Selective fluorogenic β-glucocerebrosidase substrates for convenient analysis of GCase activity in cell and tissue homogenates

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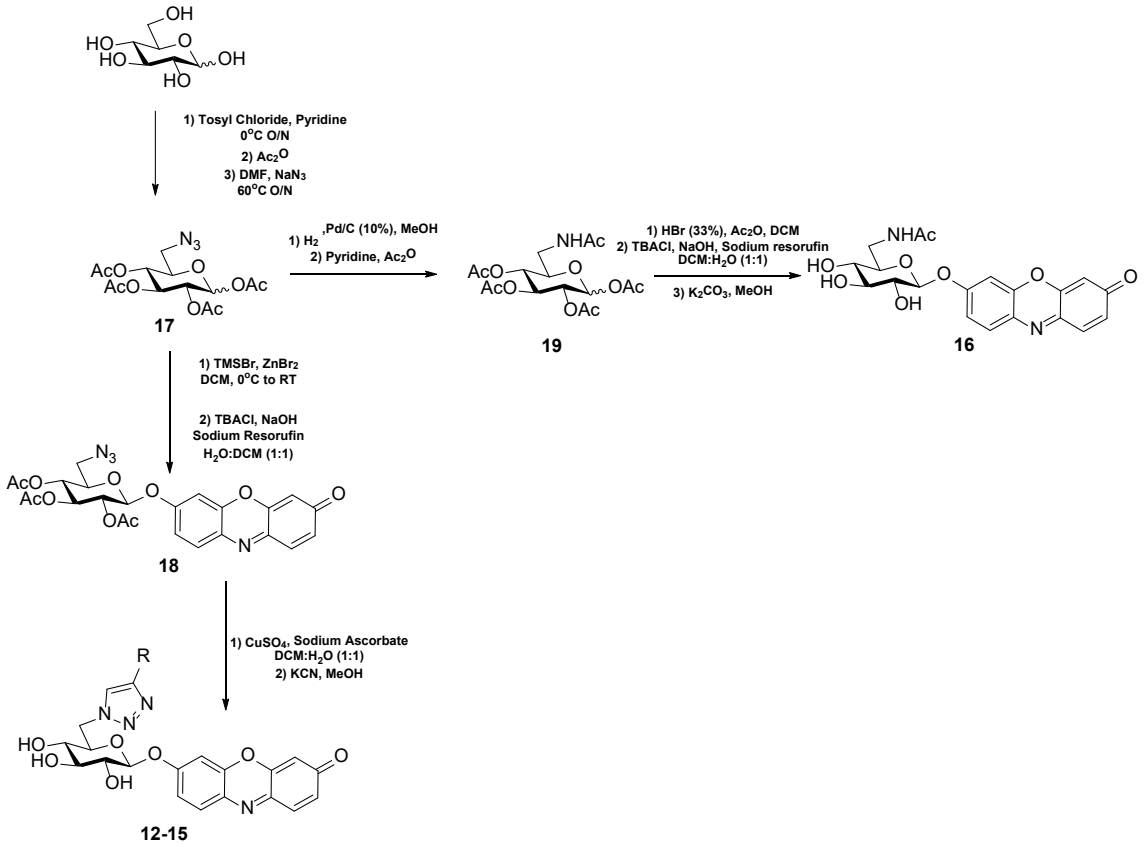
Supplementary information content list:

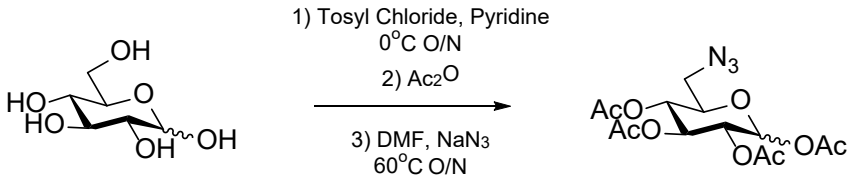


Scheme S1. The synthetic route used to access C-6 triazole and C-6 acetamido Resorufin-β-D-Glucopyranosides substrates

General Information: All solvents, reagents, and buffer salts were used as purchased from Sigma-Aldrich, Carbosynth, or Alfa Aesar. Merck pre-coated silica gel plates using combinations of ethyl acetate/n-hexane (EtOAc/Hexanes), or methanol/dichloromethane (MeOH/DCM) solvent elution systems were used to monitor the progression of all reactions. TLCs were visualized by illuminating with a short-wave UV lamp (254 nM) and soaking in Seebach stain (2.5% w/v Phosphomolybdic acid, 1% Cerium sulfate w/v, in 1 M H_2SO_4) and subsequently charred with a heat gun.

Flash chromatography was performed either under positive pressure with Fisher Scientific silica gel (230-400 mesh) or using RediSep© Gold normal phase columns (4 g, 24 g, or 40 g) on a Combiflash© RF+ instrument. HPLC purification was performed on an Agilent 1100 series instrument with an Eclipse XDB C18 column (5.0 μm, 9.4×250 mm) using HPLC grade solvents. High resolution mass spectrometry (HRMS) analysis was performed using a Bruker maXis TOF LC/MS/MS instrument using positive or negative electrospray ionization (ESI). ^{1}H NMR and ^{13}C NMR spectra were recorded using either a Bruker Avance 500 (500 MHz/126 MHz), Bruker Avance 400 (400 MHz/101 MHz), or a Bruker Avance II 600 (601 MHz/151 MHz). Chemical shifts (ppm) are reported relative to deuterated solvents (Cambridge Isotope Laboratories Inc.) residual peaks. Abbreviations used to describe the observed peaks: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet and bs, broad singlet.

1,2,3,4-Tetra-O-acetyl-6-azido-6-deoxy-D-glucopyranose (17)



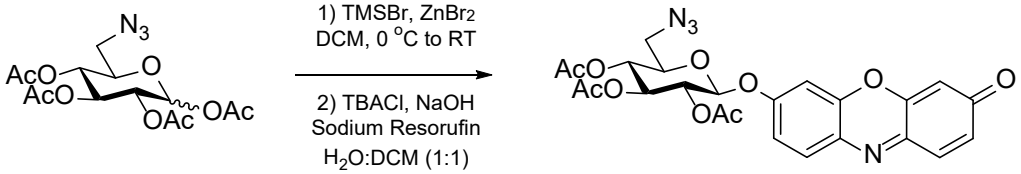
D-Glucose (5.00 g, 27.8 mmol) was suspended in anhydrous pyridine (90 mL) and cooled to 0°C using an ice bath. Tosyl chloride (5.83 g, 30.6 mmol) was added in a portion wise fashion. After stirring overnight at 0°C acetic anhydride (15.8 mL, 166.8 mmol) was added dropwise. After completing the addition, the reaction mixture was allowed to warm to room temperature and stirred for another 4 hours. Once the reaction was complete as judged by TLC the solvent was removed *in vacuo.* The resulting residue was dissolved in 250 mL dichloromethane, which was subsequently washed with water (200 mL), $NaHCO_3$ (200 mL), and brine (200 mL). The organic layer was collected, dried over sodium sulfate, filtered and the filtrate concentrated *in vacuo*. Under an atmosphere of argon, the crude mixture was dissolved in anhydrous DMF (90 mL). Sodium azide (5.42 g, 83.4 mmol) was added in one portion, after which the reaction was heated to 60°C for 16 hours. After cooling to room temperature, the reaction mixture was diluted with water (75 mL) and subsequently extracted with ethyl acetate (3 x 100 mL). The combined organic layers were dried over sodium sulfate, filtered, and the filtrate concentrated *in vacuo*. Pure compound (4.12 g, 40% yield) was isolated as a white solid following silica gel flash column chromatography (15:85), Rf: 0.3 (EtOAc:Hex (15:85)) (α:β 3:1)

^{1}H NMR (500 MHz, Chloroform-*d*) δ 6.37 (d, *J* = 3.7 Hz, 1H, αH-1), 5.74 (d, *J* = 8.3 Hz, 1H, βH-1), 5.48 (t, *J* = 10.3, 9.3 Hz, 1H, αH-3), 5.26 (t, *J* = 9.4 Hz, 1H, βH-3), 5.18 – 5.05 (m, 4H, αH-2, αH-4, βH-2, βH-4), 4.09 (ddd, *J* = 10.1, 5.4, 2.7 Hz, 1H, αH-5), 3.82 (ddd, *J* = 9.9, 5.3, 3.3 Hz, 1H, βH-5), 3.45 – 3.28 (m, 4H,αH-6,βH-6), 2.20– 2.00 (m, 12H, 4xαAc-CH_3, 4xβAc-CH_3).

^{13}C NMR (126 MHz, $CDCl_3$) δ 170.22-168.68 (4 x α-CH_3*C*=O, 4 x β-CH_3*C*=O) 91.5 (β-C1), 88.9 (α-C1), 73.8 (β-C5), 72.7(β-C3), 70.9 (α-C5), 70.1 (β-C2), 69.7 (α-C3), 69.1 (α-C2), 69.0 (β-C4), 68.9 (β-C4), 50.7 (α-C6), 50.6 (β-C6), 20.86-20.44 (4 x α-*C*H_3C=O, 4 x β-*C*H_3C=O)

HRMS: $[M+NH_4]^+$ Calc'd for $C_{14}H_{23}N_4O_9$ 391.1462, found 391.1460.

Resorufin 2,3,4-tri-O-acetyl-6-azido-6-deoxy-β-D-glucopyranoside (18)



Compound **17** was dissolved in anhydrous dichloromethane (30 mL) under an argon atmosphere. The solution was cooled to 0°C in an ice bath after which $ZnBr_2$ (1.58 g, 5.73 mmol) was added in a portion wise fashion. Trimethylsilyl bromide (756 µL, 5.73 mmol) was added dropwise to the solution. The reaction mixture was stirred at room temperature for 2 hours after which it was diluted with dichloromethane (100 mL). The organic layer was collected and subsequently washed with cold sat. $NaHCO_3$ (200 mL) and water (200 mL). The organic layer was then collected, dried over sodium sulfate, filtered and the filtrate

was concentrated *in vacuo* yielding crude 2,3,4-tri-O-acetyl-6-azido-6-deoxy-β-D-glucopyranosyl bromide (1.10 g) which was used directly in the next reaction.

The crude glucopyranosyl bromide was dissolved in a mixture of dichloromethane (20 mL) and water (20 mL). After addition of *t*-butyl ammonium chloride (913 mg, 3.35 mmol), sodium hydroxide (112 mg, 2.79 mmol), and sodium resorufin salt (787 mg, 3.35 mmol), the reaction mixture was stirred vigorously overnight. The resulting mixture was diluted with ethyl acetate (200 mL) and washed with sat. $NaHCO_3$ (150 mL) and water (150 mL). The resulting organic layer was collected, dried over sodium sulfate, filtered, and the filtrate was concentrated *in vacuo*. The crude mixture was purified using silica gel flash column chromatography using a gradient of EtOAc:DCM (20:80→30:70) yielding the pure product as an orange solid (431.1 mg, 29%), Rf: 0.6 (EtOAc:DCM (1:1)).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.33 (d, *J* = 8.7 Hz, 1H), 9.05 (d, *J* = 9.7 Hz, 1H), 8.69 – 8.60 (m, 2H), 8.40 (dd, *J* = 9.8, 2.1 Hz, 1H), 7.88 (d, *J* = 2.0 Hz, 1H), 7.03 (dt, *J* = 7.7, 2.0 Hz, 1H), 6.98 – 6.78 (m, 2H), 6.67 (ddd, *J* = 9.4, 8.4, 1.6 Hz, 1H), 6.20 (s, 9H), 5.62 (dddd, *J* = 15.5, 7.3, 3.9, 1.6 Hz, 1H), 5.00 (dt, *J* = 6.5, 2.0 Hz, 2H), 3.69 – 3.50 (m, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 190.95, 174.34, 173.83, 173.64, 164.09, 154.07, 149.18, 139.07, 137.98, 135.67, 133.56, 119.00, 110.25, 107.32, 101.57, 77.30, 76.45, 74.97, 73.07, 64.43, 54.79, 23.94, 23.90 X 2.

$[M+Na]^+$: Calc'd for $C_{24}H_{22}N_4NaO_{10}$ 549.1230, found 549.1228

General click reaction and deprotection procedure for the synthesis of resorufin 6-deoxy-6(1H-1,2,3-triazol-1-yl)-β-D-glucopyranosides

Resorufin 2,3,4-tri-O-acetyl-6-azido-6-deoxy-β-D-glucopyranoside (**18**) (1.0 equiv.) and the desired alkyne (1.2 equiv.) were dissolved in a mixture of solvents (2:1:1 - tetrahydrofuran: *t*-butanol: H_2O). The reaction flask was purged with argon. Sodium ascorbate (0.6 equiv.) and anhydrous $CuSO_4$ (0.3 equiv.) were added to the reaction mixture, which was then stirred vigorously until the reaction was complete as judged by TLC. The reaction mixture was diluted with dichloromethane and washed with sat. $NaHCO_3$ and water. The organic layer was then dried over sodium sulfate and concentrated *in vacuo*. The crude material was then used directly in the subsequent deprotection reaction.

The crude triazole product was dissolved in methanol (Concentration = 0.1 M) at room temperature. Potassium cyanide (0.5 equiv.) was added in one batch and the reaction mixture was stirred until the reaction was complete as judged by TLC. The reaction mixture was then diluted with dichloromethane and filtered through a pad of celite. The filtrate was concentrated and the resulting amorphous solid was washed with either MeOH or a mixture of MeOH:DCM three times to yield pure product.

Resorufin 6-deoxy-6-(4-(hydroxymethyl)-1H-1,2,3-triazol-1-yl)-β-D-glucopyranoside (12)

The product was synthesized according to the general method. The resulting amorphous solid was washed with methanol to yield **12** as an orange film (4.22 mg, 77% yield)

^{1}H NMR (601 MHz, DMSO-d_6) δ 7.74 – 7.67 (m, 2H), 7.52 (d, *J* = 9.8 Hz, 1H), 6.87 (dd, *J* = 8.8, 2.6 Hz, 1H), 6.84 – 6.75 (m, 2H), 6.24 (d, *J* = 2.1 Hz, 1H), 5.07 (d, *J* = 7.6 Hz, 1H), 4.79 (dd, *J* = 14.4, 2.3 Hz, 1H), 4.47 (d, *J* = 12.9 Hz, 1H), 4.43 – 4.35 (m, 2H), 3.92 (td, *J* = 9.4, 2.3 Hz, 1H), 3.39 – 3.29 (m, 2H), 3.19 – 3.14 (m, 2H).

^{13}C NMR (151 MHz, DMSO) δ 185.88, 160.80, 150.12, 146.30, 145.28, 135.31, 134.31, 131.50, 129.00, 123.68, 115.10, 106.00, 102.86, 99.88, 76.16, 74.96, 73.25, 71.34, 55.31, 51.11.

$[M+H]^+$: Calc'd for $C_{22}H_{21}N_4O_8$ 457.1354, found 457.1365

Resorufin 6-deoxy-6-(4-(phenyl)-1H-1,2,3-triazol-1-yl)-β-D-glucopyranoside (13)

The product was synthesized according to the general method. The resulting amorphous solid was washed with methanol to yield **13** as an orange film (6.34 mg, 66% yield).

^{1}H NMR (601 MHz, DMSO-d_6) δ 8.09 (s, 1H), 7.64 (d, *J* = 8.8 Hz, 1H), 7.50 – 7.45 (m, 2H), 7.41 (d, *J* = 9.8 Hz, 1H), 7.09 (dd, *J* = 8.2, 7.0 Hz, 2H), 7.04 – 6.93 (m, 2H), 6.72 (dd, *J* = 9.8, 2.1 Hz, 1H), 6.66 (d, *J* = 2.6 Hz, 1H), 5.64 (d, *J* = 5.0 Hz, 12H), 5.63 (d, *J* = 2.1 Hz, 1H), 5.59 (s, 1H), 5.40 – 5.36 (m, 1H), 5.21 – 5.11 (m, 1H), 4.89 (dd, *J* = 14.3, 2.3 Hz, 1H), 4.35 (dd, *J* = 14.4, 10.0 Hz, 1H), 3.94 (td, *J* = 9.8, 2.3 Hz, 1H), 3.41 – 3.33 (m, 2H), 3.23 (dq, *J* = 9.2, 4.0 Hz, 1H).

^{13}C NMR (151 MHz, DMSO) δ 185.09, 159.96, 148.68, 145.96, 145.64, 144.46, 134.68, 133.73, 130.85, 130.45, 128.55, 128.19, 127.24, 124.32, 122.36, 115.21, 105.50, 101.81, 98.52, 75.99, 74.48, 72.85, 71.42, 51.16

$[M+Na]^+$: Calc'd for $C_{26}H_{22}N_4NaO_7$ 525.1362, found 525.1281

Resorufin 6-deoxy-6-(4-(1-hydroxycyclohexyl)-1H-1,2,3-triazol-1-yl)-β-D-glucopyranoside (14)

The product was synthesized according to the general method. The resulting amorphous solid was washed with methanol to yield **14** as an orange film (2.3 mg, 88% yield).

^{1}H NMR (601 MHz, DMSO-d_6) δ 7.72 (d, *J* = 8.7 Hz, 1H), 7.56 (s, 1H), 7.54 (d, *J* = 9.8 Hz, 1H), 7.02 – 6.86 (m, 2H), 6.81 (dd, *J* = 9.8, 2.1 Hz, 1H), 6.22 (d, *J* = 2.0 Hz, 1H), 5.58 (d, *J* = 25.7 Hz, 2H), 5.17 (d, *J* = 7.6 Hz, 1H), 4.76 (dd, *J* = 14.5, 2.3 Hz, 1H), 4.39 – 4.23 (m, 1H), 3.99 (td, *J* = 9.5, 2.3 Hz, 1H), 3.36 (t, *J* = 8.9 Hz, 1H), 3.16 (t, *J* = 12.7 Hz, 1H), 1.88 – 0.50 (m, 10H).

^{13}C NMR (151 MHz, DMSO) δ 185.30, 160.53, 149.52, 145.77, 144.88, 134.91, 133.96, 131.23, 128.47, 121.60, 114.83, 105.82, 102.07, 99.16, 75.75, 74.43, 72.79, 71.24, 67.76, 50.81, 37.86, 37.37, 25.13, 21.45, 21.37.

$[M+H]^+$: Calc'd for $C_{26}H_{29}N_4O_8$ 525.1980, found 457.1999

Resorufin 6-deoxy-6-(4-(morpholinomethyl)-1H-1,2,3-triazol-1-yl)-β-D-glucopyranoside (15)

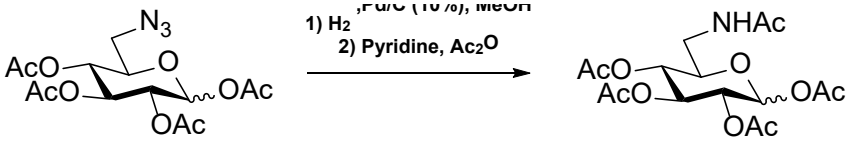
The product was synthesized according to the general method. The resulting amorphous solid was washed with 8:2 DCM:MeOH to yield to **15** as an orange film (2.8 mg, 44% yield).

^{1}H NMR (601 MHz, DMSO-d_6) δ 7.72 (d, *J* = 8.9 Hz, 2H), 7.55 (d, *J* = 9.8 Hz, 1H), 6.89 (dd, *J* = 8.9, 2.6 Hz, 1H), 6.84 – 6.79 (m, 2H), 6.25 (d, *J* = 2.1 Hz, 1H), 5.12 (d, *J* = 7.6 Hz, 1H), 4.77 (dd, *J* = 14.4, 2.4 Hz, 1H), 4.38 (dd, *J* = 14.4, 9.3 Hz, 1H), 3.97 (td, *J* = 9.5, 2.4 Hz, 1H), 3.50 – 3.41 (m, 1H), 3.43 – 3.33 (m, 5H), 3.15 (t, *J* = 9.2 Hz, 1H), 2.24 – 2.19 (m, 4H).

^{13}C NMR (151 MHz, DMSO) δ 185.31, 172.06, 160.42, 149.62, 145.86, 144.78, 142.70, 134.97, 134.01, 131.08, 128.49, 124.60, 114.69, 105.72, 102.39, 99.31, 75.88, 74.38, 72.88, 71.18, 65.96, 52.95, 52.71, 50.79.

$[M+H]^+$: Calc'd for $C_{25}H_{28}N_5O_8$ 526.1927, found 526.1932

6-Acetamido-1,2,3,4-tetra-O-acetyl-6-deoxy-D-glucopyranose (19)



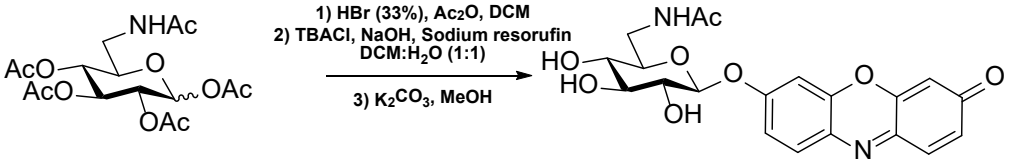
Compound **17** (758 mg, 2.68 mmol) was suspended in methanol (30 mL), the reaction flask was then flushed thoroughly with argon. In one portion Pd/C (10 wt%) (50.0 mg) was added to the suspension. The reaction flask was flushed with H_2 and stirred for 16 hours. After the reaction was complete, as judged by TLC, the reaction mixture was concentrated, and the resulting syrup was suspended in pyridine (15 mL). After cooling to 0°C, acetic anhydride was added to the solution in a dropwise fashion. The reaction mixture was then warmed to RT and stirred for 4 hours, after which it was concentrated to dryness by co-evaporation with toluene. The crude material was purified using silica gel flash column chromatography EtOAc:Hex (35→7:3)→ DCM:MeOH (10:1). The pure product (473 mg, 45% yield) was isolated as a white amorphous solid.

^{1}H NMR (600 MHz, Chloroform-*d*) δ 6.29 (d, *J* = 3.7 Hz, 1H), 5.82 (t, *J* = 6.1 Hz, 1H), 5.78 (t, *J* = 6.1 Hz, 0H), 5.67 (d, *J* = 8.3, 0.9 Hz, 0H), 5.46 (t, *J* = 9.4, 8.6 Hz, 1H), 5.24 (t, *J* = 9.4 Hz, 0H), 5.15 – 5.09 (m, 0H), 5.05 (dd, *J* = 10.3, 3.7 Hz, 1H), 4.99 – 4.90 (m, 1H), 4.06 – 3.95 (m, 1H), 3.76 – 3.67 (m, 0H), 3.60 (ddd, *J* = 14.5, 6.2, 2.6 Hz, 0H), 3.54 (ddd, *J* = 14.6, 6.0, 2.7 Hz, 1H), 3.46 – 3.33 (m, 1H), 2.18 (d, *J* = 0.9 Hz, 3H), 2.13 (d, *J* = 0.8 Hz, 1H), 2.08 (d, *J* = 0.9 Hz, 3H), 2.07 (d, *J* = 0.8 Hz, 1H), 2.04 (d, *J* = 0.9 Hz, 1H), 2.03 (d, *J* = 0.9 Hz, 6H), 2.01 (d, *J* = 0.9 Hz, 1H), 1.99 (d, *J* = 1.0 Hz, 4H).

^{13}C NMR (151 MHz, $CDCl_3$) δ 170.30, 170.26, 170.23, 170.17, 169.88, 169.81, 169.39, 169.10, 169.08, 92.03, 89.11, 73.74, 72.82, 70.70, 70.43, 69.82, 69.49, 68.83, 68.68, 39.19, 39.00, 23.31, 21.01, 20.93, 20.79, 20.77, 20.69, 20.68, 20.58.

HRMS: $[M+H]^+$ Calc'd for $C_{16}H_{24}NO_{10}$ 390.1398, found 390.1395

Resorufin 6-acetamido-6-deoxy-β-D-glucopyranoside (16)



Compound **19** (94.2 mg, 0.24 mmol) was dissolved in DCM (2.0 mL) and the resulting solution was cooled to 0°C in an ice bath. Addition of acetic anhydride (0.1 mL) to the reaction solution was followed by addition of 33% HBr in acetic acid (1.0 mL) in a dropwise fashion. The solution was stirred at 0°C for 15 minutes after which it was removed from the ice bath and stirred at room temperature for 3 hours. After the reaction was complete, as judged by TLC, the mixture was poured into ice water and subsequently diluted with DCM (10 mL). The organic layer was collected and washed with $NaHCO_3$ (10 mL), H_2O (10 mL), and brine (10 mL). The organic layer was then collected, dried over sodium sulphate, filtered and the filtrate was concentrated *in vacuo* to yield the crude 6-acetamido-6-deoxy-glucopyranosyl bromide

The resultant crude bromide was dissolved in DCM (2.0 mL) and H_2O (1.0 mL). Resorufin sodium salt (61.8 mg, 0.29 mmol), *t*-butyl ammonium chloride (80.6 mg, 0.29 mmol), and sodium hydroxide (12 mg, 0.29 mmol) were added to the solution which was subsequently stirred vigorously for 3 hours. The reaction mixture was then diluted with ethyl acetate (30 mL). The organic layer was collected and washed with $NaHCO_3$ (10 mL), H_2O (10 mL), and brine (10 mL). The organic layer was collected, dried over sodium sulfate, filtered and the filtrate was concentrated *in vacuo*. The product was enriched using silica gel flash column chromatography EtOAc:DCM (1:1 → 8:2).

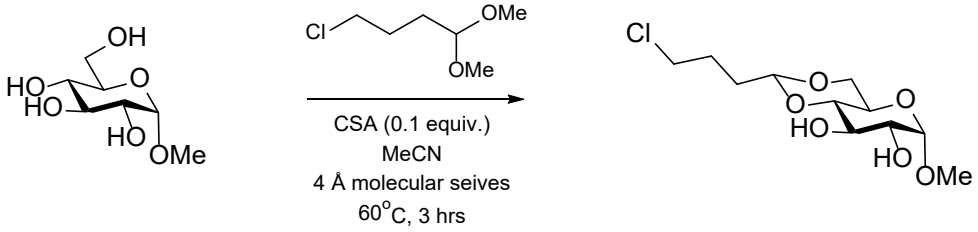
The crude tri-O-acetylated glycoside was dissolved in MeOH (1.5 mL). Catalytic amounts of K_2CO_3 (~1 mg) were added to the reaction solution which was then stirred for 2 hours at room temperature, after which it was diluted with dichloromethane (10 mL) and filtered through a thin pad of celite. The filtrate was concentrated, and the crude material was suspended in MeOH (3mL) and then centrifuged in a 15 mL falcon tube at 3000 rpm. The supernatant was removed, and this wash procedure was repeated 2 more times to yield the final pure product as an orange solid (6.1 mg, 20% yield)

^{1}H NMR (600 MHz, Methanol-d_4) δ 7.83 (d, *J* = 8.8 Hz, 1H), 7.58 (d, *J* = 9.8 Hz, 1H), 7.22 – 7.15 (m, 2H), 6.91 – 6.86 (m, 1H), 6.34 (dd, *J* = 2.1, 0.7 Hz, 1H), 5.13 – 5.08 (m, 1H), 3.68 (dd, *J* = 14.2, 2.8 Hz, 1H), 3.63 (ddd, *J* = 9.8, 7.0, 2.7 Hz, 1H), 3.57 – 3.49 (m, 2H), 3.45 (dd, *J* = 14.2, 7.2 Hz, 1H), 3.33 (p, *J* = 1.7 Hz, 21H), 3.30 – 3.24 (m, 1H), 2.01 (d, *J* = 0.7 Hz, 3H).

^{13}C NMR (151 MHz, MeOD) δ 188.38, 173.88, 162.94, 151.93, 147.19, 146.77, 136.59, 134.91, 132.70, 130.64, 116.58, 106.85, 104.20, 101.73, 77.39, 76.25, 74.77, 72.78, 41.60, 22.52.

HRMS: $[M+H]^+$ Calc'd for $C_{20}H_{21}N_2O_8$ 417.1292, found 517.1292

Methyl 4,6-O-(4-chlorobutylidene)-α-D-glucopyranoside (1)



Methyl α-D-glucopyranoside (25.0 g, 128 mmol) was suspended in anhydrous acetonitrile (500 mL) under an argon atmosphere. Camphorsulfonic acid (2.97 g, 12.8 mmol) and four angstrom molecular sieves (5.0 g) were added to the mixture the flask which was then flushed with argon. Using a dropping funnel 4-

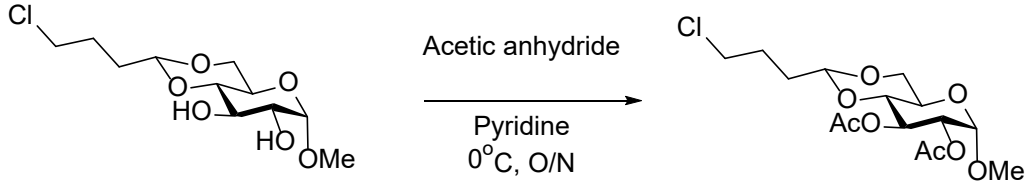
chlorobutyraldehyde dimethyl acetal (18.8 mL, 128 mmol) was added to the suspension which was subsequently heated to 60°C for 3 hours. Upon cooling to room temperature, the reaction was quenched with saturated $NaHCO_3$ (100 mL) and the acetonitrile was removed *in vacuo*. The resulting residue was dissolved in dichloromethane (500 mL) and washed with sat. $NaHCO_3$ (300 mL), water (300 mL), and brine (300 mL). The organic layer was dried over sodium sulfate and concentrated *in vacuo*. Pure **1** (25.2 g, 70% yield) was obtained as a white solid following silica column chromatography (DCM:MeOH (95:5)) Rf: 0.3 (DCM:MeOH (9:1))

^{1}H NMR (600 MHz, Chloroform-*d*) δ 4.78 (d, *J* = 3.9 Hz, 1H), 4.63 (t, *J* = 4.8 Hz, 1H), 4.14 (ddd, *J* = 10.3, 4.9, 0.9 Hz, 1H), 3.86 (td, *J* = 9.3, 1.9 Hz, 1H), 3.65 (td, *J* = 9.9, 4.9 Hz, 1H), 3.62 – 3.53 (m, 3H), 3.55 – 3.44 (m, 1H), 3.45 (s, 2H), 3.29 (dd, *J* = 9.8, 9.0 Hz, 1H), 2.69 (d, *J* = 2.1 Hz, 1H), 2.25 (d, *J* = 9.8 Hz, 1H), 2.00 – 1.87 (m, 2H), 1.89 – 1.79 (m, 1H).

^{13}C NMR (151 MHz, $CDCl_3$) δ 101.80, 99.80, 80.42, 77.37, 77.16, 76.95, 73.13, 72.03, 68.58, 62.56, 55.70, 44.93, 31.62, 27.22.

HRMS: $[M+Na]^+$ Calc'd for $C_{11}H_{19}ClNaO_6$ 305.0765, found 305.0762

Methyl 2,3-di-O-acetyl-4,6-O-(4-chlorobutylidene)-α-D-glucopyranoside (2)



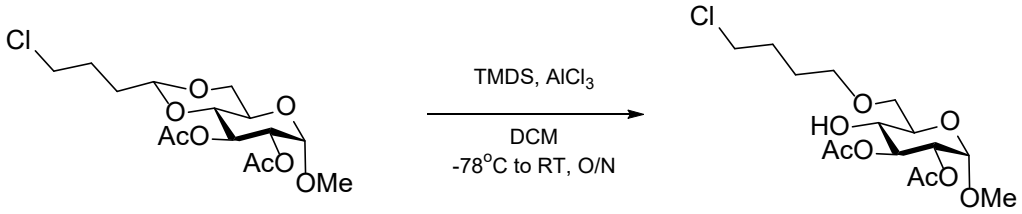
Compound **1** (6.5 g, 23 mmol) was dissolved in pyridine (22.2 mL, 270mmol) at room temperature. After cooling the resulting solution to 0°C in an ice bath, acetic anhydride (21.7 ml, 230 mmol) was added dropwise over 15 minutes. The ice bath was removed, and the solution was stirred at room temperature for 4 hours after which the crude mixture was co-concentrated with toluene (3 x 200 mL). The resulting syrup was dissolved in dichloromethane (250 mL) and washed with water (200 mL), sat. $NaHCO_3$ (200 mL), and brine (200 mL). The organic layer was collected, dried over sodium sulfate, filtered and the filtrate was concentrated *in vacuo*. The mixture was purified by silica gel flash column chromatography (EtOAc: Hex (2:8)) yield the product (16.47 g, quant.) as a clear viscous oil. Rf: 0.65 (EtOAc:Hex (35:65))

^{1}H NMR (600 MHz, Chloroform-*d*) δ 5.48 (td, *J* = 9.9, 0.8 Hz, 1H), 4.91 (d, *J* = 3.7 Hz, 1H), 4.86 (ddd, *J* = 9.9, 3.7, 0.9 Hz, 1H), 4.60 – 4.55 (m, 1H), 4.19 – 4.11 (m, 1H), 3.77 (td, *J* = 9.9, 4.9 Hz, 1H), 3.58 – 3.49 (m, 3H), 3.44 – 3.38 (m, 4H), 2.08 (td, *J* = 11.0, 0.8 Hz, 6H), 1.95 – 1.76 (m, 4H).

^{13}C NMR (151 MHz, $CDCl_3$) δ 169.87, 169.31, 101.33, 97.07, 78.40, 76.76, 76.55, 76.34, 71.15, 71.00, 68.46, 67.88, 61.83, 59.89, 54.86, 44.18, 30.83, 26.68, 20.55, 20.28, 20.26, 13.71.

HRMS: $[M+NH_4]^+$ Calc'd for $C_{15}H_{27}ClNO_8$ 384.1415, found 384.1420

Methyl 2,3,4-tri-O-acetyl-6-O-(4-chlorobutyl)-β-D-glucopyranoside (3)



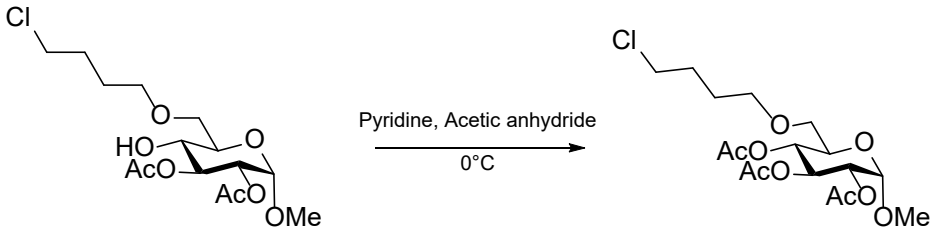
Compound **2** (2.06 g, 5.6 mmol) was dissolved in DCM (56 mL) under an argon atmosphere. After addition of $AlCl_3$ (1.12 g, 8.43 mmol) the solution was cooled to -78°C in a dry ice/acetone bath and 1,1,3,3 Tetramethyldisiloxane (993 µL, 5.62 mmol) was added in a dropwise fashion. After stirring at -78°C for 20 minutes the reaction mixture was warmed to room temperature and stirred overnight. The crude solution was diluted with dichloromethane (200 mL) and subsequently washed with 1 N HCl (150 mL) and water (150 mL). The organic layer was collected, dried over sodium sulfate, filtered and the filtrate was concentrated *in vacuo*. The mixture was purified by silica gel flash column chromatography (EtOAc: Hexanes (35:65 →1:1)) to yield product (1.36 g, 66%) as a viscous oil. Rf:0.5 (EtOAc:Hex (1:1))

^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.29 (dd, *J* = 10.1, 9.0 Hz, 1H), 4.90 (d, *J* = 3.7 Hz, 1H), 4.85 (dd, *J* = 10.1, 3.7 Hz, 1H), 3.85 – 3.65 (m, 4H), 3.65 – 3.47 (m, 4H), 3.40 (s, 3H), 2.10 (s, 3H), 2.08 (s, 3H), 1.91 – 1.81 (m, 2H), 1.80 – 1.69 (m, 2H).

^{13}C NMR (101 MHz, Chloroform-*d*) δ 171.82, 170.44 , 96.90 , 73.39 , 71.14 , 70.82 , 70.56 , 70.22 , 70.11 , 55.34 , 44.96 , 29.44 , 27.01 , 21.01 , 20.87 .

HRMS: $[M+Na]^+$ Calc'd for $C_{15}H_{25}ClNaO_8$ 391.1129, found 391.1130

Methyl 2,3,4-tri-O-acetyl-6-O-(4-chlorobutyl)-β-D-glucopyranoside (4)



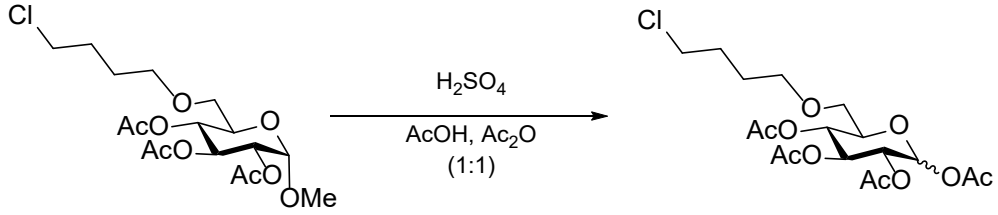
Compound **3** (2.59 g, 7.03 mmol) was dissolved in pyridine (20 mL) and the resulting solution was cooled to 0°C in an ice bath. Acetic anhydride (6.0 mL) was added to the reaction in a dropwise fashion at 0°C. After the addition was complete the reaction mixture was warmed to room temperature and stirred overnight. After the reaction was complete, as judged by tlc, the solvent was removed by co-evaporation with toluene. The resulting syrup was dissolved in DCM (100 mL) and the resulting solution was washed with sat. $NaHCO_3$ (50 mL), water (50 mL), and brine (50 mL). The resulting organic layer was collected, dried over sodium sulfate, filtered and the filtrate was concentrated *in vacuo.* Purification by silica flash column chromatography (EtOAc:Hexanes (2:8 → 35:65)) yielded **4** as a pure viscous syrup (2.68 g, 93% yield) Rf: 0.65 (EtOAc:Hex (1:1))

^{1}H NMR (600 MHz, Chloroform-*d*) δ 5.48 (dd, *J* = 10.3, 9.4 Hz, 1H), 5.09 (dd, *J* = 10.2, 9.3 Hz, 1H), 4.96 (d, *J* = 3.6 Hz, 1H), 4.90 (dd, *J* = 10.2, 3.7 Hz, 1H), 3.91 (ddd, *J* = 10.2, 4.9, 2.5 Hz, 1H), 3.65 – 3.38 (m, 10H), 2.08 (s, 10H), 2.04 (s, 3H), 2.02 (s, 3H), 1.93 – 1.80 (m, 2H), 1.80 – 1.70 (m, 2H).

^{13}C NMR (151 MHz, $CDCl_3$) δ 170.30, 170.28, 169.71, 96.74, 71.04, 70.96, 70.40, 69.25, 69.16, 68.39, 55.44, 45.11, 45.11, 29.34, 26.96, 20.88, 20.85.

HRMS: $[M+Na]^+$ Calc'd for $C_{17}H_{27}ClNaO_{10}$ 461.1195, found 461.1185

1,2,3,4-Tetra-O-acetyl-6-O-(4-chlorobutyl)-β-D-glucopyranose (5)



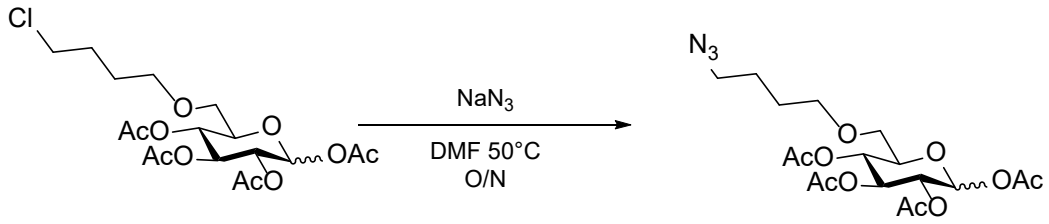
Compound **4** (2.68 g, 6.5 mmol) was dissolved in a mixture of AcOH: Ac_2O (1:1) (50 mL) and the resulting solution was cooled to 0^{o}C in an ice bath. H_2SO_4 (0.05 mL, 0.05 equiv.) was added to the reaction mixture and the reaction was stirred overnight at room temperature. After the reaction was complete, as judged by TLC, it was concentrated under reduced pressure. The resultant syrup was dissolved in DCM (200 mL) and the resulting solution was washed with water (100 mL), sat. $NaHCO_3$ (100 mL), and brine (100 mL). The organic layer was collected, dried over sodium sulfate, filtered and the filtrate was concentrated *in vacuo.* Purification by silica gel flash column chromatography (EtOAc:Hexanes (30:70)) yielded **5** as a white solid (2.38 g, 85% yield)

^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.34 (d, *J* = 3.6 Hz, 1H), 5.70 (d, *J* = 8.2 Hz, 0H), 5.53 – 5.43 (m, 1H), 5.25 (t, *J* = 9.4 Hz, 0H), 5.18 (dd, *J* = 10.2, 9.5 Hz, 1H), 5.14 (d, *J* = 8.3 Hz, 0H), 5.09 (dd, *J* = 10.3, 3.7 Hz, 1H), 4.04 (ddd, *J* = 10.3, 4.3, 2.7 Hz, 1H), 3.76 (ddd, *J* = 9.8, 4.6, 2.7 Hz, 0H), 3.66 – 3.44 (m, 6H), 3.42 (dt, *J* = 9.5, 6.3 Hz, 1H), 2.18 (s, 3H), 2.11 (s, 1H), 2.06 (s, 3H), 2.04 (s, 0H), 2.04 (s, 3H), 2.02 (s, 3H), 1.94 – 1.77 (m, 2H), 1.81 – 1.66 (m, 2H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.29, 170.17, 169.33, 169.24, 169.00, 168.84, 91.76, 89.14, 74.05, 73.04, 71.16, 71.00, 70.92, 70.36, 70.03, 69.30, 68.94, 68.62, 68.57, 44.93, 29.20, 26.81, 20.89, 20.83, 20.69, 20.66, 20.66, 20.60, 20.56, 20.45.

HRMS: $[M+Na]^+$ Calc'd for $C_{18}H_{27}ClNaO_{10}$ 461.1185, found 461.1190

1,2,3,4-Tetra-O-acetyl-6-O-(4-azidobutyl)-β-D-glucopyranose (6)



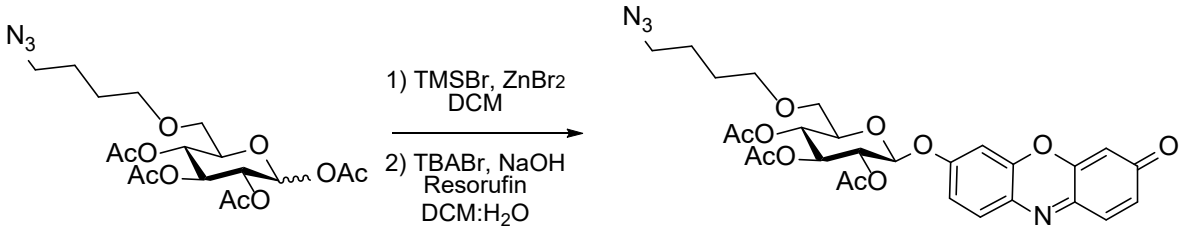
Compound **5** (990 mg, 2.26 mmol) was dissolved in dimethylformamide (6 mL) and sodium azide (440 mg, 6.77 mmol) was added in one batch to the resulting solution. The reaction mixture was warmed to 50°C and stirred for 16 hours, after which it was concentrated to dryness by co-evaporation with toluene. The resulting residue was diluted with $NaHCO_3$ (100 mL) and extracted with ethyl acetate (3 X 100 mL). The organic layers were collected, dried over sodium sulfate, filtered and the filtrate was concentrated *in vacuo.* The resulting product (**6**) was isolated as a white solid (868.3 mg, 86%) without purification.

^{1}H NMR (600 MHz, Chloroform-*d*) δ 6.33 (d, *J* = 3.7 Hz, 1H), 5.69 (d, *J* = 8.3 Hz, 0H), 5.47 (dd, *J* = 10.3, 9.5 Hz, 1H), 5.24 (t, *J* = 9.5 Hz, 0H), 5.18 (dd, *J* = 10.2, 9.5 Hz, 1H), 5.16 – 5.10 (m, 0H), 5.08 (dd, *J* = 10.3, 3.7 Hz, 1H), 4.03 (ddd, *J* = 10.2, 4.3, 2.6 Hz, 1H), 3.76 (ddd, *J* = 9.9, 4.6, 2.6 Hz, 0H), 3.61 – 3.34 (m, 5H), 3.34 – 3.23 (m, 3H), 2.18 (s, 3H), 2.11 (s, 1H), 2.04 (d, *J* = 7.6 Hz, 4H), 2.03 (s, 4H), 2.01 (d, *J* = 1.1 Hz, 3H), 1.70 – 1.62 (m, 4H).

^{13}C NMR (151 MHz, $CDCl_3$) δ 170.43, 169.77, 169.45, 168.98, 91.81, 89.20, 74.12, 73.09, 71.30, 71.22, 70.37, 70.06, 69.34, 68.98, 68.61, 68.55, 51.33, 51.32, 26.77, 26.76, 25.70, 25.69, 21.02, 20.95, 20.82, 20.78, 20.72, 20.69, 20.58.

HRMS: $[M+Na]^+$ Calc'd for $C_{18}H_{27}N_3NaO_{10}$ 468.1589, found 468.1605

Resorufin 2,3,4-tri-O-acetyl-6-O-(4-azidobutyl)-β-D-glucopyranoside (7)



Compound **6** (606 mg, 1.36 mmol) was dissolved in anhydrous dichloromethane (13.6 mL) under an atmosphere of argon and the resulting solution was cooled to 0°C in an ice bath. After Zinc Bromide (613 mg, 2.72 mmol) was added to the solution in one portion, trimethyl silyl bromide (359 µL, 2.72 mmol) was added in a slow dropwise fashion. The reaction mixture was stirred for 2 hours before subsequently warming slowly to RT and stirred for another 2 hours. After the reaction was complete, as judged by TLC, it was quenched by being poured into ice cold sat. $NaHCO_3$ (50 mL). The resulting bi-phasic mixture was diluted with dichloromethane (50 mL), and the organic layer was collected and subsequently washed with H_2O (50 mL) and brine (50 mL). The organic layer was collected, dried over sodium sulphate, filtered and the filtrate was concentrated *in vacuo.* The crude glucopyranosyl bromide material was carried forward without any purification

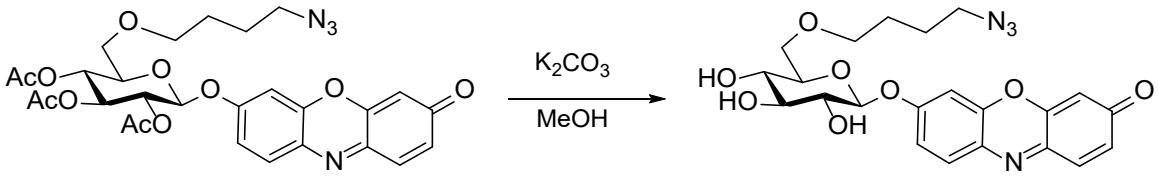
The crude glucopyranosyl bromide (634.1 mg, 1.36 mmol) was dissolved in dichloromethane (12.5 mL) and H_2O (12.5 mL). Resorufin sodium salt (384 mg, 1.63 mmol), sodium hydroxide (65.2 mg, 1.63 mmol) and *t*-butyl ammonium chloride (445 mg, 1.63 mmol) were added to the reaction mixture which was then stirred vigorously for 16 hours. After the reaction was complete, as judged by TLC, the mixture was diluted with ethyl acetate (75 mL) and H_2O (50 mL). The organic layer was collected and washed with sat. $NaHCO_3$ (50 mL) and brine (50 mL). The aqueous layers were collected, combined, and back extracted with dichloromethane (100 mL). The organic layers were collected, combined, dried over sodium sulphate, filtered and the filtrate was concentrated *in vacuo.* The final product (**7**) (198 mg, 24% yield) was purified by silica gel flash column chromatography (Hex:EtOAc 9:1 → 8:2).

^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.75 (d, *J* = 8.8 Hz, 1H), 7.45 (d, *J* = 9.8 Hz, 1H), 7.03 (dd, *J* = 8.8, 2.6 Hz, 1H), 6.98 (d, *J* = 2.6 Hz, 1H), 6.87 (dd, *J* = 9.8, 2.0 Hz, 1H), 6.35 (d, *J* = 2.0 Hz, 1H), 5.38 – 5.29 (m, 2H), 5.20 (d, *J* = 7.3 Hz, 1H), 5.16 (dd, *J* = 10.0, 8.9 Hz, 1H), 3.89 (ddd, *J* = 10.0, 6.3, 2.5 Hz, 1H), 3.62 (dd, *J* = 10.9, 2.5 Hz, 1H), 3.58 – 3.50 (m, 1H), 3.49 – 3.39 (m, 1H), 3.32 – 3.17 (m, 2H), 2.09 (d, *J* = 0.7 Hz, 6H), 2.07 (s, 3H), 1.72 – 1.56 (m, 4H).

^{13}C NMR (151 MHz, $CDCl_3$) δ 186.43, 170.38, 169.60, 169.40, 160.08, 149.70, 146.95, 145.28, 134.93, 134.81, 131.74, 129.66, 115.00, 107.08, 103.55, 98.43, 74.17, 72.78, 71.36, 71.12, 69.51, 68.79, 51.29, 26.88, 25.75, 20.84, 20.81, 20.79.

HRMS: $[M+Na]^+$ Calc'd for $C_{28}H_{30}N_4NaO_{11}$ 621.1804, found 621.1803

Resorufin 6-O-(4-azidobutyl)-β-D-glucopyranoside (11)



Compound **7** (15.4 mg, 0.026 mmol) was dissolved in methanol (2 mL). Potassium carbonate (1.7 mg, 0.013 mmol) was added to the resulting solution in one portion reaction and the reaction mixture was stirred vigorously for 3 hours. The reaction was quenched by addition of ammonium acetate (50 mg) and the solvent was removed under reduced pressure. The pure products were obtained via purification via silica gel flash column chromatography (95:5 → 9:1) to yield the product **11** as an orange solid (10.0 mg, 82% yield).

^{1}H NMR (600 MHz, Methanol-d_4) δ 7.82 (d, *J* = 9.6 Hz, 1H), 7.57 (d, *J* = 9.8 Hz, 1H), 7.30 – 7.09 (m, 2H), 6.88 (dd, *J* = 9.8, 2.1 Hz, 1H), 6.36 (d, *J* = 2.1 Hz, 1H), 5.21 – 5.03 (m, 1H), 3.86 (dd, *J* = 11.0, 1.9 Hz, 1H), 3.70 (dd, *J* = 7.2, 2.5 Hz, 1H), 3.64 – 3.48 (m, 5H), 3.41 – 3.35 (m, 1H), 3.27 (td, *J* = 5.6, 4.9, 3.1 Hz, 2H), 1.69 – 1.63 (m, 4H).

^{13}C NMR (151 MHz, Methanol-d_4) δ 188.39 , 163.00 , 151.94 , 147.09 , 146.78 , 136.58 , 134.86 , 132.72 , 130.64 , 116.66 , 106.86 , 104.15 , 101.58 , 77.85 , 77.26 , 74.61 , 71.99 , 71.53 , 71.26 , 52.29 , 27.96 , 26.89 .

HRMS: $[M+Na]^+$ Calc'd for $C_{22}H_{24}N_4NaO_8$ 495.1484, found 495.1486

General synthetic methods for the synthesis of compounds 8-10:

Compound **7** and the necessary alkyne was dissolved in a biphasic mixture of DCM:H_2O (1:1) or a mixture of *t*-butanol: tetrahydrofuran: H_2O (1:1:1), or *t*-butanol:H_2O(1:1). The reaction flask was purged with argon. Sodium ascorbate (0.6 equiv.) and anhydrous $CuSO_4$ (0.3 equiv.) were added to the reaction mixture, which was then stirred vigorously. After the reaction was complete, as judged by TLC, it was diluted with dichloromethane and the organic layer was collected and subsequently washed with minimal sat. $NaHCO_3$, H_2O, and brine. The organic layer was collected, dried over sodium sulphate, filtered and the filtrate was concentrated *in vacuo.* The crude tri-O-acetylated glycoside was then deprotected without further purification

The crude triazole product was dissolved in methanol (2 mL) and potassium carbonate (0.5 equiv.) was added to the solution all at once. The reaction was stirred vigorously for 3 hours. The reaction was quenched with ammonium acetate (6 equiv.) and the reaction mixture was subsequently concentrated under reduced pressure. The pure product was obtained as an orange solid via purification via silica gel flash column chromatography (DCM:MeOH).

Resorufin 6-O-(4-(1-hydroxymethyl)-1H-1,2,3-triazol-1-yl)butyl-β-D-glucopyranoside (8)

The product was synthesized according to the general method. Purification was performed a using a gradient of MeOH:DCM (90:10 → 85:15→ 8:2) to yield **8** as an orange film (12.0 mg, 57% yield)

^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.75 (s, 1H), 7.68 (d, *J* = 8.7 Hz, 1H), 7.48 (dd, *J* = 9.8, 1.2 Hz, 1H), 7.14 – 7.07 (m, 2H), 6.79 (dd, *J* = 9.7, 1.9 Hz, 1H), 6.23 (d, *J* = 1.9 Hz, 1H), 5.06 (dd, *J* = 5.5, 2.1 Hz, 1H), 4.55 (d, *J* = 10.0 Hz, 2H), 4.34 (t, *J* = 7.2 Hz, 2H), 3.78 (dd, *J* = 11.0, 1.9 Hz, 1H), 3.69 – 3.61 (m, 1H), 3.57 (dd, *J* = 10.9, 6.4 Hz, 1H), 3.55 – 3.51 (m, 1H), 3.50 – 3.41 (m, 3H), 3.38 – 3.32 (m, 1H), 2.11 – 1.80 (m, 3H), 1.75 – 1.45 (m, 2H).

^{13}C NMR (151 MHz, $CDCl_3$) δ 188.37, 162.93, 151.80, 147.06, 146.66, 136.56, 134.86, 134.85, 132.72, 132.70, 130.55, 116.48, 106.89, 106.87, 104.15, 101.61, 77.83, 77.22, 74.64, 71.86, 71.47, 71.22, 56.47, 51.15, 28.60, 27.47.

HRMS: $[M+Na]^+$ Calc'd for $C_{25}H_{28}N_4NaO_9$ 551.1748, found 551.1748

Resorufin 6-O-(4-(1-hydroxycyclohexyl)-1H-1,2,3-triazol-1-yl)butyl-β-D-glucopyranoside (9)

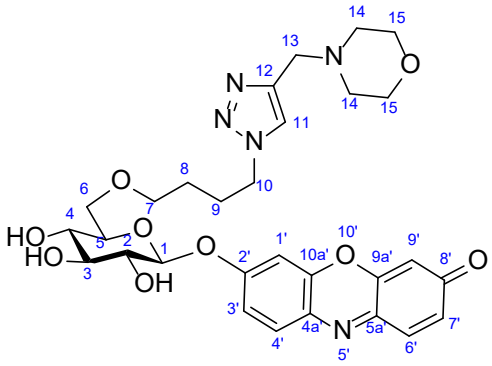
The product was synthesized according to the general method. Purification was performed a using a gradient of MeOH:DCM (95:5 → 90:10) to yield **9** as an orange film (6.7 mg, 34% yield)

^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.76 (d, *J* = 8.7 Hz, 1H), 7.74 (s, 1H), 7.54 (d, *J* = 9.8 Hz, 1H), 7.19 – 7.15 (m, 2H), 6.85 (dd, *J* = 9.8, 2.1 Hz, 1H), 6.30 (d, *J* = 2.1 Hz, 1H), 5.10 (d, 1H), 4.86 (s, 18H), 4.37 (t, *J* = 7.2 Hz, 2H), 3.82 (dd, *J* = 10.9, 2.0 Hz, 1H), 3.69 (ddd, *J* = 9.9, 6.4, 2.0 Hz, 1H), 3.65 – 3.53 (m, 2H), 3.54 – 3.47 (m, 3H), 3.43 – 3.35 (m, 1H), 3.31 (p, *J* = 1.6 Hz, 7H), 1.97 (dt, *J* = 14.0, 4.4 Hz, 2H), 1.83 – 1.69 (m, 4H), 1.63 – 1.55 (m, 3H), 1.51 – 1.44 (m, 2H), 1.40 – 1.33 (m, 1H).

^{13}C NMR (151 MHz, $CDCl_3$) δ 188.39, 162.99, 151.89, 147.12, 146.73, 136.60, 134.89, 134.85, 132.74, 130.62, 122.27, 116.51, 116.44, 106.90, 104.28, 104.21, 101.71, 77.84, 77.25, 74.66, 71.89, 71.45, 71.20, 70.20, 51.10, 38.85, 28.58, 27.52, 26.57, 23.06.

HRMS: $[M+Na]^+$ Calc'd for $C_{30}H_{36}N_4NaO_9$ 619.2374, found 619.2374

Resorufin 6-O-(4-(morpholinomethyl)-1H-1,2,3-triazol-1-yl)butyl β-D-glucopyranoside (10, Morsel-X)



The product was synthesized according to the general method. Purification was performed using a gradient of MeOH:DCM (90:10 → 85:15) to yield compound **10** (**Morsel-X**) as an orange film (4.8 mg, 24% yield).

^{1}H NMR (600 MHz, Methanol-d_4) δ 7.85 (s, 1H, H-11), 7.77 (d, *J* = 8.5 Hz, 1H, H-4'), 7.55 (d, *J* = 9.8 Hz, 1H, H-6'), 7.20 – 7.14 (m, 2H, H-1', H-3'), 6.86 (dd, *J* = 9.8, 2.0 Hz, 1H, H-7'), 6.31 (d, *J* = 2.1 Hz, 1H,H-9'), 5.22 – 5.07 (m, 1H, H-1), 4.41 (t, *J* = 7.2 Hz, 2H, H-10), 3.84 (dd, *J* = 10.9, 1.9 Hz, 1H, H-6^{a}), 3.73 – 3.56 (m, 9H, H-4, H-6^{b},H-7^{a}, H-13, H-15), 3.54 – 3.45 (m, 3H, H-2, H-3, H-7^{b}), 3.42 – 3.37 (m, 1H, H-5), 2.54 (s, 4H, H-14), 2.05 – 1.92 (m, 2H, H-8), 1.62 (dt, *J* = 7.9, 6.2 Hz, 2H, H-9).

^{13}C NMR (151 MHz, MeOD) δ 188.34 (C-5a'), 162.94 (C-2'), 151.82 (C-4a'), 147.10 (C-8'), 146.69 (C-9a'), 143.45 (C-12), 136.57 (C-6'), 134.87 (C-7'), 132.71 (C-4'), 130.58 (C-10a'), 125.50 (C-11), 116.53 (C-3'), 106.88 (C-9'), 104.12 (C-1'), 101.65 (C-1), 77.82 (C-3), 77.22 (C-5), 74.63 (C-2), 71.85 (C-7), 71.46 (C-4), 71.22 (C-6), 67.36 (C-15), 54.06 (C-14), 53.82 (C-13) 51.20 (C-10), 28.56 (C-8), 27.46 (C-9).

HRMS: $[M+Na]^+$ Calc'd for $C_{29}H_{35}N_5NaO_9$ 620.2322, found 620.2327

Recombinant GCase, GBA2, and GBA3 activity assays

Enzymatic activity of the recombinant human β-glucosidases, GCase (Purchased from R&D systems: 7410-GHB), GBA2, and GBA3 (Purchased from R&D systems: 5969-GH) were performed using conditions similar to those previously reported for GBA1[1]. Briefly, Michalis-Menten experiments and all enzyme and substrate dilutions were performed in McIlvaine buffer (150 mM, pH 5.2, 0.1% (v/v) Triton X-100 , and 0.25% (w/v) sodium deoxy-taurocholate with a fixed dimethyl sulfoxide (DMSO) concentration of 1% (v/v)). 100 µL of each of the five resorufin selective substrates at concentrations from 800 µM to 3.12 µM was added to 100 µL of GBA1, GBA2 or GBA3 yielding a 200 µL reaction volume containing final substrate concentrations ranging from 400 µM to 1.56 µM and final enzyme concentration of 1nM. Reaction solution was mixed with a pipette then aliquoted in 45 µL triplicates to a NUNC black 384 well plate. Fluorescence signal was measured continuously for 20 minutes at 25°C in a Spectramax i3X microplate reader set at excitation and emission wavelengths of 572 nm and 610 nm, respectively. Max reaction rates (in RFU s^{-1}) were calculated within Softmax Pro software and background fluorescence signal from controls containing each **s**ubstrate diluted to the same concentration in assay buffer were subtracted from each reaction rate. A resorufin standard curve (concentrations ranging from 2500 nM to 0.15 nM in the reaction buffer) was used to interpolate RFU values to quantitative reaction rate units of (µM s^{-1} nM^{-1}. The resulting michaelis-menten curves were fit using Graphpad Prism5 software.

Cell Lysate Activity Assays

Frozen cell pellet were resuspended in lysis buffer (*freshly* prepared 20 mg mL^{-1} EDTA-free complete Mini in M-PER) to $50x10^6$ c mL^{-1}. The suspension was cooled on ice and vortexed for 5 minutes, then sonicated with the microtip for 10 seconds at 10% amplitude. The total protein concentration was determined using the Nanodrop 2000 with lysis buffer as a blank control. The lysate was diluted to 20 mg mL^{-1} with the M-PER & protease inhibitor mixture.

Cell lysate activity assays were completed in McIlvaine buffer (150 mM, pH 5.2, 0.1 (v/v) Triton X-100, and 0.25% (w/v) sodium deoxy-taurocholate (1% (v/v) DMSO fixed across all assays). The fluorescence signal of a reaction mixture containing 0.25 mg mL^{-1} cell lysate, 100 µM of either resorufin-β-D-glucopyranoside or Morsel-X, and either 10 µM AT3375 or no inhibitor, was measured continuously for 20 minutes at 25°C in a Spectramax i3X microplate reader set at excitation and emission wavelengths of 572 nm and 610 nm, respectively. Max reaction rates in RFU s^{-1} were calculated within Softmax Pro software and background fluorescence signal from negative controls were subtracted from each reaction rate.

Brain tissue homogenate activity assays

Mouse brain homogenates were prepared by mechanical homogenization in 10X weight/volume of homogenization buffer (250 mM Sucrose, 10 mM Tris pH = 7.5, 1 mM EDTA, 0.25% (v/v) Triton X-100, and protease inhibitors), they were then sonicated for 15 seconds and incubated for 30 minutes at 4°C. The resultant Homogenate was centrifuged at 14,000 g for 1 hour at 4°C the supernatant was collected and used for all proceeding experiments. Protein concentration was determined using the Bio-Rad DC™ Protein Assay kit. Homogenates were kept on ice until they were used for the activity assays which were completed on the same day as homogenization.

Assays using resorufin-β-D-glucopyranoside and Morsel-X

In a 96 well mixing plate, 50 µL of 6 mg mL^{-1} brain homogenate was added to a 50 µL solution of AT3375 (20 µM), miglustat (20 µM), or both AT3375 and miglustat (20 µM of both) in McIlvaine buffer (150 mM, pH 5.4, containing 0.16% (w/v) sodium deoxy-taurocholate or no sodium deoxy-taurocholate). The inhibitor and homogenate mixtures were incubated at 25°C for 5 minutes. After incubation, 100 µL of the appropriate substrate (Res-Glc or Morsel-X: 200 µM), in the assay buffer, was added and the reactions were redistributed to a NUNC black 384 well plate in 45 µL triplicates. The Fluorescence signal was either measured continuously for 10 minutes at 25°C in a BioTek Neo 2 Plate reader set at excitation and emission wavelengths of 572 nm and 610 nm, respectively, or the reaction was stopped by addition of 30 µL of 1.0M Tris buffer (pH 9.0) and read as an endpoint with excitation and emission wavelengths of 572 nm and 610 nm, respectively. Background hydrolysis rates for Res-Glc were calculated based upon incubation of 100 µM substrate in either 6 mg mL^{-1} brain homogenate containing both AT3375 and miglustat (10 µM) or in lysis buffer. Background hydrolysis rates for Morsel-X were calculated based upon the median fluorescent signal of the WT samples that had been treated with AT3375 (10 µM) and miglustat (10 µM). The calculated background hydrolysis rates were subtracted from the raw values and the max reaction rates were calculated within Gen5 BioTek reader software and graphed using Graphpad prism 5 software. Measurement of GCase activity using Res-Glc was done by comparison of the uninhibited samples and those treated with the GCase selective inhibitor AT3375, the activity difference between these two sample groups was attributed to GCase.

Assays using 4-methylumbelliferyl β-D-glucopyranoside (4MU-Glc)

Assays using 4-methylumbelliferyl-β-D-glucopyranoside were done in a similar fashion to those with Res-Glc and Morsel-X. Briefly, in a 96 well mixing plate, 50 µL of 6 mg mL^{-1} brain homogenate was added to a 50 µL solution of AT3375 (20 µM), miglustat (20 µM), or both AT3375 and miglustat (20 µM of both) in McIlvaine buffer (150 mM, pH 5.4, containing 0.16% (w/v) sodium deoxy-taurocholate or no sodium deoxy-taurocholate). The inhibitor and homogenate mixtures were incubated at 25°C for 5 minutes. After incubation, 100 µL of 4-MU-Glc (2 mM), in the assay buffer with 1% DMSO, was added and the reactions were redistributed to a NUNC black 384 well plate in 45 µL triplicates. The plate was then incubated 15 minutes at 25°C after which the reaction was stopped by addition of 30 µL of 1.0M Tris buffer (pH 9.0) to each reaction well and the fluorescent signal was read as an endpoint with excitation and emission wavelengths of 360 nm and 460 nm, respectively. Background hydrolysis rates for 4-MU-Glc were measured by incubation of 1 mM 4-MU-Glc in a blank containing lysis buffer instead of 0.6 mg mL^{-1} brain homogenate. The background hydrolysis was used to normalize the raw values and the max reaction rates were calculated within Gen5 BioTek reader software and graphed using Graphpad prism 5 software. Measurement of GCase activity using 4-MU-Glc was done by comparison of the uninhibited samples and those treated with the GCase selective inhibitor AT3375, the activity difference between these two sample groups was attributed to GCase.

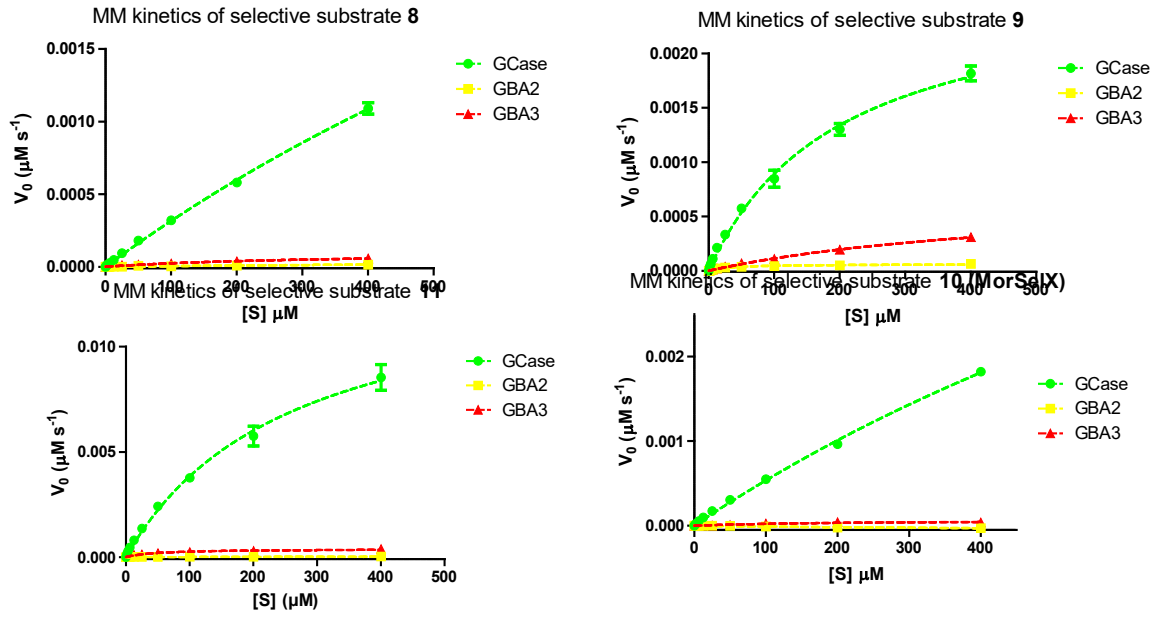
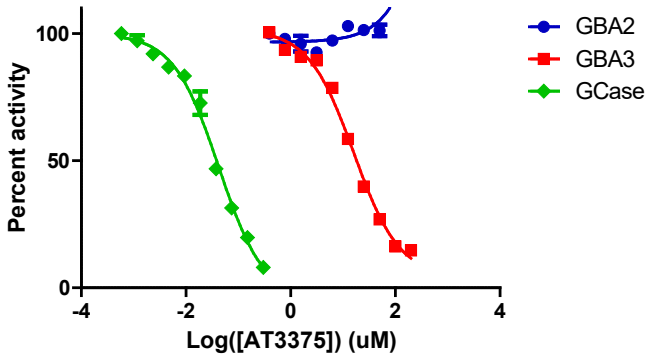


Figure S1. Michaelis-Menten plots of the O-6 alkylated derivatives of Res-Glc with the three functionally related human β-glucosidases (GCase, GBA2, and GBA3)



	GCASE	GBA2	GBA3
AT3375 IC_{50}	43 nM	n.d.	16 µM

Figure S2. IC_{50} curves of the AT3375 against the human β-glucosidases, GCase, GBA2, and GBA3 (n.d. = no detectable inhibition

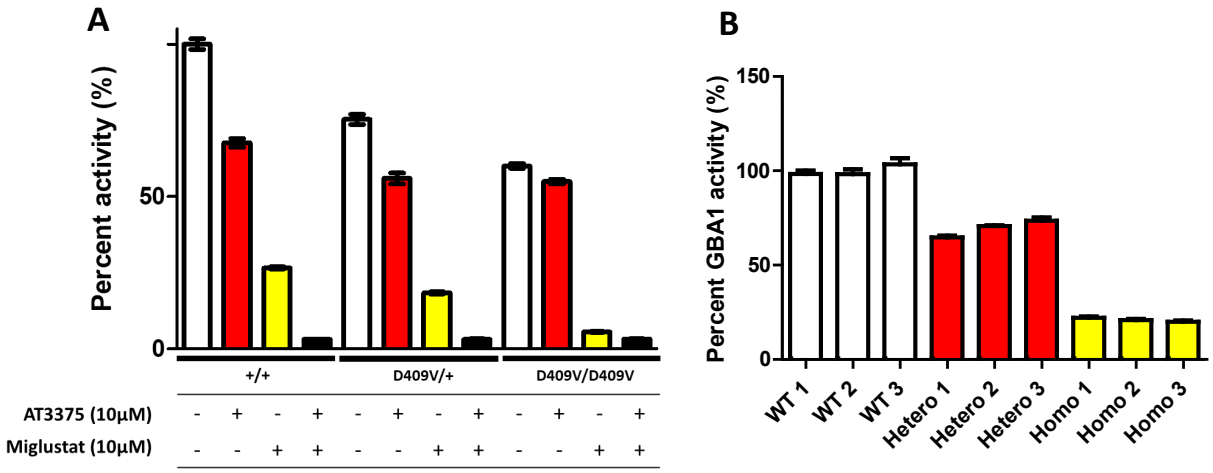


Figure S3. Determination of residual GCase activity in transgenic mice. A) Subtractive β-glucosidase activities assays of tissue homogenates from mice (+/+, D409V/+, D409V/D409V) using the non-selective substrate 4-methylumbelliferyl-β-D-glucopyranoside (Error bars represent SEM for the mean values obtained over three replicates of each of three independent biological mouse brain samples (n = 3 in each group). B) Calculated levels of residual GCase activity based upon the subtractive assay using AT3375

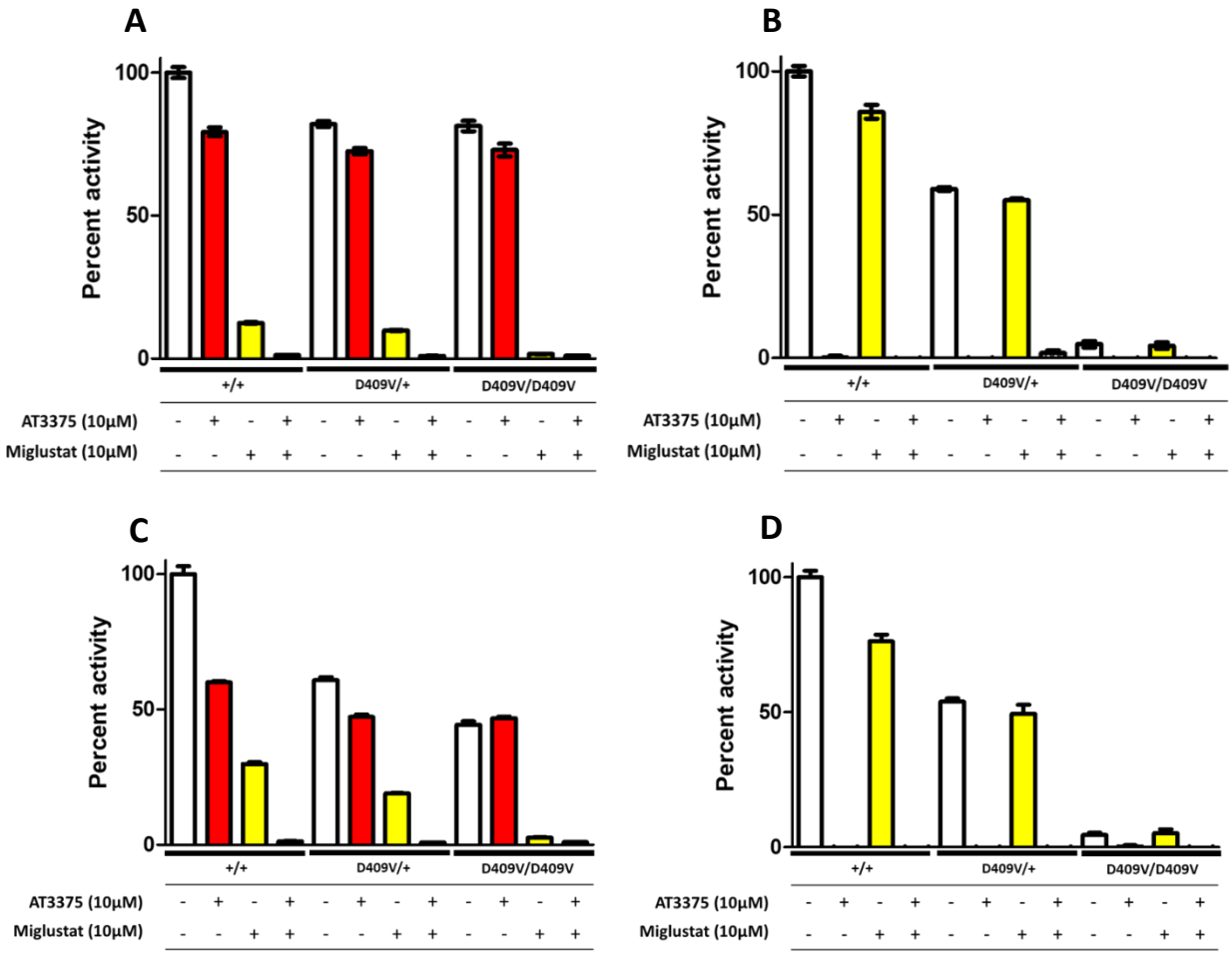


Figure S4. A comparison of results from subtractive β-glucosidase activities assays of tissue homogenates from mice (+/+, D409V/+, D409V/D409V (performed in triplicate, Error bars are SEM) with (Panel C,D) and without (panel A/B) sodium deoxy-taurocholate (0.16%) in the assay buffer. Miglustat and AT3375, selective inhibitors, were used to control for each of the human β-glucosidases (AT3375 for GCase, miglustat for GBA2, and a combination of both for GBA3. A) Measured β-glucosidase activity in tissue homogenates using Res-Glc (100 µM) in the absence of sodium deoxy-taurocholate. B) Measured β-glucosidase activity using Morsel-X (100 µM) in the absence of sodium deoxy-taurocholate. C) Measured β-glucosidase activity in tissue homogenates using Res-Glc (100 µM) in the presence of 0.16% sodium deoxy-taurocholate. D) Measured β-glucosidase activity using Morsel-X (100 µM) in the presence of 0.16% sodium deoxy-taurocholate

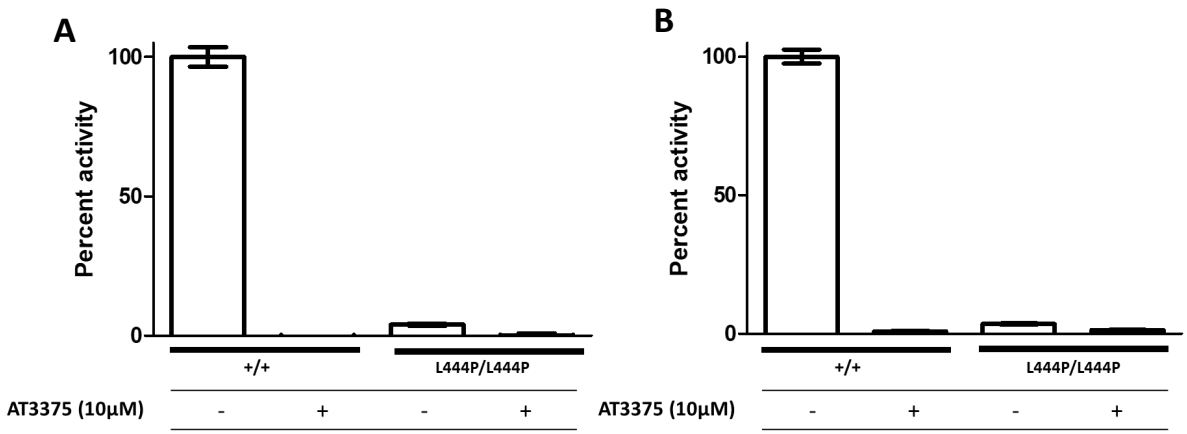


Figure S5. Measurement of residual GCase activity in fibroblast lysates from Gaucher's patient (Type II, L444P/L444P, GM01260). AT3375 (10 µM) was used to control for GBA2 activity. A) Measurement of β-glucosidase activity in WT fibroblasts and homozygous L444P/L444P mutant fibroblasts using Res-Glc in the assay buffer containing 0.25% sodium deoxy-taurocholate. AT3375 (10 µM) was used to control for GBA2 activity. B) Measurement of β-glucosidase activity in WT fibroblasts and homozygous L444P mutant fibroblasts using Morsel-X in the assay buffer containing 0.25% sodium deoxy-taurocholate.

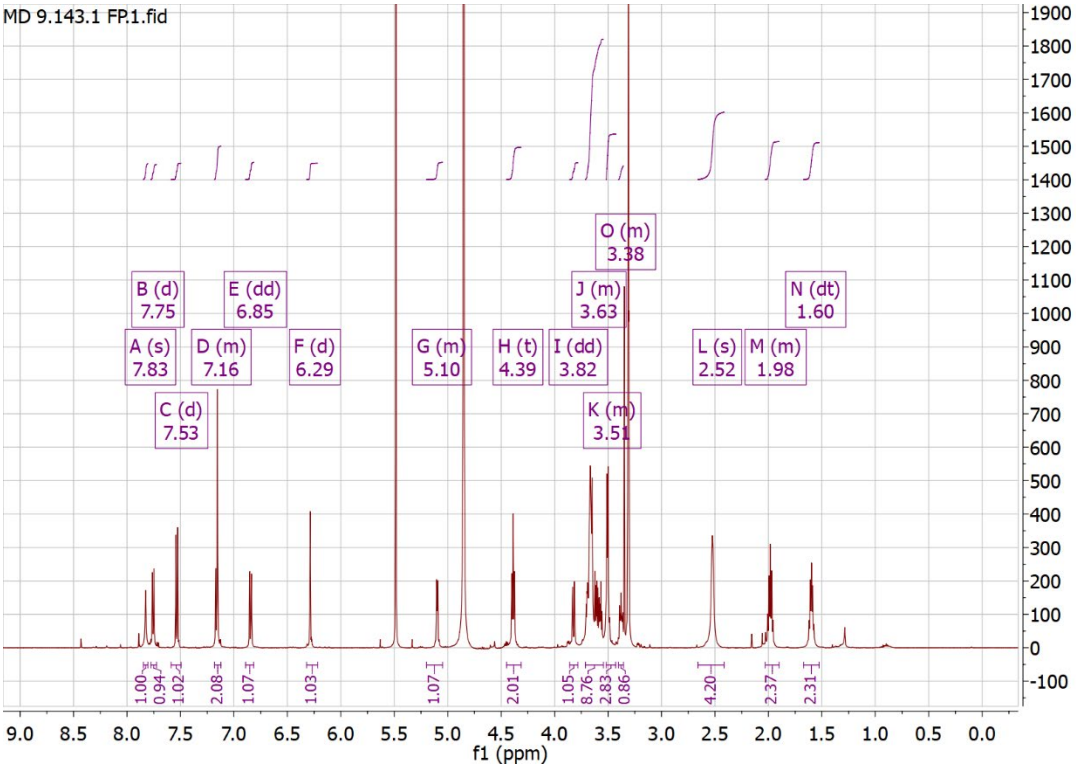


Figure S6. ^{1}H NMR spectrum of compound **10** (Morsel-X)

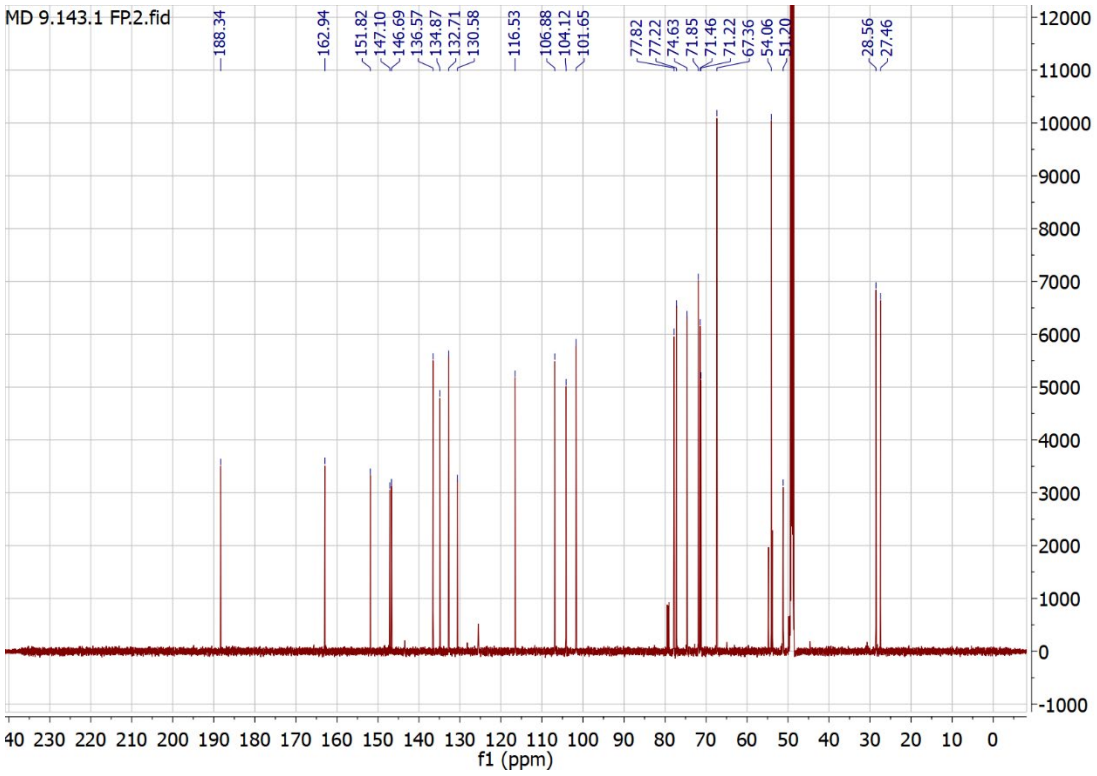


Figure S7. ^{13}C NMR spectrum of compound **10** (Morsel-X)

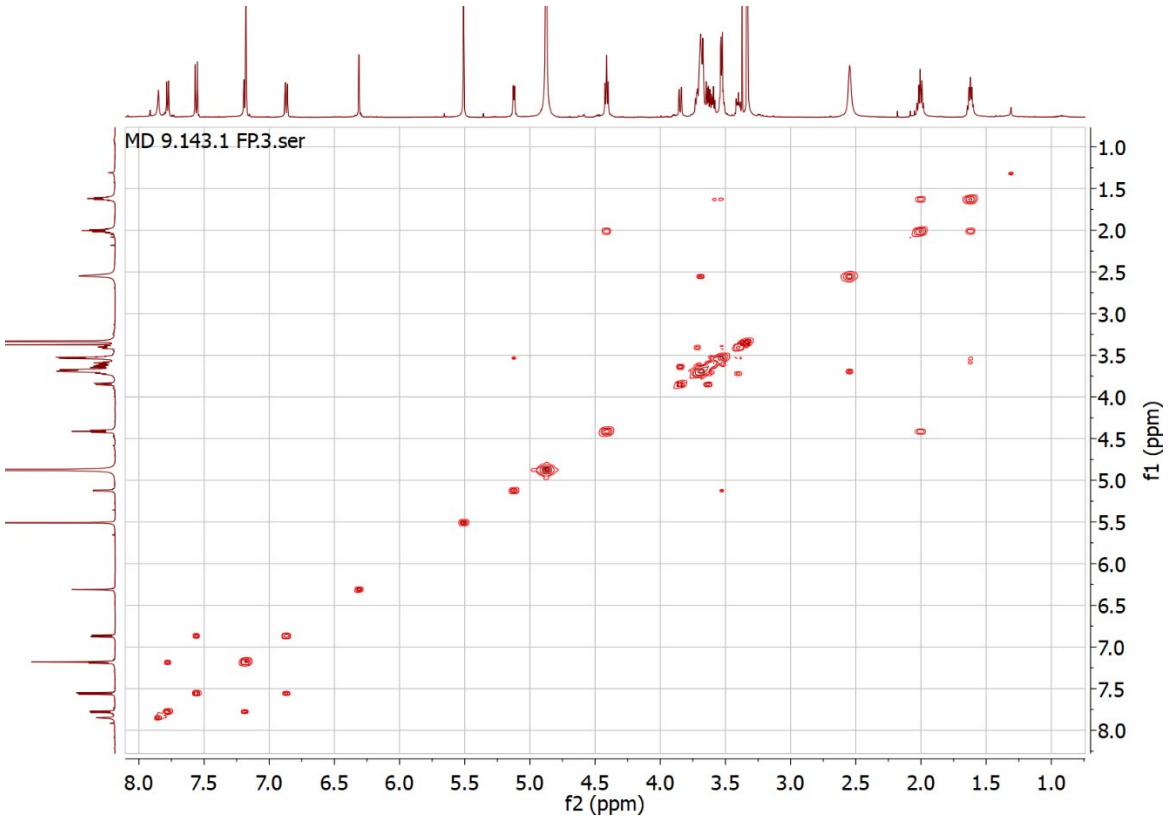


Figure S8. 2D COSY NMR spectrum of compound **10** (Morsel-X)

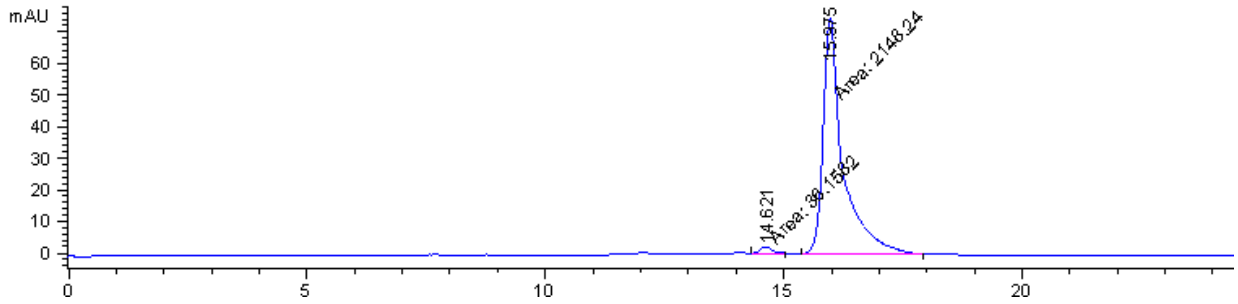


Figure S9. HPLC chromatogram of a solution of Morsel-X on an Agilent 1100 HPLC using an Eclipse XDB C18 semi-preparatory column (solvents used: A = 20 mM NH_4OAc (pH 6.8), B = 8:2 (MeOH:A)) and a gradient of 50-70 % solvent B over 15 minutes at a flow rate of 2 mL min^{-1}.

References:

(1) Ben Bdira, F.; Kallemeijn, W. W.; Oussoren, S. V.; Scheij, S.; Bleijlevens, B.; Florea, B. I.; van Roomen, C. P. A. A.; Ottenhoff, R.; van Kooten, M. J. F. M.; Walvoort, M. T. C.; et al. Stabilization of Glucocerebrosidase by Active Site Occupancy. *ACS Chem. Biol.* **2017**, *12* (7), 1830–1841.