Contribution of shape and charge to the inhibition of a family GH99 endo-α-1,2-mannanase

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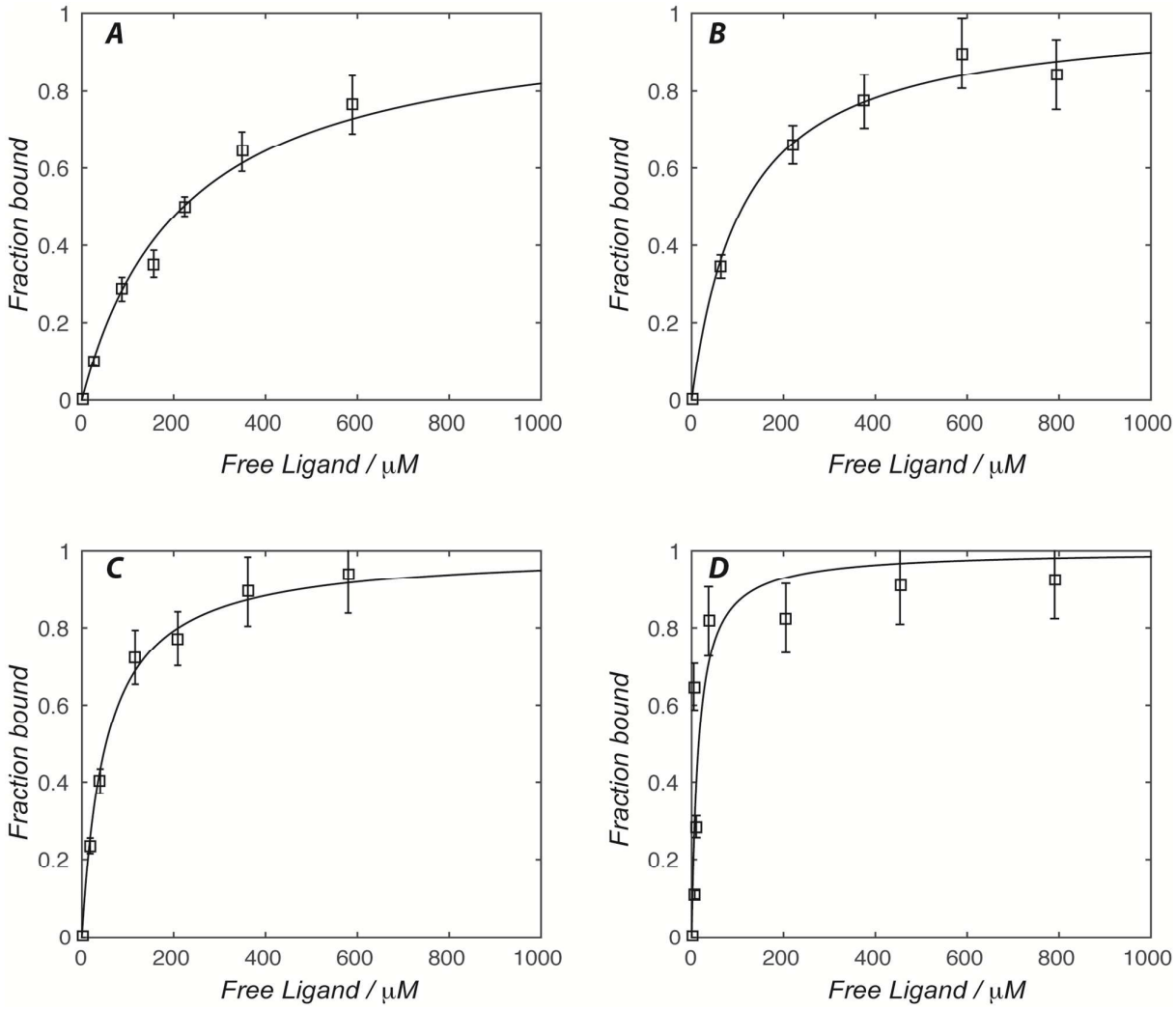

Figure S1. NMR titration curves for binding of ManddMan and ManGlucal to ^{15}N-labelled *Bt*GH99 and *Bx*GH99.

Binding of (A) ManddMan to *Bx*GH99, (B) ManGlucal to *Bx*GH99, (C) ManddMan to *Bt*GH99, and (D) ManGlucal to *Bt*GH99. Nε-Hε of R295 (*Bx*GH99) and R291 (*Bt*GH99) were used as binding reporters. Duplicate experimental points at three different ligand concentrations were used for error-bar estimation.

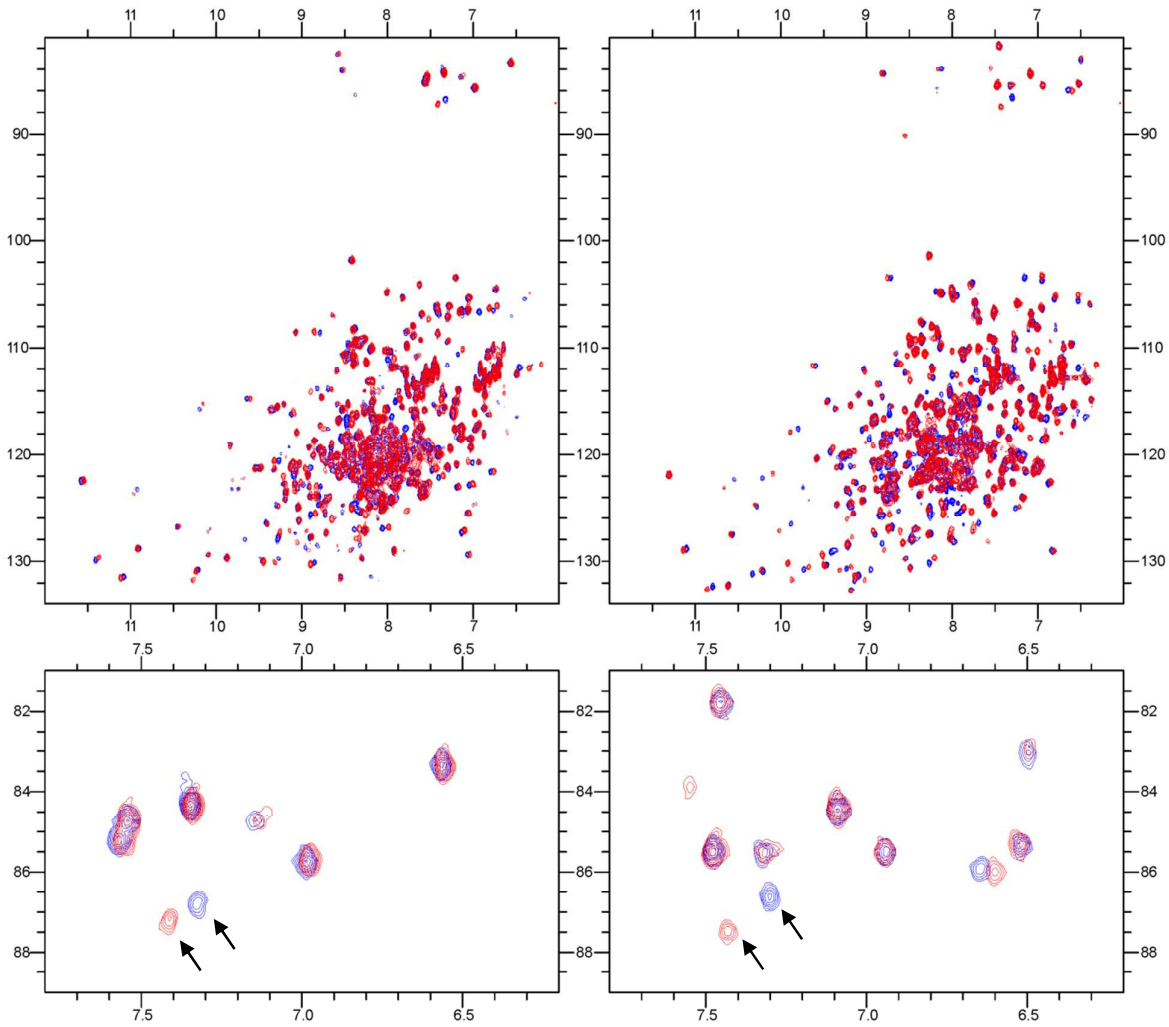

Figure S2. 2D SOFAST-HMQC spectra for binding of ManGlucal to ^{15}N-labelled *Bt*GH99 and *Bx*GH99.

2D SOFAST-HMQC NMR spectra of ^{15}N-labelled *Bt*GH99 (left) or *Bx*GH99 (right) in the absence (blue) or presence of an excess of ManGlucal (**7**; red). The arrows highlight the chemical shift perturbation observed for the signal corresponding to Nε-Hε of R291 (*Bt*GH99) and R295 (*Bx*GH99).

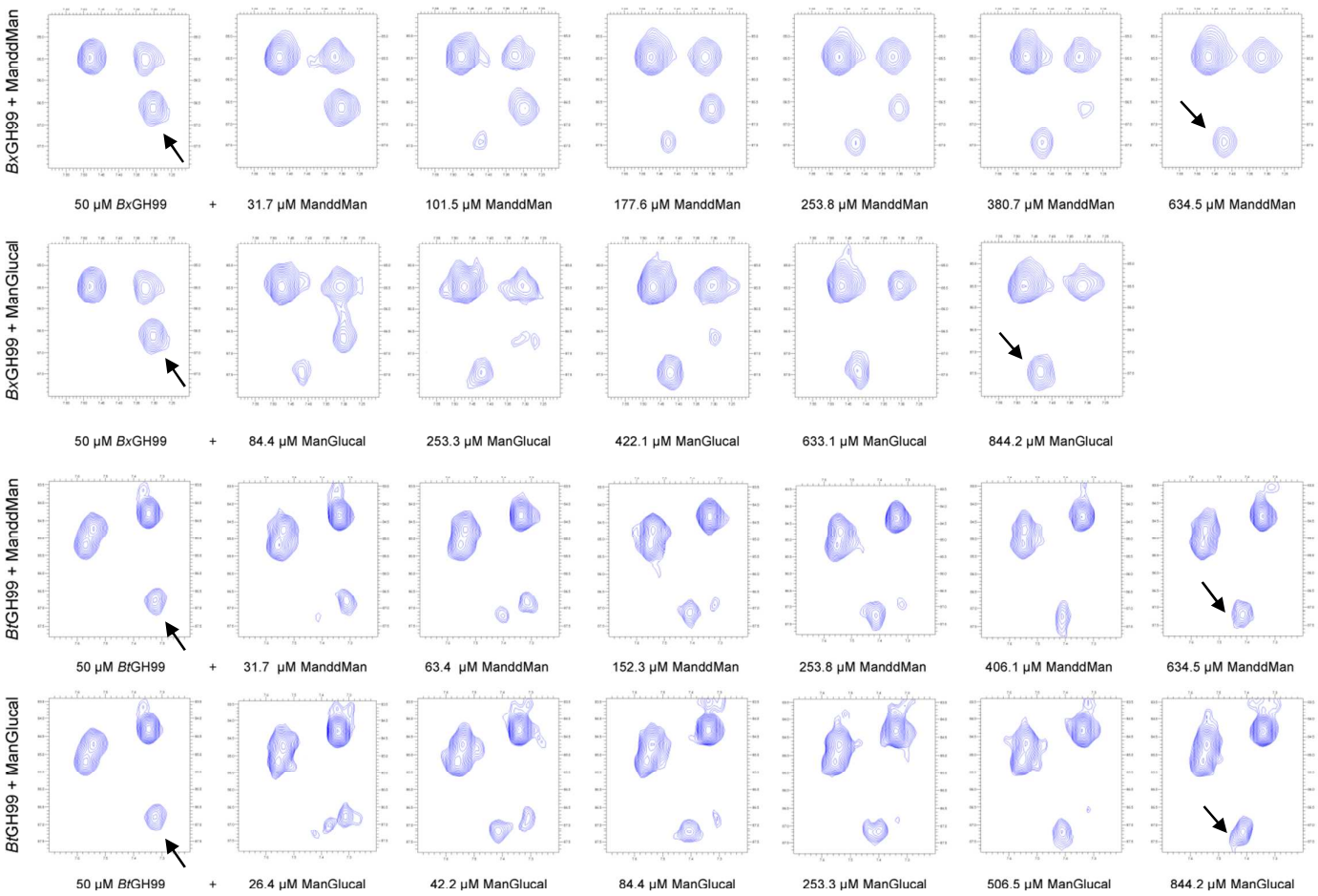

Figure S3. Raw NMR spectra for titration curves reported in Figure S1 for binding of binding of ManddMan and ManGlucal to ^{15}N-labelled *Bt*GH99 and *Bx*GH99.

Nε-Hε of R295 (*Bx*GH99) and R291 (*Bt*GH99) were used as binding reporters. Arrows denote the signals used for monitoring the titration.

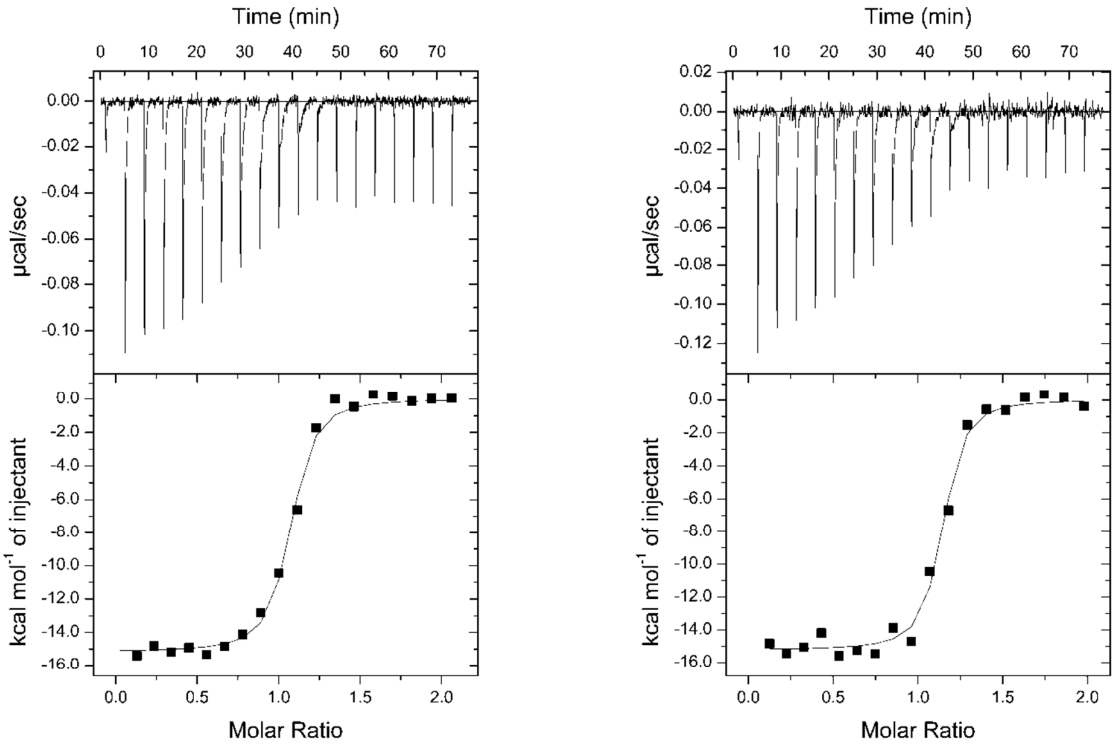

Figure S4. Isothermal titration calorimetry of ManNOE binding to (left) *Bt*GH99 and (right) *Bx*GH99.

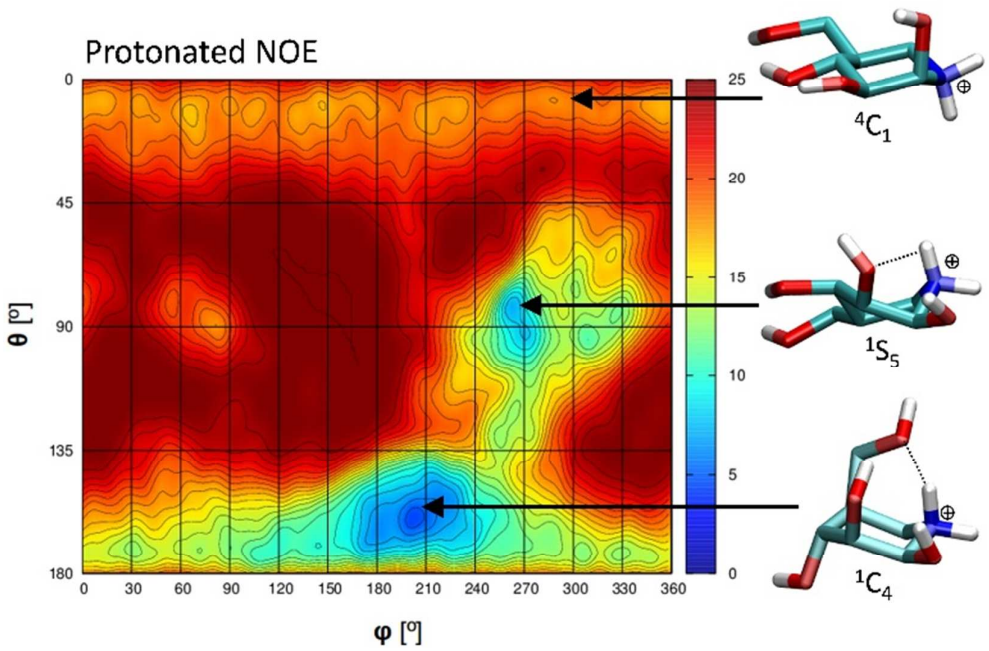

Figure S5. Conformational free-energy landscape for protonated NOE.
Conformational free-energy landscape contoured at 1 kcal mol^{-1}. The FEL for protonated NOE broadly exhibits the same topology as for neutral NOE, but differs in relative energies of the global and local minima. For protonated NOE the $^1C_4$ conformation is the global minimum, and the $^1S_5$ conformation is stabilized relative to that for the neutral NOE FEL. The extracted structures (shown at right) for the $^4C_1$, $^1S_5$ and $^1C_4$ conformations of protonated NOE that suggest the main reason for these changes are the presence of transannular intramolecular hydrogen bonds between the charged group and the 3- or 6-OH groups in the last two conformers that are absent in the first.

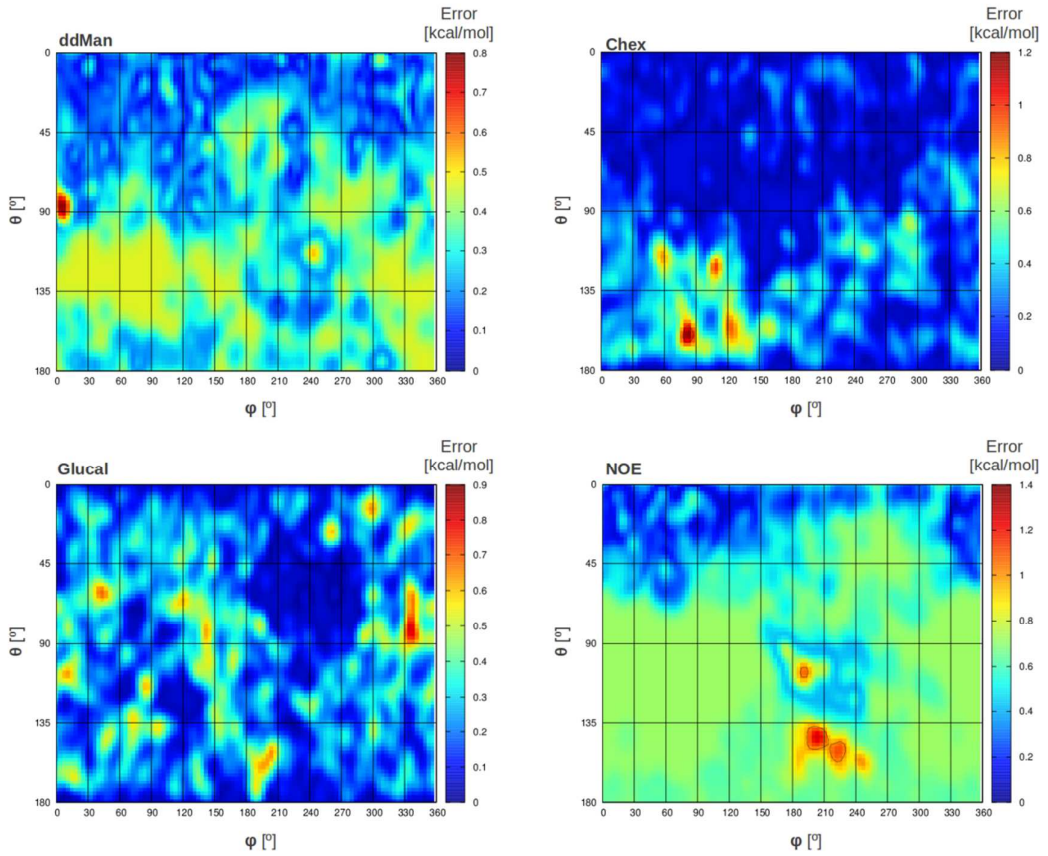

Figure S6. Error surfaces for all inhibitors.
Plots were calculated according to the time-independent free energy estimator described by Tiwary and Parrinello.[1] The energy surface for NOE is for the neutral molecule.

Table S1. Data collection and refinement statistics for *Bx*GH99 complexes

	E333Q –GlcChex	*Bx*GH99–ManddMan	*Bx*GH99–ManddMan–1,2-α-mannobiose	*Bx*GH99–ManGlucal	*Bx*GH99–ManGlucal–2α-mannobiose	*Bx*GH99–ManNOE	*Bx*GH99–ManNOE–2α-mannobiose
Data collection							
Space group	I 4	I 4	I 4	I 4	I 4	I 4	I 4
Beamline	Diamond i04-1	Diamond i02	Diamond i02	Diamond i02	Diamond i02	Diamond i24	Diamond i04
Wavelength (Å)	0.920	0.979	0.979	0.979	0.979	0.969	0.979
Cell dimensions							
a, *b*, *c* (Å)	108.4, 108.4, 67.8	108.6, 108.6, 67.7	108.4, 108.4, 67.6	108.7, 108.7, 67.6	108.6, 108.6, 67.7	108.5, 108.5, 67.6	108.0, 108.0, 67.5
α, β, γ (°)	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90
Resolution (Å)	39.54-1.2 (1.22-1.2)*	76.77-1.03 (1.05-1.03)	76.65-1.04 (1.06-1.04)	76.85-1.07 (1.09-1.07)	57.45-1.18 (1.12-1.18)	76.73-1.14 (1.16-1.14)	57.21-1.05 (1.07-1.05)
R_{merge}	0.059 (0.955)	0.052 (0.989)	0.052 (1.063)	0.052 (1.748)	0.052 (0.790)	0.051 (1.158)	0.054 (1.314)
R_{pim}	0.022 (0.446)	0.024 (0.566)	0.023 (0.634)	0.023 (0.874)	0.028 (0.447)	0.022 (0.855)	0.023 (0.915)
CC(1/2)	0.999 (0.543)	0.994 (0.479)	0.994 (0.427)	0.997 (0.33)	0.998 (0.706)	0.999 (0.387)	0.999 (0.308)
$I / \sigma I$	15.3 (1.7)	14.4 (1.2)	13 (1)	10.9 (0.6)	9.1(0.9)	10.6 (0.7)	10.8 (0.7)
Completeness (%)	98.7 (82.7)	97.5 (73.2)	99 (87.3)	99.8 (96.5)	99.7 (95.5)	94.6 (61.5)	99.3 (89.1)
Redundancy	7.9 (5.3)	6 (3.6)	6 (3.5)	6.3 (4.9)	4.4 (4.0)	5.6 (2.3)	5.9 (2.7)
Refinement							
Resolution (Å)	39.54-1.20	76.78-1.03	76.65-1.04	76.85-1.07	57.45-1.18	76.73-1.14	57.21-1.05
No. reflections all/free	121915/6060	188468/9325	184685/9151	172107/8530	128509/6396	134703/6708	178944/8872
R_{work} / R_{free}	0.118/0.139	0.117/0.131	0.119/0.134	0.126/0.143	0.136/0.157	0.125/0.144	0.115/0.133
Non-H atoms							
Protein	3152	3211	3231	3150	3163	3227	3166
Inhibitor/Man2	21	21	21/34	21	21/23	22	22/34
Water	350	423	374	404	358	428	480
B-factors (Å^2)							
Protein	16.8	15.0	15.5	17.1	17.0	17.3	15.6
Inhibitor/Man2	15.5	12.5	12.8/18.4	14.5	14.7/24.9	16.7	12.3/15.2
Water	31.5	33.3	32.6	35.7	33.8	35.4	35.9
R.m.s. deviations							
Bond lengths (Å)	0.0122	0.0103	0.0111	0.0109	0.0102	0.0106	0.0131
Bond angles (°)	1.586	1.53	1.609	1.535	1.458	1.502	1.673
PDB ID	5MEL	5M17	5M3W	5M5D	5MC8	5LYR	5M03

*Values in parentheses are for highest-resolution shell.

Protein expression, purification, and crystallization

Gene expression and protein purification

E. coli BL21 (DE3) cells were transformed by plasmids reported previously[2] containing *Bt*GH99 (pETYSBLic), *Bx*GH99 wild-type, E333Q and E336Q (pET28a). Single clones were shaken at 180 rpm and 37 °C in 50 ml tubes (Falcon) containing 5 ml LB media with 50 μg ml^{-1} kanamycin. Large-scale selective LB cultures in 2 l conical flasks were inoculated 1:1000 with starter cultures and shaken at 180 rpm and 37 °C until the OD_{600} was higher than 1, induced using then switched to 16 °C for 24 h. Cell pellets were separated from the media by centrifugation, resuspended in 25 mM HEPES pH 7.0, 300 mM NaCl, 20 mM imidazole and lysed by sonication. The lysate was centrifuged twice and the supernatant was applied to a pre-equilibrated 5 ml HisTrap FF or FF crude column (GE). Protein bound to the column was eluted using imidazole gradient (up to 500 mM). Fractions containing purest protein were combined and concentrated using Amicon Ultra 30 kDa concentrator (Millipore). Further purification was done using a Sepharose S75 16/60 or 16/600 column equilibrated in 25 mM HEPES pH 7.0, 100 mM NaCl, 1 mM DTT, which was also used as the buffer for storage. Fractions containing pure GH99 proteins were combined, concentrated as previously, aliquoted, flash frozen in liquid nitrogen and stored at -80 °C.

Crystallization and Data Collection

*Bx*GH99 wild-type and E333Q variant were crystallized in darkness at 19 °C using 24-well plates (Greiner Bio-One) using hanging drop vapour diffusion method. Each 0.5 ml reservoir solution contained 3 M sodium acetate at pH 6.4-7.4. The hanging drop was created by mixing 1 volume of protein solution (30 mg ml^{-1}) with 1 volume of reservoir solution. Soaking was performed by adding 0.5-1 droplet volume of 20 mM ligand stock to the droplet with grown *Bx*GH99 crystals. Initial cryoprotection with 20% ethylene glycol before flash freezing in liquid nitrogen was omitted for ManNOE soaks because the diffraction of X-rays by the crystals was not adversely affected.

Synthetic chemistry

General

^{1}H and ^{13}C NMR were recorded using 400, 500 or 600 MHz instruments. All signals were referenced to solvent peaks ($CDCl_3$: δ 7.26 ppm for ^{1}H or 77.16 ppm for ^{13}C; D_2O: δ 4.80 ppm for ^{1}H). TLC analysis used aluminium backed Merck Silica Gel 60 F_{254} sheets, detection was achieved using UV light or 10% H_2SO_4 in MeOH. Flash chromatography was performed using Geduran silica gel according to the method of Still *et al*.[3] THF and CH_2Cl_2 were dried according to the method of Pangborn *et al*.[4]

2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→3)-1,2,4,6-tetra-*O*-acetyl-α,β-D-mannopyranose (11)

Acetic anhydride (0.61 mL) was added slowly to a solution of 1,3-α-mannobiose **10** (50.8 mg, 0.148 mmol) and DMAP (18.1 mg, 0.148 mmol) in pyridine (1.22 mL) at 0 °C. The mixture was warmed to rt and stirred for 16 h. The mixture was diluted with EtOAc (20 mL) and washed with water (2 × 20 mL), aq. sat. $NaHCO_3$ (4 × 10 mL), aq. 1 M HCl (4 × 10 mL), and brine (2 × 10 mL), then dried ($MgSO_4$) and concentrated to afford the crude octaacetate **11** (97.7 mg, 97%; *α/β* 1.0:0.4) as a pale yellow oil, α anomer; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.99–2.26 (24 H, 8 × s, Ac), 3.94 (1 H, m, H5), 4.04 (1 H, m, H5'), 4.08 (2 H, m, H6a,6a'), 4.17 (1 H, dd, $J_{2,3}$ = 3.5, $J_{3,4}$ = 10.0 Hz, H3), 4.26 (2 H, m, H6b,6b'), 5.00 (2 H, m, H1',2'), 5.19 (1 H, dd, $J_{2',3'}$ = 3.0, $J_{3',4'}$ = 10.0 Hz, H3'), 5.23 (1 H, dd, $J_{1,2}$ = 2.0, $J_{2,3}$ = 3.5 Hz, H2), 5.27 (1 H, t, $J_{3',4'}$ = $J_{4',5'}$ = 10.0 Hz, H4'), 5.36 (1 H, t, $J_{3,4}$ = $J_{4,5}$ = 10.0 Hz, H4), 6.08 ppm (1 H, d, $J_{1,2}$ = 2.0 Hz, H1); ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.6-21.0 (8 C, 8 × Me), 62.3, 62.5 (2 C, C6,6'), 66.0, 67.2, 68.3, 69.7, 69.8, 70.1, 70.9, 75.0 (8 C, C2,3,4,5,2',3',4',5'), 90.8 (1 C, C1'), 99.2 (1 C, C1), 168.0-170.8 ppm (8 C, 8 × C=O).

(2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→3)-(2,4,6-tri-*O*-acetyl-α-D-mannopyranosyl) bromide (12)

The octaacetate **11** (96.9 mg, 0.142 mmol) was dissolved in 33% w/v HBr/AcOH (0.1 mL, 0.507 mmol) and stirred for 3 h. The mixture was diluted with EtOAc (20 mL), poured into ice water and quickly washed with $NaHCO_3$ (3 × 10 mL), and brine (2 × 10 mL), then dried ($MgSO_4$) and co-evaporated with toluene under reduced pressure to afford the crude glycosyl bromide **12** as a viscous brown oil, which was used directly in the next step; ^{1}H NMR (400 MHz, $CDCl_3$), partial spectrum: δ 4.60 (1 H, dd, $J_{1,2}$ = 12.0, $J_{2,3}$ = 4.0 Hz, H2), 5.38 (1 H, t, $J_{3,4}$ = $J_{4,5}$ = 8.0 Hz, H4), 6.34 ppm (1 H, d, $J_{1,2}$ = 1.4 Hz, H1).

2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→3)-4,6-di-*O*-acetyl-1,5-anhydro-2-deoxy-D-*arabino*-hex-1-enitol (13)

Activated Zn (46.7 mg, 0.714 mmol), NH_4Cl (38.2 mg, 0.714 mmol), and VO(salen)[5] (0.478 mg, 7.10 μmol) were added to the crude bromide **12** (99.9 mg, 0.143 mmol) in methanol (1.5 mL) and the mixture was stirred for 30 min. The mixture was filtered through Celite and concentrated to give a light-brown residue that was diluted with EtOAc (20 mL) and washed with water (2 × 20 mL), aq. sat. $NaHCO_3$ (2 × 10 mL), and brine (2 × 10 mL), then dried ($MgSO_4$) and concentrated to give a pale-yellow residue. Flash chromatography (EtOAc/pet. spirits/Et_3N 50:49.5:0.5) afforded the protected glucal **13** (18.1 mg, 23% over two steps) as a golden-yellow oil; $[\alpha]_D^{23}$ –2.62 (*c* 0.31, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.99 (3 H, s, Ac), 2.04 (3 H, s, Ac), 2.10 (3 H, s, Ac), 2.11 (6 H, 2 × s, Ac), 2.15 (3 H, s, Ac), 4.08 (1 H, m, H5'), 4.11 (1 H, dd, $J_{5,6a}$ = 2.5, $J_{6a,6b}$ = 12.2 Hz, H6a), 4.18 (1 H, dd, $J_{5',6a'}$ = 4.0, $J_{6a',6b'}$ = 12.0 Hz, H6a'), 4.21 (1 H, m, H3), 4.25 (1 H, m, H5), 4.27 (1 H, dd, $J_{5,6b}$ = 5.0, $J_{6a,6b}$ = 15.0 Hz, H6b), 4.43 (1 H, dd, $J_{5',6b'}$ = 6.2, $J_{6a',6b'}$ = 11.9 Hz, H6b'), 4.87 (1 H, dd, $J_{1,2}$ = 6.2, $J_{2,3}$ = 3.6 Hz, H2), 5.05 (1 H, d, $J_{1',2'}$ = 1.7 Hz, H1'), 5.12 (1 H, dd, $J_{1',2'}$ = 2.8, $J_{2',3'}$ = 1.9 Hz, H2'), 5.19 (1 H, dd, $J_{3,4}$ = 5.0, $J_{4,5}$ = 6.7 Hz, H4), 5.28 (2 H, m, H3',4'), 6.44 ppm (1 H, dd, $J_{1,2}$ = 6.2, $J_{1,3}$ = 1.3 Hz, H1); ^{13}C NMR (125 MHz, $CDCl_3$) δ 20.8–21.0 (6 C, 6 × Me), 61.3 (1 C, C6'), 62.7 (1 C, C6), 66.3 (1 C, C4'), 68.1 (1 C, C4), 68.8 (1 C, C3'), 69.2 (1 C, C5'), 69.9 (1 C, C2'), 72.2 (1 C, C3), 73.9 (1 C, C5), 97.8 (1 C, C1'), 99.8 (1 C, C2), 145.2 (1 C, C1), 169.8-170.8 ppm (6 C, 6 × C=O); HRMS (ESI)$^+$ *m/z* 578.2080 [$C_{24}H_{34}NO_{15}$ $(M+NH_4)^+$ requires 578.2079].

2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→3)-(4,6-di-*O*-acetyl-1,2-dideoxy-D-mannopyranose (14)

A mixture of the glucal **13** (5.60 mg, 9.99 μmol) and 5% Pd/C (8.5 mg) in methanol (1.5 mL) was stirred under a hydrogen atmosphere for 2 h. The mixture was filtered through Celite and concentrated, followed by acetylation with acetic anhydride (0.5 mL) in pyridine (0.5 mL). Flash chromatography (EtOAc/pet. spirits 30:70) afforded the dideoxy compound **14** (4.50 mg, 80%) as a colourless oil; $[\alpha]_D^{23}$ +16 (*c* 0.42, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): δ 1.90 (1 H, dddd, $J_{1a,2a}$ = 5.0, $J_{1b,2a}$ = 11.6, $J_{2a,2b}$ = 12.8, $J_{2a,3}$ = 12.8 Hz, H2a), 1.99 (3 H, s, Ac), 2.04 (3 H, s, Ac), 2.08 (3 H, s, Ac), 2.09 (1 H, m, H2b), 2.10 (3 H, s, Ac), 2.12 (3 H, s, Ac), 2.15 (3 H, s, Ac), 3.45 (2 H, m, H1a,5), 3.75 (1 H, ddd, $J_{1a,1b}$ = 9.2, $J_{1b,2a}$ = 11.6, $J_{1b,2b}$ = 5.1 Hz, H1b), 4.01–4.15 (4 H, m, H3,5',6b,6b'), 4.19 (1 H, dd, $J_{5,6a}$ = 5.15, $J_{6a,6b}$ = 12.3 Hz, H6a), 4.25 (1 H, dd, $J_{5',6a'}$ = 5.6, $J_{6a',6b'}$ = 12.3 Hz, H6a'), 4.96 (2 H, m, H4,1'), 5.04 (1 H, m, H2'), 5.27 ppm (2 H, m, H3',4'); ^{13}C NMR (150 MHz, $CDCl_3$) δ 20.8-21.0 (6 C, 6 × Me), 29.8 (1 C, C5), 32.9 (1 C, C2), 62.7 (1 C, C6'), 63.0 (1 C, C6), 65.6 (1 C, C1), 66.5 (1 C, C4'), 68.7 (1 C, C3'), 69.1 (1 C, C5'), 70.2 (1 C, C2'), 70.6 (1 C, C4), 79.7 (1 C, C3), 98.8 (1 C, C1'), 169.8-171.0 ppm (6 C, 6 × C=O); HRMS (ESI)$^+$ *m/z* 580.2234 [$C_{24}H_{38}NO_{15}$ $(M+NH_4)^+$ requires 580.2236].

α-D-Mannopyranosyl-(1→3)-1,2-dideoxy-D-mannopyranose (ManddMan; 5)

Sodium methoxide in methanol (0.217 M, 25.0 μL, 5.40 μmol) was added to compound **14** (8.00 mg, 0.0142 mmol) in methanol (1.3 mL) and the solution was stirred for 2 h. The mixture was neutralised using Dowex-50 resin (H^+ form) and filtered. Reverse phase chromatography (H_2O, 100%) afforded ManddMan **5** (3.50 mg, 80%) as a colourless oil; $[\alpha]_D^{25}$ +49.8 (*c* 0.19, CH_3OH); ^{1}H NMR (400 MHz, D_2O): δ 1.74 (1 H, dddd, $J_{1a,2a}$ = 5.0, $J_{1b,2a}$ = 11.7, $J_{2a,2b}$ = 12.8, $J_{2a,3}$ = 12.8 Hz, H2a), 2.16 (1 H, m, H2b), 3.34 (1 H, m, H5), 3.42 (1 H, t, $J_{3,4}$ = $J_{4,5}$ = 8.0 Hz, H4), 3.54 (1 H, m, H1a), 3.63-3.90 (8 H, m, H1b,3',4',5',6a,6a',6b,6b'), 3.97 (1 H, m, H3), 4.03 (1 H, dd, $J_{1',2'}$ = 3.4, $J_{2',3'}$ = 1.8 Hz, H2'), 5.13 ppm (1 H, d, $J_{1',2'}$ = 1.6 Hz, H1'); ^{13}C NMR (150 MHz, D_2O) δ 32.3 (1 C, C2), 60.98 (1 C, C3), 60.99 (1 C, C6), 65.3 (1 C, C1), 66.8 (1 C, C4'), 70.1 (1 C, C2'), 70.4 (2 C, C4,6'), 73.1 (1 C, C3'), 80.2 (1 C, C5'), 80.4 (1 C, C5), 101.7 ppm (1 C, C1'); HRMS (ESI)$^+$ *m/z* 333.1152 [$C_{12}H_{22}O_9Na$ $(M+Na)^+$ requires 333.1156].

α-D-Mannopyranosyl-(1→3)-1,5-anhydro-2-deoxy-D-*arabino*-hex-1-enitol (ManGlucal; 7)

Sodium methoxide in methanol (0.217 M, 8.00 μL, 1.74 μmol) was added to a solution of the glucal **13** (8.20 mg, 0.0146 mmol) in methanol (0.64 mL) and the resulting solution was stirred for 2 h. The mixture was concentrated, followed by reverse phase chromatography (H_2O 100) to afford the deprotected disaccharide **7** (3.90 mg, 87%) as a colourless oil; $[\alpha]_D^{23}$ +18 (*c* 0.145, CH_3OH); ^{1}H NMR (400 MHz, D_2O): δ 3.64-3.94 (8 H, m, H4,5,6a,6b,3',4',6a',6b'), 3.99 (1 H, dd, $J_{1',2'}$ = 3.4, $J_{2',3'}$ = 1.8 Hz, H2'), 4.02 (1 H, ddd, $J_{4,5}$ = 8.5, $J_{5',6a'}$ = 5.6, $J_{5',6b'}$ = 2.9 Hz, H5'), 4.30 (1 H, m, H3), 4.93 (1 H, dd, $J_{1,2}$ = 6.15, $J_{2,3}$ = 2.9 Hz, H2), 5.17 (1 H, d, $J_{1',2'}$ = 1.7 Hz, H1'), 6.48 ppm (1 H, dd, $J_{1,2}$ = 6.1, $J_{1,3}$ = 1.3 Hz, H1); ^{13}C NMR (100 MHz, D_2O) δ 59.7 (1 C, C6), 60.9 (1 C, C5), 66.7 (1 C, C4'), 67.1 (1 C, C4), 70.1 (1 C, C2'), 70.3 (1 C, C3'), 73.1 (1 C, C6'), 74.3 (1 C, C3), 78.3 (1 C, C5'), 100.3 (1 C, C1'), 100.7 (1 C, C2), 144.6 ppm (1C, C1); HRMS (ESI)$^+$ *m/z* 331.1000 [$C_{12}H_{20}O_9Na$ $(M+Na)^+$ requires 331.1000].

Benzyl 2-*O*-(2-*O*-acetyl-3,4,5-tri-*O*-benzyl-α-mannopyranosyl)-(1→2)-(4-cyano-4-deoxy-β-D-arabinopyranoside) (17)

TfOH (4.4 μL, 0.050 mmol) was added to a mixture of acceptor **16** (125 mg, 0.50 mmol), 2-*O*-acetyl-3,4,5-tri-*O*-benzyl-α-mannopyranosyl trichloroacetimidate[6] **15** (372 mg, 0.625 mmol) and 4 Å mol. sieves in CH_2Cl_2 at -40 ˚C. The mixture was stirred for 15 min, warmed to 0 ˚C and quenched with Et_3N (7.0 μL, 5.07 mmol then concentrated under reduced pressure. Flash chromatography (EtOAc/pet. spirits 40:60) gave the disaccharide **17** (168 mg, 46%) as a colourless oil; $[\alpha]_D^{23}$ –44, (*c* 0.9, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.21 (3 H, s, Ac), 3.17-3.18 (1 H, m, H4), 3.32 (1 H, s, OH), 3.5 (1 H, dd, $J_{5',6a'}$ = 6.5, $J_{6a',6b'}$ = 10.5 Hz, H6a'), 3.60 (1 H, dd, $J_{5',6b'}$ = 1.5, $J_{6a',6b'}$ = 10 Hz, H6b'), 3.67-3.96 (6 H, m, H2,3',4',5a,5b,5'), 4.18-4.22 (1 H, m, H3),

4.44-4.61 (6 H, m, 6 × C**H**$_2$Ph), 4.69 (1 H, dd, J = 11 Hz, C**H**$_2$Ph), 4.84 (1 H, dd, J = 11 Hz, C**H**$_2$Ph), 5.18 (1 H, dd, $J_{1,2}$ = 3.5 Hz, H1), 5.23 (1 H, d, $J_{1',2'}$ = 1.5 Hz, H1'), 5.39-5.40 (1 H, m, H2'), 7.14-7.32 ppm (20 H, m, Ph); ^{13}C NMR (125 MHz, $CDCl_3$): δ 21.1 (1 C, Ac), 36.5 (1 C, C4), 58.5 (1 C, C5), 64.3 (1 C, C3), 68.7, 69.3, 69.8, 72.1, 72.1, 73.7, 74.4, 74.9, 78.1, 78.9 (C2,2',3',4,5',6',4 × CH_2Ph), 97.9 (1 C, C1), 99.1 (1 C, C1'), 127.6, 127.8, 127.9, 128.1, 128.2, 128.3, 128.4, 128.5, 128.7 ppm (24 C, 4 × Ph); HRMS (ESI)$^+$ *m/z* 741.3401 [$C_{42}H_{45}NO_{10}$ (M+NH_4^+) requires 741.3382].

Benzyl 2-*O*-(2-*O*-acetyl-3,4,5-tri-*O*-benzyl-α-mannopyranosyl)-(1→2)-(3-*O*-acetyl-4-cyano-4-deoxy-β-D-arabinopyranoside)

A mixture of acetic anhydride (250 µL) and disaccharide **17** (20 mg, 0.028 mmol) in pyridine (500 µL) was stirred at rt for 18 h. The solution was concentrated under reduced pressure and the residue was azeotroped with toluene (3 × 10 mL). Flash chromatography (EtOAc/pet. spirits 45:55) of the residue gave the title compound (10.8 g, 49%) as a colourless oil; $[\alpha]_D^{24}$ –66 (*c* 0.29, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.13 (3 H, s, Ac), 2.19 (3 H, s, Ac), 3.41-3.42 (1 H, m, H4), 3.50 (1 H, dd, $J_{5',6a'}$ = 1.5, $J_{6a',6b'}$ = 10.5, H6a'), 3.55-3.70 (2 H, m, H5',6b'), 3.78-3.82 (2 H, m, H4',5a), 3.90 (1 H, dd, $J_{2',3'}$ = 3.5, $J_{3',4'}$ = 9.4 Hz, H3'), 3.97-4.03 (2 H, m, H2,5b), 4.45-4.67 (7 H, m, C**H**$_2$Ph), 4.86 (1 H, d, J = 10.9 Hz, C**H**$_2$Ph), 5.02 (1 H, d, $J_{1',2'}$ = 1.5 Hz, H1'), 5.08 (1 H, d, $J_{1,2}$ = 3.6 Hz, H1), 5.21 (1 H, dd, $J_{2,3}$ = 5.5, $J_{3,4}$ = 10.4 Hz, H3), 5.24-5.25 (1, m, H2), 7.12-7.37 ppm (20 H, m, 4 × Ph); ^{13}C NMR (125 MHz, $CDCl_3$): δ 20.9 (1 C, Ac), 21.2 (1 C, Ac), 34.5 (1 C, C4), 58.2 (1 C, C5), 67.7 (1 C, C3), 68.9 (2 C, C2', **C**H_2Ph), 70.7 (1 C, **C**H_2Ph), 72.1 (1 C, **C**H_2Ph), 72.4 (1 C, C5'), 73.7 (1 C, **C**H_2Ph), 74.3 (1 C, C4'), 74.9 (1 C, C2), 75.1 (1 C, **C**H_2Ph), 77.9 (1 C, C3'), 97.2 (1 C, C1), 100.0 (1 C, C1'), 127.7, 127.9, 128.1, 128.2, 128.3, 128.4, 128.5, 128.6, 128.8 ppm (24 C, 4 × Ph); HRMS (ESI)$^+$ *m/z* 788.3063 [$C_{44}H_{47}NaO_{11}$ (M+Na)$^+$ requires 788.3041]. The downfield shift of H3' relative to that in compound **17** provided evidence for the regiochemistry of the glycosylation reaction.

Benzyl 2-*O*-(3,4,5-tri-*O*-benzyl-α-mannopyranosyl)-(1→2)-(4-cyano-4-deoxy-β-D-arabinopyranoside) (18)

Acetyl chloride (860 µL) was added to disaccharide **17** (127 mg, 0.175 mmol) in MeOH (20 mL) and the mixture was stirred for 18 h at rt. The reaction was quenched with Et_3N (1.67 mL, 12 mmol) and the solvent evaporated under reduced pressure. Flash chromatography (EtOAc/pet. spirits 50:50) of the residue gave the diol **18** (84 mg, 70 %) as a colourless oil; $[\alpha]_D^{23}$ –62, (*c* 2.26, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.59 (1 H, s, OH), 3.15 (1 H, m, H4), 3.30 (1 H, s, OH), 3.48, (1 H, dd, $J_{5',6a'}$ = 5.4, $J_{6a',6b'}$ = 8.6 Hz, H6a'), 3.57 (1 H, dd, $J_{5',6b'}$ = 1.6, $J_{6a',6b'}$ = 8.5 Hz, H6b'), 3.69 (1 H, t, H4), 3.78 (1 H, dd, $J_{4,5a}$ = 1.5, $J_{5a,5b}$ = 10.0 Hz, H5a), 3.83-5.85 (3 H, m, H2,3',5b), 3.93 (1 H, m, H5'), 3.99 (1 H, m, H2'), 4.19 (1 H, dd, $J_{3,4}$ = 4.6, $J_{2,3}$ = 8.2 Hz, H3), 4.45-4.67 (7 H, m, C**H**$_2$Ph), 4.80 (1 H, d, J = 9.3 Hz, C**H**$_2$Ph), 5.20 (1 H, d, $J_{1,2}$ = 3.0 Hz, H1), 5.27 (1 H, d, $J_{1',2'}$ = 1.5 Hz, H1'), 7.23-7.33 ppm (24 H, m, 4 × Ph); ^{13}C NMR (125 MHz, $CDCl_3$): δ 36.4 (1 C, C4), 58.5 (1 C, C5), 64.9 (1 C, C3), 68.5, 69.3, 69.8, 71.8, 72.5, 73.6, 74.4, 74.8, 78.8, 79.8 (10 C, C2,2',3',4',5',6',4 × **C**H_2Ph), 98.0 (1 C, C1), 100.6 (1 C, C1'), 127.7, 127.8, 1280, 128.1, 128.4, 128.5, 128.7 ppm (20 C, 4 × Ph); HRMS (ESI)$^+$ *m/z* 699.3304 [$C_{40}H_{43}NO_9$ (M+NH_4)$^+$ requires 399.3276].

Benzyl 2-*O*-(2-*O*-acetyl-2,4,5-tri-*O*-benzyl-α-mannopyranosyl)-(1→2)-(4-*C*-[(*tert*-butoxycarbonyl)amino]methyl-4-deoxy-β-D-arabinopyranoside) (19)

Borane dimethyl sulfide complex (35.7 µL, 0.379 mmol) was added dropwise to nitrile **18** (57.1 mg, 0.0838 mmol) in anhydrous THF (3 mL), and the mixture was stirred under reflux for 24 h. MeOH (3 mL) was added dropwise and reflux continued for an additional 18 h. The solvent was evaporated under reduced pressure and the residue was azeotroped with MeOH (3 × 20 mL) and evaporated to dryness under reduced pressure. The residue was dissolved in THF/MeOH (1:3, 12 mL) and treated with 10% aq. Na_2CO_3 (2 mL) and $(Boc)_2O$ (36.6 mg, 0.168 mmol). After 2 h the mixture was filtered and the filtrate concentrated under

reduced pressure. Flash chromatography (EtOAc/pet. spirits 50:50) of the residue gave the carbamate **19** (44.7 mg, 68 %) as a colourless oil; $[\alpha]_D^{23}$ –26 (*c* 1.2, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.45 (9 H, s, 3 × CH_3), 2.11 (1 H, m, H4), 2.60 (1 H, m, OH), 3.24 (1 H, m, H6a'), 3.39-3.48 (4 H, m, H5a,6b',C**H**$_2$NHBoc), 3.56 (1 H, dd, $J_{4,5b}$ = 4.5, $J_{5a,5b}$ = 11 Hz, H5b) , 3.76-4.15 (6 H, H2, 2',3,3',4',5'), 4.41-4.80 (8H, m, 4 x C**H**$_2$Ph), 5.01 (2 H, m, H1', NH), 5.15 (1 H, s, H1), 7.15-7.35 (20 H, m, 4 × Ph); ^{13}C NMR (125 MHz, $CDCl_3$): δ 28.5 (3 C, CH_3), 38.1 (1 C, C6'), 40.7 (1 C, C4), 61.4 (C5), 66.9, 68.8, 69.0, 69.8, 71.3, 72.3, 73.6, 74.4, 74.7, 76.3, 79.9, 80.1 (10 C, C2,2',3,3',4',5',CH_2NHBoc, 4 × **C**H_2Ph), 98.0 (1 C, C1), 99.8 (1 C, C1'), 127.6, 127.7, 127.8, 127.9, 128.0, 128.2, 128.3, 128.4, 128.5, 128.6 (24 C, 4 × Ph); HRMS (ESI)$^+$ *m/z* 803.4132 [$C_{45}H_{55}NO_{11}$ $(M+NH_4)^+$ requires 803.4113].

α-D-Mannopyranosyl-(1→3)-noeuromycin hydrochloride (ManNOE.HCl; 9)

The carbamate **19** (21.7 mg, 00276 mmol) in THF/H_2O (1:1, 5 mL) was treated with $Pd(OH)_2$/C (10%, 10 mg) and H_2 (15 atm, 18 h). The suspension was filtered, concentrated and subject to flash chromatography ($CHCl_3$/MeOH/H_2O 70:27:1) to give a colourless oil. The oil was treated with 1 M aq. HCl (2 mL) and stirred for 30 min. The solution was concentrated under reduced pressure to give ManNOE.hydrochloride **9** as a colourless residue. NMR data of the noeuromycins ('glc' has an equatorial 2-OH, 'man' has an axial 2-OH, '*p*-glc' and '*p*-man' are the pyran*ose* tautomers); $[\alpha]_D^{25}$ +38, 0.5 M; ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.95-2.05 (m, H5-man, H5-glc), 2.96-3.94 (m, H2',3,3',4,4',5',6a,6b,6a',6b'), 4.07 (dd, $J_{1',2'}$ = 1.5, $J_{2',3'}$ = 3 Hz, H2'-glc), 4.09 (dd, $J_{1',2'}$ = 1.5, $J_{2',3'}$ = 3.5 Hz, H2'-man), 4.66 (m, H2-*p*-glc), 4.72 (m, H2-glc), 5.04 (d, $J_{1',2'}$ = 1.5 Hz, H1'-*p*-man), 5.12 (d, $J_{1',2'}$ = 1.5 Hz, H1'-man), 5.18 (d, $J_{1',2'}$ = 1.5 Hz, H1'-*p*-glc), 5.24 (d, $J_{1',2'}$ = 1.5 Hz, H1'-glc), 5.36 (d, $J_{2,3}$ = 3.5 Hz, H2-*p*-man), 5.43 ppm (d, $J_{2,3}$ = 3.5 Hz, H2-man); ^{13}C NMR (125 MHz, $CDCl_3$): δ 37.3, 38.1, 40.7, 40.7, 40.9, 41.2, 55.4, 58.6, 58.8, 59.0, 60.6, 60.8, 60.9, 65.9, 66.3, 66.4, 66.5, 66.7, 69.7, 69.9, 70.0, 70.2, 70.3, 70.4, 72.3, 72.9, 73.2, 73.5, 77.4, 78.9, 80.0, 80.2, 91.8, 101.4. 101.6, 102.5 ppm; HRMS (ESI)$^+$ *m/z* 326.1444 [$C_{12}H_{35}NO_9$ $(M+ NH_4)^+$ requires 326.1446].

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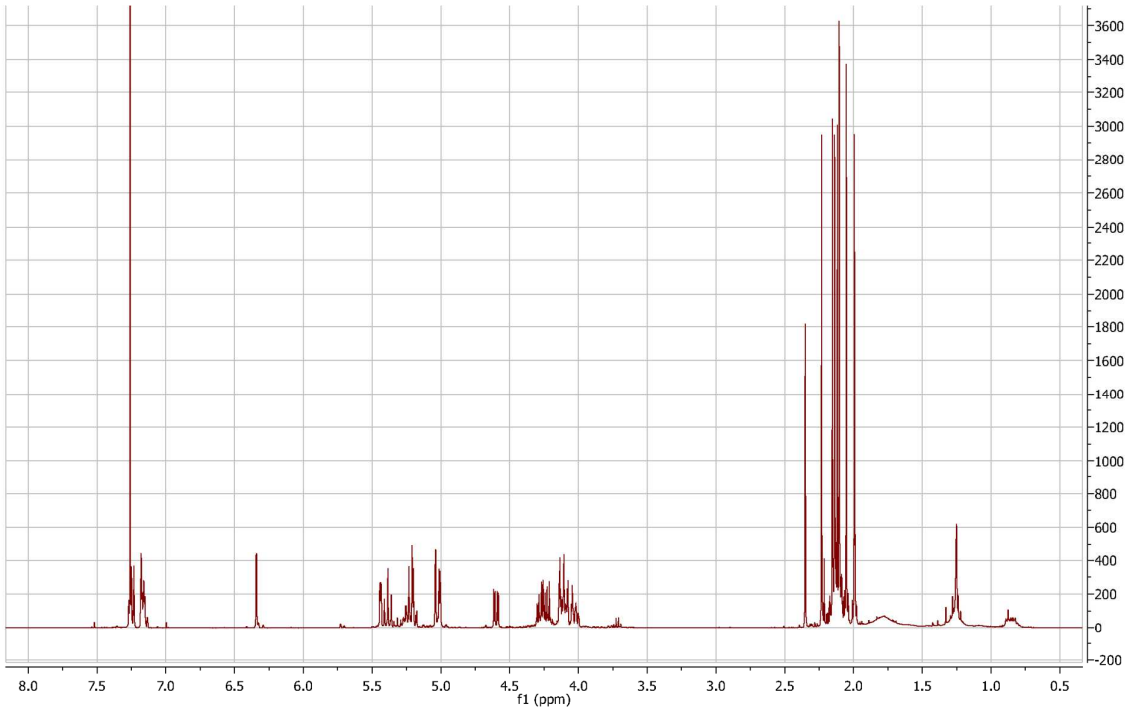
^{1}H and ^{13}C NMR spectra

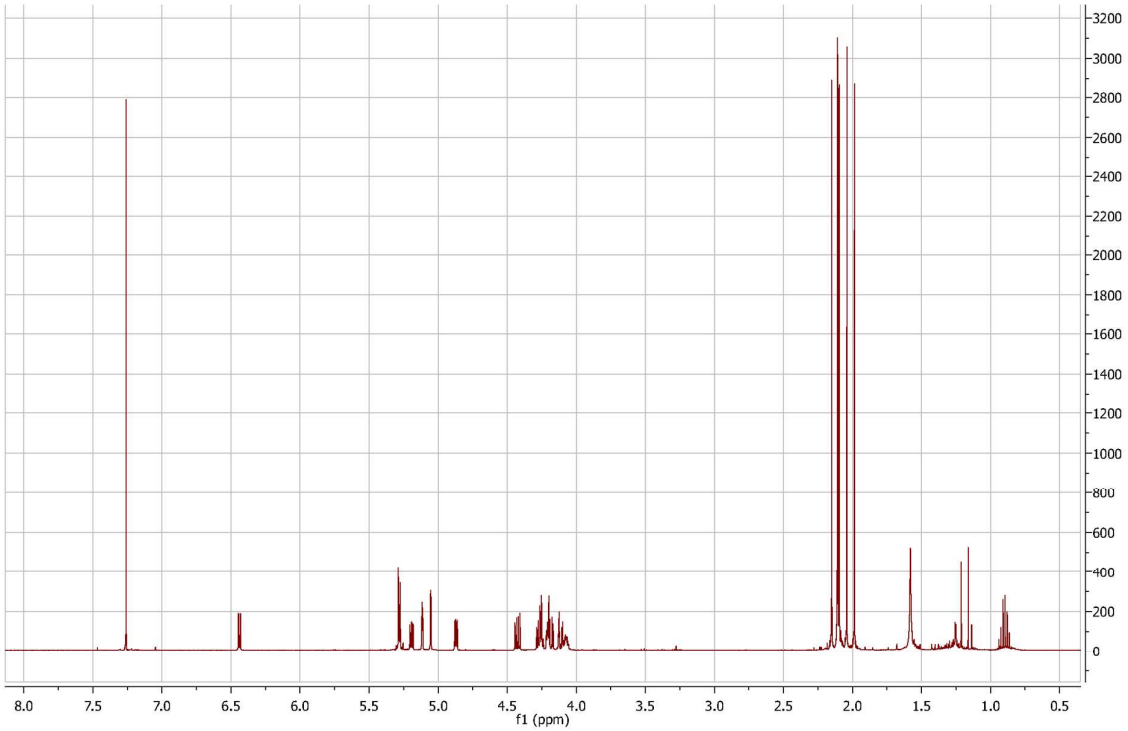
(2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→3)-(2,4,6-tri-*O*-acetyl-α-D-mannopyranosyl) bromide (12)

^{1}H NMR

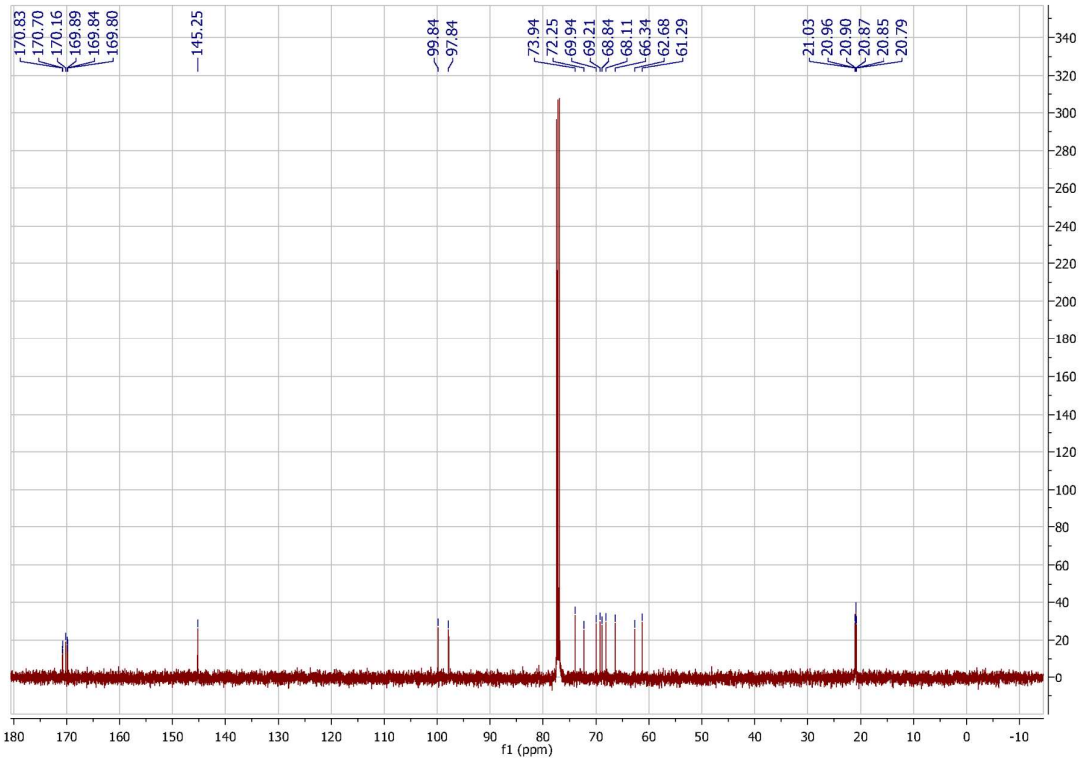


2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→3)-4,6-di-*O*-acetyl-1,5-anhydro-2-deoxy-D-*arabino*-hex-1-enitol (13)

^{1}H NMR

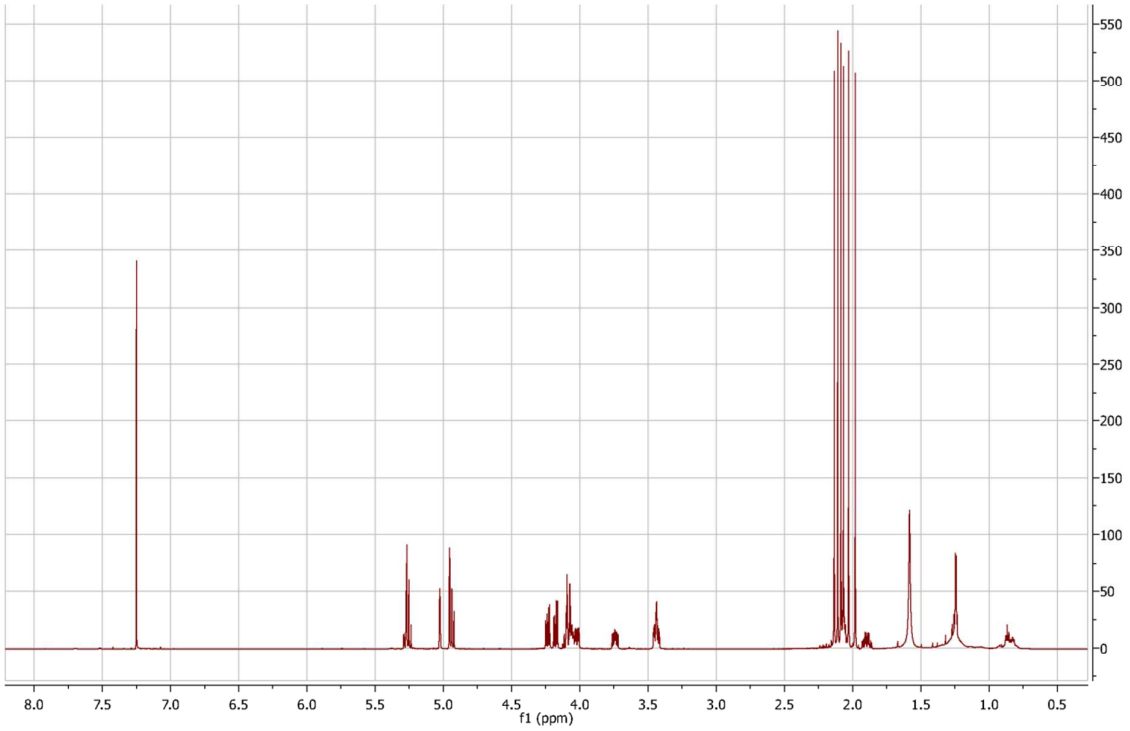


^{13}C NMR

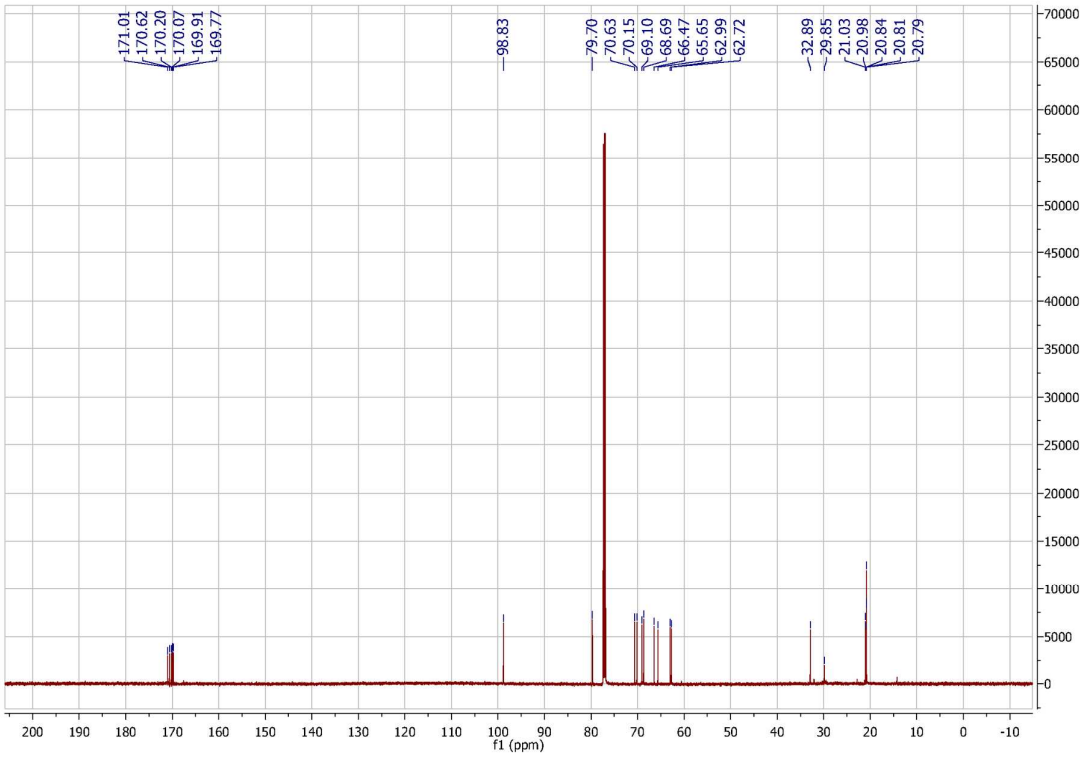


2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→3)-(4,6-di-*O*-acetyl-1,2-dideoxy-D-mannopyranose (14)

^{1}H NMR

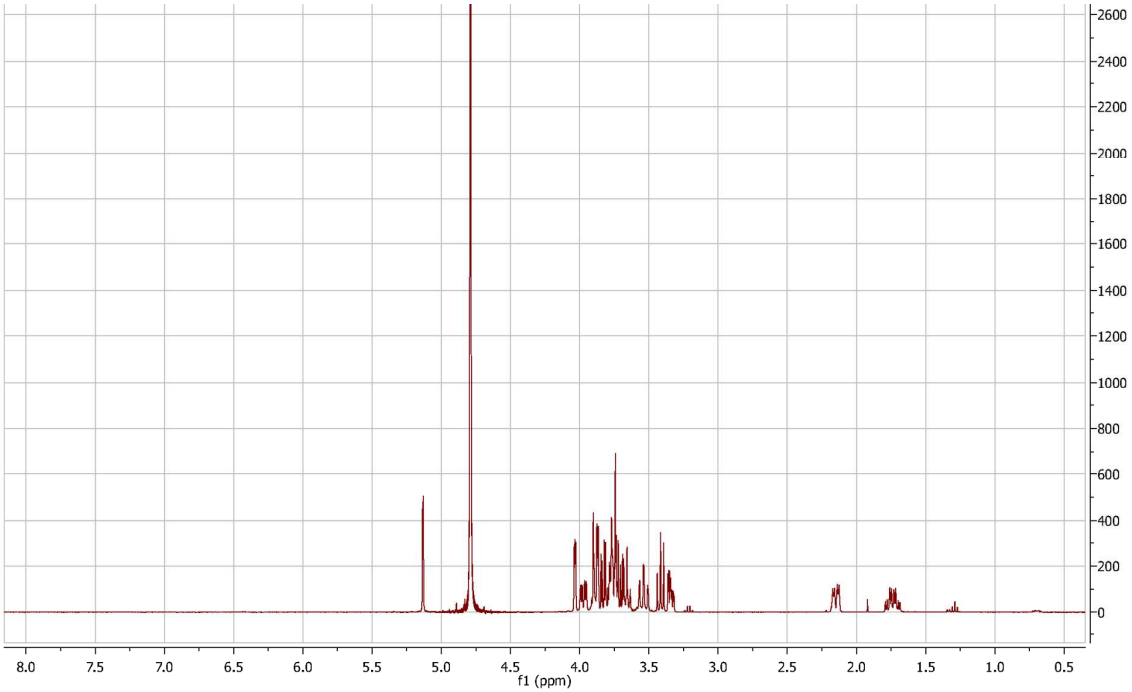


^{13}C NMR

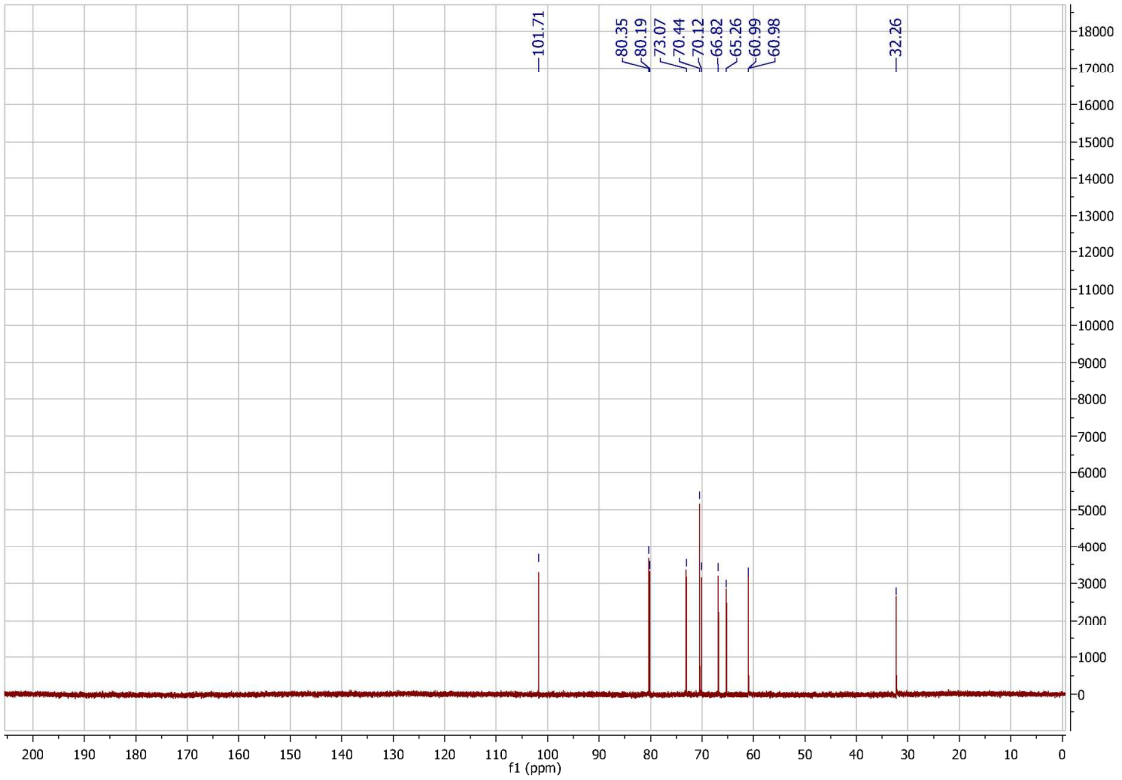


α-D-Mannopyranosyl-(1→3)-1,2-dideoxy-D-mannopyranose (ManddMan; 5)

^{1}H NMR

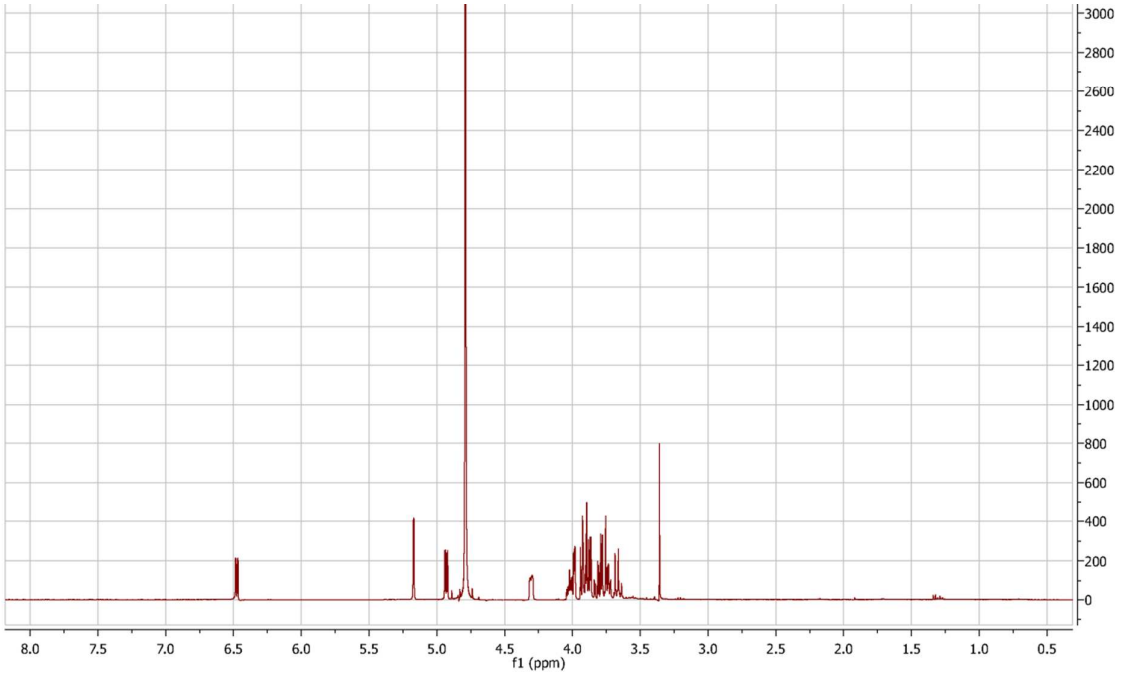


^{13}C NMR

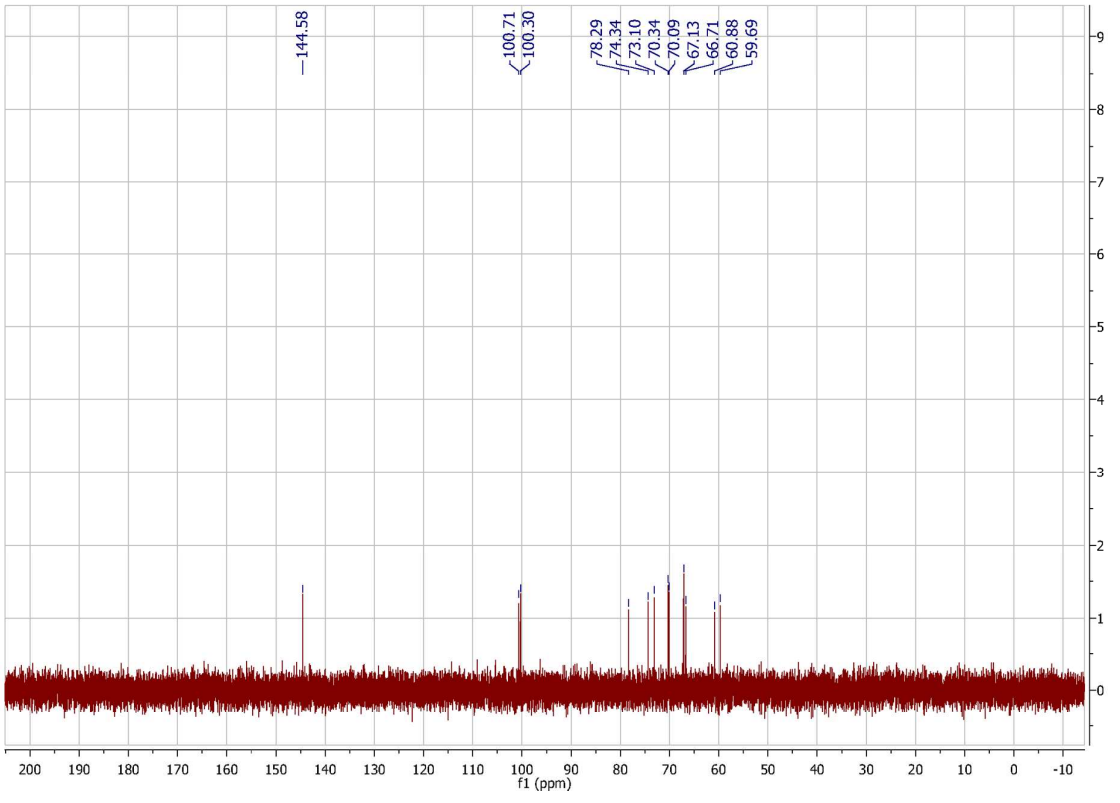


α-D-Mannopyranosyl-(1→3)-1,5-anhydro-2-deoxy-D-*arabino*-hex-1-enitol (ManGlucal; 7)

^{1}H NMR

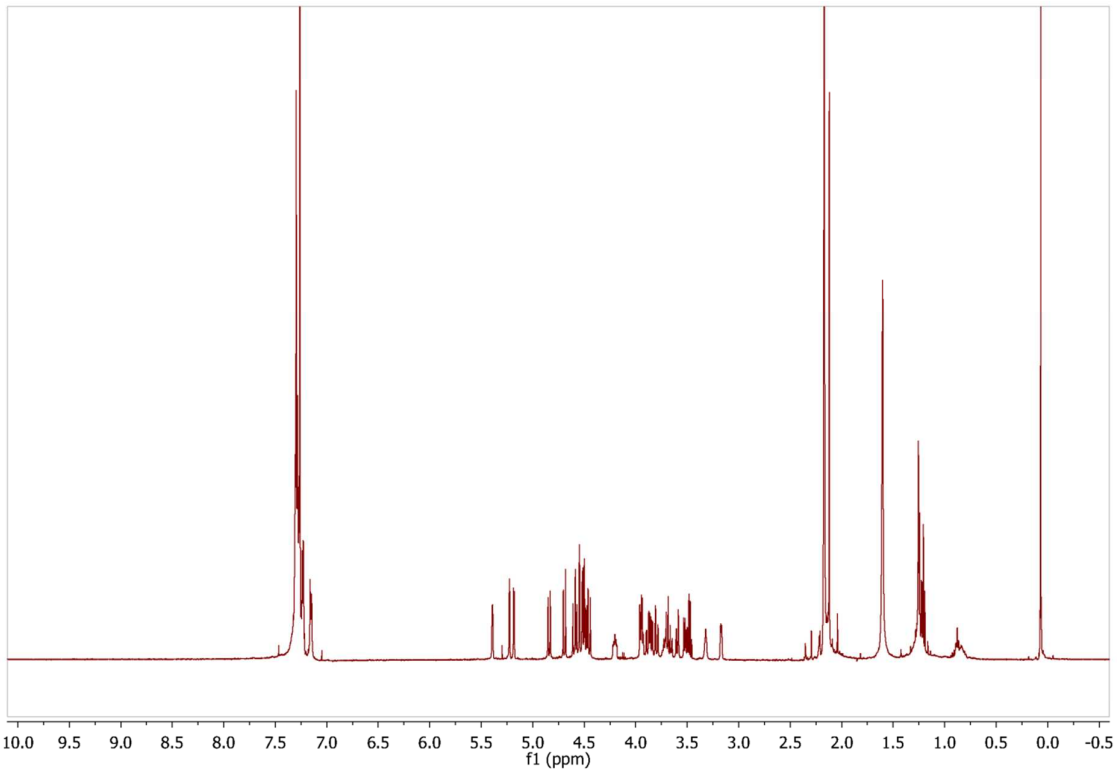


^{13}C NMR

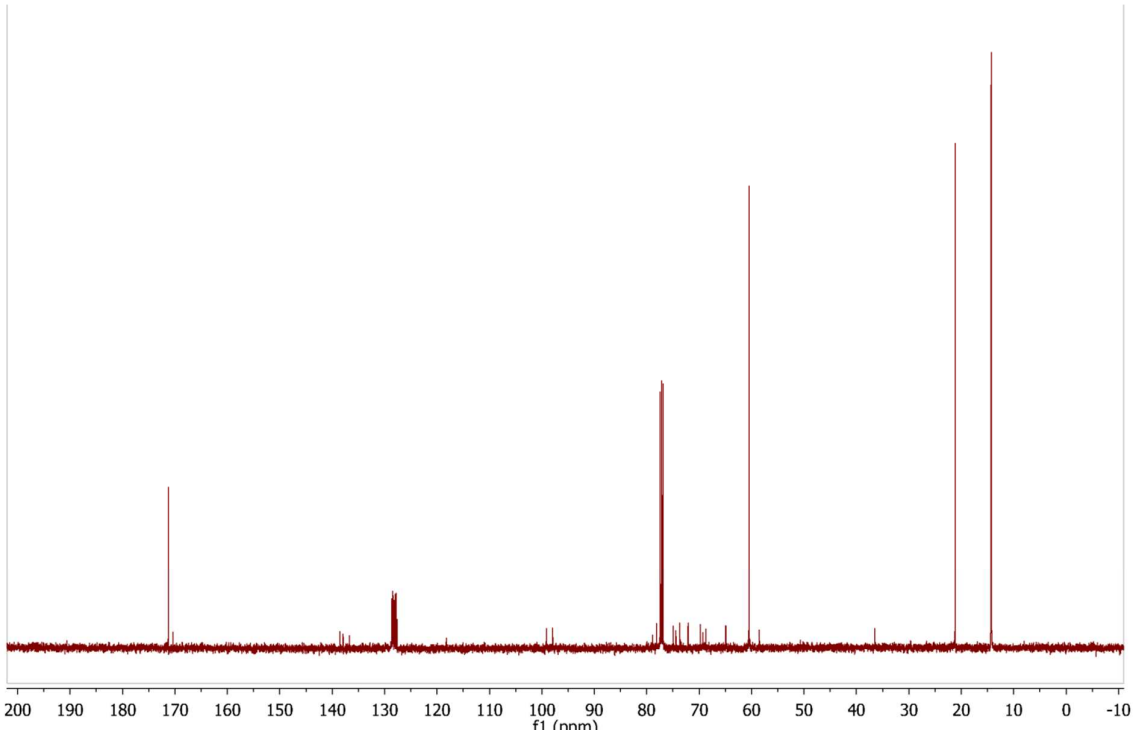


Benzyl 2-*O*-(2-*O*-acetyl-3,4,5-tri-*O*-benzyl-α-mannopyranosyl)-(1→2)-(4-cyano-4-deoxy-β-D-arabinopyranoside) (17)

^{1}H NMR

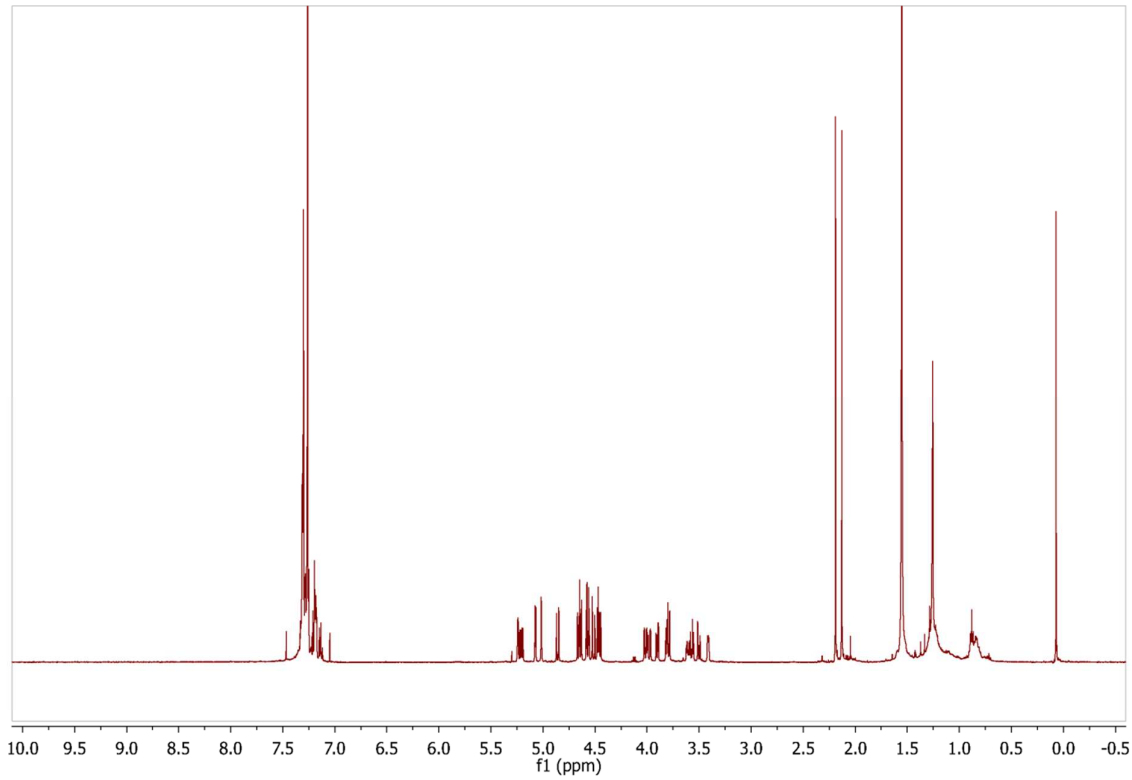


^{13}C NMR

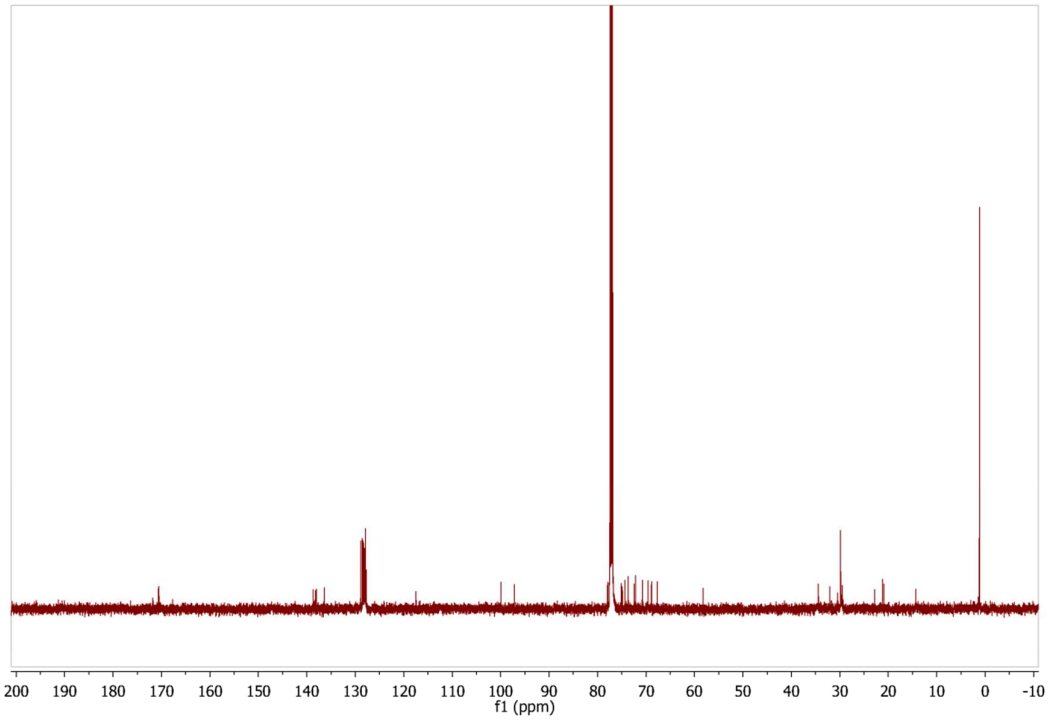


Benzyl 2-*O*-(2-*O*-acetyl-3,4,5-tri-*O*-benzyl-α-mannopyranosyl)-(1→2)-(3-*O*-acetyl-4-cyano-4-deoxy-β-D-arabinopyranoside)

^{1}H NMR

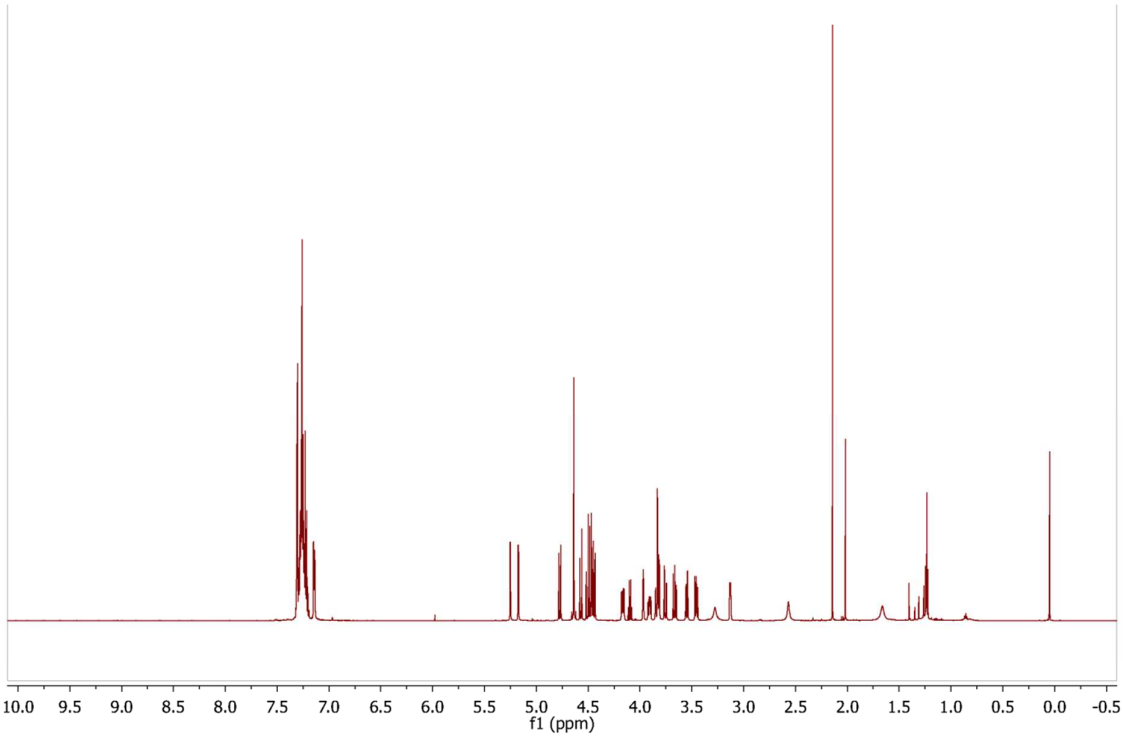


^{13}C NMR

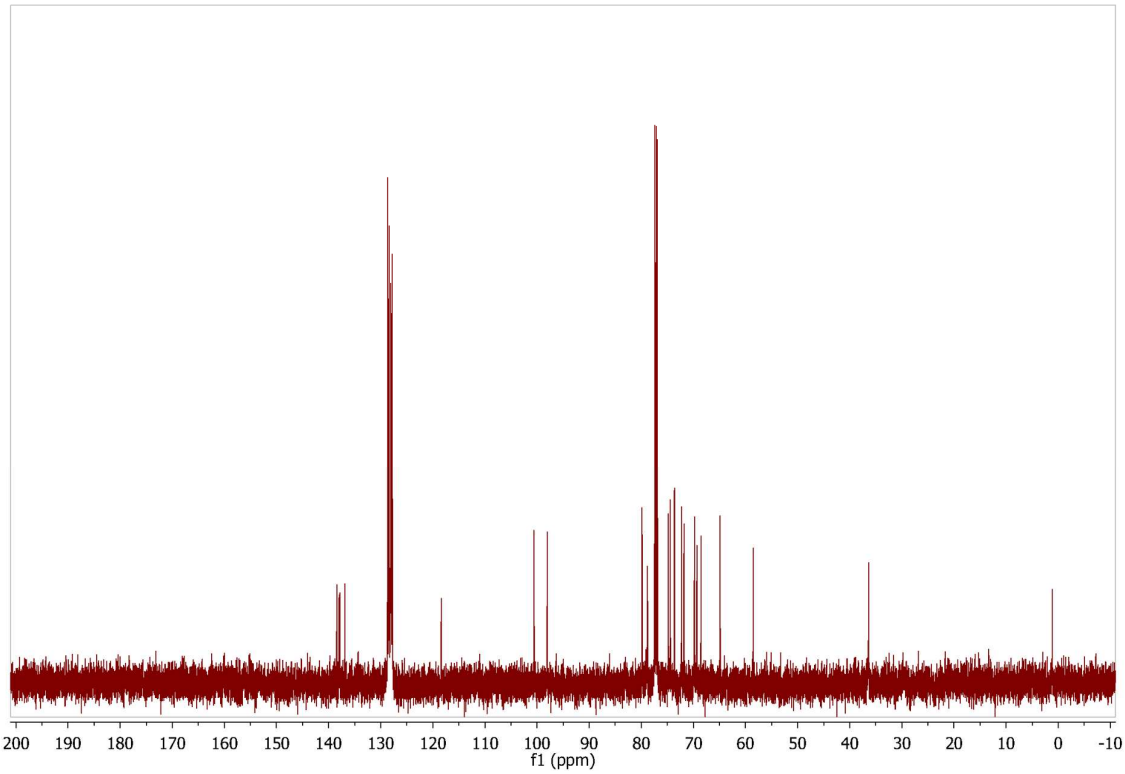


Benzyl 2-*O*-(3,4,5-tri-*O*-benzyl-α-mannopyranosyl)-(1→2)-(4-cyano-4-deoxy-β-D-arabinopyranoside) (18)

^{1}H NMR

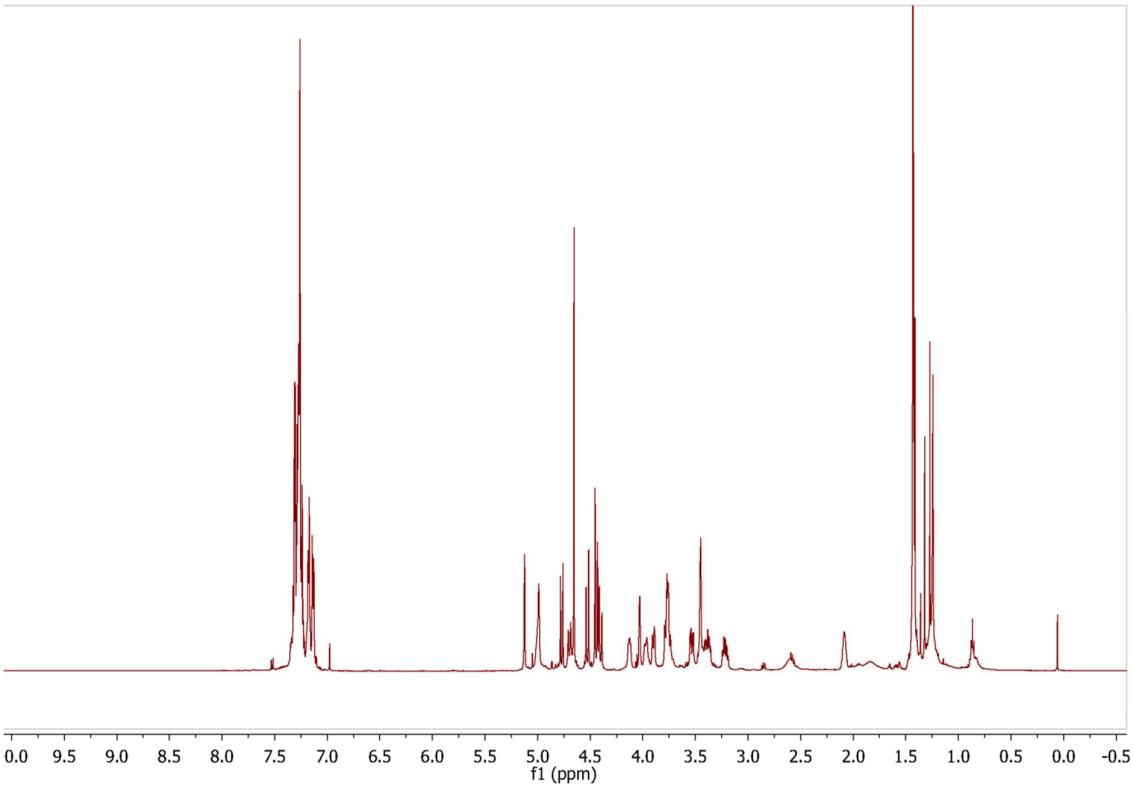


^{13}C NMR



Benzyl 2-*O*-(2-*O*-acetyl-2,4,5-tri-*O*-benzyl-α-mannopyranosyl)-(1→2)-(4-*C*-[(*tert*-butoxycarbonyl)amino]methyl-4-deoxy-β-D-arabinopyranoside) (19)

^{1}H NMR



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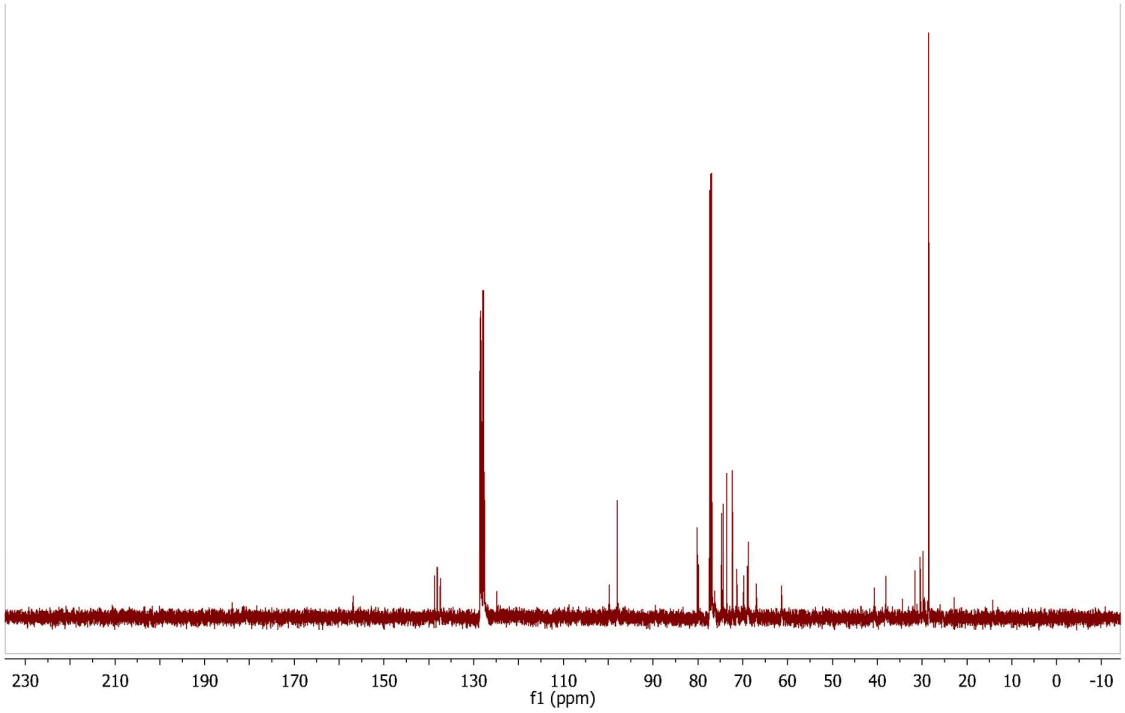

α-D-Mannopyranosyl-(1→3)-noeuromycin hydrochloride (ManNOE.HCl; 9)

^{1}H NMR