Electronic Supplementary Material (ESI) for Medicinal Chemistry Communications.

Supporting Information

A Mechanistic Study on the α-*N*-Acetylgalactosaminidase from *E. meningosepticum*: A Family 109 Glycoside Hydrolase

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Supporting Information Contents:

List of Abbreviations

app	apparent
br	Broad
BSA	Bovine serum albumin
c	concentration
CHES	N-Cyclohexyl-2-aminoethanesulfonic acid
d	Doublet
DTT	Dithiothreitol
GH	Glycosyl hydrolase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LB	Luria Broth
m	multiplet
MES	2-(*N*-morpholino)ethanesulfonic acid
NAD^+	Nicotinamide adenine dinucleotide
NMR	Nuclear magnetic resonance
PCR	Polymerase chain reaction
PNPαGalNAc	4-nitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside
t	triplet
TB	Terrific Broth
TBAF	Tetrabutyl ammonium fluoride
TCEP	Tris-(carboxyethyl)phosphine
TLC	Thin-layer chromatography
TMS	Tetramethylsilane
Trunc	truncated

General Information

All chemicals were of analytical grade or better and were purchased from Sigma-Aldrich unless noted otherwise. Milli-Q water (18.2 MΩ cm^{-1}) was used for all kinetic experiments. All pH values were measured using a standard pH electrode attached to a VWR pH meter. All NMR spectra were acquired on either a Bruker 400, 500 or 600 MHz spectrometer. Chemical shifts are reported in parts per million downfield from signals for TMS. The signal residues from deuterated chloroform and external TMS salts (D_2O) were used for ^1H NMR spectral references; for ^{13}C NMR spectra, natural abundance signals from $CDCl_3$ and external TMS salts (D_2O) were used as references. Coupling constants (*J*) are reported in hertz. Melting points were determined on a Gallenkamp melting point apparatus and are not corrected. Optical rotations were measured on a Perkin-Elmer 341 polarimeter and are reported in units of deg cm^2 g^{-1} (concentrations reported in units of g per 100 mL). PNPαGalNAc (3a) was purchased from Sigma-Aldrich and used without further purification.

Experimental Details

Typical procedure for the synthesis of substrates: all aryl 2-acetamido-2-deoxy-α-D-galactopyranosides were synthesized from 2-deoxy-2-(methoxycarbonylamino)-1,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside using $SnCl_4$ as activator in a solvent of CH_2Cl_2. In a typical procedure, 2-deoxy-2-(methoxycarbonylamino)-1,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside **1** (1 g, 2.4 mmol) and the appropriate phenol (4.9 mmol) were dissolved in anhydrous CH_2Cl_2 (50 mL) and then $SnCl_4$ (0.3 mL, 2.4 mmol) was added, and the reaction mixture was stirred at ambient temperature under an inert atmosphere for 48 h. Following the addition of water (40 mL) the reaction mixture was neutralized by adding saturated $NaHCO_3$ (30 mL). The crude product was extracted from the aqueous layer using CH_2Cl_2 (3 × 40 mL), and the combined organic layers were washed with brine (2 × 25 mL), dried (Na_2SO_4) and concentrated under reduced pressure (typical α:β ratios 4:1 to 5:1). This crude material was purified by column chromatography using EtOAc-Hexane (35:65, EtOAc:Hexane) as the eluent to obtain the pure α-galactoside (yields 55–65%). Removal of the carbamate was accomplished using TBAF according to a reported procedure to give crude aryl 2-amino-2-deoxy-α-D-galactopyranosides (yields 45–50%).[1] The resultant crude product was acetylated under standard conditions: pyridine (10 mL) and acetic anhydride (10 mL). The peracetylated product was extracted from the aqueous layer using CH_2Cl_2 (3 × 35 mL), and the combined organic layers were washed with brine (2 × 20 mL), dried (Na_2SO_4) and concentrated under reduced pressure and the excess pyridine was removed azeotropically using toluene. Purification of the final product was achieved by performing a column chromatography in 5% MeOH-CH_2Cl_2, to obtain the pure per-acetylated product (quantitative yields). Finally, the substrates were obtained by deprotection of the peracetylated material using Zemplen conditions (NaOMe/MeOH) followed by neutralization

using Amberlite (H^+) resin to obtain the pure substrates in the series of aryl 2-acetamido-2-deoxy-α-D-galactopyranosides in 20–30% overall yield over six steps (individual overall yields are reported with the physical data for compounds **3b–f**). Characterization data for the panel of substrates used in this study are given below:

3-Nitrophenyl 1,3,4,6-tetra-O-acetyl-2-deoxy-2-(methoxycarbonylamino)-α-D-galactopyranoside (**2b**) $[\alpha]^{20}_D$ = +29.3 (*c* 1.0, $CHCl_3$) ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.95 (s, 1H, Ar), 7.92 (d, *J* = 8.1, 1H, Ar), 7.47 (t, 1H, Ar), 7.39 (d, *J* = 8.2, 1H, Ar), 5.67 (d, *J* = 3.1, 1H, H-1), 5.45 (app.d, 1H, H-4), 5.34 (dd, *J* = 11.4, 2.9, 1H, H-3), 4.97 (d, *J* = 9.5, 1H, NH), 4.48 - 4.52 (m, 1H, H-2), 4.26 (t, *J* = 6.5, 1H, H-5), 4.11 (m, 2H, H-6, 6′), 3.68 (s, 3H, OCH_3, $NHCOOCH_3$), 2.17 (s, 3H), 2.03 (s, 3H), 1.91 (s, 3H) (3× $OCOCH_3$); ^{13}C NMR (151 MHz, $CDCl_3$) δ 170.70, 170.25, 170.08, 156.52, 149.10, 130.25 (Ar C), 122.89 (Ar C), 117.91(Ar C), 111.87 (Ar C), 97.23 (C-1), 67.98 (C-5), 67.81(C-3), 66.98 (C-4), 61.60 (C-6), 52.48 (OCH_3, $NHCOOCH_3$), 49.55 (C-2), 20.63, 20.58, 20.38 (3 × OCH_3).

3-Chlorophenyl 1,3,4,6-tetra-O-acetyl-2-deoxy-2-(methoxycarbonylamino)-α-D-galactopyranoside (**2c**) $[\alpha]^{20}_D$ = +22.9 (*c* 0.8, $CHCl_3$) ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.23 (d, *J* = 8.2, 1H, ArH), 7.13 (s, 1H, ArH), 7.06 (d, *J* = 8.0, 1H, ArH), 6.96 (dd, *J* = 8.1, 1.7, 1H, ArH), 5.58 (d, *J* = 3.0, 1H, H-1), 5.45 (app d, 1H, H-4), 5.32 (dd, *J* = 11.3, 2.8, 1H, H-3), 4.94 (d, *J* = 9.6, 1H, NH), 4.44 - 4.48 (m, 1H, H-2), 4.26 (t, *J* = 6.3, 1H, H-5), 4.14 – 4.02 (m, 2H, H-6, 6′), 3.67 (s, 3H, OCH_3, $NHCOOCH_3$), 2.19 (s, 3H), 2.04 (s, 3H), 1.97 (s, 3H) (3 × $OCOCH_3$); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.77, 170.38, 170.21(3×CO, 3×$OCOCH_3$), 156.58, 130.45 (ArC), 123.34(ArC), 117.23 (ArC), 115.06 (ArC), 96.76 (C-1), 68.11 (C-3), 67.80 (C-5), 67.18 (C-4), 61.70 (C-6), 52.53 (OCH_3, $NHCOOCH_3$), 49.63 (C-2), 20.74, 20.68, 20.55 (3×OCH_3).

4-Chlorophenyl 1,3,4,6-tetra-O-acetyl-2-deoxy-2-(methoxycarbonylamino)-α-D-galactopyranoside (**2d**) $[\alpha]^{20}_{D}$ = +20.9 (*c* 0.32, $CHCl_3$) ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.26 (d, *J* = 8.9, 2H, ArH), 7.00 (d, *J* = 9.0, 2H, ArH), 5.56 (d, *J* = 3.4, 1H, H-1), 5.44 (d, 1H, H-4), 5.32 (dd, *J* = 11.3, 2.9, 1H, H-3), 4.98 (d, *J* = 9.8, 1H, NH), 4.45 (m, 1H, H-2), 4.24 (t, *J* = 6.6, 1H, H-5), 4.07 (m, 2H, H-6, 6′), 3.66 (s, 3H, OCH_3, $NHCOOCH_3$), 2.18 (s, 3H), 2.04 (s, 3H), 1.94 (s, 3H) (3× $OCOCH_3$); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.76, 170.26, 170.18 (3×CO, 3×$OCOCH_3$), 156.53, 154.57, 129.57 (ArC), 117.97 (ArC), 96.92 (C-1), 68.10 (C-3), 67.67 (C-5), 67.14 (C-4), 61.59 (C-6), 52.49 (OCH_3, $NHCOOCH_3$), 49.61(C-2), 20.72, 20.66, 20.53 (3×OCH_3).

Phenyl 1,3,4,6-tetra-O-acetyl-2-deoxy-2-(methoxycarbonylamino)-α-D-galactopyranoside (**2e**) $[\alpha]^{20}_{D}$ = +19.5 (*c* 1.12, $CHCl_3$) ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.29 - 7.33 (m, 2H, Ar), 7.05 - 7.08 (m, 3H, Ar) 5.60 (d, *J* = 3.4, 1H, H-1), 5.45 (app.d, 1H, H-4), 5.35 (dd, *J* = 11.4, 3.0, 1H, H-3), 5.0 (d, *J* = 9.8, 1H, NH), 4.48 - 4.43 (m, 1H, H-2), 4.28 (t, *J* = 6.5, 1H, H-5), 4.03 - 4.13 (m, 2H, H-6, 6′), 3.65 (s, 3H, OCH_3, $NHCOOCH_3$), 2.18 (s, 3H), 2.03 (s, 3H), 1.92 (s, 3H) (3× $OCOCH_3$); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.75, 170.30, 170.23 (3 × CO), 156.56, 156.02, 129.65 (ArC), 116.57 (ArC), 96.64(C-1), 68.29 (C-5), 67.50 (C-3), 67.23 (C-4), 61.57 (C-6), 52.44 (OCH_3, $NHCOOCH_3$), 49.64(C-2), 20.74, 20.68, 20.53 (3×OCH_3).

4-Methoxyphenyl 1,3,4,6-tetra-O-acetyl-2-deoxy-2-(methoxycarbonylamino)-α-D-galactopyranoside (**2f**) $[\alpha]^{20}_{D}$ = +20.5 (*c* 0.58, $CHCl_3$) ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.99 (d, *J* = 9.2, 2H, ArH), 6.83 (d, *J* = 9.1, 2H, ArH), 5.47 (d, *J* = 3.3, 1H, H-1), 5.45 (app.d, *J* = 2.8, 1H, H-4), 5.32 (dd, *J* = 11.3, 3.0, 1H, H-3), 5.01 (d, *J* = 9.9, 1H, NH), 4.43-4.442 (m, 1H, H-2), 4.32 (t, *J* = 6.6, 1H, H-5), 4.13-4.04 (m, 2H, H-6, 6′), 3.77 (s, 3H, OCH_3), 3.66 (s, 3H, OCH_3, $NHCOOCH_3$), 2.17 (s, 3H), 2.03 (s, 3H), 1.96 (s, 3H) (3× $OCOCH_3$); ^{13}C NMR (151 MHz,

$CDCl_3$) δ 170.74, 170.30, 170.24 (3×CO, 3×$OCOCH_3$), 156.58, 155.50, 150.06, 118.00 (ArC), 114.67 (ArC), 97.63 (C-1), 68.34 (C-3), 67.40 (C-5), 67.31(C-4), 61.69 (C-6), 55.64 (C, OCH_3), 52.42 (OCH_3, $NHCOOCH_3$), 49.69 (C-2), 20.72, 20.67, 20.57 (3×OCH_3).

3-Nitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside (**3b**) Yield 25% (6 steps); Mpt = 159–161 °C, $[\alpha]^{20}_D$ =+271.4 (*c* 0.29, H_2O); ^{1}H NMR (600 MHz, D_2O) δ 7.97 (t, *J* = 2.2, 1H, ArH), 7.95 (d, *J* = 7.8, 1H, ArH), 7.56 (t, *J* = 8.2, 1H, ArH), 7.51 (dd, *J* = 8.3, 2.3, 1H, ArH), 5.73 (d, *J* = 3.6, 1H, H-1), 4.37 (dd, *J* = 11.0, 3.6, 1H, H-2), 4.15 (dd, *J* = 11.1, 3.2, 1H, H-3), 4.05- 4.02 (m, 2H, H-4, H-5), 3.71 (m, 2H, H-6, 6′), 2.02 (s, 3H, $NHCOCH_3$); ^{13}C NMR (151 MHz, D_2O) δ 174.77 (CO, $NHCOCH_3$), 156.35 (ArC), 148.67 (ArC), 130.51(ArCH), 123.84 (ArCH), 117.85 (ArCH), 111.93 (ArCH), 96.26 (C-1), 72.16 (C-5), 68.38 (C-4), 67.58 (C-3), 61.06 (C-6), 49.59 (C-2), 21.91 (OCH_3, $NHCOCH_3$); HRMS expected for $C_{14}H_{18}N_2O_8$ is 365.0961 ($M^+ + Na^+$): Found 365.0953.

3-Chlorophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside (**3c**) Yield 25% (6 steps); Mpt = 180-181 °C (decomp.), $[\alpha]^{20}_D$ =+210.5 (*c* 0.63, H_2O) ^{1}H NMR (600 MHz, D_2O) δ 7.32 (t, *J* = 8.0, 1H, ArH), 7.20 (t, *J* = 2.2, 1H, ArH), 7.14 – 7.10 (m, 1H, ArH), 7.06 (dd, *J* = 8.4, 2.4, 1H, ArH), 5.61 (d, *J* = 3.7, 1H, H-1), 4.33 (dd, *J* = 11.1, 3.7, 1H, H-2), 4.12 (dd, *J* = 11.0, 3.2, 1H, H-3), 4.05 – 4.02 (m, 2H, H-4, H-5), 3.74 – 3.68 (m, 2H, H-6, 6′), 2.02 (s, 3H, $NHCOCH_3$); ^{13}C NMR (151 MHz, D_2O) δ 174.77 (CO, $NHCOCH_3$), 156.83 (ArC), 134.30 (ArC), 130.78 (ArCH), 122.99 (ArCH), 117.35 (ArCH), 115.49 (ArCH), 96.22 (C-1), 71.94 (C-5), 68.42 (C-4), 67.60 (C-3), 61.06 (C-6), 49.67 (C-2), 21.89 (OCH_3, $NHCOCH_3$); HRMS expected for $C_{14}H_{18}ClNO_6$ 354.0720 ($M^+ + Na^+$): Found 354.0714 ($M^+ + Na^+$).

4-Chlorophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside (**3d**) Yield 20% (6 steps); Mpt = 218–220 °C, $[\alpha]^{20}_D$ =+190.5 (*c* 0.8, H_2O) ^{1}H NMR (600 MHz, D_2O) δ 7.40 (d, *J* = 9.0, 2H), 7.14

(d, *J* = 9.0, 2H), 5.63 (d, *J* = 3.6, 1H, H-1), 4.37 (dd, *J* = 11.1, 3.7, 1H, H-2), 4.16 (dd, *J* = 11.1, 3.2 Hz, H-3), 4.09 (m, 2H, H-4, H-5), 3.79 – 3.72 (m, 2H, H-6, 6′), 2.07 (s, 3H, OCH3, $NHCOCH_3$); ^{13}C NMR (151 MHz, D_2O) δ 174.25 (CO, $NHCOCH_3$), 154.35 (ArC), 129.02 (ArC), 126.82 (ArCH), 118.08 (ArCH), 95.85 (C-1), 71.37 (C-5), 67.90 (C-4), 67.11(C-3), 60.56 (C-6), 49.20 (C-2), 21.40 (OCH_3, $NHCOCH_3$); HRMS expected for $C_{14}H_{18}ClNO_6$ 354.0720 ($M^+ + Na^+$): Found 354.0717 ($M^+ + Na^+$).

Phenyl 2-acetamido-2-deoxy-α-D-galactopyranoside (**3e**) Yield 21% (6 steps); Mpt = 249–250 °C (decomp.), $[\alpha]^{20}_D$ =+220.0 (*c* 0.56, H_2O) ^1H NMR (600 MHz, D_2O) δ 7.43 (t, *J* = 8.0, 2H), 7.17 (dd, *J* = 12.6, 7.7, 3H), 5.66 (d, *J* = 3.6, 1H, H-1), 4.37 (dd, *J* = 11.1, 3.5, 1H, H-2), 4.19 (dd, *J* = 11.2, 3.2, 1H, H-3), 4.13 (t, *J* = 6.1, 1H, H-5), 4.10 (app d, 1H, H-4), 3.75-3.77 (m, 2H, H-6, 6′), 2.07 (s, 3H, OCH_3, $NHCOCH_3$); ^{13}C NMR (151 MHz, D_2O) δ 174.27 (CO, $NHCOCH_3$), 155.69, 129.39 (ArC), 122.64 (ArC), 116.70 (ArC), 95.73 (C-1), 71.26 (C-5), 67.94 (C-4), 67.15 (C-3), 60.56 (C-6), 49.28 (C-2), 21.40 (OCH_3, $NHCOCH_3$); HRMS expected for $C_{14}H_{19}NO_6$ 320.1110 ($M^+ + Na^+$): Found 320.1103 ($M^+ + Na^+$).

4-Methoxyphenyl 2-acetamido-2-deoxy-α-D-galactopyranoside (**3f**) Yield 30% (6 steps); Mpt = 175–177 °C, $[\alpha]^{20}_D$ =+200.8 (*c* 0.35, H_2O) ^1H NMR (600 MHz, D_2O) δ 7.05 (d, *J* = 9.1, 2H, ArH), 6.92 (d, *J* = 9.1, 2H, ArH), 5.43 (d, *J* = 3.7, 1H, H-1), 4.26 (dd, *J* = 10.9, 3.5, 1H, H-2), 4.09 – 4.05 (m, 2H, H-3, H-5), 4.01 (d, *J* = 2.5, 1H, H-4), 3.74 (s, 3H, OCH_3), 3.68 (app. d, 2H, H-6, 6′), 2.00 (s, 3H, $NHCOCH_3$); ^{13}C NMR (151 MHz, D_2O) δ 174.26 (CO, $NHCOCH_3$), 154.16 (ArC), 150.03 (ArC), 118.40 (ArCH), 114.56 (ArCH), 96.80 (C-1), 71.21(C-5), 67.96 (C-4), 67.13 (C-3), 60.59 (C-6), 55.31(OCH_3), 49.33 (C-2), 21.41(OCH_3, $NHCOCH_3$); HRMS expected for $C_{15}H_{21}NO_7$ 350.1216 ($M^+ + Na^+$): Found 350.1210 ($M^+ + Na^+$).

Cloning of *E. meningosepticum* α-N-acetylgalactosaminidase (α-NAGAL)

A truncated version of *E. meningosepticum* α-*N*-acetylgalactosaminidase (EMBL Nucleotide Sequence Database accession number AM039444) lacking the first 17 amino acids, which is the same truncation as that reported by Lui et al.,[2] was amplified by PCR from genomic DNA (ATCC No. 51720D) using the Thermo Scientific Phusion High-Fidelity PCR system with primers NAGAL-EcoRI: 5'CCGGAATTCAAAAAGGTAAGAATAGCTTTTT3' and NAGAL-HindIII: 5'CCCAAGCTTGTAGTCGTCATTTATTGCAAATG3' introducing the EcoRI and HindIII restriction sites in the forward and reverse primer respectively. The PCR fragment was digested with *EcoRI* and *HindIII* and inserted into correspondingly digested pET28a vector (Strategene) for expression of a C-termius His_6 tagged protein. The construct was used to transform *Escherichia coli* BL21 (DE3 Gold) (Novagen) competent cells. Transformants were plated onto LB plates containing 100 μg/mL kanamycin. Plasmid from a single colony was purified and DNA sequencing by Macrogen using T7 promoter and T7 terminator primers provided the nucleotide composition of the amplified gene. The nucleotide sequence was aligned with AM039444, which was amplified from genomic DNA (ATCC 13253),[2] using BioEdit and the amino acid differences were identified (Figure S1).

Expression and purification of *E. meningosepticum* α-*N*-acetylgalactosaminidase (α-NAGAL)

The tagged enzyme was grown in TB supplemented with 100 μg/ml kanamycin. Expression was induced at OD_{600} ~ 0.6 by addition of isopropyl β-D-1-thiogalactoside (IPTG) at a final concentration of 1 mM. Cell pellet from 1 L culture was resuspended in 50 mL of lysis buffer, 40 mM $NaPO_4$, pH 6.8, 100 mM NaCl, 1% lysozyme and a protease inhibitor cocktail tablet (Roche Diagnostics) followed by sonication (20 sec ON/ 60 sec OFF at 50% capacity). The lysate was

centrifuged for 30 min at 13000 rpm and the supernatant was loaded onto a HisTrap column (GE Life Sciences). The protein was eluted by increasing the concentration of imidazole in the elution buffer. The fractions containing enzyme as determined by SDS-PAGE were dialyzed against Tris buffer (40 mM containing NaCl 100 mM) overnight at 4 °C. These fraction were then concentrated using a 10 kDa Micorcon centrifugal filter (Millipore). The protein was then concentrated by centrifugation through a 10 kDa filter and its concentration was assessed (Bradford Assay).

Typical Conditions for the Measurement of Michaelis–Menten Parameters. The concentration of α-NAGAL was chosen such that less than 10% of the total substrate was consumed during the assay. For each assay, the enzyme was incubated in the appropriate buffer at 37 °C for 5 min. After which the reaction was initiated by the addition of substrate. The initial rate of hydrolysis was followed spectrophotometrically at the wavelength of maximal absorbance change. Typically, the substrate concentration was varied between 40 μM and 500 mM and the measured initial rate versus concentration data were fit to a standard Michaelis-Menten equation. To determine the effect of pH on enzymatic activity, kinetics parameters were measured over a pH range of 6.3–8.8. The buffers used were MES (20 mM, MES-NaOH, pH 6.0–6.7), HEPES (20 mM, pH 6.5–8.2), and CHES (20 mM, pH 8.5–9.5). Typical assay conditions were: α-NAGAL (final concentration of 0.23 μg/mL) was incubated at 37 °C with the appropriate buffer containing NaCl (50 mM) and BSA (0.1 % w/v) for 5 min prior to addition of substrate PNPαGalNAc and the hydrolysis reaction was monitored by UV-vis spectroscopy. The difference in extinction coefficients (Δε) for PNPαGalNAc and the released 4-nitrophenolate was determined at each pH value and, the initial rate measurements were fit to a standard Michaelis-Menten equation.

The so obtained values for k_{cat} and k_{cat}/K_m and the associated pH values were fit to the equation given below using the computer program prism.

$$k = \frac{k_L}{1 + \left(K_1/[H^+]\right)^{h1} + \left(K_1K_2/[H^+]^2\right)^{h2}} + \frac{k_M}{1 + \left([H^+]/K_1\right)^{h1} + \left(K_2/[H^+]\right)^{h2}} + \frac{k_H}{1 + \left([H^+]^2/K_1K_2\right)^{h1} + \left([H^+]/K_2\right)^{h2}}$$

Where k is the observed rate constant (k_{cat} or k_{cat}/K_m), k_L and k_H are the rate constants at low and high pH, while k_M is the rate constant for the active form of the enzyme. K_1 and K_2 are the two apparent equilibrium constants for protonation and *h1* and *h2* are the Hill coefficients. In both fits (k_{cat} vs. pH and k_{cat}/K_m vs. pH) the fitted value for k_H was within error equal to zero.

Kinetic Investigation of Cofactor Dependence *E. meningosepticum* α-*N*-acetylgalactosaminidase (α-NAGAL)

A sample of α-NAGAL (final concentration of 0.23 μg/mL) in buffer (50 mM HEPES, pH 7.5) was pre-incubated with NAD^+ (5–100 μM) at 37 °C for 5 min. PNPαGalNAc was added to the solution to initiate the hydrolysis reaction at 400 nm. Similar experiment was carried out for the dependence on reducing agent DTT, (5–100 μM) and divalent metal ion Mn^{2+} (10–100 μM). The enzyme showed no enhancement in catalytic rate of hydrolysis upon addition of either of the cofactors.

Product Studies.

^{1}H NMR spectroscopy (500 MHz) was employed to identify the stereochemical course of the enzyme-catalyzed reaction. The reaction conditions involved incubation of enzyme α-NAGAL (3.0 μg/ml) in buffer (50 mM HEPES, pH 7.5) containing CD_3OD (5 M) at 37 °C,. After addition of PNPαGalNAc (2.1 mg) the reaction was allowed to proceed at 37 °C for ~10 h until TLC analysis (1:4 v/v MeOH:EtOAc) showed no remaining starting material. Removal of the enzyme by centrifugal ultra-filtration (10KDa filtration unit) at 4 °C was followed by

lyophilization of the resultant solution. The resultant white solid was dissolved D_2O and a ^1H NMR spectrum was acquired.

Linear Free Energy Relationship – Brønsted Analysis

A series of substrates with varying leaving groups were synthesized to perform a Brønsted analysis. Full Michaelis-Menten curves were measured, in buffer (50 mM HEPES, pH 7.5) at 37 °C, for each substrate using the above protocol.

Figure S1: Protein sequence alignment for the *E. meningosepticum* α-*N*-acetylgalactosaminidase used in this study and that reported by Liu et al. (ATCC 13253).[2]

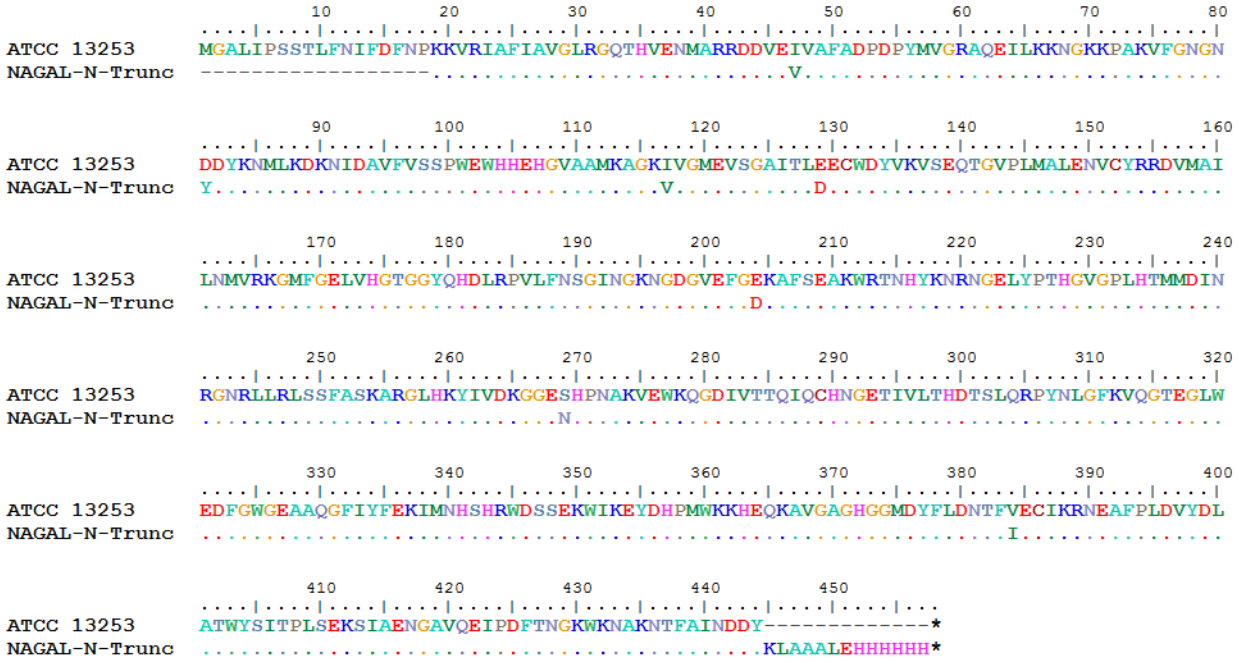


Figure S2: DNA sequence alignment for the *E. meningosepticum* α-*N*-acetylgalactosaminidase gene used in this study and that reported by Liu et al. (ATCC 13253).[22] _ENREF_2

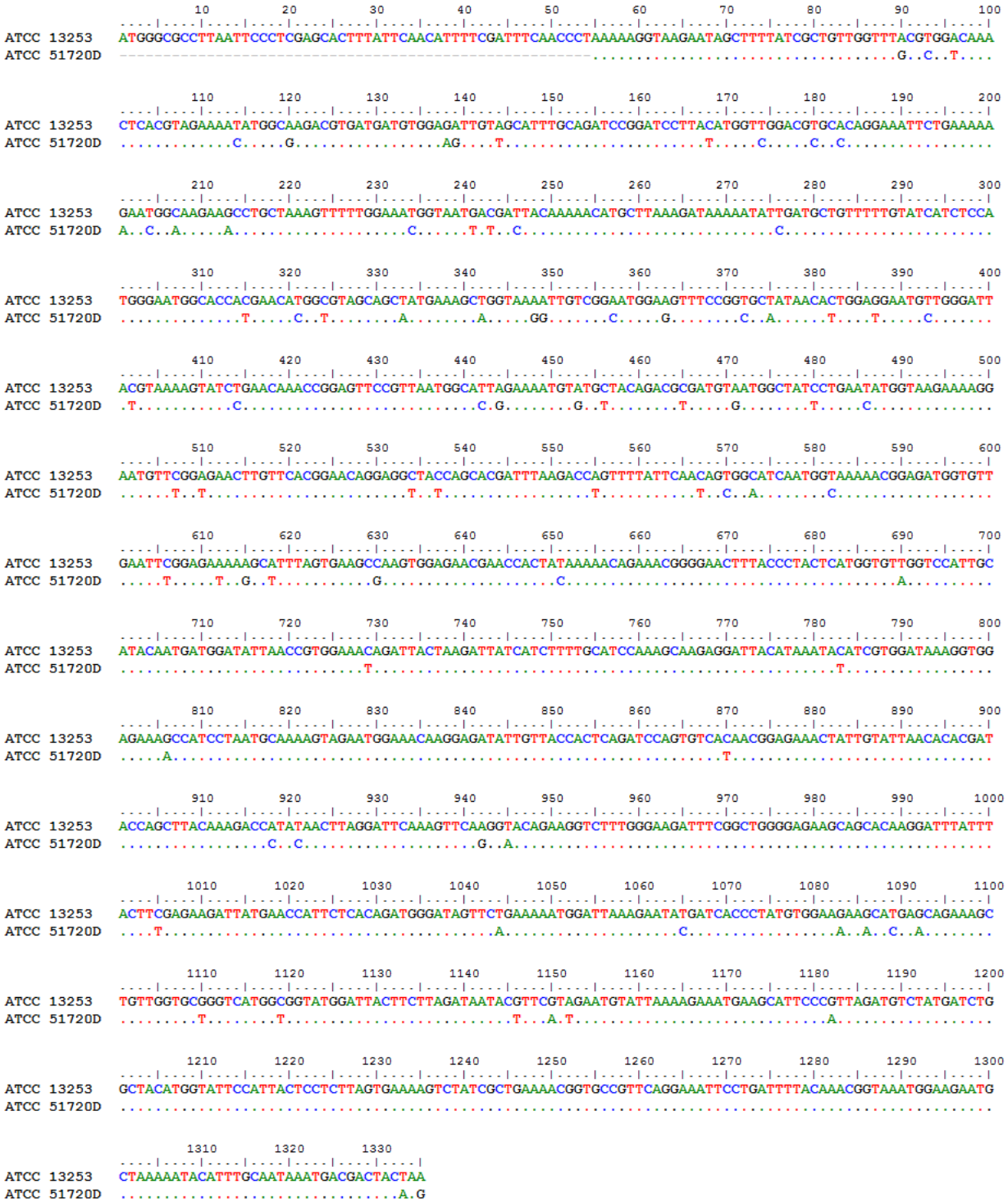


Fig. S3 Comparison of the active sites for GH4 (left) and GH109 (right) enzymes. Key features of the GH4 enzyme are the Mn^{2+} cation, tyrosine 265, and aspartic acid 172, which acidify the C-2 proton, abstract the C-2 proton and catalyse aglycone departure, respectively. Key features of the GH109 enzyme are: methionine 375 and tyrosine 307, which bind to the *N*-acetyl group and tyrosine 179 that is the closest possible base to the C-2 proton.

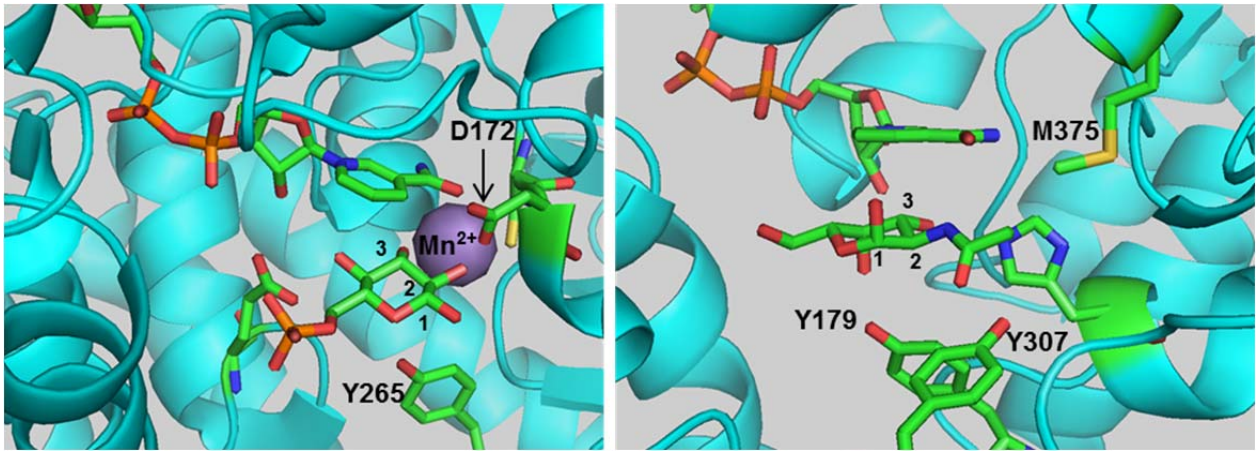


Figure S4. 3-Chlorophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside (600 MHz, D_2O).

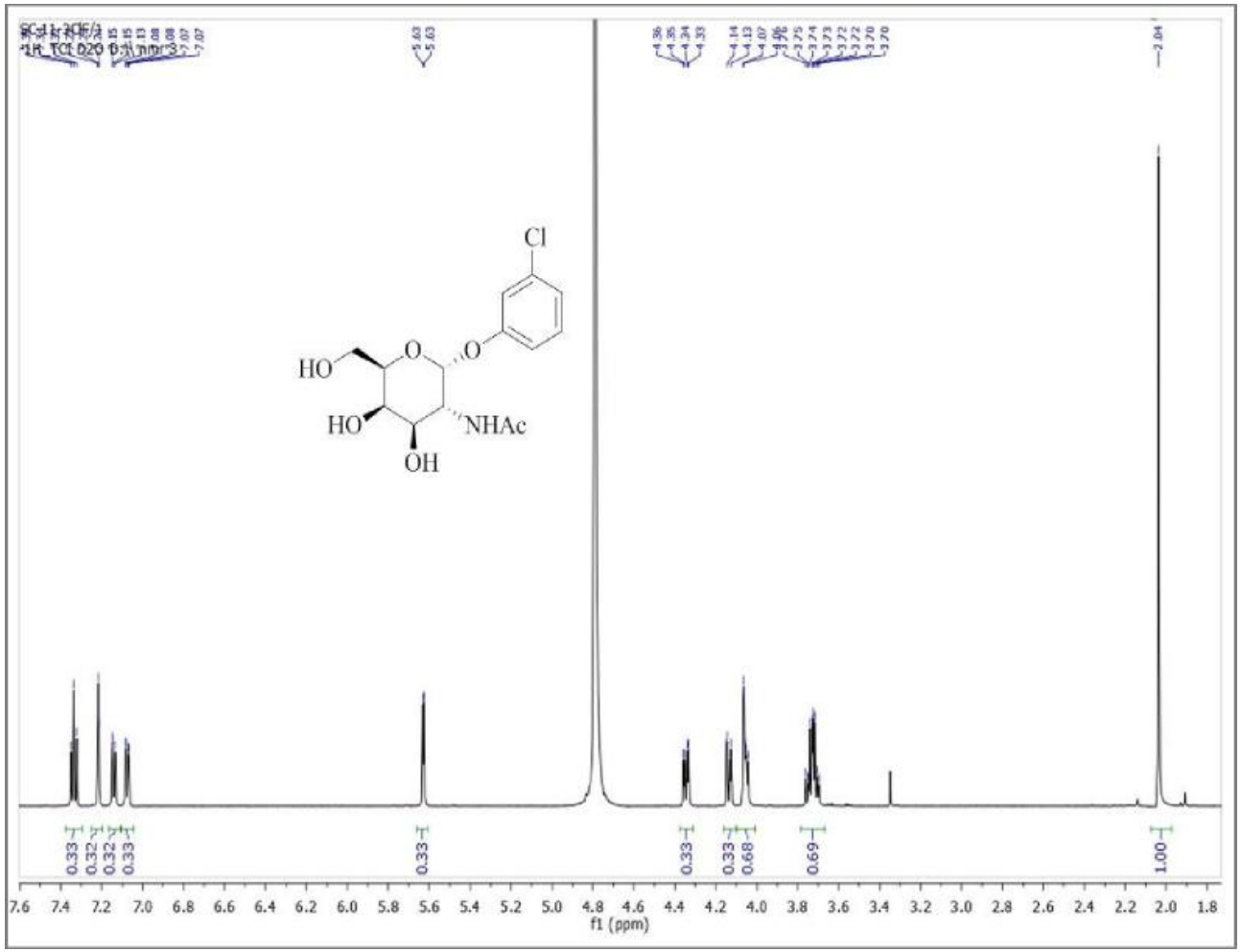


Figure S5. 3-Nitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside (600 MHz, D_2O).

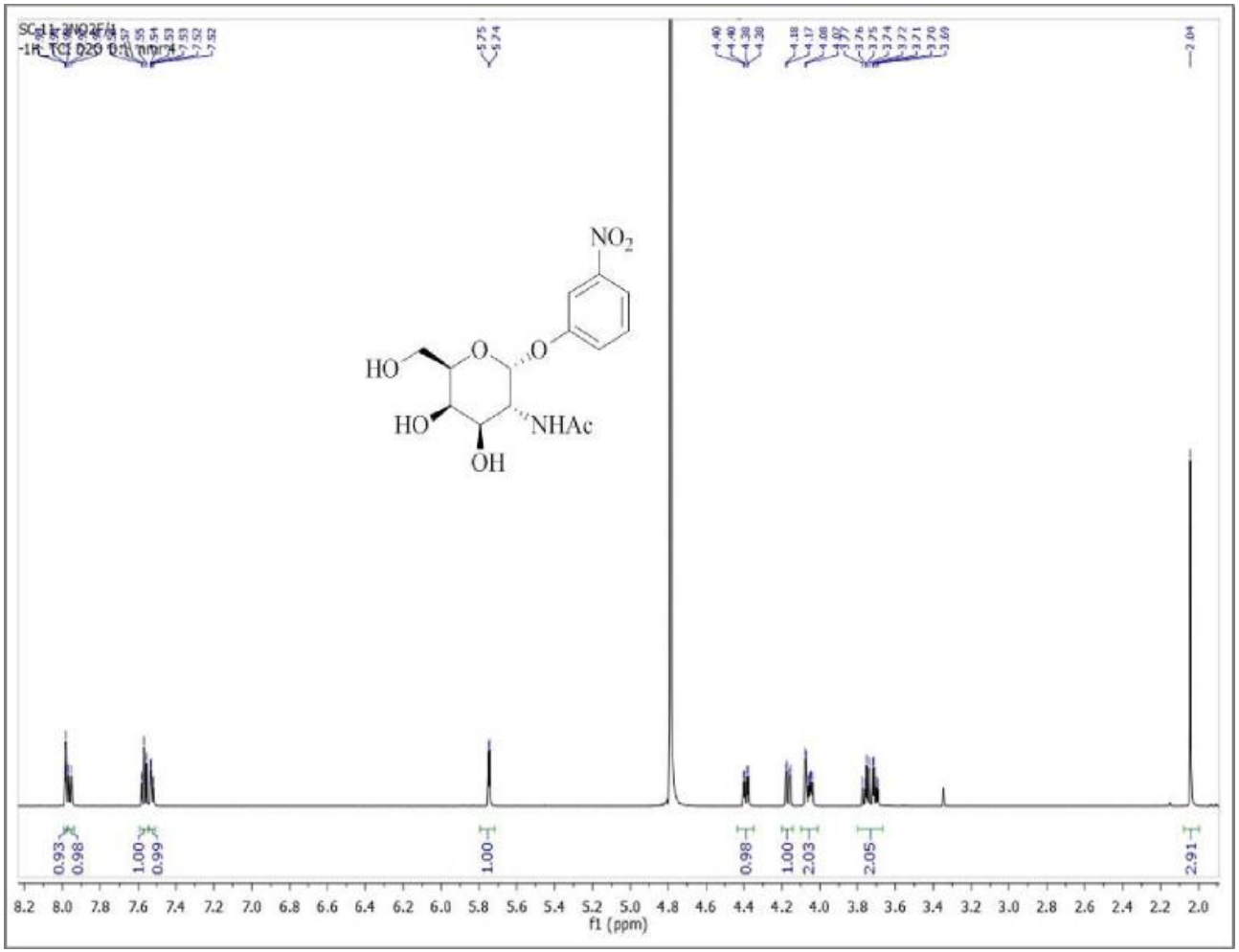


Figure S6. 4-Methoxyphenyl 2-acetamido-2-deoxy-α-D-galactopyranoside (600 MHz, D_2O).

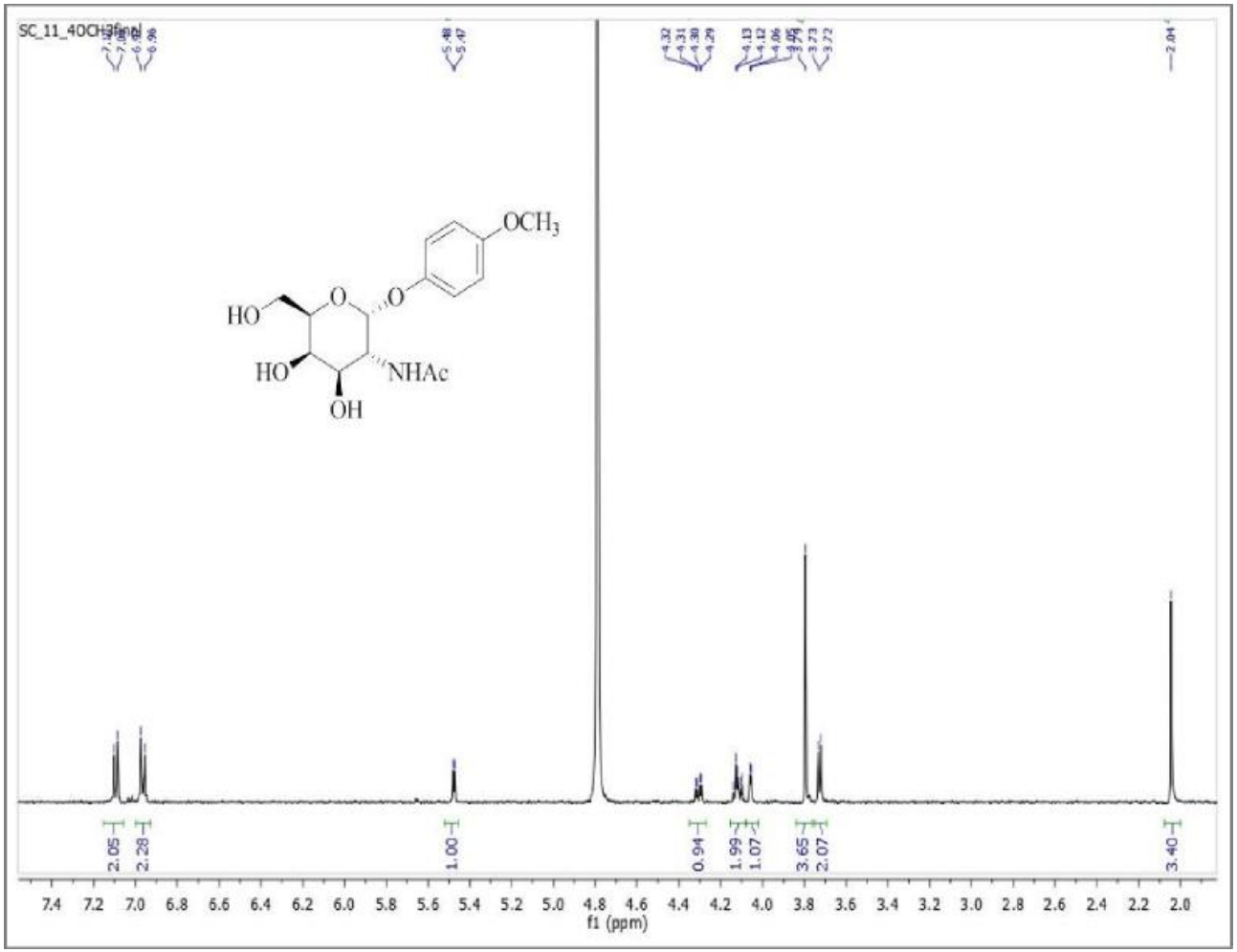
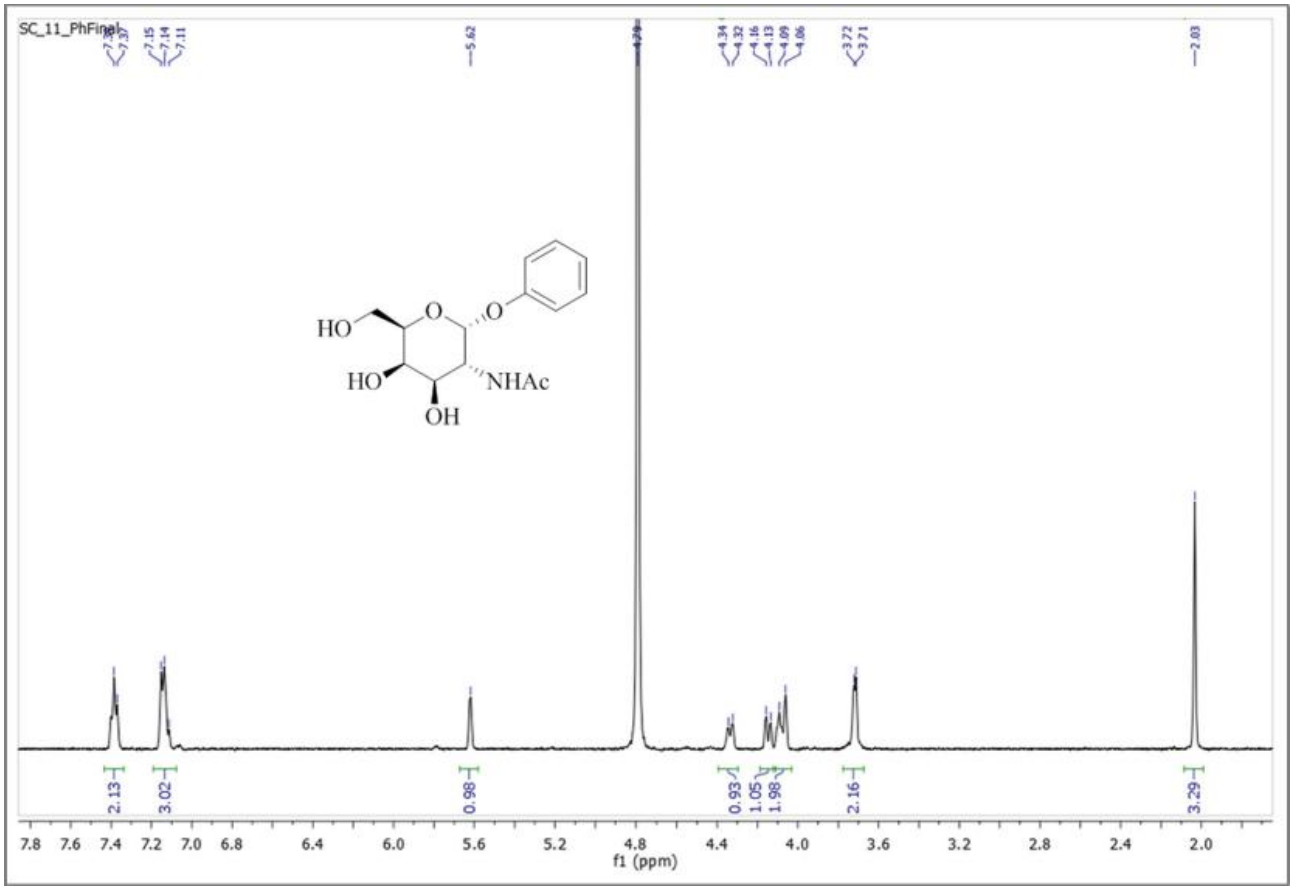


Figure S7. Phenyl 2-acetamido-2-deoxy-α-D-galactopyranoside (600 MHz, D_2O).



References:

1. U. Jacquemard, V. Beneteau, M. Lefoix, S. Routier, J. Y. Merour and G. Coudert, *Tetrahedron*, 2004, **60**, 10039.
2. Q. Y. P. Liu, G. Sulzenbacher, H. P. Yuan, E. P. Bennett, G. Pietz, K. Saunders, J. Spence, E. Nudelman, S. B. Levery, T. White, J. M. Neveu, W. S. Lane, Y. Bourne, M. L. Olsson, B. Henrissat and H. Clausen, *Nat. Biotechnol.*, 2007, **25**, 454.