 Hz, $J_{5,6b}$ 2.2 Hz, H6b), 4.02 (1H, d, J_{gem} 17.1 Hz, NHC(O)C<u>H</u>HN$_3$), 4.01-3.92 (1H, m, H2), 3.96 (1H, d, J_{gem} 16.9 Hz, NHC(O)CH<u>H</u>N$_3$), 3.89 (1H, ddd, $J_{4,5}$ 10.1 Hz, $J_{5,6a}$ 5.0 Hz, $J_{5,6b}$ 2.3 Hz, H5), 2.11 (s, 3H, C(O)C<u>H</u>$_3$), 2.05 (s, 6H, 2x C(O)C<u>H</u>$_3$); ^{13}C-APT NMR (101 MHz, CDCl3): δ_C 170.8 (C=O),

169.4 (C=O), 167.7 (C=O), 88.0 (C1), 73.8 (C5), 71.8 (C3), 68.2 (C4), 61.9 (C6), 54.0 (C2), 52.5 (NHC(O)$\underline{C}H_2N_3$), 20.7, 20.5 (OC(O)$\underline{C}H_3$); HRMS (ESI$^+$) Calcd. For $C_{14}H_{19}N_7O_8$ (M+Na$^+$) 436.1187. Found 436.1184.

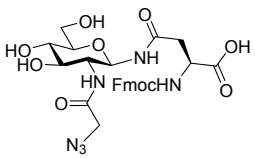
1,3,4,6-tetra-*O*-acetyl-2-chloroacetamido-2-deoxy-β-D-glucopyranose (6)



1,3,4,6-tetra-*O*-acetyl-β-D-glucosamine hydrochloride (**5**)[5] (5.0 mmol, 1.92 g) was suspended in DCM (25 mL) and Et_3N (10 mmol, 1.4 mL, 2 eq) was added. The resulting clear solution was cooled in an ice bath and chloroacetic anhydride (7.5 mmol, 1.28 g, 1.5 eq) was added. After 10 minutes, the ice bath was removed and the reaction mixture was stirred at room temperature for 3.5 hours. The reaction mixture was then diluted with DCM and the organic layer was washed twice with a 1 M aqueous solution of HCl. The organic layer was dried over $MgSO_4$, filtered and concentrated. Silica gel column chromatography (40% → 50% ethyl acetate in pentane)[6] yielded the desired compound 1,3,4,6-tetra-*O*-acetyl-2-chloroacetamido-2-deoxy-β-D-glucopyranose (**6**) (1.91 g, 4.5 mmol, 90%) as a white solid.

$[\alpha]_D^{25°C}$ = +11.9 (c = 1.0 in $CHCl_3$); IR (Thin film): ν = 1743 cm^{-1}, 1663 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 7.14 (1H, d, $J_{2,NH}$ 9.5 Hz, NH), 5.87 (1H, d, $J_{1,2}$ 8.7 Hz, H1), 5.43 (1H, t, $J_{2,3}$ = $J_{3,4}$ 10.0 Hz, H3), 5.14 (1H, t, $J_{3,4}$ = $J_{4,5}$ 9.6 Hz, H4), 4.41–4.24 (2H, m, H2, H6a), 4.16 (1H, dd, J_{gem} 12.5 Hz, $J_{5,6b}$ 2.0 Hz, H6b), 4.01 (2H, s, NHC(O)C$\underline{H}_2$Cl), 3.95 (1H, ddd, $J_{4,5}$ 9.9 Hz, $J_{5,6a}$ 4.9 Hz, $J_{5,6b}$ 2.7 Hz, H5), 2.23–1.98 (m, 12H, 4x C(O)C$\underline{H}_3$); ^{13}C-APT (101 MHz, $CDCl_3$): δ_C 171.1, 170.7, 169.44, 169.41, 166.8 (C=O), 92.0 (C1), 72.7 (C5), 72.1 (C3), 68.2 (C4), 61.8 (C6), 53.1 (C2), 42.4 (NHC(O)$\underline{C}H_2$Cl), 20.9, 20.7, 20.61, 20.58 (OC(O)$\underline{C}H_3$); HRMS (ESI$^+$) Calcd. For $C_{16}H_{22}ClNO_{10}$ (M+Na$^+$) 446.0824. Found 446.0821.

N^{γ}-[2-azidoacetamido-2-deoxy-β-D-glucopyranosyl]-N^{α}-fluorenylmethoxycarbonyl-L-asparagine (10)



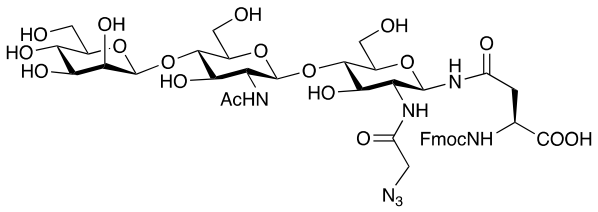
N^{γ}-[3,4,6-tri-*O*-Acetyl-2-azidoacetamido-2-deoxy-β-D-glucopyranosyl]-N^{α}-fluorenylmethoxycarbonyl-L-asparagine (**2**) (0.2 mmol, 150 mg) was suspended in MeOH (8.6 mL) and KCN (0.31 mmol, 1.5 eq) was added. The suspension turned into a clear solution after which a precipitate started to form. After 24 hours, the precipitate was isolated by filtration to afford the desired compound N^{γ}-[2-azidoacetamido-2-deoxy-β-D-glucopyranosyl]-N^{α}-fluorenylmethoxycarbonyl-L-asparagine (**10**) (112 mg, 0.18 mmol, 90%) as a white solid.[7]

^{1}H NMR (300 MHz, DMSO-d6): δ_H 8.76 (1H, d, $J_{1,NH}$ 8.9 Hz, N$^{\gamma}$H-Asn), 8.24 (1H, d, $J_{2,NH}$ 7.6 Hz, N$\underline{H}$C(O)CH_2N_3), 7.88 (2H, d, *J* 7.5 Hz, Fmoc-Ar), 7.69 (2H, d, *J* 7.2 Hz, Fmoc-Ar), 7.41

(2H, t, *J* 7.4 Hz, Fmoc-Ar), 7.33 (2H, d, *J* 7.3 Hz, Fmoc-Ar), 6.50 (1H, d, *J* 6.7 Hz, $N^{\alpha}H$), 5.30 (1H, d, *J* 4.2 Hz, 3O<u>H</u>), 5.17 (2H, d, *J* 4.2 Hz, 4O<u>H</u>), 4.91 (1H, t, $J_{1,NH}$ =$J_{1,2}$ 8.9 Hz, H1), 4.84-4.75 (1H, m, 6OH), 4.27-4.09 (3H, m, CH-Fmoc, CH_2-Fmoc), 3.90 (1H, d, J_{gem} 15.8 Hz, NHC(O)C<u>H</u>HN$_3$), 3.88-3.79 (1H, m, H2), 3.72 (1H, d, J_{gem} 15.7 Hz, NHC(O)CH<u>H</u>N$_3$), 3.69-3.60 (1H, m, H6a), 3.54-3.37 (3H, m, H2, H3, H6b), 3.17-3.03 (2H, m, H4, H5), 2.49-2.31 (2H, m, C<u>H$_2$</u>-Asn); HRMS (ESI$^+$) Calcd. For $C_{27}H_{30}N_6O_{10}$ (M+H$^+$) 599.2096. Found 599.2093.

Enzymatic glycosylation

***N*$^{\gamma}$-[β-D-Mannopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-azidoacetamido-2-deoxy-β-D-glucopyranosyl]-*N*$^{\alpha}$-fluorenylmethoxycarbonyl-L-asparagine (14)**



Method 1: Using WT Endo A

Disaccharide oxazoline (**11**) (0.3 mg, 0.8 μmol) and glycosyl amino acid acceptor (**10**) (0.15 mg, 0.26 μmol) were incubated with WT Endo A (1.0 μL, 20 μg) in sodium phosphate buffer (84 μL, 100 mM, pH 6.5) containing 20% v/v DMSO (21 μL) at 30 °C. After 4 h, HPLC analysis revealed that 94 % of the glycosyl amino acid acceptor (**10**) (t_R 14.0 min) had been converted to the trisaccharide product (**14**) (t_R 12.7 min). The product was purified by RP-HPLC; ^{1}H NMR (400 MHz, D_2O): δ_H 7.79 (2H, d, *J* 7.0 Hz, Fmoc-Ar), 7.59 (2H, d, 7.0 Hz, Fmoc-Ar), 7.37 (2H, at, *J* 7.8 Hz, Fmoc-Ar), 7.32 (2H, at, 7.0 Hz, Fmoc-Ar), 4.94 (1H, d, $J_{1a,2a}$ 9.4 Hz, H1a), 4.68 (1H, s, H1c), 4.51 (1H, s, H1b), 4.22 (2H, m, C<u>H$_2$</u>N$_3$), 4.11-4.22 (3H, m, CH-Asn, CH_2-Fmoc), 3.92 (1H, br s, H2c), 3.30-3.80 (15H, m, H2a, H2b, H3b, H3c, H4a, H4b, H4c, H5b, H6a, H6b, H6c, H6′a, H6'b, H6'c, C<u>H</u>-Fmoc), 3.06 (2H, m, H3a, H5a), 2.37 (2H, m, C<u>H$_2$</u>-Asn), 1.91 (3H, s, OC(O)C<u>H$_3$</u>); HRMS (ESI$^+$) Calcd. For $C_{41}H_{54}N_7O_{20}$ (M+H$^+$) 964.3418. Found 964.3455.

Method 2: Using WT Endo M

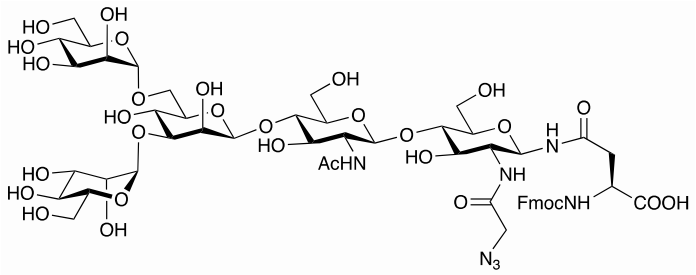
Disaccharide oxazoline (**11**) (0.3 mg, 0.8 μmol) and glycosyl amino acid acceptor (**10**) (0.15 mg, 0.26 μmol) were incubated with WT Endo M (2.0 μL, 2 mU) in sodium phosphate buffer (84 μL,100 mM, pH 6.5) containing 20% v/v DMSO (21 μL) at 30 °C. After 4h, HPLC analysis

revealed that 90% of the glycosyl amino acid acceptor (**10**) (t_R 14.0 min) had been converted to the trisaccharide product (**14**) (t_R 12.7 min).

Method 3: Using Endo-M-N175Q

Disaccharide oxazoline (**11**) (0.3 mg, 0.8 μmol) and glycosyl amino acid acceptor (**10**) (0.15 mg, 0.26 μmol) were incubated with Endo M N175Q (2.0 μL, 2 mU) in sodium phosphate buffer (84 μL,100 mM, pH 6.5) containing 20% v/v DMSO (21 μL) at 30 °C. After 4h, HPLC analysis revealed that 6% of the glycosyl amino acid acceptor (**10**) (t_R 14.0 min) had been converted to the trisaccharide product (**14**) (t_R 12.7 min).

***N*$^{\gamma}$-[α-D-Mannopyranosyl-(1→3)-[α-D-mannopyranosyl-(1→6)-]-β-D-mannopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-azidoacetamido-2-deoxy-β-D-glucopyranosyl]-*N*$^{\alpha}$-fluorenylmethoxycarbonyl-L-asparagine (15)**



Method 1: Using Endo A

Tetrasaccharide oxazoline (**12**) (0.6 mg, 0.9 μmol) and glycosyl amino acid acceptor (**10**) (0.2 mg, 0.3 μmol) were incubated with WT Endo A (1 μL, 20 μg) in sodium phosphate buffer (96 μL, 100 mM, pH 6.5) containing 20% v/v DMSO (24 μL) at 30 °C. After 15 min, HPLC analysis revealed that 63% of the glycosyl amino acid acceptor (**10**) (t_R 14.0 min) had been converted to the pentasaccharide product (**15**) (t_R 12.1 min). The product was purified by RP-HPLC and characterized by HRMS (ESI$^+$) Calcd. For $C_{53}H_{74}N_7O_{30}$ (MH^+) 1288.4475. Found 1288.4489.

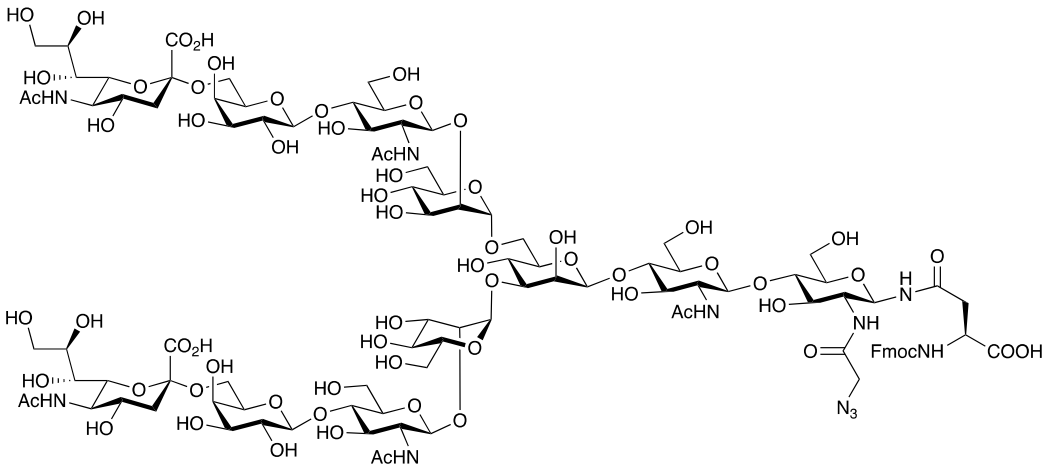
Method 2: Using WT Endo M

Tetrasaccharide oxazoline (**12**) (0.6 mg, 0.9 μmol) and glycosyl amino acid acceptor (**10**) (0.2 mg, 0.3 μmol) were incubated with WT Endo M (2 μL, 2mU) in sodium phosphate buffer (96 μL, 100 mM, pH 6.5) containing 20% v/v DMSO (24 μL) at 30 °C. After 30 min, HPLC analysis revealed that 16% of the glycosyl amino acid acceptor (**10**) (t_R 14.0 min) had been converted to the pentasaccharide product (**15**) (t_R 12.1 min).

Method 3: Using Endo-M-N175Q

Tetrasaccharide oxazoline (**12**) (0.6 mg, 0.9 μmol) and glycosyl amino acid acceptor (**10**) (0.2 mg, 0.3 μmol) were incubated with Endo-M-N175Q (2 μL, 2mU) in sodium phosphate buffer (100 mM, pH 6.5) containing 20% of DMSO at 37 °C. After 30 min, HPLC analysis revealed that 60% of the glycosyl amino acid acceptor (**10**) (t_R 14.0 min) had been converted to the pentasaccharide product (**15**) (t_R 12.1 min)

N^{γ}-[(NeuAc-Gal-GlcNAc-Man)$_2$-Man-GlcNAc-GlcNAz]-N^{α}-fluorenylmethoxycarbonyl-L-asparagine (16)



Method 1: Using WT Endo M

Complex bi-antennary oxazoline (**13**) (0.30 mg, 0.15 μmol) and glycosyl amino acid acceptor (**10**) (0.2 mg, 0.3 μmol) were incubated with WT Endo M (2 μL, 2mU) in sodium phosphate buffer (16 μL, 100 mM, pH 6.5) containing 20% v/v DMSO (4 μL) at 37 °C. After 30 min, HPLC analysis revealed that 20% of the glycosyl amino acid acceptor (**10**) (t_R 14.0 min) had been converted to the undecasaccharide product (**16**) (t_R 10.8). The product was purified by RP-HPLC and characterized by HRMS (ESI^+). Calcd. For $C_{103}H_{154}N_{11}O_{66}$ (MH^+) 2600.9027. Found 2600.9173.

Method 2: Using Endo M-N175Q

Complex bi-antennary oxazoline (**13**) (0.30 mg, 0.15 μmol) and glycosyl amino acid acceptor (**10**) (0.2 mg, 0.3 μmol) were incubated with Endo-M-N175Q (2 μL, 2mU) in sodium phosphate buffer (16 μL, 100 mM, pH 6.5) containing 20% v/v DMSO (4 μL) at 37 °C. After 30 min, HPLC

analysis revealed that 78% of the glycosyl amino acid acceptor (**10**) (t_R 14.0 min) had been converted to the undecasaccharide product (**16**) (t_R 10.8).

Time Course Studies

Figure S1. Production of ManGlcNAcGlcNAz-Fmoc-Asn (14):

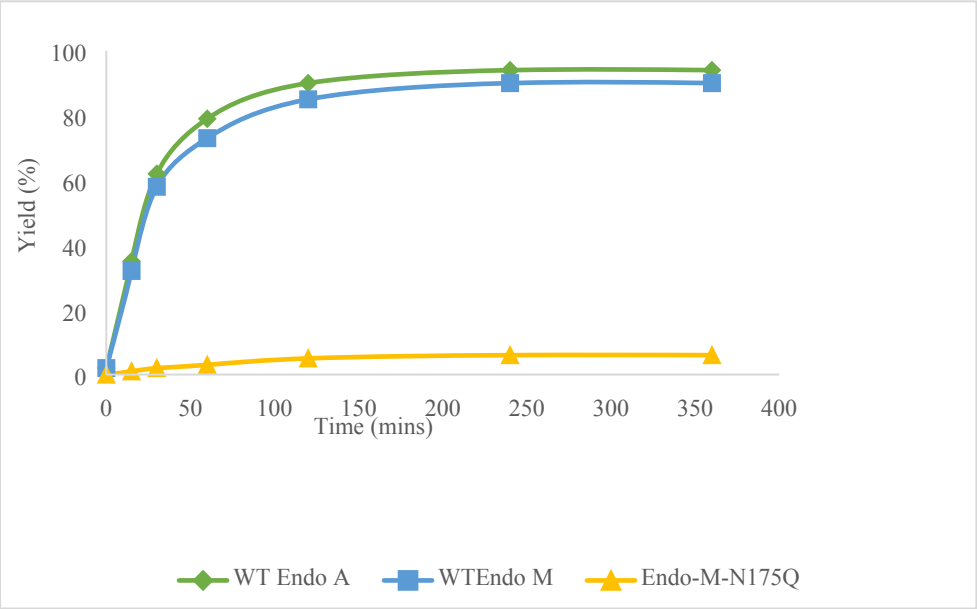


Figure S2. Production of Man_2ManGlcNAcGlcNAz-Fmoc-Asn (15):

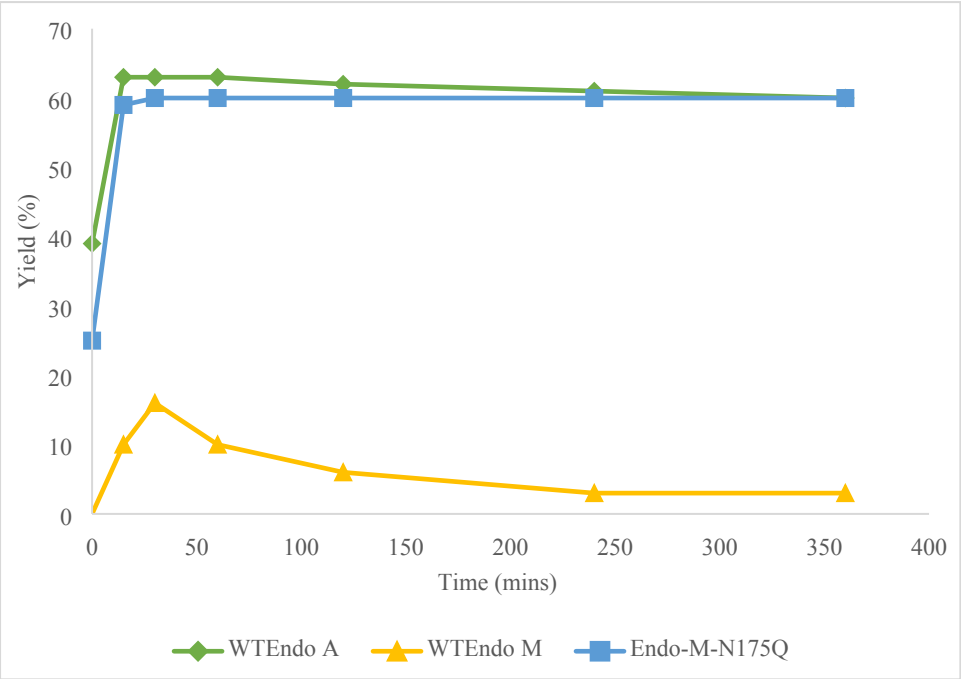
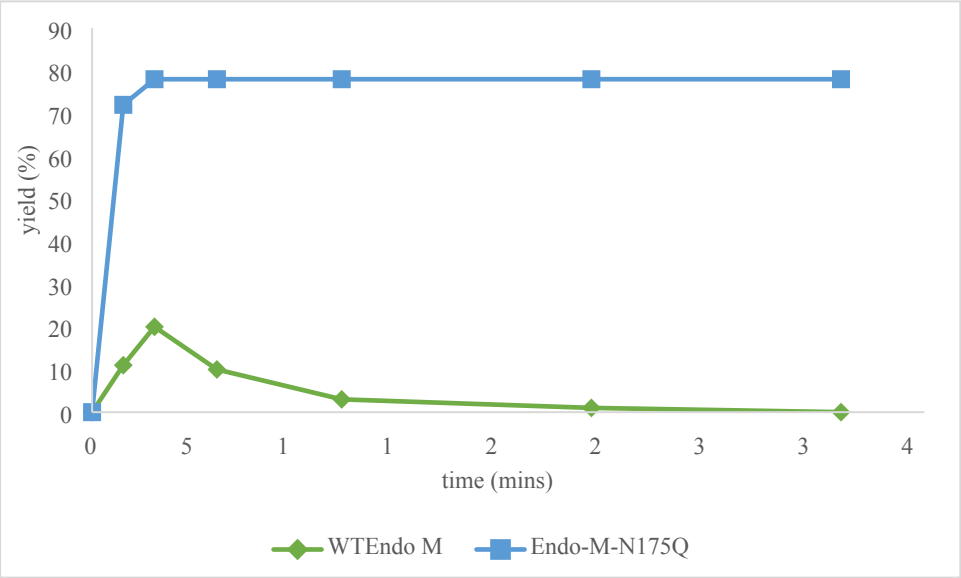
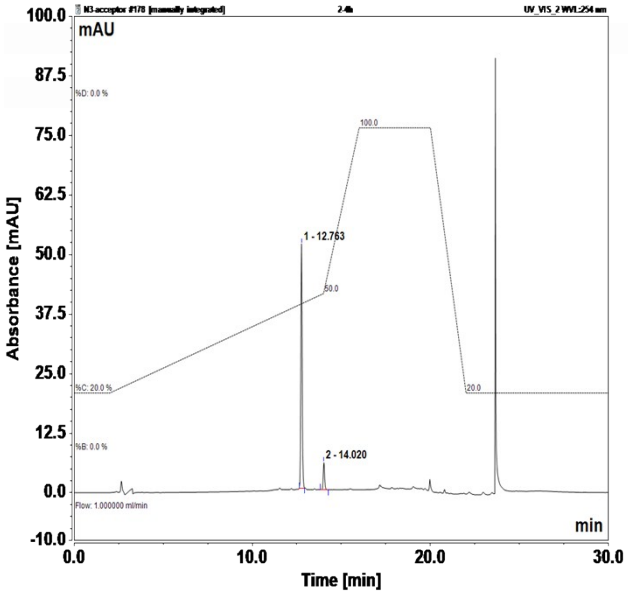

70
60
50
40
30
20
10
0
Yield (%)
0
50
100
150
200
250
300
350
400
Time (mins)
WTEndo A
WTEndo M
Endo-M-N175Q

Figure S3. Production of (NeuAcGalGlcNAcMan)$_2$ManGlcNAcGlcNAz-Fmoc-Asn (16)

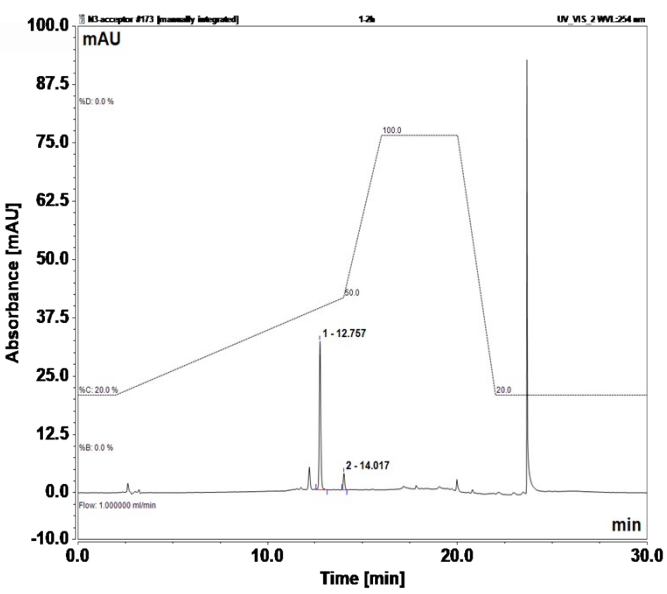


HPLC and ESI-MS analysis of ENGase-catalysed glycosylation reactions

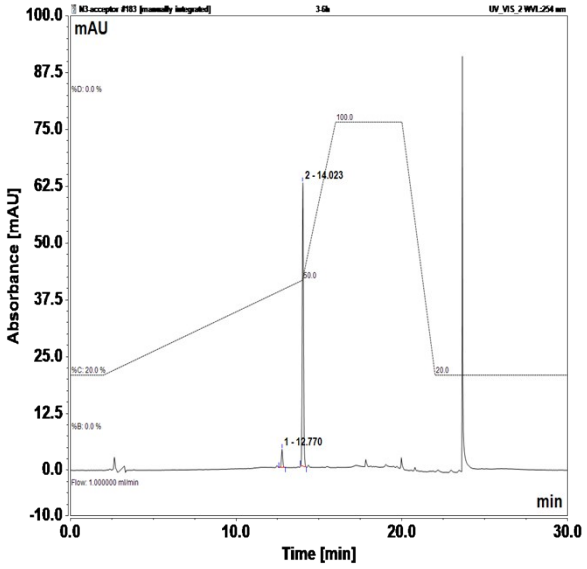
ManGlcNAcGlcNAz-Fmoc-Asn (14)



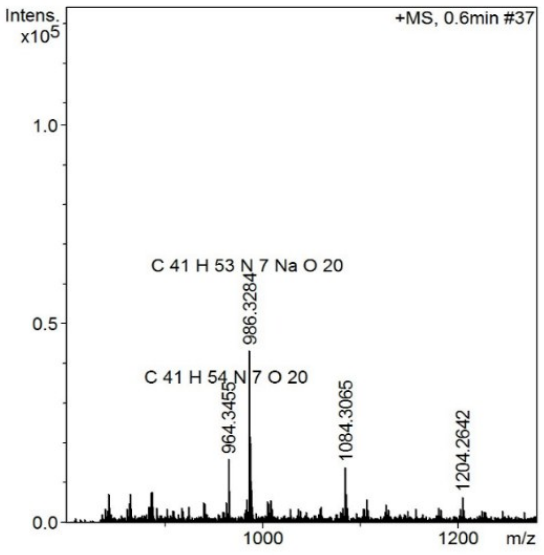
1. WT Endo A



2. WT Endo M

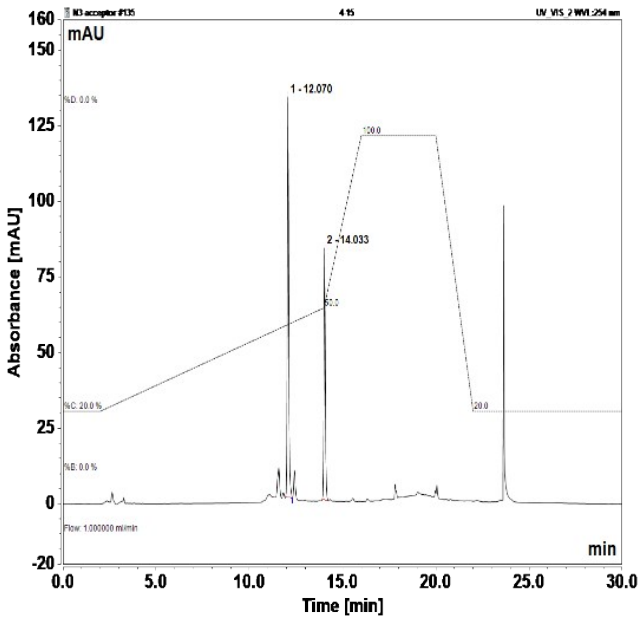


3. Endo M N175Q

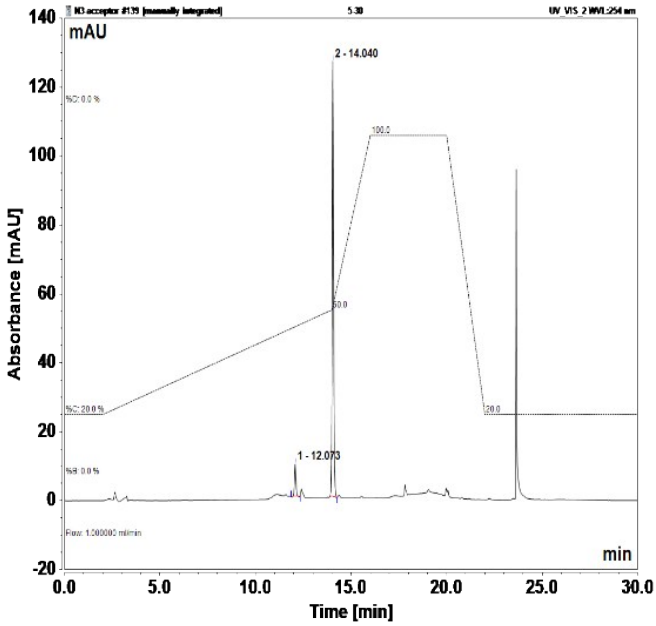


HRMS

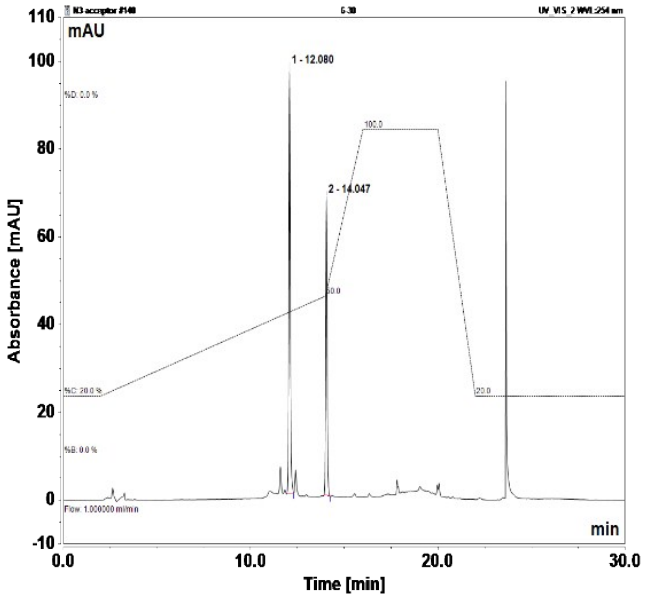
$Man_2ManGlcNAcGlcNAz$-Fmoc-Asn (15):

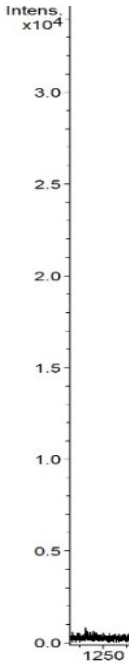


1. WT Endo A



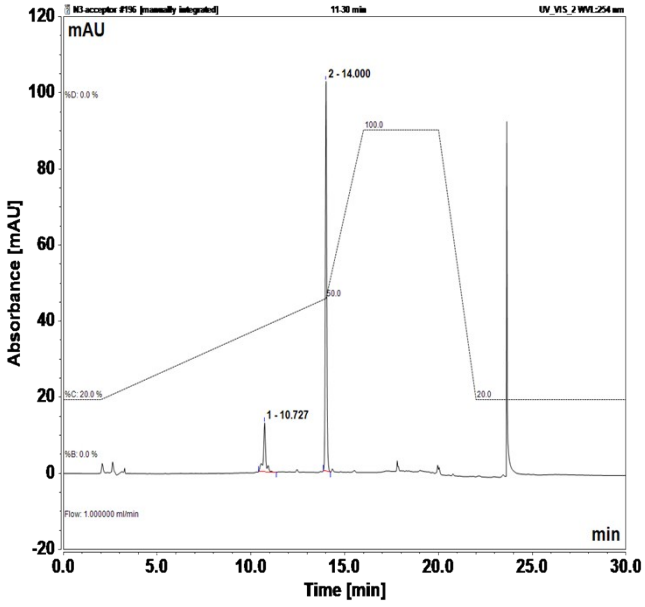
2. WT Endo M



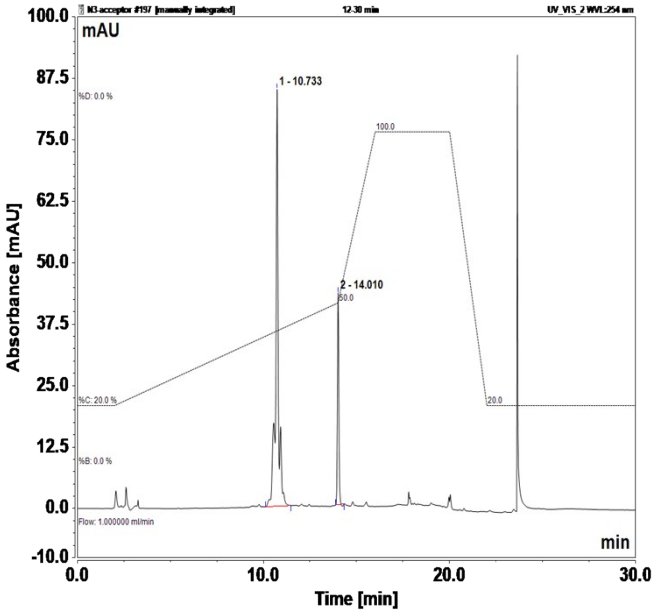


3. Endo N175Q

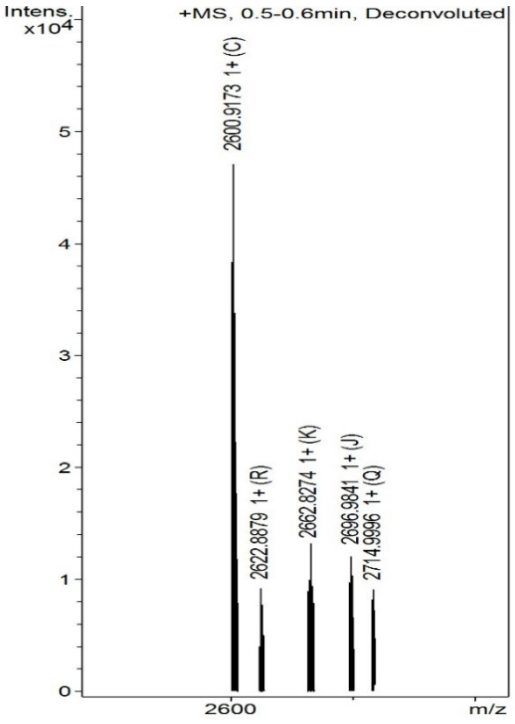
$(NeuAcGalGlcNAcMan)_2ManGlcNAcGlcNAz$-Fmoc-Asn (16)



1. WT-Endo M



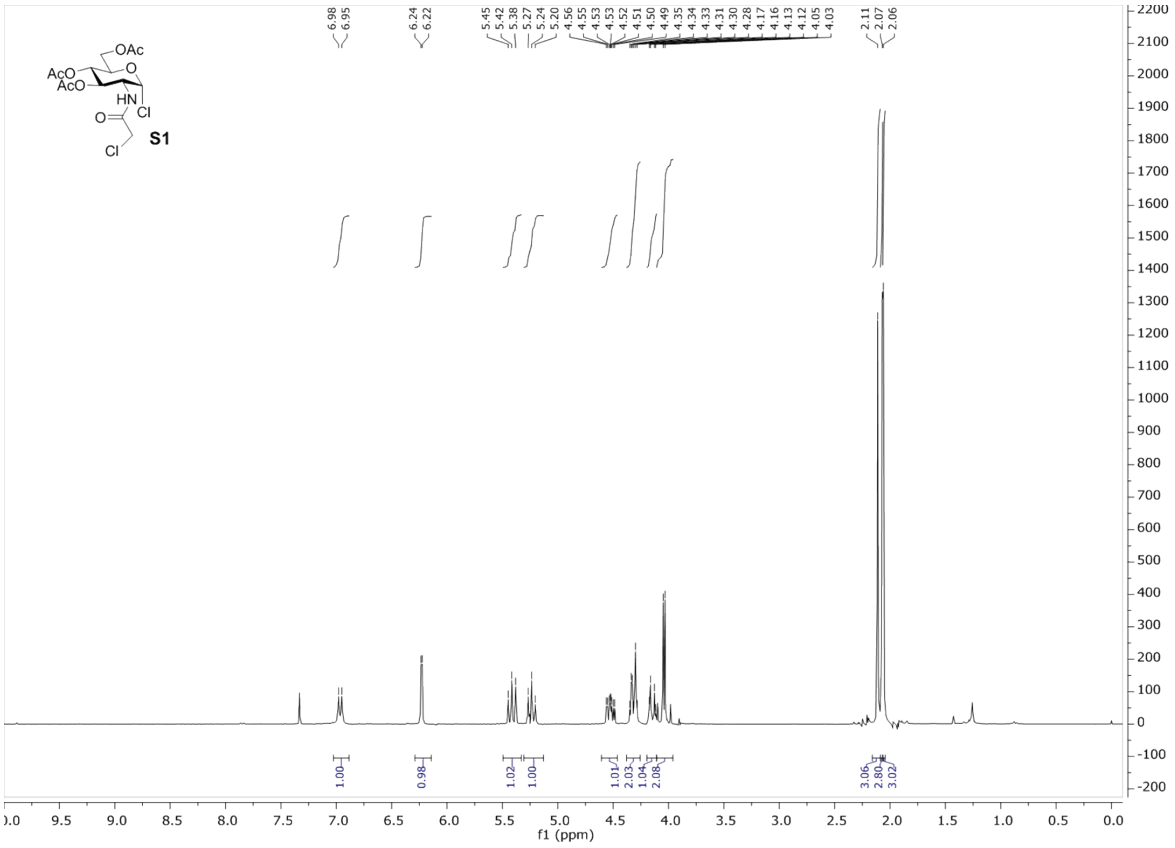
2. Endo M N175Q

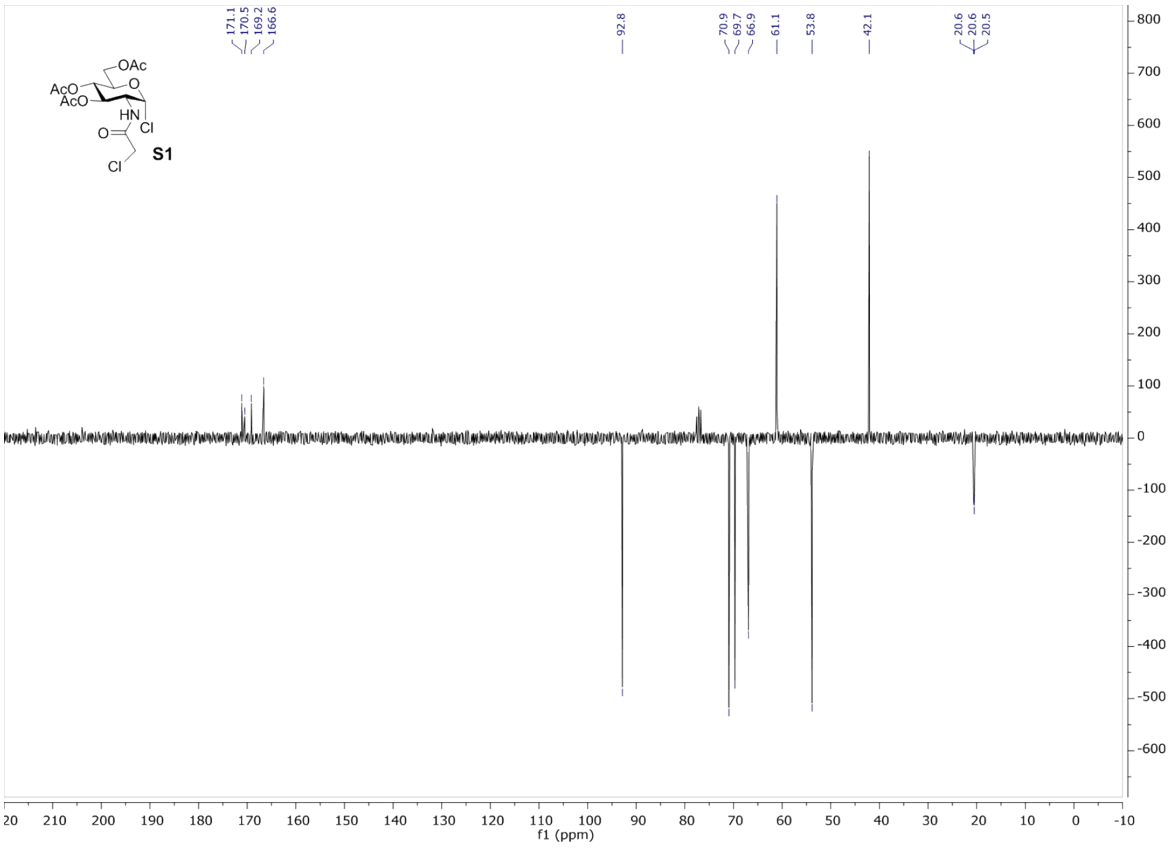


HRMS

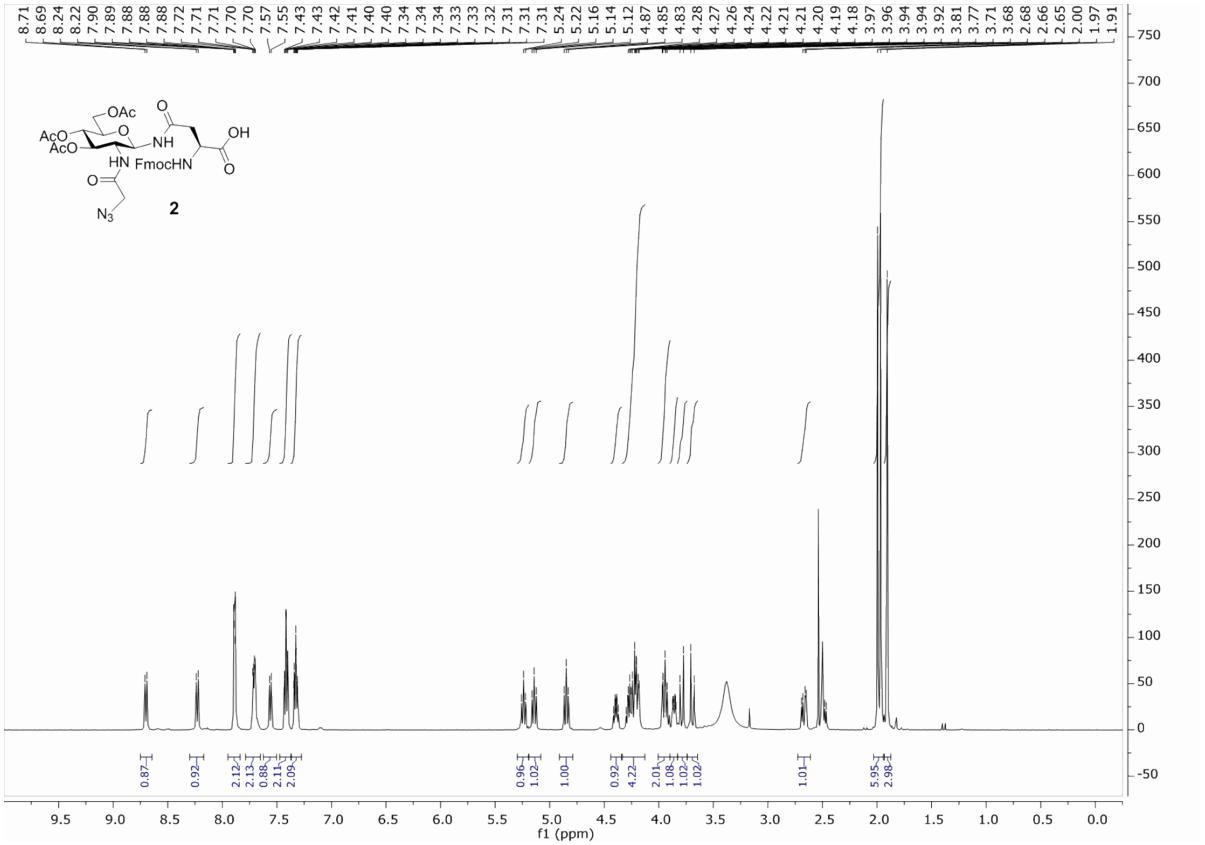
NMR Spectra

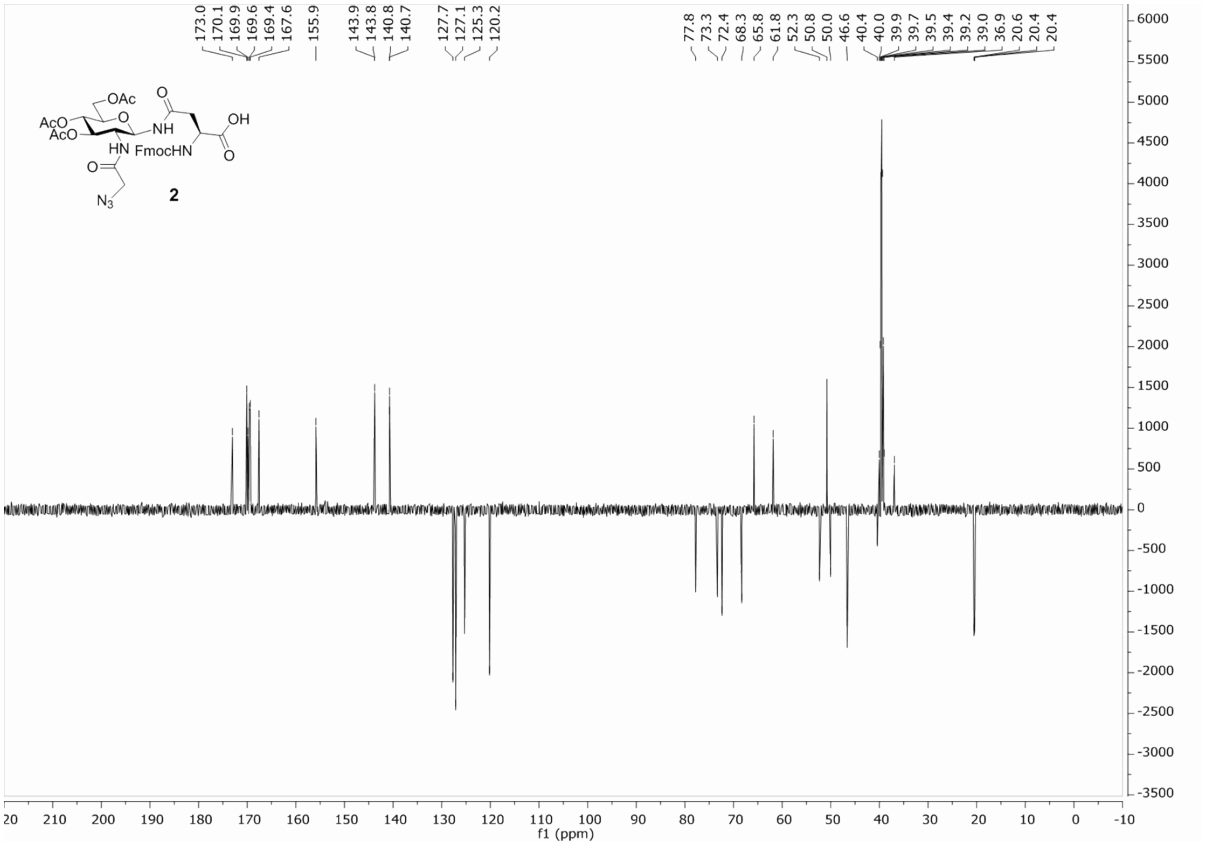
3,4,6-tri-*O*-acetyl-2-chloroacetamido-2-deoxy-α-D-glucopyranosyl chloride (S1)



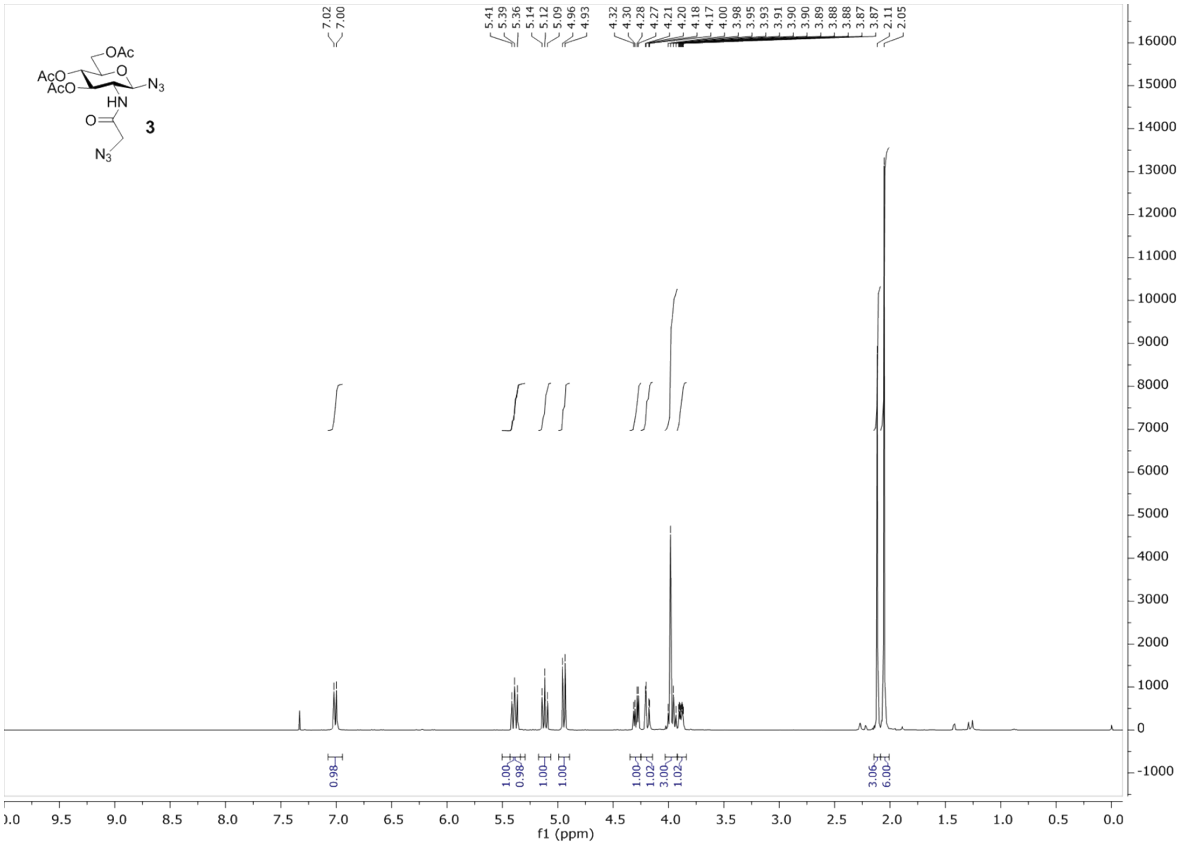


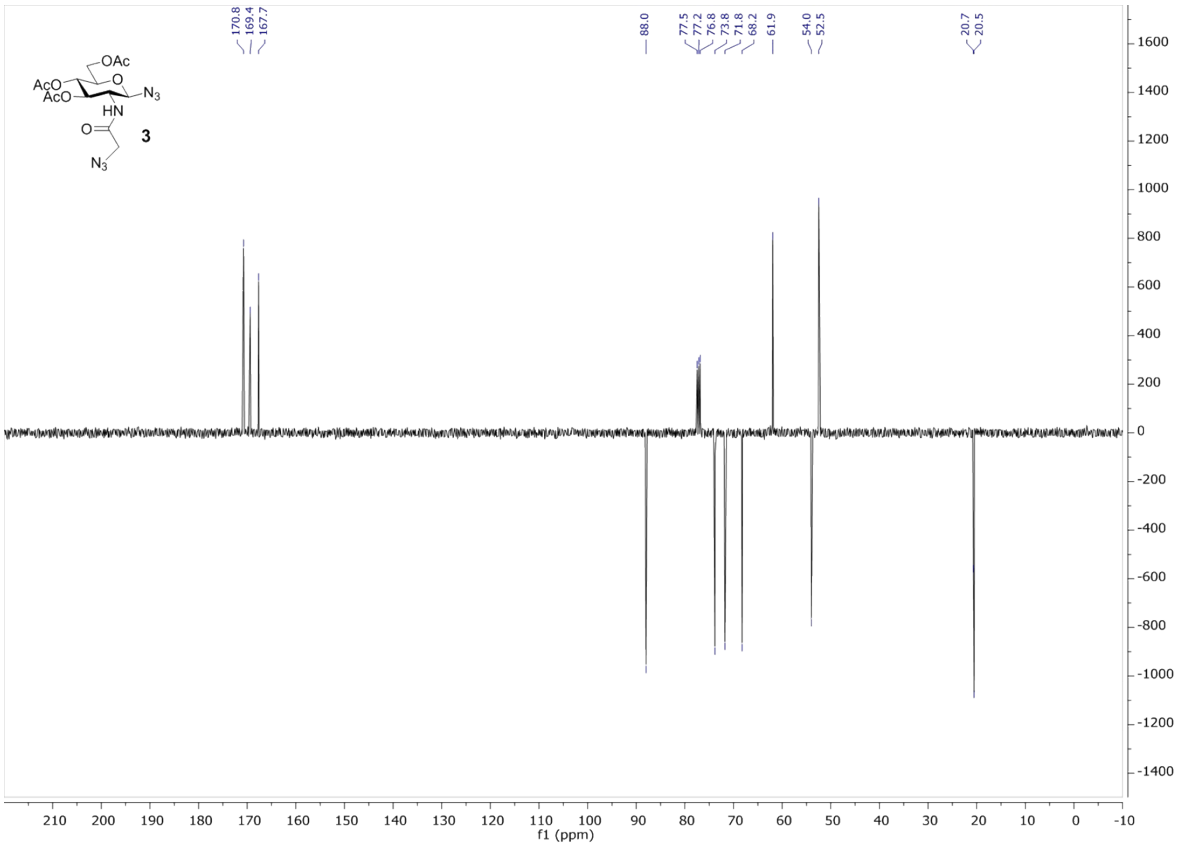
N^{γ}-[3,4,6-tri-*O*-Acetyl-2-azidoacetamido-2-deoxy-β-D-glucopyranosyl]-N^{α}-fluorenylmethoxycarbonyl-L-asparagine (2)



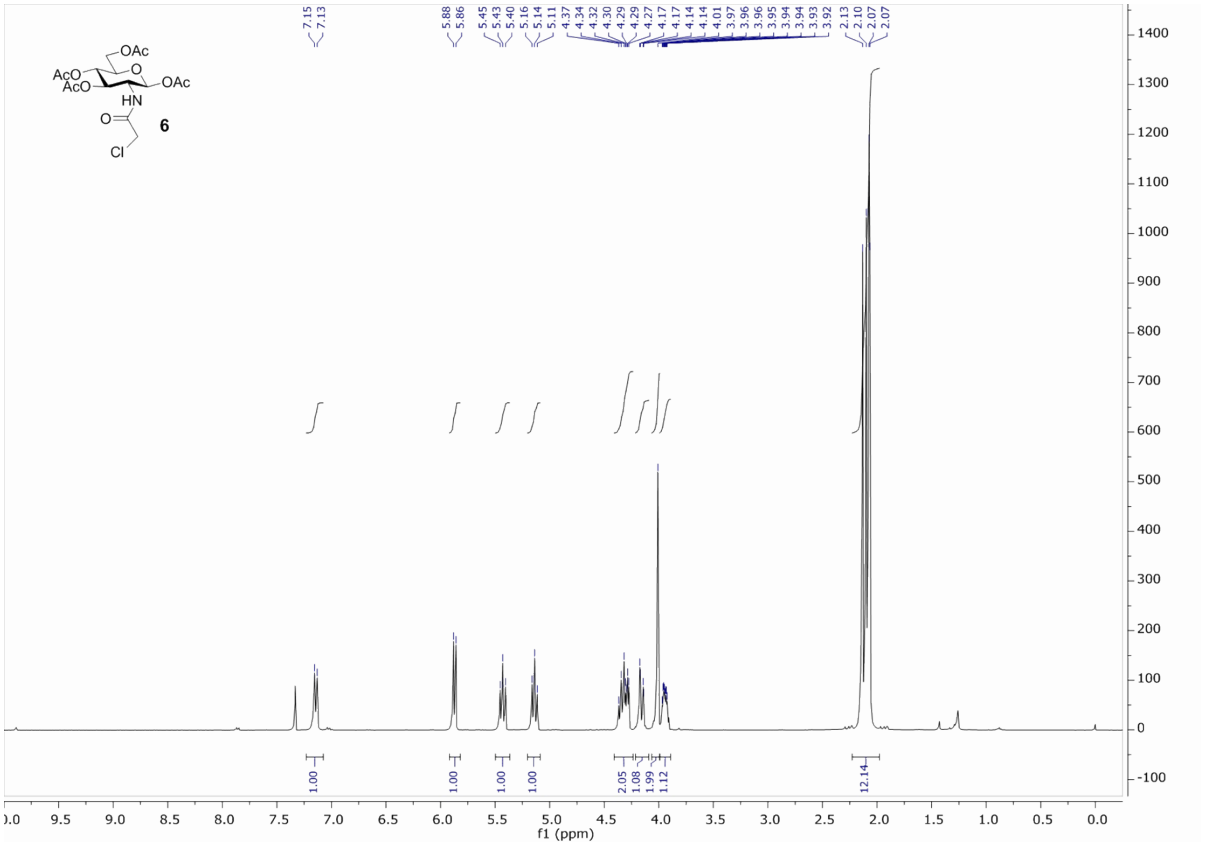


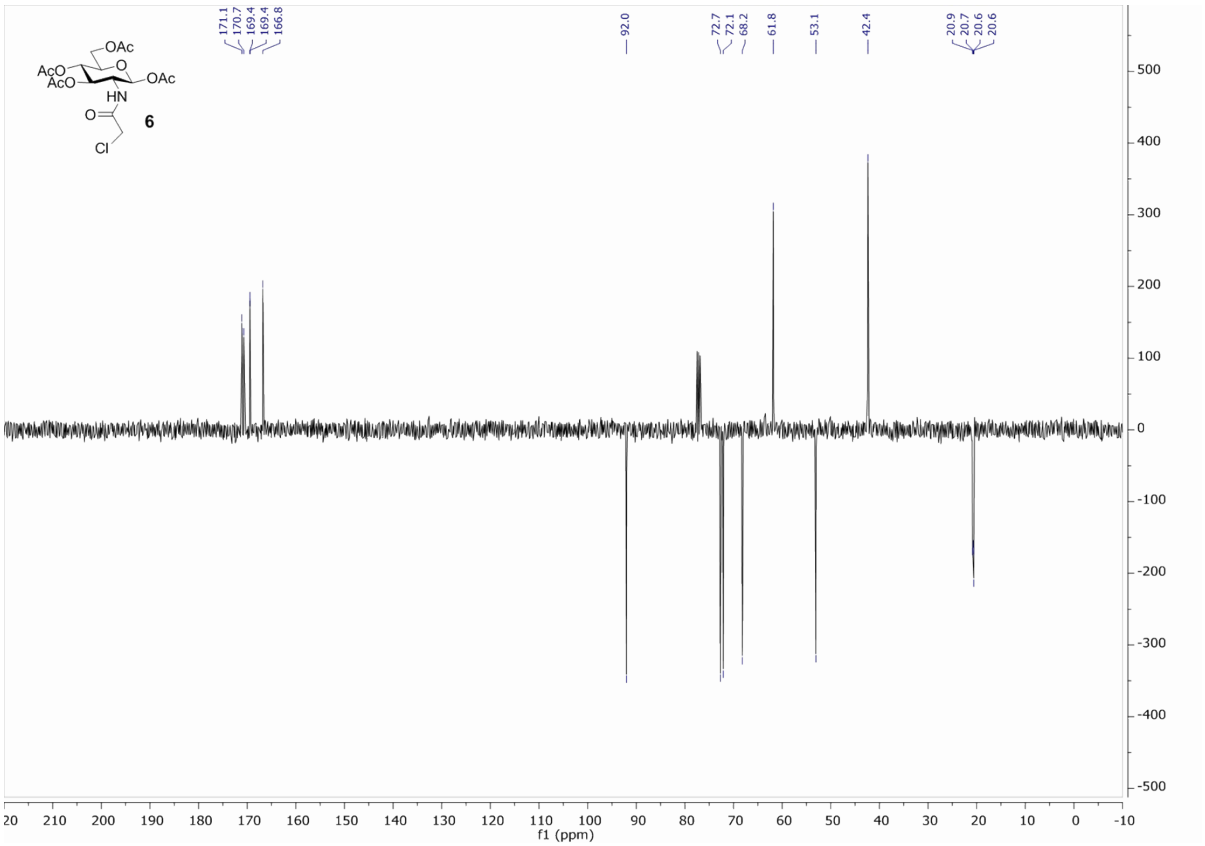
3,4,6-tri-*O*-acetyl-2-azidoacetamido-2-deoxy-β-D-glucopyranosyl azide (3)



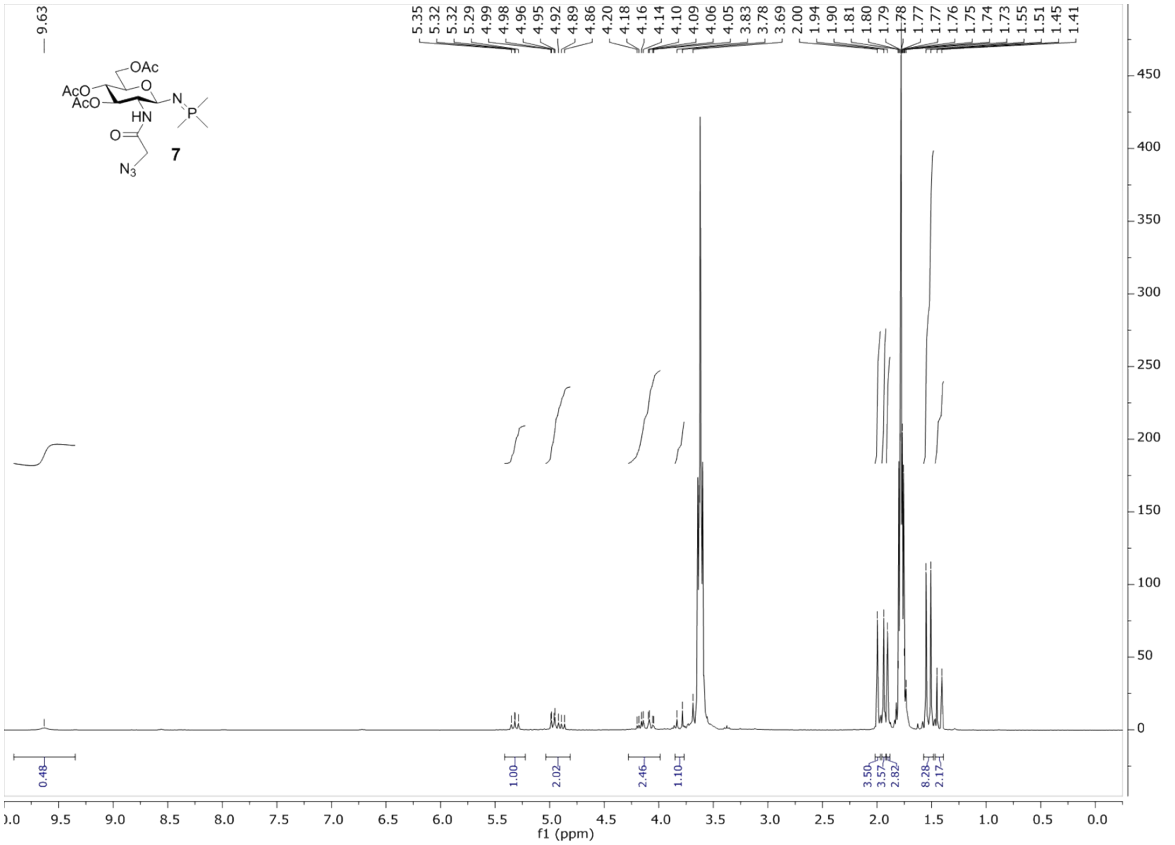


1,3,4,6-tetra-*O*-acetyl-2-chloroacetamido-2-deoxy-β-D-glucopyranose (6)

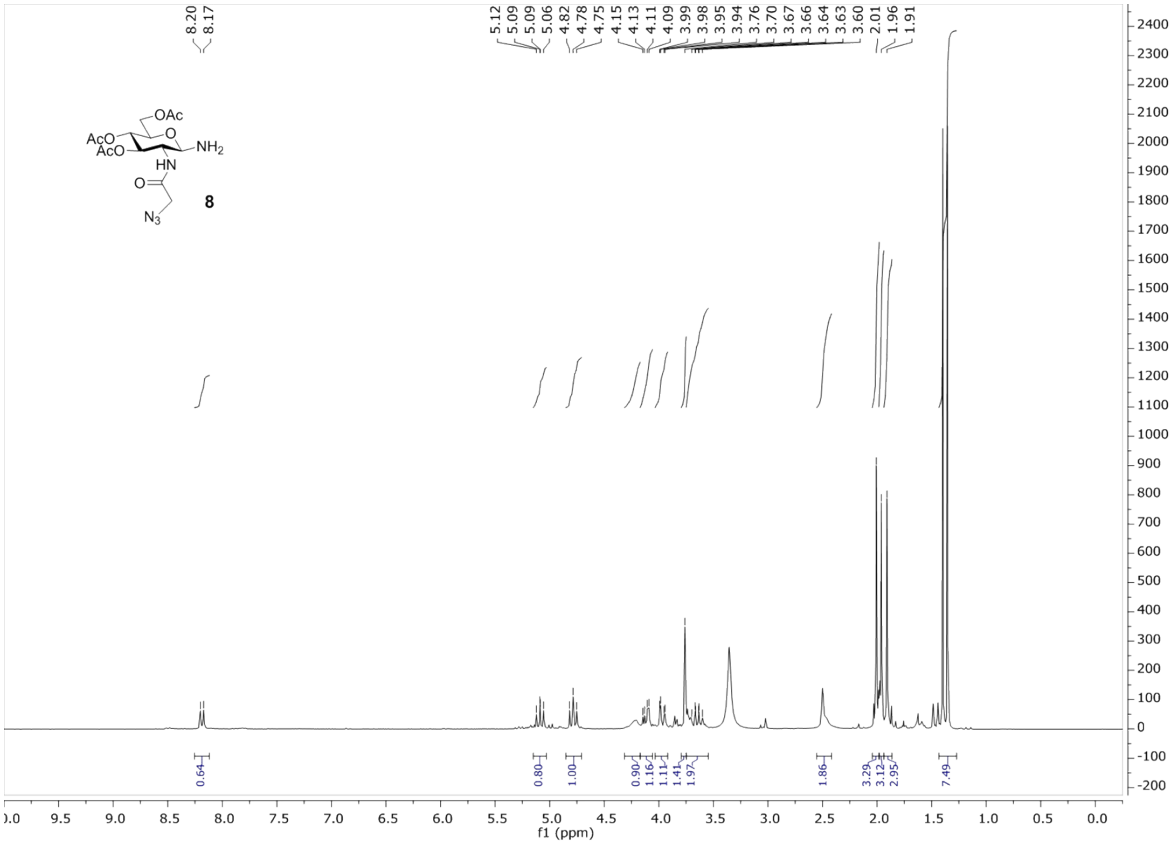




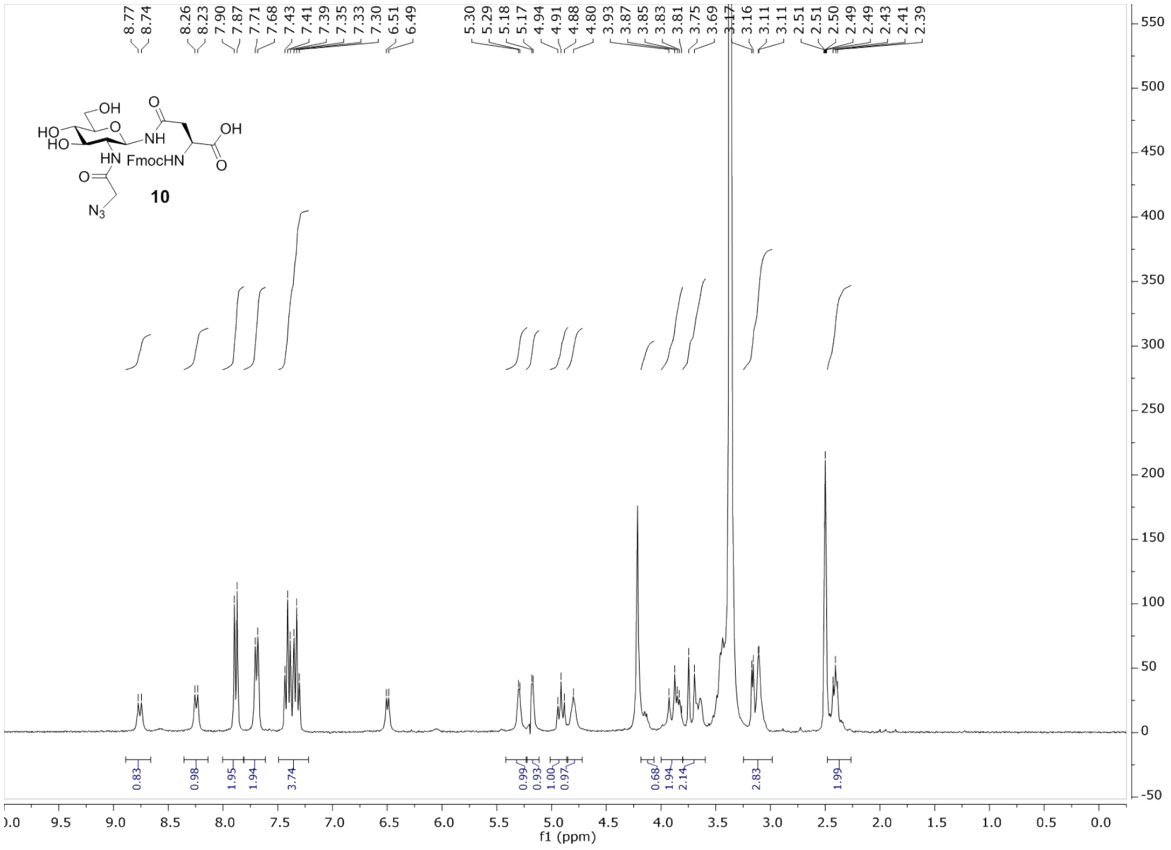
3,4,6-tri-*O*-acetyl-2-azidoacetamido-2-deoxy-β-D-glucopyranosyl trimethylphosphinimide (7)



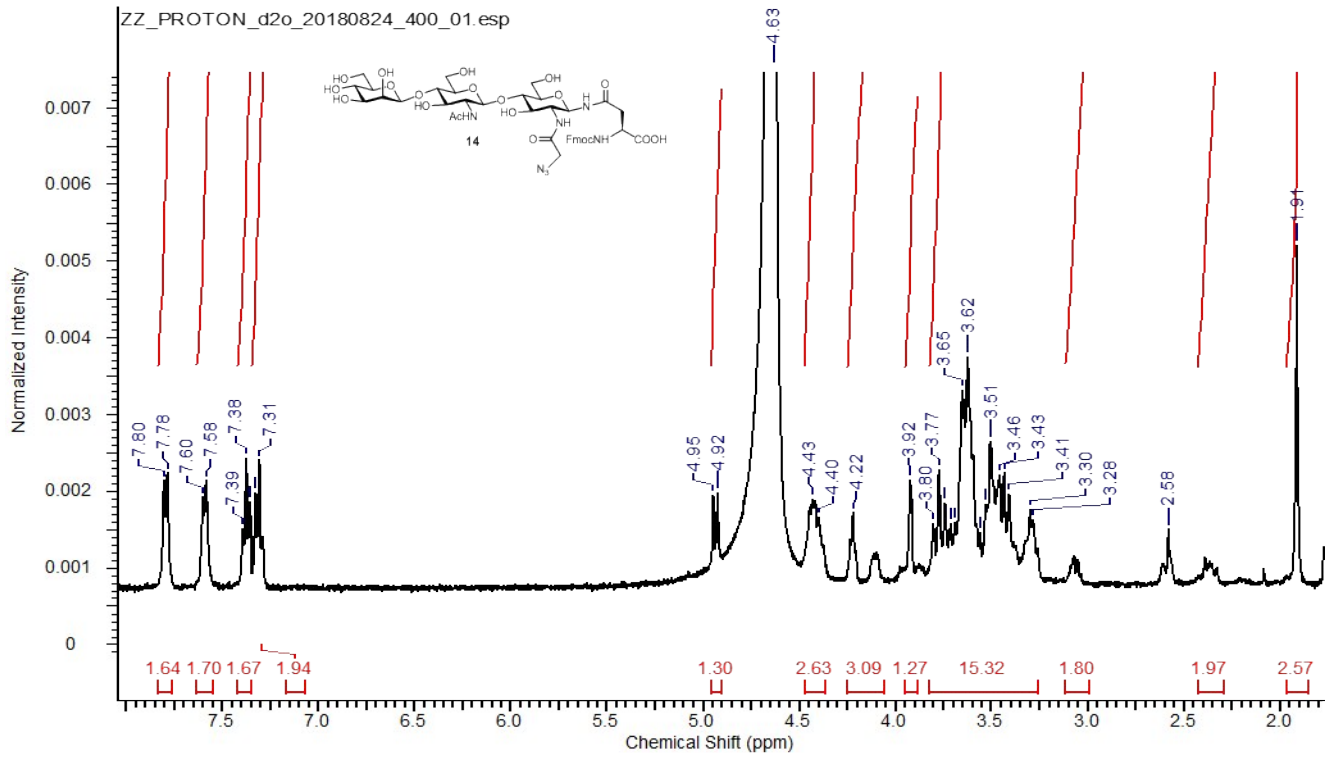
3,4,6-tri-*O*-acetyl-2-azidoacetamido-2-deoxy-β-D-glucopyranosyl amine (8)



N^{γ}-[2-azidoacetamido-2-deoxy-β-D-glucopyranosyl]-N^{α}-fluorenylmethoxycarbonyl-L-asparagine (10)



ManGlcNAcGlcNAz-Fmoc-Asn (14):



References

[1] C. D. Heidecke, Z. Ling, N. C. Bruce, J. W. B. Moir, T. B. Parsons, and A. J. Fairbanks, *ChemBioChem*, 2008, **9**, 2045-2051.
[2] H,P coupling constant in agreement with those reported for similar compounds in L. Kovács, E. Ősz, V. Domokos, W. Holzer and Z. Györgydeák, *Tetrahedron*, 2001, **57**, 4609–4621.
[3] F. M. Ibatullin and S. I. Selivanov, *Tetrahedron Lett.*, 2009, **50**, 6351–6354.
[4] These signals (corresponding to the quarternary carbon atoms of the Fmoc) were double, presumably due to their diastereotopic nature, an observation made for several unrelated Fmoc-protected compounds in our lab.
[5] M. Bergmann and L. Zervas, *Ber. Dtsch. Chem. Ges.*, 1931, **64**, 975–980.
[6] For larger scale, we prefer a different eluent system (0 →10 → 20% EtOAc in DCM) for solubility reasons.
[7] This compound was poorly soluble in both H_2O and DMSO, though soluble enough for enzymatic glycosylation.