Investigation of acyl transfer auxiliary-assisted glycoconjugation for glycoprotein semi-synthesis

Kudakwashe Nyandoro, Charles M. G. Lamb, Haoran Yu, Jian Shi, and Derek Macmillan *

Supporting information

Sugar-Linked Auxiliary Synthesis:

Chemicals were purchased from Sigma-Aldrich, Acros, Fischer Scientific and Nova Biochem, and used without further purification. Anhydrous solvents were purchased from Fischer Scientific and Sigma-Aldrich and were used as supplied. Flash chromatography was carried out on silica gel 60 Å (35-70 microns) from Merck. Thin layer chromatography was carried out on aluminium sheets coated (0.25 mm layer coating) with Merck silica gel 60 Å F_{254}. When required TLC staining was performed with *p*-anisaldehyde dip. All nuclear magnetic resonance (NMR) experiments were recorded at room temperature on Bruker AMX 400, 500 and 600 MHz instruments. Chemical shifts (δH and δC) are recorded in ppm with the stated residual solvent as the internal standard.

*Synthesis of sugar linked auxiliary **7***: Benzaldehyde **3** (1.21g, 3.47 mmol), glycosyl amine **2a** (1.00 g, 2.89 mmol) and sodium triacetoxyborohydride (1.22 g, 5.78 mmol) were dissolved in CH_2Cl_2 (15 ml) and stirred at room temperature for 45 min. The mixture was then diluted with CH_2Cl_2 (15 ml) and neutralised with sat. aq. $NaHCO_3$ (15 ml). The mixture was separated and the aqueous phase was extracted with CH_2Cl_2 (3 x 15ml). The combined organic extracts were collected and washed with brine (25 ml), dried over $MgSO_4$, filtered and evaporated under vacuum to afford crude product. The crude product was flash chromatographed on silica gel using a solvent gradient from 1:1 EtOAc/Pet ether to 100% EtOAc to afford **6** as a white solid (36%). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H/ppm: 7.10 (2H, d, *J*= 8.6 Hz, PMB), 6.80 (2H, d, *J*= 8.6 Hz, PMB), 6.60 (1H, s, Ar), 5.32 (1H, t, *J*= 9.3 Hz, NH), 4.27-4.23 (1H, dd, *J*= 4.50, 12.1 Hz, H-6_a), 4.11 (1H, d, *J* = 9.5 Hz, H-1), 4.07-3.80 (6H, m, H-6_b, H-2, CH_2, H-3, H-4), 3.97 (3H, s, CH_3), 3.85 (3H, s, CH_3), 3.84 (3H, s, CH_3), 3.78 (3H, s, CH_3), 3.74 (2H, s, CH_2), 3.60 (1H, m, H-5), 2.08 (3H, s, CH_3), 2.00 (6H, s, 2 x CH_3), 1.78 (3H, s, CH_3). ^{13}C NMR (101 MHz, CD_3OD) δ_C (ppm): 171.8, 171.6,170.6, 169.9 (4xC=O), 152.8, 152.7, 152.5, 141.8, 131.1, 129.7, 129.2, 127.8, 114.8, 113.5, 89.9, 74.7, 73.7, 70.7, 63.6, 62.0, 61.1, 56.6, 55.5, 54.9, 43.0, 40.5, 22.8, 20.7, 20.6, 20.5. ESI-HRMS: m/z calculated for $C_{32}H_{42}N_2O_{12}S$ [M+H] 679.2537, found $[M+H]^+$ 679.2538.

To a solution of **6** (0.5 g, 0.70 mmol) in 2,2,2-trifluroethanol (20 ml) was added a solution of 3-nitro-2-pyridinesufenyl chloride (0.23 g, 1.2 mmol) in CH_2Cl_2 (5 ml). The reaction was stirred at room temperature for 1 h and more 3-nitro-2-pyridinesufenyl chloride (50 mg, 0.26 mmol) was added. The mixture was stirred for another 30 min and concentrated under vacuum to give crude **7** which was purified by flash chromatography on silica using a solvent gradient from 1:1 EtOAc/Pet ether to 100% EtOAc to afford **7** as a yellow oil (0.21 g, 41%). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H/ppm: 8.84 (1H, dd, *J*= 1.5, 4.4 Hz, Pyr), 8.53 (1H, dd, *J*= 1.5, 8.2 Hz, Pyr), 7.42-7.33 (1H, m, Pyr), 7.04 (1H, s, ArH), 5.58 (1H, d, *J* = 8.9 Hz, NH), 5.11 (1H, apt, *J* = 9.6 Hz, H-3), 5.00 (1H, apt, *J* = 9.7 Hz, H-4), 4.35-4.19 (2H, m, H-1, H-6_a), 4.17-4.00 (4H, m, H-6_b, H-2, CH_2), 3.84 (3H, s, OMe), 3.83 (3H, s, OMe), 3.75 (3H, s, OMe), 3.63-3.61 (1H, m, H-5), 2.10 (3H, s, CH_3), 2.06-1.97 (6H, m, 2 x CH_3), 1.87 (3H, s, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ_C/ppm: 171.5, 170.9, 170.8, 169.4 (4xC=O), 159.0, 153.6, 152.8, 142.3, 133.7, 131.2, 130.1, 121.2, 110.2, 89.3, 73.6, 72.4, 74.3, 68.6, 62.5, 61.4, 60.9, 60.4, 56.1, 53.7, 49.2, 42.5, 41.5, 23.3, 20.9, 20.8. ESI-MS: m/z calculated for $C_{29}H_{36}N_4O_{13}S_2$ $[M]^+$ 712.17, found $[M+H]^+$, 713.18.

*Synthesis of sugar-linked auxiliary **10***: Glycosyl amine **2b** (0.50 g, 2.25 mmol) was dissolved in pyridine (10 ml). To the mixture was added **8** (0.6 g) and stirred overnight at 50 °C. The solvent was evaporated, and the crude product was flash chromatographed on silica gel with EtOAc/MeOH to afford **9** (0.50g, 40 %) as a white solid. ^{1}H NMR (500 MHz, CD_3OD) δ_H/ppm: 7.09 (2H, d, *J*= 8.6 Hz, PMB), 6.80 (2H, d, *J*= 8.6

Hz, PMB), 6.67 (1H, s, ArH), 3.87 (1H, m, H-6_b), 3.87-3.68 (2H, m, CH_2), 3.85 (3H, s, CH_3), 3.81 (1H, m, H-2), 3.80 (3H, s, CH_3), 3.74 (3H, s, CH_3), 3.70 (3H, s, CH_3), 3.69 (2H, m, H-1, H-6_a), 3.60-3.52 (1H, m, H-3), 3.35-3.32 (1H, m, H-4), 3.35-3.31 (2H, m, CH_2), 3.21-3.17 (1H, m, H-5), 1.86 (3H, s, CH_3). ESI-HRMS: m/z calculated for $C_{26}H_{36}N_2O_9S$ $[M]^+$ 552.2142, found $[M+H]^+$ 553.2205.

To a solution of **9** (55.3 mg, 0.10 mmol) in 2,2,2-trifluroethanol (10 ml) was added a solution of 3-nitro-2-pyridinesufenyl chloride (22.9 mg, 0.11 mmol) in CH_2Cl_2. The reaction was stirred at room temperature for 1 h and more 3-nitro-2-pyridinesufenyl chloride (10 mg) was added. The mixture was stirred for another 30 min and concentrated in vacuo to give **10** as an orange solid. **10** appeared unstable to chromatography and was used without further purification ESI-HRMS: m/z calculated for $C_{23}H_{31}N_4O_{10}S_2$ $[M+H]^+$ 587.1476, found $[M+H]^+$ 587.1451.

*Synthesis of sugar-linked auxiliary **11***: Anhydrous EtOH/THF (5:3**,** 8 ml), Et_3N (0.78 ml, 5.6 mmol), and di-*tert*butyl dicarbonate (1 M in THF, 1.68 ml, 1.68 mmol) were added to glycosyl nitrile (**12**, 0.2 g, 0.56 mmol) and 10% Pd/C (0.1 g, 0.5 g/g **12**) under nitrogen. The reaction mixture was evacuated and then stirred under a hydrogen atmosphere at room temperature. After 16 h TLC confirmed complete consumption of starting material. The reaction mixture was filtered through celite and the filtrate concentrated under vacuum. The crude material was dissolved in CH_2Cl_2 (15 ml), and then washed with saturated $NaHCO_3$ (15 ml), brine (15 ml), water (15 ml), dried over $MgSO_4$, filtered, and concentrated under vacuum. The crude product was purified by flash column chromatography over silica (100% EtOAc) to afford **13** as a white powder (0.21 g, 0.46 mmol, 82%). R_f = 0.37 (100% EtOAc). ^{1}H NMR (600MHz, $CDCl_3$) δ_H/ppm: 5.75 (1H, d, *J* = 9.1 Hz, NH), 5.15-4.95 (3H, m, H-3, H-4, NH), 4.23 (1H, dd, *J* = 12.2, 4.6 Hz, H-6_a), 4.10 (1H, dd, J = 12.3, 2.4 Hz, H-6_b), 4.00-3.88 (1H, q, *J* = 9.8 Hz , H-2), 3.65-3.50 (2H, m, H-5, H-1), 3.50-3.34 (1H, m, CH_{2a}), 3.08-2.98 (1H, m, CH_{2b}), 2.08 (3H, s, $COCH_3$), 2.02 (6H, s, 2 x $COCH_3$), 1.95 (3H, s, $COCH_3$), 1.43 (9H, s, 3 x CH_3). ^{13}C NMR (151 MHz, $CDCl_3$) δ_C/ppm: 171.5, 170.8, 170.6, 169.4 (4x C=O), 156.0, 79.7, 78.1, 75.7, 73.9, 68.4, 62.1, 51.8, 41.7, 28.3, 23.3, 20.8 , 20.7, 20.6. ESI-HRMS: m/z calculated for $C_{15}H_{25}N_2O_8$ $[M+H]^+$, 461.1611 found $[M+H]^+$, 461.1602.

13 (0.3 g, 0.65 mmol) was dissolved in dichloromethane (5 ml) and to it was added TFA (1.3 ml) and the reaction was stirred for 2 h at room temperature. Solvents were evaporated under vacuum and the crude material triturated with ether to yield glycosyl methylamine (0.21 g, 94%) which was used without further purification. ^{1}H NMR (600MHz, $CDCl_3$) δ_H/ppm: 5.81 (1H, d, *J* = 9.2 Hz, NH), 5.11- 4.99 (2H, m, H-3, H-4), 4.24 (1H, dd, *J* = 12.3, 4.7 Hz, H-6a), 4.11-4.03 (2H, m, H-6b, H-2), 3.67-3.60 (1H, m, H-5), 3.59 - 3.51 (1H, m, H-1), 2.78 (2H, d, *J* = 4.8 Hz, CH_2N), 2.08 (3H, s, CH_3), 2.02 (6H, s, 2 x CH_3), 1.94 (3H, s, CH_3). ^{13}C NMR (151 MHz, $CDCl_3$) δ_C/ppm: 171.6, 170.9, 170.5, 169.6 (C=O), 78.6 (C5), 75.9 (C4), 74.2 (C3), 68.5 (C1), 62.4 (C6), 51.9 (C2), 50.3 (CH_2), 23.4 (NAc), 21.0, 20.9, 20.8 (OAc). ESI-MS: m/z calculated for $C_{15}H_{25}N_2O_8$ [M + H], 361.1611 found [M+H], 361.1602. Benzaldehyde **3** (0.42 g, 1.2 mmol) was dissolved in a mixture of 2% AcOH in CH_2Cl_2 (6 ml). Glycosyl methylamine (0.36 g, 1.0 mmol) was then added to the mixture and followed by sodium cyanoborohydride (0.15 g, 2.4 mmol). The reaction was stirred at room temperature and monitored by TLC. After 2 the mixture was diluted with CH_2Cl_2 (10 ml) and neutralised with sat. aq. $NaHCO_3$ (10 ml). The organic layer was separated and washed with brine (2 x 5 ml), dried over $MgSO_4$, filtered and evaporated under vacuum. The crude product was flash chromatographed on silica gel with EtOAc/MeOH 9:1 solvent system to afford **14** (0.47 g, 68%) as a yellow oil. ^{1}H NMR (600MHz, $CDCl_3$) δ_H/ppm: 7.10 (2H, d, *J*= 8.6 Hz, PMB), 6.80 (2H, d, *J*= 8.6 Hz, PMB), 6.60 (1H, s, ArH), 5.70 (1H, s, NH), 5.04 (2H, m, H-3, H-4), 4.18 (1H, dd, *J* = 12.3, 4.7 Hz, H-6_a), 4.04 (1H dd, *J* = 12.3, 2.2 Hz, H-6_b) 4.01-3.92 (3H, m, H-2, CH_2), 3.86-3.79 (1H, m, H-1), 3.86 (3H, s, CH_3), 3.83 (3H, s, CH_3), 3.79 (3H, s, CH_3), 3.78 (3H, s, CH_3), 3.72 (2H, s, CH_2), 3.58-3.55 (2H, m, H-5, CH_{2a}), 2.78 – 2.70 (3H, m, CH_{2b}, CH_2), 2.04-1.98 (12H, 4x s, 4 x CH_3). ^{13}C NMR (151 MHz, $CDCl_3$) δ_C/ppm: 171.6, 170.9, 170.5, 169.6 (4x C=O), 159.0, 152.9, 152.5, 141.7, 130.4, 130.2, 129.4, 114.2, 112.4, 102.0, 77.3, 75.7, 74.3, 68.5, 62.5, 61.4, 60.9, 56.0, 55.4, 52.2, 49.7, 45.6, 40.4, 23.4, 20.9, 20.8. ESI-HRMS: m/z calculated for $C_{33}H_{45}N_2O_{12}S$ $[M+H]^+$ 693.2693, found $[M+H]^+$ 693.2692.

To a solution of **14** (68 mg, 0.10 mmol) in 2,2,2-trifluroethanol (8 ml) was added a solution of 3-nitro-2-pyridinesufenyl chloride (20 mg, 0.11 mmol) in CH_2Cl_2 (2 ml). The reaction was stirred at room temperature for 2 h and more 3-nitro-2-pyridinesufenyl chloride (10 mg, 52 µmol) was added. The mixture was stirred for another 30 min and concentrated in vacuo. The crude product was flash chromatographed on silica gel with EtOAc/MeOH 9:1. Two disulfide products were obtained as a mixture from the column and may be used in the next step without further purification. Alternatively

mixed disulfide products (100 mg, 0.18 mmol) were dissolved in ligation buffer (6M Gn·HCl in 0.1 M sodium phosphate buffer pH 7.0) (10 ml), and TCEP was added to final concentration of 30 mM. The reaction mixture was purified by preparative HPLC and lyophilised to yield the thiol **15** as a yellow oil (35 mg, 40%). ^{1}H NMR (600MHz, $CDCl_3$) δ_H/ppm: 6.78 (1H, s, ArH), 5.15-5.07 (1H, m, H-3), 5.05-4.99 (1H, m, H-4), 4.40 (1H, d, *J*=13.4, CH_{2a}), 4.25 (1H, d, *J*=13.4, CH_{2b}), 4.20-4.1(2H, m, H-1, H-6_a), 4.03-3.95 (5H, m, H-2, , H-6_b, CH_3), 3.86 (3H, s, CH_3), 3.81 (3H, s, CH_3), 3.58-3.55 (2H, m, H-5, CH_{2a}), 3.39 (1H, d, *J* = 12.8 Hz, CH_{2a}), 2.98 (1H, dd, *J* = 12.8, 8.1 Hz, CH_{2b}), 2.10-1.80 (12H, s, 4 x CH_3). ^{13}C NMR (151 MHz, $CDCl_3$) δ_C/ppm: 172.0, 171.1, 170.8, 169.5 (4xC=O), 155.0, 152.7, 140.9, 125.6, 116.4, 112.7, 76.0, 74.5, 73.3, 68.1, 62.0, 61.5, 61.0, 56.3, 51.8, 48.4, 46.2, 23.1, 20.8. ESI-HRMS: m/z calculated for $C_{25}H_{37}N_2O_{11}S$ $[M+H]^+$ 573.2118, found $[M+H]^+$ 573.2120.

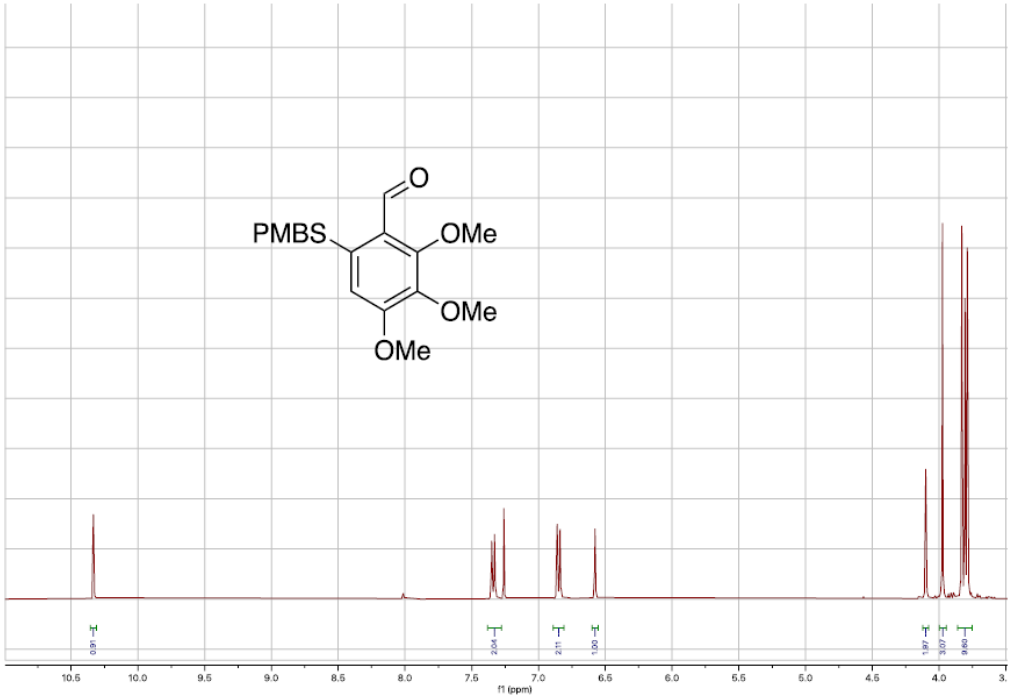
*Synthesis of sugar-linked auxiliary **19***. Boc protected sugar **13** (160 mg, 0.35 mmol) was dissolved in DCM (6 mL) and TFA (1.2 mL) was added dropwise to the stirring solution. The yellow reaction mixture was stirred for 3 h at room temperature until TLC showed complete consumption of the starting material, then concentrated under vacuum to yield an oil, which was used without further purification. ^{1}H NMR (600MHz, $CDCl_3$) δ_H/ppm: 5.81 (1H, d, *J* = 9.2 Hz, NH), 5.11 – 4.99 (2H, m, H-3, H-4), 4.24 (1H, dd, *J* = 12.3, 4.7 Hz, H-6_a), 4.11 – 4.03 (2H, m, H-6_b, H-2), 3.67 – 3.60 (1H, m, H-5), 3.59 - 3.51 (1H, m, H-1), 2.78 (2H, d, *J* = 4.8 Hz, CH_2N), 2.08 (3H, s, $COCH_3$), 2.02 (6H, s, 2 x $COCH_3$), 1.94 (3H, s, $COCH_3$). ^{13}C NMR (151 MHz, $CDCl_3$) δ_C/ppm: 171.6, 170.9, 170.5, 169.6 (C=O), 78.6 (C_5), 75.9 (C_4), 74.2 (C_3), 68.5 (C_1), 62.4 (C_6), 51.9 (C_2), 50.3 (CH_2), 23.4 (NAc), 21.0, 20.9, 20.8 (OAc). ESI-MS: m/z calculated for $C_{15}H_{25}N_2O_8$ [M + H], 361.1611 found [M+H], 361.1602.

The oil (121 mg, 0.34 mmol) was dissolved in a mixture of 2% AcOH in dichloromethane (6 ml). 2-mercapto-2-phenethyl auxiliary **20** (166 mg, 0.42 mmol) was added along with sodium triacetoxyborohydride (0.36 g, 1.68 mmol). The reaction mixture was stirred at room temperature for 4 hours until TLC showed complete consumption of the starting material. The mixture was then diluted with dichloromethane (10 mL), washed with sat. aq. $NaHCO_3$ (10 mL) and sat. aq. NaCl (2 x 10 mL). The organic phase was then dried over $MgSO_4$, filtered under gravity and concentrated *in vacuo* to produce a pale-yellow oil. The crude product was purified by flash chromatography with EtOAc as the eluant, ramping up to 9:1 EtOAc/MeOH. The eluted product was concentrated to produce **21** as a viscous yellow oil (132 mg, 50%). ^{1}H NMR (600 MHz, $CDCl_3$) δH/ppm 7.40-7.35 (12H, m, ArH), 7.27-7.15 (22H, m, ArH), 7.07-7.03 (4H, m, ArH), 5.01-4.86 (4H, m, H-3, H-4), 4.14-4.04 (4H, m,H-6a, H-6b), 3.93 – 3.86 (4H, m, H-2), 3.62 (1H, m, SCH), 3.50 – 3.39 (2H, m, H-5), 3.36 – 3.30 (2H, 2 x d, H-1), 3.28 – 3.20 (1H, m, CH2), 3.16 – 3.10 (1H, m, CH2), 2.79 – 2.71 (1H, m, CH2), 2.66 – 2.60 (1H, m, CH2), 2.42 – 2.33 (2H, m, CH2), 2.28 – 2.23 (1H, dd, CH2), 2.21 – 2.15 (1H, m, CH2), 2.05 (24H, m, OAc). ^{13}C NMR (126 MHz, CDCl3) δ_C/ppm 171.7, 170.8, 170.0, 169.4, 146.8, 144.8, 129.9 128.6, 128.5, 128.2, 128.1, 128.0, 127.9, 127.2, 126.8, 126.8, 75.6, 74.5, 74.4, 68.9, 68.4, 62.5, 62.4, 55.4, 53.6, 52.3, 52.1, 50.4, 49.7, 31.6, 30.5, 30.3, 29.8, 23.4, 23.3, 20.8, 20.8. ESI-HRMS: m/z calculated for $C_{42}H_{47}N_2O_8S$ $[M+H]^+$ 739.3053, found $[M+H]^+$, 739.3030

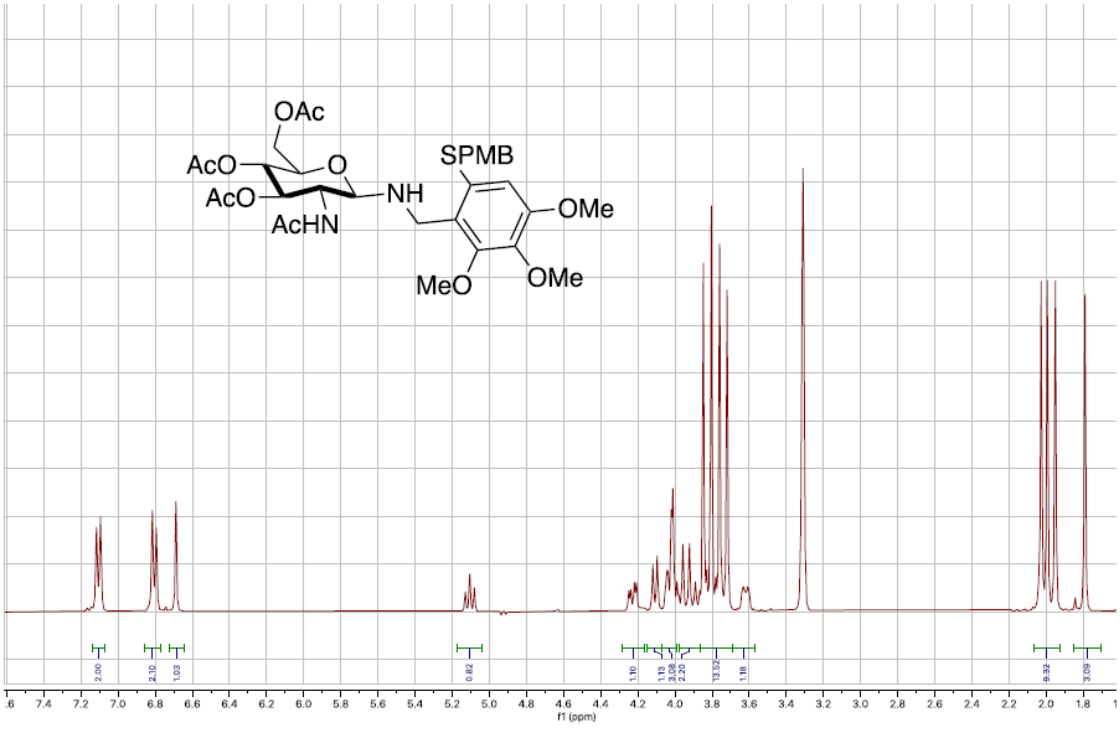
21 (7 mg, 9.47 µmol) was dissolved in 95:5 DCM/Triethyl silane (1.9 mL) and TFA (0.1 mL). The mixture was stirred at room temperature. After 2 hours, TLC showed complete consumption of the starting material and the reaction was concentrated in vacuo. The resulting orange oil was washed with diethyl ether (3 x 5 mL) and again concentrated in vacuo to yield **19** (4.1 mg, 8.26 µmol, 96%) as a clear viscous oil which was used in ligation reactions without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H/ppm (observed as an epimeric mixture) 7.42-7.26 (10H, m, ArH), 5.15-5.00 (4H, m, H-3, H-4), 4.47 – 4.28 (2H, m, H-1), 4.22 - 4.10 (2H, m,H-6a, H-6b), 4.02 – 3.25 (14H, m, 2 x CH2, H-2, H-5, SCH), 2.12-1.92 (24H, m, COCH3). 13C NMR (126 MHz, CDCl3) δC/ppm 175.5, 174.4, 173.9, 173.6, 140.3, 140.1, 130.4, 130.3, 129.7, 129.6, 127.8, 127.7, 75.6, 75.5, 74.2, 74.1, 73.8, 69.2, 57.9, 54.8, 54.6, 52.0, 49.9, 41.4, 40.0, 39.8, 22.6, 22.6, 20.9, 20.8, 20.71. ESI-HRMS: m/z calculated for $C_{23}H_{33}N_2O_8S$ $[M+H]^+$ 497.1958, found ESI [M+H], 497.1952

Selected Spectra:

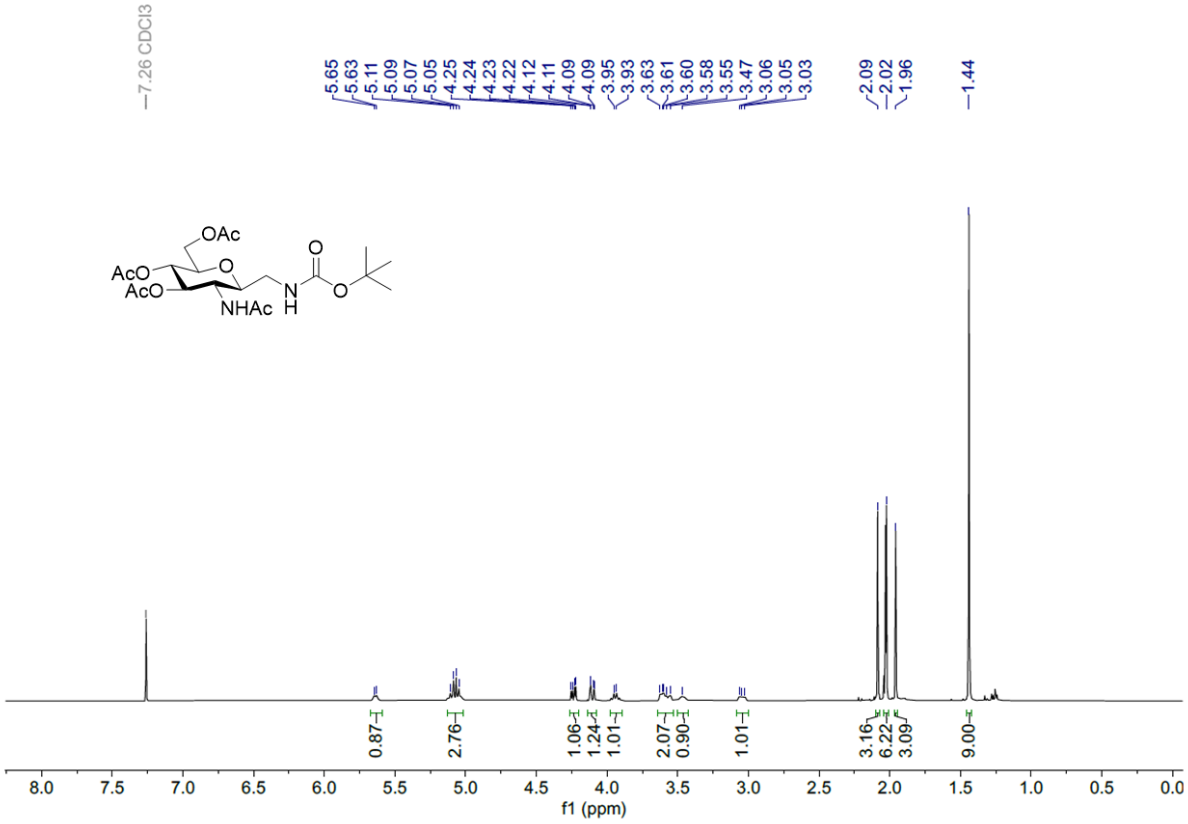
Aldehyde **3**

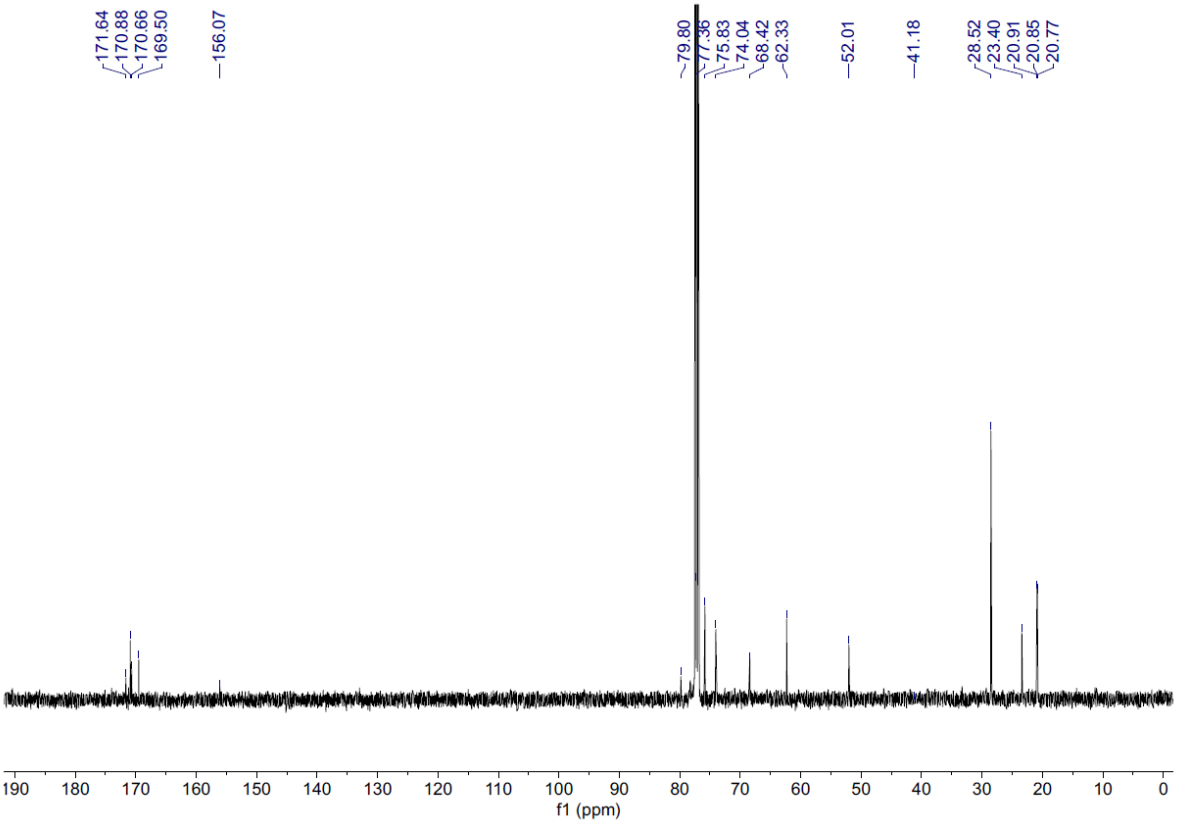


Glycosyl amine **6**

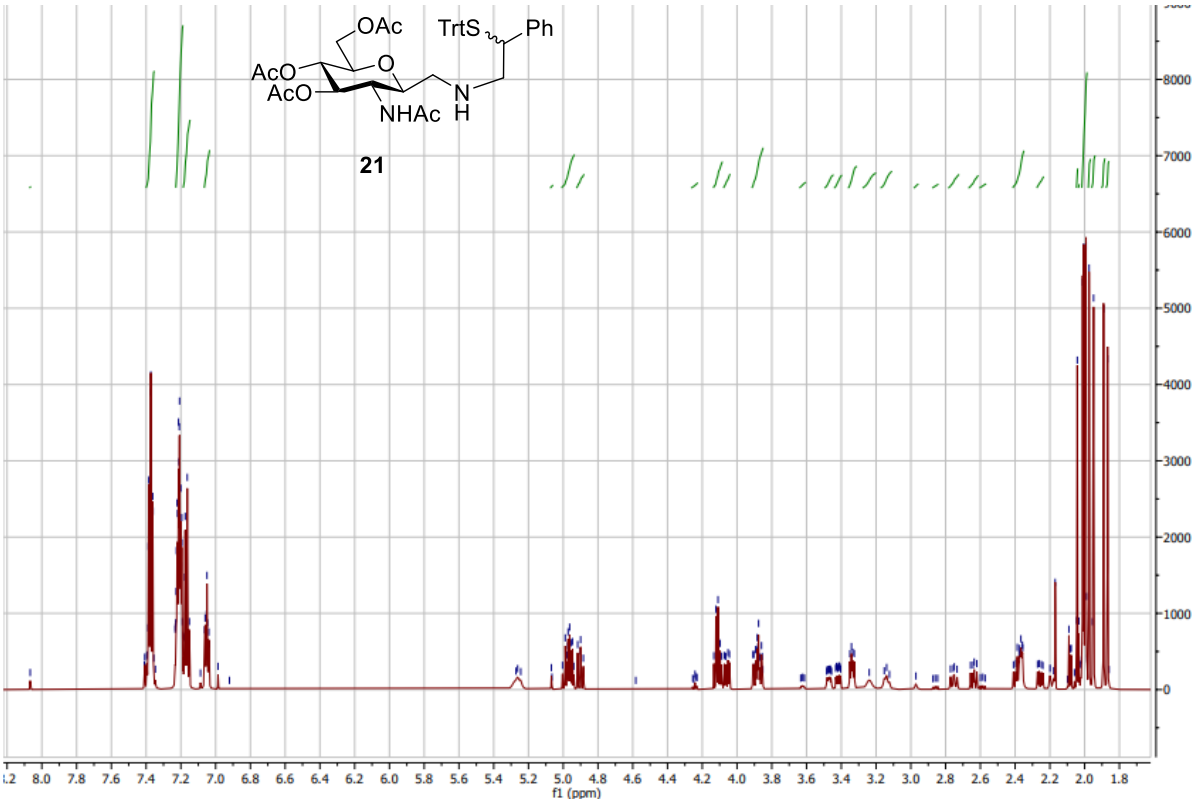


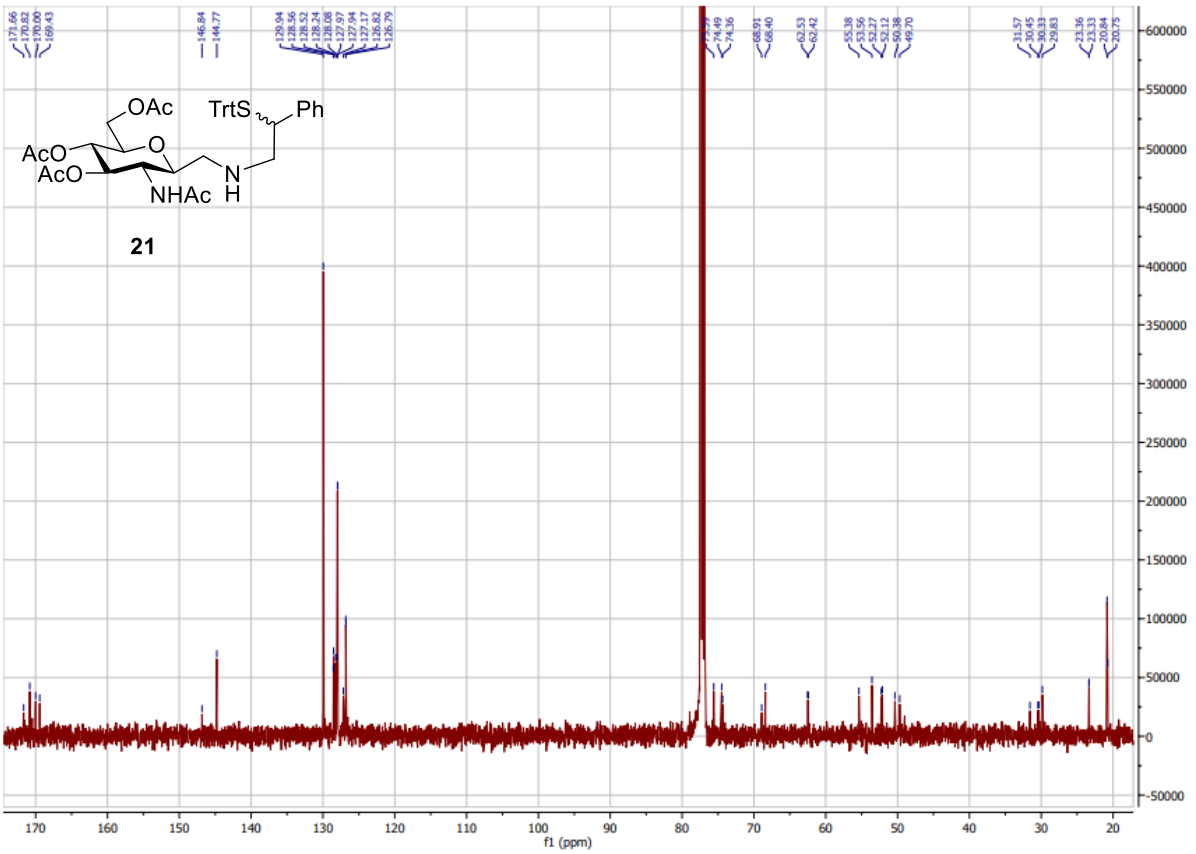
Boc protected glycosyl methylamine **13**:



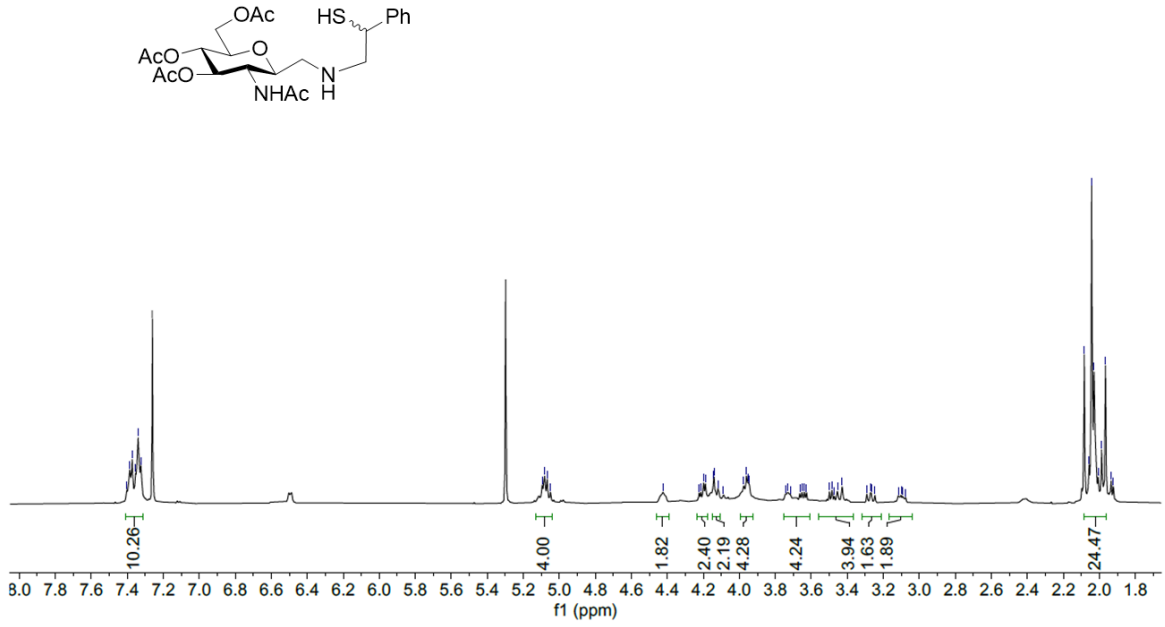


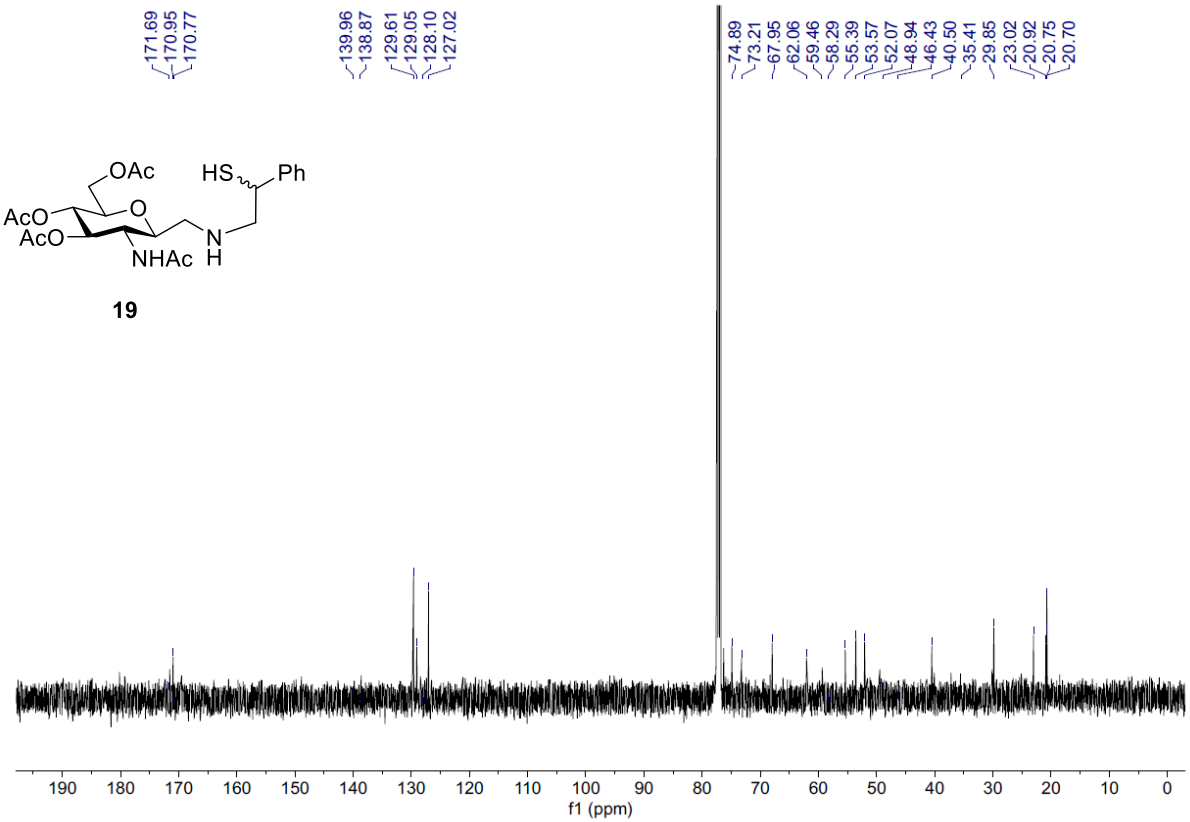
Trityl protected sugar-linked auxiliary **21**.





Sugar-linked auxiliary **19**

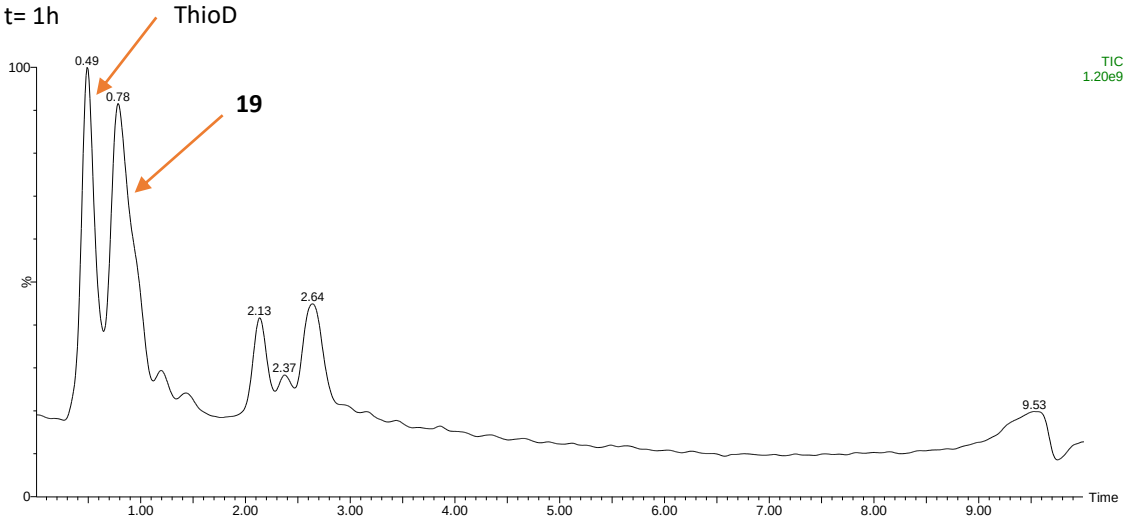


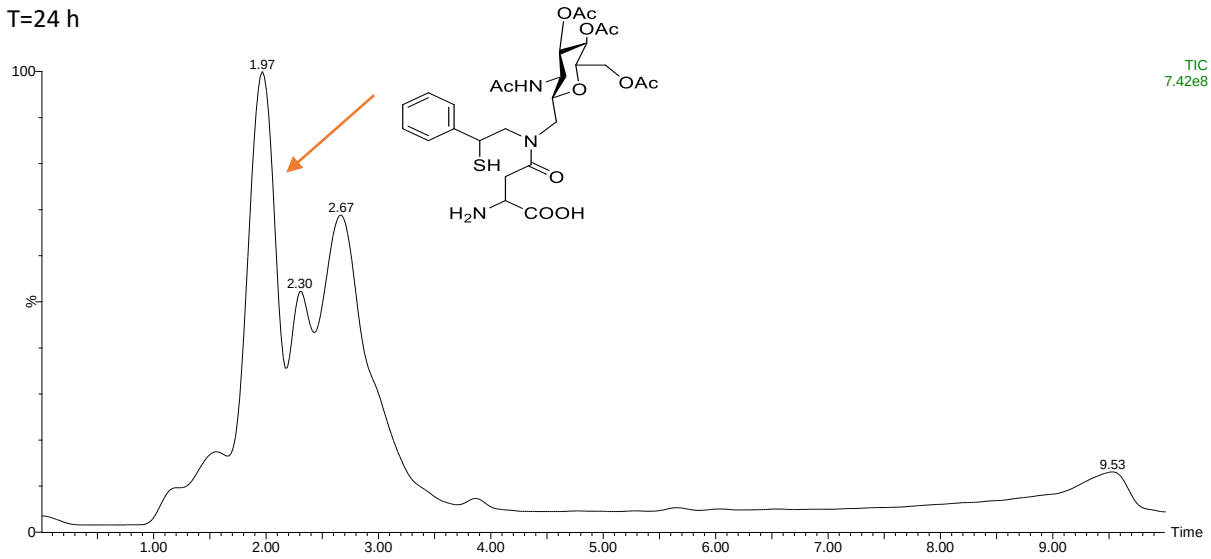


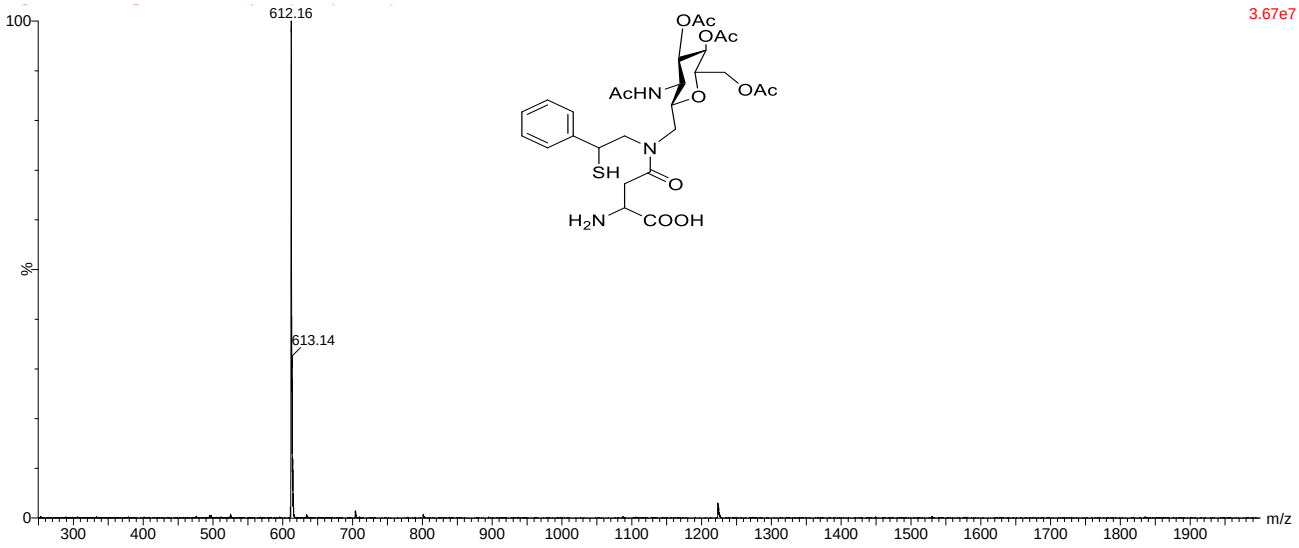
19

Reaction between **19** and ThioD.

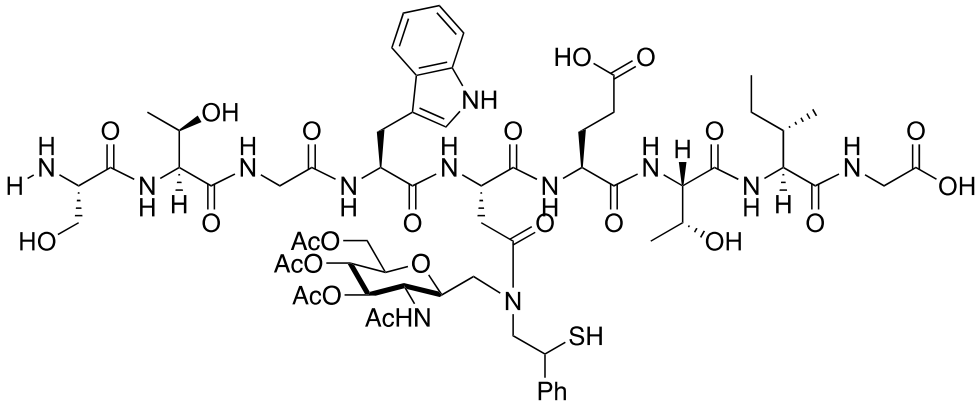
ThioD (1 mg, 3.3 µmol) was dissolved in a ligation buffer comprised of 0.1 M sodium phosphate buffer pH 7 and 6.0 M guanidinium chloride to a concentration of 2 mg/mL. To this was added **19** (2.1 mg, 4.3 µmol, 1.3 equiv), MPAA (17 mg, final concentration 0.2 M) and TCEP (7.5 mg, final concentration 60 mM), adjusting the pH to 7 with 1 M NaOH. The reaction was then shaken on a thermomixer (750 rpm) at 25 °C and monitored via LC-MS. After 24 hours the reaction was complete.

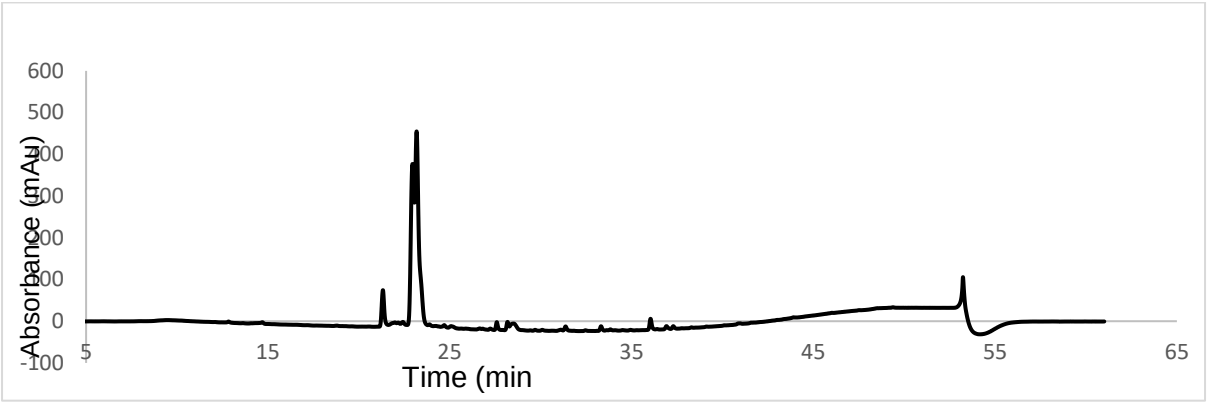


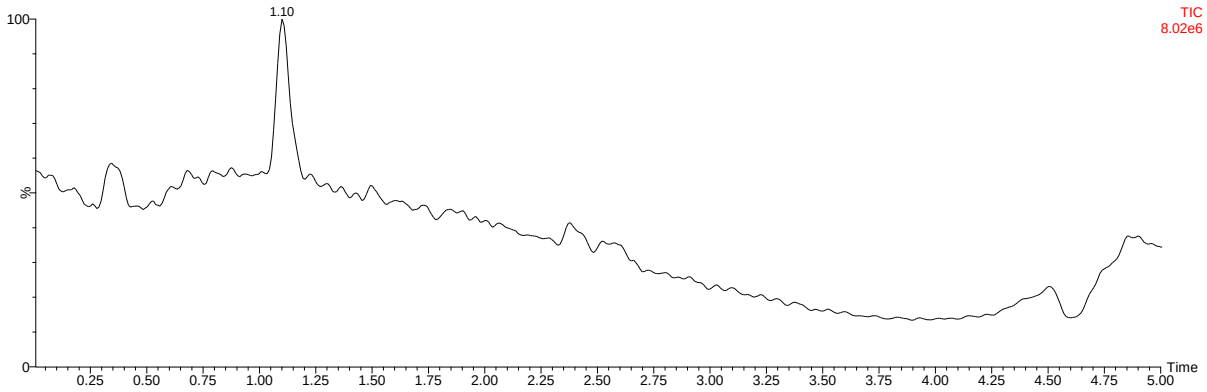

T=24 h
TIC
7.42e8
100
%
0
1.97
2.30
2.67
9.53
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
Time
OAc
OAc
AcHN
O
OAc
N
SH
O
H2N
COOH


3.67e7
100
%
0
612.16
613.14
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
1700
1800
1900
m/z
OAc
OAc
AcHN
O
OAc
N
SH
O
H2N
COOH

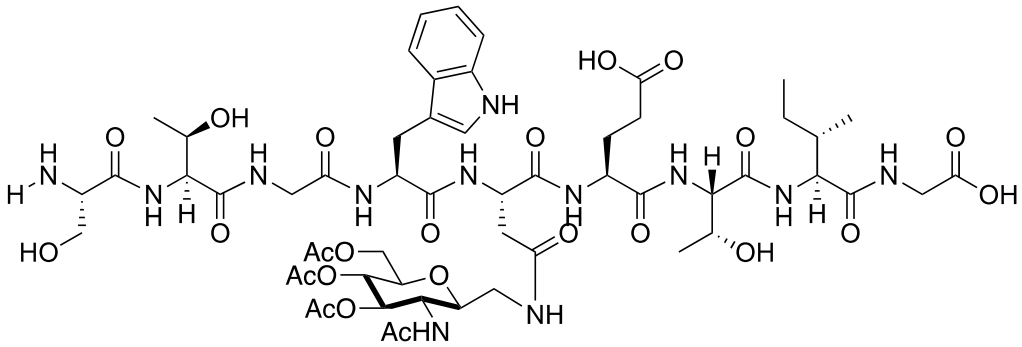
Reactions of peptide **22**



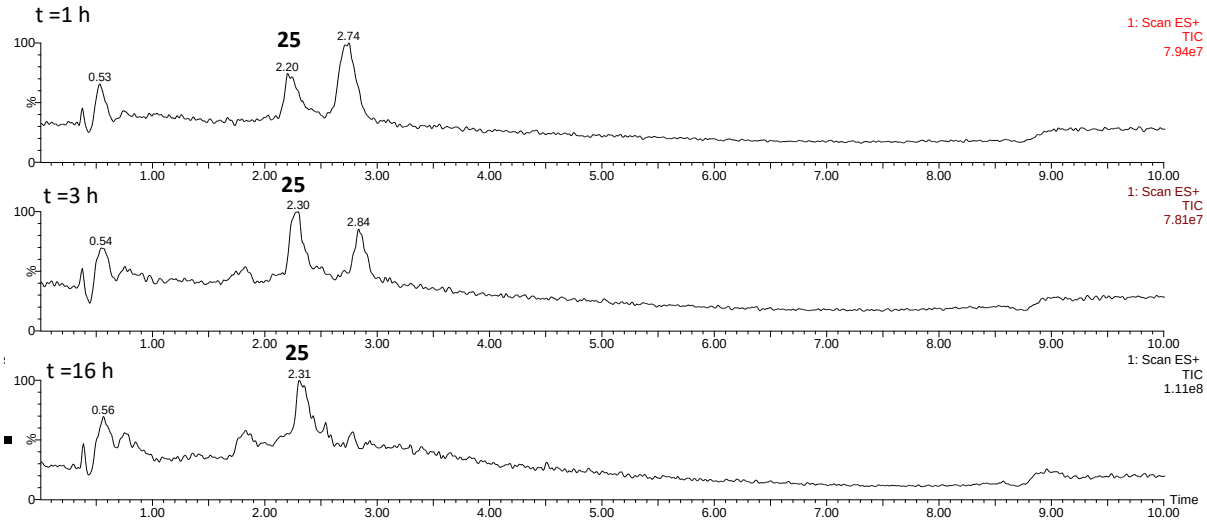


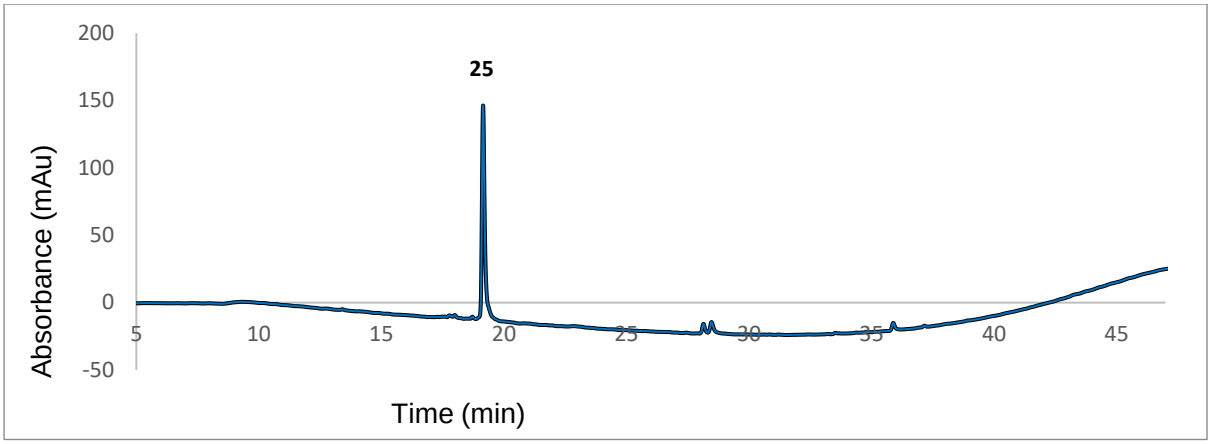


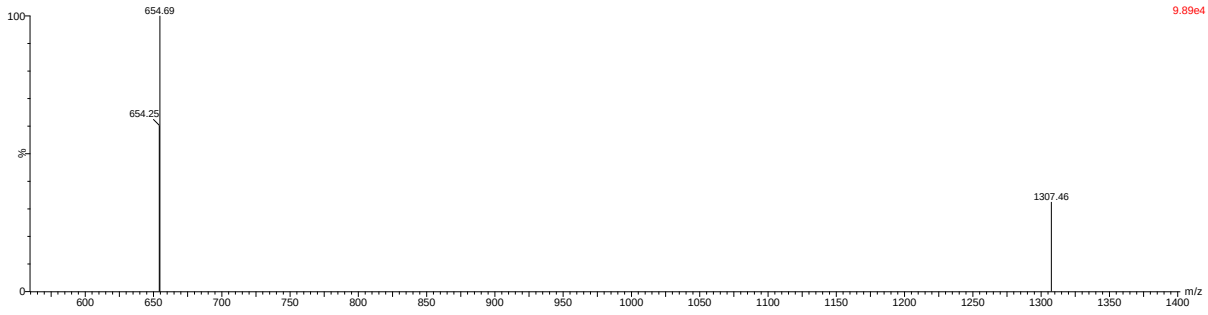
Peptide **25**:



LC-MS analysis of auxiliary removal at 1 h, 3 h, and 16 h.





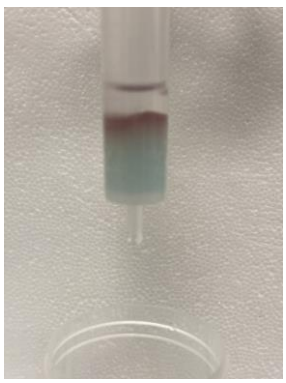


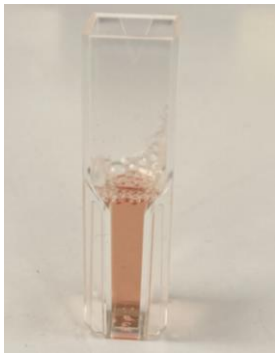
Protein concentration calculations and images:

Calculation for protein yield/concentration after 4-6 h protein induction (from 0.5 L cell culture).

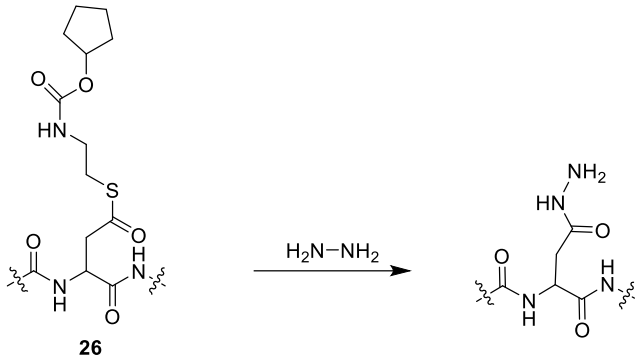
$C=A_{409}/\varepsilon$ = 0.530/164000
C=3.23 µM (in an eluted volume of 7.5mL, before concentration)
= 2.42×10^{-8} mol
mass protein obtained = 2.42×10^{-8} x 18555 (approx.) = 4.49×10^{-4} g
= 0.45 mg (corresponds to approx. 1 mg/L)

After concentration to 1.0 mL A_{409}= 3.036
C= 3.036/164000
= 1.85×10^{-5} M (18.5 µM) and 0.34 mg per reaction.



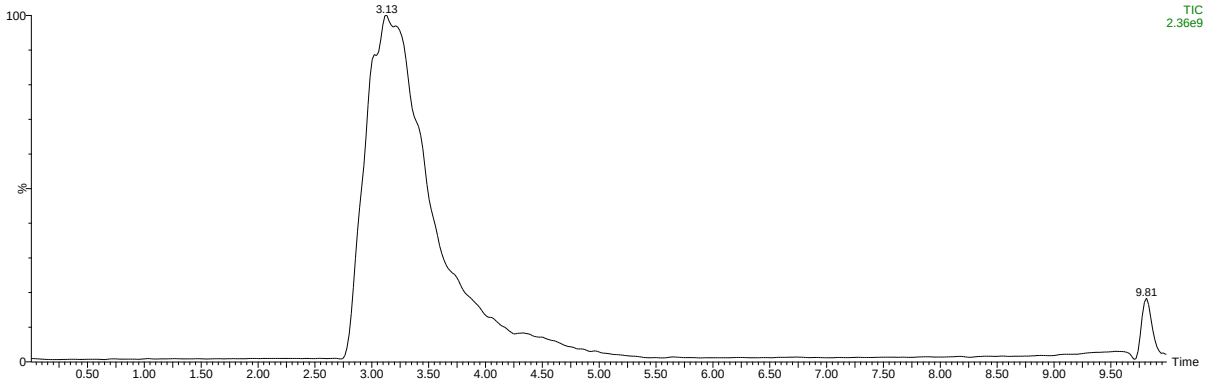


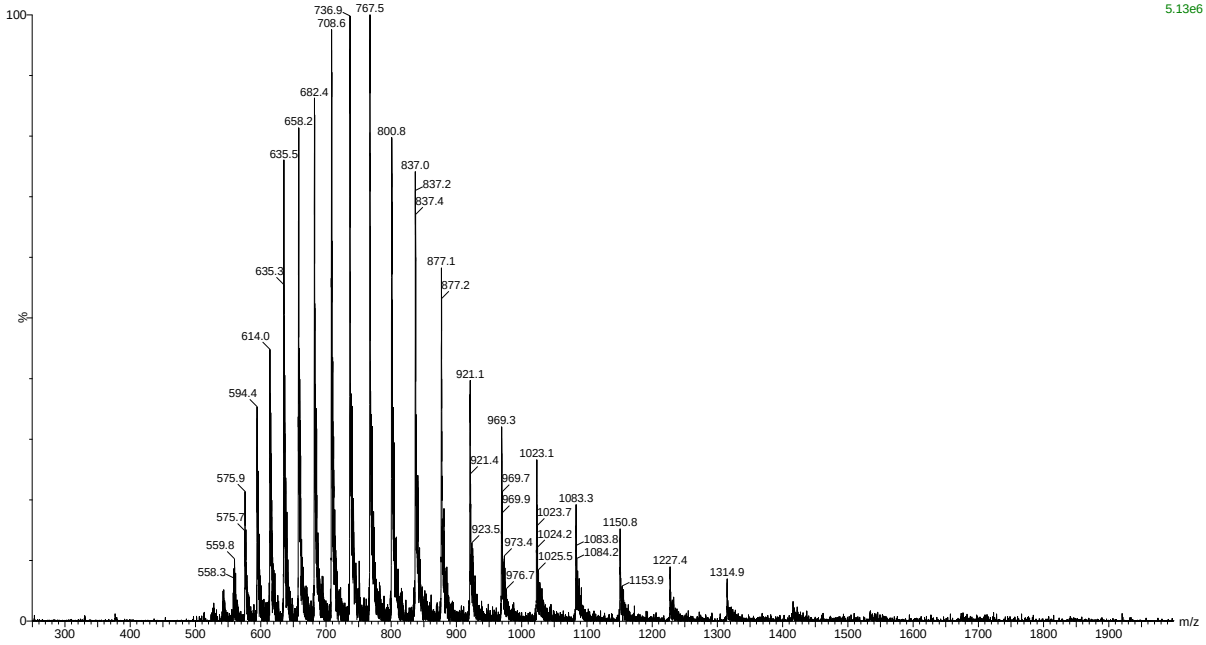
Left: protein loaded into Ni^{2+} affinity column prior to elution. **Right:** Protein sample after concentration.

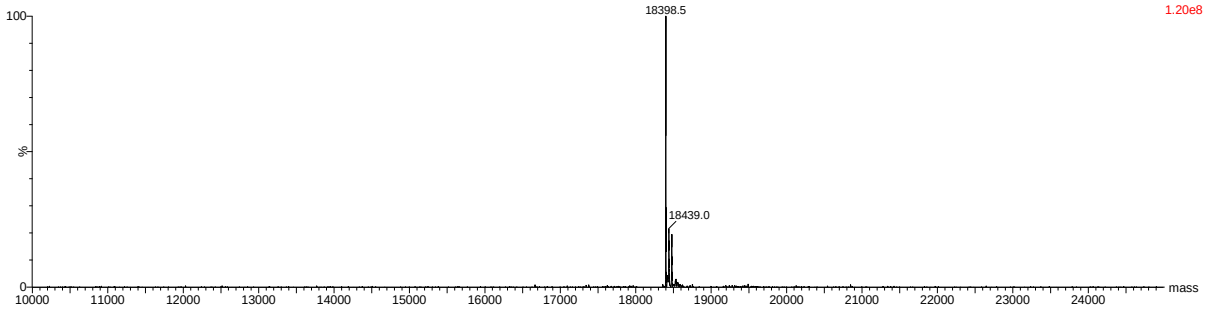


26

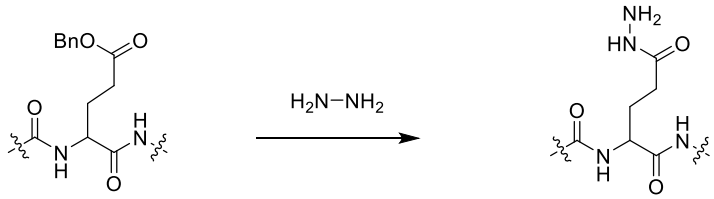
Hydrazinolysis of Myo4ThioD. Hydrazine hydrate 2.5% v/v was added to concentrated (18.5 µM) samples of **26** in 50 mM Na phosphate buffer (pH 8.0). The reaction was shaken in an Eppendorf thermomixer (25°C, 750 rpm), monitored by LC-MS and conversion was judged to be complete within 1 h at room temperature. Samples were then snap-frozen in liquid nitrogen and lyophilised. Expected mass M^+ 18397.1 Da, found M^+ 18398.5 Da.





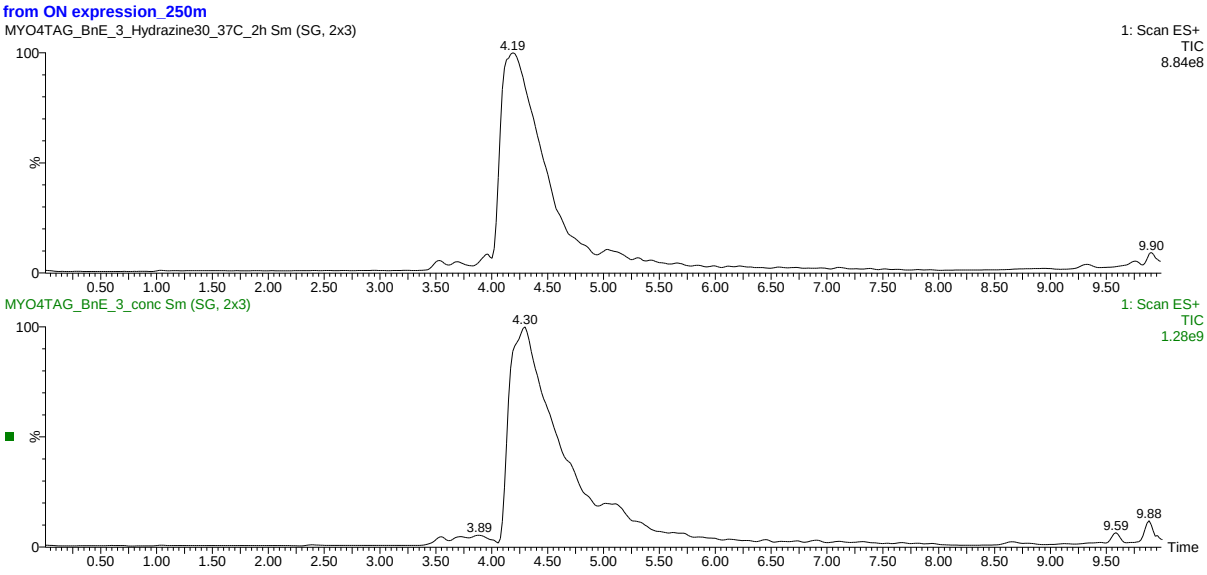


Hydrazinolysis of Myo4BnE:

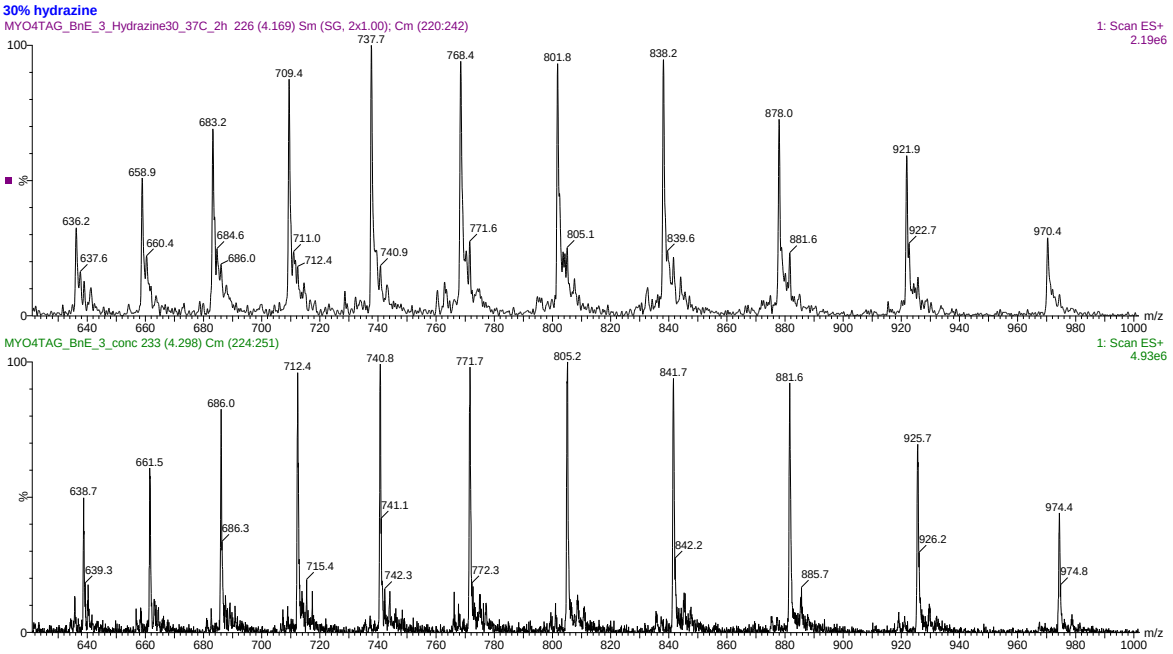


2

Hydrazinolysis: upper trace is TIC for reaction with 30% v/v hydrazine after 2 h at 37 °C. Lower trace is starting material.



Comparing mass spectra:



Deconvolution: