Probing Synergy Between Two Catalytic Strategies in the Glycoside Hydrolase *O*-GlcNAcase using Multiple Linear Free-energy Relationships.

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General Procedures for Synthesis of Compounds:

Starting materials were obtained from Sigma, unless indicated, and used without further purification. The starting material (D-Glucosamine) was obtained from Bioshop. Dicloromethane was dried by distillation over CaH_2 prior to use. Synthetic reactions were monitored by TLC using Merck Kieselgel 60 F_{254} aluminium-backed sheets. Compounds were detected by a combination of UV absorption and charring with 2 M H_2SO_4 in ethanol and heating. Flash chromatography under a positive pressure was performed with Merck Kieselgel 60 (230–400 mesh) using the specified eluants. ^{1}H NMR spectra were recorded on a Varian AS500 Unity Innova spectrometer at 500 MHz.

Synthesis and characterization of substrates:

Synthesis of 2-acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy-β-D-glucopyranose (**2**) – 1,3,4,6-tetra-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranose hydrochloride(**1**) was prepared as described previously(1). This hydrochloride salt (10 g) was added to 100 ml of DCM and cooled on ice. Triethylamine (2.2 equivalents) was added and this solution was stirred until everything had dissolved. Acetyl chloride (1.2 equivalents) was then added slowly and the reaction mixture was stirred on ice for several minutes and then slowly allowed to warm to room temperature after which time the reaction was judged complete by TLC analysis. The reaction was extracted with two washes with water and once with saturated sodium chloride. The organic extracts were dried over $MgSO_4$ and filtered, and the solvent was partially removed *in vacuo*. The desired product was then recrystallized from DCM and hexanes and the product was obtained with an 85 % yield. Characterization of 1,3,4,6-tetra-*O*-acetyl-2-acetamido-2-deoxy-β-D-glucopyranose has been described previously.

Synthesis of 1,3,4,6-tetra-O-acetyl-2-deoxy-2-fluoroacetamido-β-D-glucopyranose (**3**) – Triethylamine (0.8 mL) and dry pyridine (20 mL) were added to a cooled (0 ^{0}C) solution of 1,3,4,6-tetra-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranose hydrochloride (**1**) (1) (5 g) in a solution of DMF (100 mL). Sodium fluoroacetate (1.8 g) was added to a stirred mixture of dry DMF (90 mL) containing dried Dowex 50-H$^+$ resin (12 g). After 1 h, DCC (3.2 g) and 30 ml of the fluoroacetic acid solution were added via cannula to the reaction vessel containing the hydrochloride salt. The resulting solution was allowed to stand for 16 h at 0 ^{0}C, after which time the reaction was judged complete by TLC analysis. The solvent was partially removed *in vacuo* and ethyl acetate (300 ml) and a solution of saturated sodium chloride (100 mL) were added. The organic layer was collected, and the aqueous layer was extracted twice with ethyl acetate. The combined organic extracts were washed successively with water, twice with saturated sodium bicarbonate, and finally with a solution of saturated sodium chloride. The organic extracts were dried over $MgSO_4$ and filtered, and the solvent was removed *in vacuo* to yield colorless syrup. The desired product was purified using flash column silica chromatography using a gradient solvent system (2:1; hexanes/ethyl acetate) to yield the partially purified desired compound. The desired product was then recrystallized from ethyl acetate and hexanes and the product was obtained with a 77 % yield.

1,3,4,6-tetra-O-acetyl-2-deoxy-2-fluoroacetamido-β-D-glucopyranose **(3)** – ^{1}H NMR (500 MHz, CD$_3$OD) δ: 5.83 (1H, d, $J_{H1,H2}$ = 8.8 Hz, H-1), 5.34 (1H, dd, J_{H3-H4} = 9.3 Hz, H-3), 5.00 (1H, dd, J_{H4-H5} = 10.0 Hz, H-4), 4.74 (2H, dd, J_{H7-F} = 47.0 Hz, H-7), 4.26 (1H, dd, $J_{H6-H6'}$ = 12.5 Hz, H-6), 4.10 (1H, dd, J_{H2-H3} = 10.5 Hz, H-2), 4.07 (1H, dd, H-6'), 3.89 (1H, ddd, J_{H5-H6} = 4.6 Hz, $J_{H5-H6'}$ = 2.3 Hz, H-5), 2.04 (3H, s, OAc), 2.01 (3H, s, OAc), 1.98 (3H, s, OAc), 1.95 (3H, s, OAc) ppm.

Synthesis of 1,3,4,6-tetra-O-acetyl-2-deoxy-2-difluoroacetamido-β-D-glucopyranose **(4)** – Triethylamine (0.8 mL) and dry pyridine (20 mL) were added to a cooled (0 ^{0}C) solution of 1,3,4,6-tetra-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranose hydrochloride (5 g) in a solution of DMF (100 mL). Dicyclohexylcarbodiimide (DCC, 3 g) and difluoroacetic acid (1.2 mL) were added to the reaction mixture via syringe. The resulting solution was allowed to stand for 16 h at 0 ^{0}C, after which time another 0.5 mL of difluoroacetic acid were added. After a further 3.5 h at room temperature, the reaction was judged complete by TLC analysis. The solvent was partially removed *in vacuo* and ethyl acetate (300 ml) and a solution of saturated sodium chloride (100 mL) were added. The organic layer was collected, and the aqueous layer was extracted twice with ethyl acetate. The combined organic extracts were washed successively with water, twice with saturated sodium bicarbonate, and finally with a solution of saturated sodium chloride. The organic extracts were dried over MgSO$_4$ and filtered, and the solvent was removed *in vacuo* to yield colorless syrup. The desired product was purified using flash column silica chromatography using a gradient solvent system (3:1; hexanes/ethyl acetate) to yield the partially purified desired compound. The desired product was then recrystallized from ethyl acetate and hexanes and the product was obtained with an 83 % yield.

1,3,4,6-tetra-O-acetyl-2-deoxy-2-difluoroacetamido-β-D-glucopyranose **(4)** – ^{1}H NMR (500 MHz, CD$_3$OD) δ: 4.96 (1H, t, J_{H7-F} = 53.6 Hz, H-7), 5.78 (1H, d, $J_{H1,H2}$ = 8.9 Hz, H-1), 5.28 (1H, dd, J_{H3-H4} = 9.3 Hz, H-3), 4.98 (1H, dd, J_{H4-H5} = 10.0 Hz, H-4), 4.23 (1H, dd, $J_{H6-H6'}$ = 12.5 Hz, H-6), 4.06 (1H, dd, J_{H2-H3} = 10.5 Hz, H-2), 4.05 (1H, dd, H-6'), 3.89 (1H, ddd, J_{H5-H6} = 2.3 Hz, $J_{H5-H6'}$ = 4.5 Hz, H-5), 2.01 (3H, s, OAc), 1.99 (3H, s, OAc), 1.95 (3H, s, OAc), 1.92 (3H, s, OAc) ppm.

Synthesis of 1,3,4,6-tetra-O-acetyl-2-deoxy-2-trifluoroacetamido-β-D-glucopyranose **(5)** – Triethylamine (0.8 mL) was added to a solution of 1,3,4,6-tetra-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranose hydrochloride (1 g) dissolved in dry dichloromethane (20 ml) and cooled (0 ^{0}C). Trifluoroacetic anhydride (0.6 mL) was added via syringe and the resulting solution was allowed to stand for 16 h at 0 ^{0}C, after which time the reaction was judged complete by TLC analysis. The solution was dilute in 50 ml of ethyl acetate and washed successively with water, twice with saturated sodium bicarbonate, and finally with a solution of saturated sodium chloride. The organic extracts were dried over MgSO$_4$ and filtered, and the solvent was removed *in vacuo* to yield a white solid. The desired product was recrystallized from ethyl acetate and hexanes and the product was obtained with a 91 % yield.

1,3,4,6-tetra-O-acetyl-2-deoxy-2-trifluoroacetamido-β-D-glucopyranose **(5)** – ^{1}H NMR (500 MHz, CD$_3$OD) δ: 5.81 (1H, d, $J_{H1,H2}$ = 8.9 Hz, H-1), 5.31 (1H, dd, J_{H3-H4} = 9.3 Hz,

H-3), 5.03 (1H, dd, J_{H4-H5} = 10.0 Hz, H-4), 4.26 (1H, dd, $J_{H6-H6'}$ = 12.5 Hz, H-6), 4.09 (1H, dd, J_{H2-H3} = 10.5 Hz, H-2), 4.08 (1H, dd, H-6'), 3.91 (1H, ddd, J_{H5-H6} = 4.6 Hz, $J_{H5-H6'}$ = 2.3 Hz, H-5), 2.04 (3H, s, OAc), 2.02 (3H, s, OAc), 1.98 (3H, s, OAc), 1.94 (3H, s, OAc) ppm.

General procedure for the synthesis of the 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl chlorides (**6-8**) – In a similar manner that has been described previously for the non-fluorinated derivative(2), acetic anhydride (10 mL) was added to the 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-fluoroacetamido-β-D-glucopyranoses (**2-4**) (1 g) and cooled (0 ^{0}C). $HCl_{(g)}$ was added until the solution was saturated and the reaction was allowed to proceed for 16 h at room temperature. As the reaction proceeded the starting material dissolved into the solvent. Upon completion of the reaction the solvent was partially removed *in vacuo* to 1/3 of the volume. Dichloromethane (40 mL) was added and this solution was washed extensively with a saturated solution of sodium bicarbonate, water, and then saturated sodium chloride. The organic extracts were dried over $MgSO_4$ and filtered, and solvent was removed *in vacuo*. The compounds were not susceptible to crystallization, however, analysis of the products by NMR showed minimal contamination with yields ranging from 70-90%. Therefore, the desired compounds were used in the following steps without further purification. Characterization of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl chloride has been described previously.

3,4,6-tri-O-acetyl-2-deoxy-2-fluoroacetamido-β-D-glucopyranosyl chloride (**7**) – ^{1}H NMR (500 MHz, CDCl$_3$) δ: 6.62 (1H, d, J_{HN-H2} = 6.6 Hz, H-N), 6.21 (1H, d, $J_{H1,H2}$ = 3.7 Hz, H-1), 5.34 (1H, dd, J_{H3-H4} = 9.3 Hz, H-3), 5.00 (1H, dd, J_{H4-H5} = 10.0 Hz, H-4), 4.79 (2H, AB$_q$, J_{H7-F} = 47.2 Hz, H-7), 4.26 (1H, dd, $J_{H6-H6'}$ = 12.5 Hz, H-6), 4.10 (1H, dd, J_{H2-H3} = 10.5 Hz, H-2), 4.07 (1H, dd, H-6'), 3.89 (1H, ddd, J_{H5-H6} = 4.6 Hz, $J_{H5-H6'}$ = 2.3 Hz, H-5), 2.04 (3H, s, OAc), 2.11 (3H, s, OAc), 2.06 (3H, s, OAc), 2.05 (3H, s, OAc) ppm.

3,4,6-tri-O-acetyl-2-deoxy-2-difluoroacetamido-β-D-glucopyranosyl chloride (**8**) – ^{1}H NMR (500 MHz, CDCl$_3$) δ: 6.73 (1H, d, H-N), 6.20 (1H, d, $J_{H1,H2}$ = 3.8 Hz, H-1), 5.89 (1H, t, J_{H7-F} = 53.9 Hz, H-7), 5.34 (1H, dd, J_{H3-H4} = 9.3 Hz, H-3), 5.00 (1H, dd, J_{H4-H5} = 10.0 Hz, H-4), 4.26 (1H, dd, $J_{H6-H6'}$ = 12.5 Hz, H-6), 4.10 (1H, dd, J_{H2-H3} = 10.5 Hz, H-2), 4.07 (1H, dd, H-6'), 3.89 (1H, ddd, J_{H5-H6} = 4.6 Hz, $J_{H5-H6'}$ = 2.3 Hz, H-5), 2.04 (3H, s, OAc), 2.11 (3H, s, OAc), 2.06 (3H, s, OAc), 2.05 (3H, s, OAc) ppm.

Synthesis of aryl 2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucopyranoses (**10a-k, 11a-k, 12a-k**) – To a mixture of the respective fluorinated 2-acetamido-3,4,6-tri-*O*-acetyl-α-D-glucopyranosyl chloride (**6-8**) (1 eq.), benzyltriethylammonium chloride (1eq.), and the acceptor phenol (2 eq.) was added to sufficient dichloromethane (1 volume) to yield a solution of 200 mM. An equal volume of 1 M NaOH was then added and the resulting mixture was stirred vigorously at room temperature. Ethyl acetate (5 volumes) was then added and the resulting organic phase was successively washed with 1 M NaOH (2 x 1 volume), water (1 volume), and saturated sodium chloride solution (1 volume). The organic layer was dried (MgSO4), filtered, and concentrated. These materials were recrystallized using a mixture of ethyl acetate and hexanes to yield the desired glycosides in yields ranging from 40 to 60 %.

Synthesis of aryl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoses (**13a-k**) – The desired products were obtained from 3,4,6-tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-β-D-glucopyranosyl bromide (**9**) (3) using AgCO₃ and the appropriate acceptor in a similar fashion as described previously(4).

Aryl 3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-β-D-glucopyranosides (**10a-k, 11a-k, 12a-k, 13a-k**) – 3,4-dinitro-phenyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (**10a**), 3-fluoro-4-nitro-phenyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (**10b**), 3-nitro-phenyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (**10g**), 4-chloro-phenyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (**10i**), and phenyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (**10j**) were made for a previous study(5). 4-nitro-phenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoroacetamido-β-D-glucopyranoside(**11c**), 4-nitro-phenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-difluoroacetamido-3β-D-glucopyranoside(**12c**), and 4-nitro-phenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-β-D-glucopyranoside(**13c**) are known and their physical characteristics were consistent with those found in the literature(4). 3-fluoro-4-nitro-phenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoroacetamido-β-D-glucopyranoside(**11b**), 3-fluoro-4-nitro-phenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-difluoroacetamido-β-D-glucopyranoside(**12b**), and 3-fluoro-4-nitro-phenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-β-D-glucopyranoside(**13b**) were prepared for another study(6). Complete ¹H NMR assignments for the rest of the compounds are located in Tables 1-8.

General Synthesis of aryl 2-acetamido-2-deoxy-β-D-glucopyranosides (**14a-k, 15a-k, 16a-k, 17 a-k**) – To a stirred solution of the aryl 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoroacetamido-β-D-glucopyranoses in anhydrous methanol was added a spatula tip of sodium methoxide. The reaction mixture was allowed to stir for approximately one hour at room temperature. After this time, the reaction was neutralized with Amberlite IR-120 resin (H₊) and filtered. The filtrate was concentrated *in vacuo* and the desired products were recrystallized using a mixture of ethanol and ether. Yields of the desired product after one recrystallization ranged from 40 to 80 %.

Aryl 2-acetamido-2-deoxy-β-D-glucopyranosides (**14a-k, 15a-k, 16a-k, 17 a-k**) – 3,4-dinitro-phenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**14a**), 3-fluoro-4-nitro-phenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**14b**), 3-nitro-phenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**14g**), 4-chloro-phenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**14i**), and phenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**14j**) were made for a previous study(5). 4-methyl-umbelliferyl 2-deoxy-2-fluoroacetamido-β-D-glucopyranoside (**15d**), 4-methyl-umbelliferyl 2-deoxy-2-difluoroacetamido-β-D-glucopyranoside (**16d**), and 4-methyl-umbelliferyl 2-deoxy-2-trifluoroacetamido-β-D-glucopyranoside (**17d**) are known and their physical characteristics were consistent with those found in the literature(7). 3-fluoro-4-nitrophenyl 2-deoxy-2-fluoroacetamido-β-D-glucopyranoside (**15b**), 3-fluoro-4-nitrophenyl 2-deoxy-2-difluoroacetamido-β-D-glucopyranoside (**16b**), and 3-fluoro-4-nitrophenyl 2-deoxy-2-trifluoroacetamido-β-D-glucopyranoside (**17b**) were prepared for another study(6). 4-nitro-phenyl 2-acetamido-2-

deoxy-β-D-glucopyranoside (**14c**) and 4-methyl-umbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**14d**) were purchased from Sigma. Complete ^{1}H NMR assignments for the rest of the compounds are located in Tables 9-16.

Synthesis of methyl 3,4,6-tri-O-acetyl-2-deoxy-2-fluroacetamido-β-D-glucopyranoside (**19b**) – Triethylamine (0.8 mL) and dry pyridine (20 mL) were added to a cooled (0 [0]C) solution of methyl 3,4,6-tri-*O*-acetyl-2-amino-2-deoxy-α-D-glucopyranoside hydrobromide (**18**) (8) (5 g) in a solution of DMF (100 mL). Sodium fluoroacetate (1.8 g) was added to a stirred mixture of dry DMF (90 mL) containing dried Dowex 50-H^{+} resin (12 g). After 1 h, DCC (3.2 g) and 30 ml of the fluoroacetic acid solution were added via cannula to the reaction vessel containing the hydrochloride salt. The resulting solution was allowed to stand for 16 h at 0 [0]C, after which time the reaction was judged complete by TLC analysis. The solvent was partially removed *in vacuo* and ethyl acetate (300 ml) and a solution of saturated sodium chloride (100 mL) was added. The organic layer was collected, and the aqueous layer was extracted twice with ethyl acetate. The combined organic extracts were washed successively with water, twice with saturated sodium bicarbonate, and finally with a solution of saturated sodium chloride. The organic extracts were dried over MgSO₄ and filtered, and the solvent was removed *in vacuo* to yield colorless syrup. The desired product was purified using flash column silica chromatography using a gradient solvent system (2:1; hexanes/ethyl acetate) to yield the partially purified desired compound that was used in the next step without further purification.

Synthesis of methyl 3,4,6-tri-O-acetyl-2-deoxy-2-difluroacetamido-β-D-glucopyranoside (**19c**) – Triethylamine (0.8 mL) and dry pyridine (20 mL) were added to a cooled (0 [0]C) solution of methyl 3,4,6-Tri-*O*-acetyl-2-amino-2-deoxy-α-D-glucopyranoside hydrobromide (5 g) in a solution of DMF (100 mL). Dicyclohexylcarbodiimide (DCC, 3 g) and difluoroacetic acid (1.2 mL) were added to the reaction mixture via syringe. The resulting solution was allowed to stand for 16 h at 0 [0]C, after which time another 0.5 mL of difluoroacetic acid were added. After a further 3.5 h at room temperature, the reaction was judged complete by TLC analysis. The solvent was partially removed *in vacuo* and ethyl acetate (300 ml) and a solution of saturated sodium chloride (100 mL) were added. The organic layer was collected, and the aqueous layer was extracted twice with ethyl acetate. The combined organic extracts were washed successively with water, twice with saturated sodium bicarbonate, and finally with a solution of saturated sodium chloride. The organic extracts were dried over MgSO₄ and filtered, and the solvent was removed *in vacuo* to yield colorless syrup. The desired product was purified using flash column silica chromatography using a gradient solvent system (3:1; hexanes/ethyl acetate) to yield the partially purified desired compound. The desired product was purified using flash column silica chromatography using a gradient solvent system (3:1; hexanes/ethyl acetate) to yield the partially purified desired compound that was used in the next step without further purification.

Synthesis of methyl 3,4,6-tri-O-acetyl-2-deoxy-2-trifluroacetamido-β-D-glucopyranoside (**19d**) – Triethylamine (0.8 mL) was added to a solution of methyl 3,4,6-tri-*O*-acetyl-2-amino-2-deoxy-α-D-glucopyranoside hydrobromide (1 g) dissolved in dry

dichloromethane (20 ml) and cooled (0 ^{0}C). Trifluoroacetic anhydride (0.6 mL) was added via syringe and the resulting solution was allowed to stand for 16 h at 0 ^{0}C, after which time the reaction was judged complete by TLC analysis. The solution was dilute in 50 ml of ethyl acetate and washed successively with water, twice with saturated sodium bicarbonate, and finally with a solution of saturated sodium chloride. The organic extracts were dried over MgSO$_4$ and filtered, and the solvent was removed *in vacuo* to yield a white solid. The desired product was purified using flash column silica chromatography using a gradient solvent system (4:1; hexanes/ethyl acetate) to yield the partially purified desired compound that was used in the next step without further purification.

General synthesis of methyl 2-deoxy-2-acetamido-β-D-glucopyranosides (**20a-d**) – A spatula tip of anhydrous sodium methoxide was added to a solution of the methyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoroacetamido-β-D-glucopyranosides in methanol. The basic solution was stirred until the reaction was judged complete by TLC analysis (typically 1 h). A solution of glacial acetic acid in methanol (1:20) was added dropwise to the reaction mixture until the pH of the solution was found to be neutral. The solvent was then removed *in vacuo*, and the desired materials were isolated by flash silica chromatography using a solvent system (ranging from 5:1ethyl acetate /methanol for the non-fluorinated compound to straight ethyl acetate for the trifluorinated compound). Removal of the solvent *in vacuo* of the desired fractions yielded the products as fine powders.

methyl 2-deoxy-2-fluoroacetamido-β-D-glucopyranoside (**20b**) – ^{1}H NMR (500 MHz, CD$_3$OD) δ: 4.79 (2H, dd, J_{H7-F} = 47.2 Hz, $J_{H7-H7'}$ = 2.2 Hz, H-7), 4.36 (1H, d, $J_{H1,H2}$ = 8.4 Hz, H-1), 3.86 (1H, dd, $J_{H6-H6'}$ = 12.0 Hz, H-6), 3.66 (1H, dd, H-6'), 3.72 (1H, dd, J_{H3-H4} = 10.2 Hz, H-3), 3.49 (1H, dd, J_{H4-H5} = 8.4 Hz, H-4), 3.43 (3H, s, OMe), 3.30 (1H, dd, J_{H2-H3} = 8.7 Hz, H-2), 3.25 (1H, ddd, J_{H5-H6} = 2.3 Hz, $J_{H5-H6'}$ = 5.7 Hz, H-5) ppm.

methyl 2-deoxy-2-difluoroacetamido-β-D-glucopyranoside (**20c**) – ^{1}H NMR (500 MHz, CD$_3$OD) δ: 6.0 (1H, t, J_{H7-F} = 54.0 Hz, $J_{H7-F'}$ = 54.0 Hz, H-7), 4.36 (1H, d, $J_{H1,H2}$ = 8.4 Hz, H-1), 3.85 (1H, dd, $J_{H6-H6'}$ = 12.0 Hz, H-6), 3.66 (1H, dd, H-6'), 3.61 (1H, dd, J_{H3-H4} = 10.3 Hz, H-3), 3.48 (1H, dd, J_{H4-H5} = 8.4 Hz, H-4), 3.43 (3H, s, OMe), 3.29 (1H, dd, J_{H2-H3} = 9.8 Hz, H-2), 3.24 (1H, ddd, J_{H5-H6} = 2.2 Hz, $J_{H5-H6'}$ = 5.7 Hz, H-5) ppm.

methyl 2-deoxy-2-trifluoroacetamido-β-D-glucopyranoside (**20d**) – ^{1}H NMR (500 MHz, CD$_3$OD) δ: 4.35 (1H, d, J_{H1-H2} = 8.4 Hz, H-1), 3.85 (1H, dd, $J_{H6-H6'}$ = 12.0 Hz, H-6), 3.66 (1H, dd, H-6'), 3.64 (1H, dd, J_{H3-H4} = 10.5 Hz, H-3), 3.48 (1H, dd, J_{H4-H5} = 8.4 Hz, H-4), 3.44 (3H, s, OMe), 3.29 (1H, dd, J_{H2-H3} = 9.8 Hz, H-2), 3.24 (1H, ddd, J_{H5-H6} = 2.3 Hz, $J_{H5-H6'}$ = 5.7 Hz, H-5) ppm.

Synthesis of 3,4,6-tri-O-acetyl-2-deoxy-2-N-pentanoyl-β-D-glucopyranose (**21**) – The desired compound was prepared as described previously(7).

Synthesis of 3,4,6-tri-O-acetyl-2-deoxy-2-N-pentanoyl-β-D-glucopyranoside chloride (**22**) – Conversion of 3,4,6-tri-*O*-acetyl-2-deoxy-2-*N*-pentanoyl-β-D-glucopyranose (**21**) to its corresponding α-chloride was accomplished in the same manner as described above for

compounds 6-8. The desired compound was used in the subsequent steps without characterization.
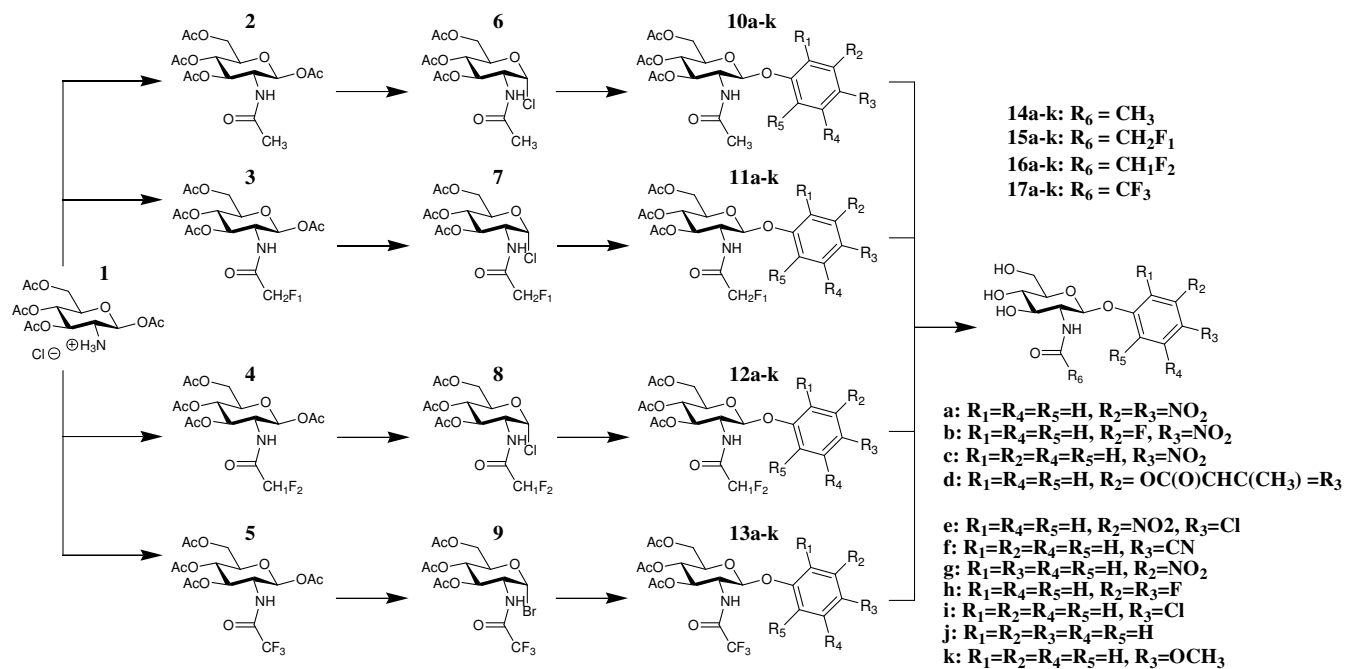
Synthesis of aryl 3,4,6-tri-O-acetyl-2-deoxy-2-N-pentanoyl-β-D-glucopyranosides (**23a-h**) – Phase transfer conditions were used to prepare the desired compounds in the same manner as described for compounds 10-12.

aryl 3,4,6-tri-O-acetyl-2-deoxy-2-N-pentanoyl-β-D-glucopyranosides (**23a-h**) – 4-methylumbelliferyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-*N*-pentanoyl-β-D-glucopyranoside was prepared for a previous study (7). 3,4-dinitrophenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-*N*-pentanoyl-β-D-glucopyranoside and 3-fluoro-4-nitrophenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-*N*-pentanoyl-β-D-glucopyranoside were used in the subsequent step without characterization. Complete ^{1}H NMR assignments for the rest of the compounds are located in Tables 17-18.

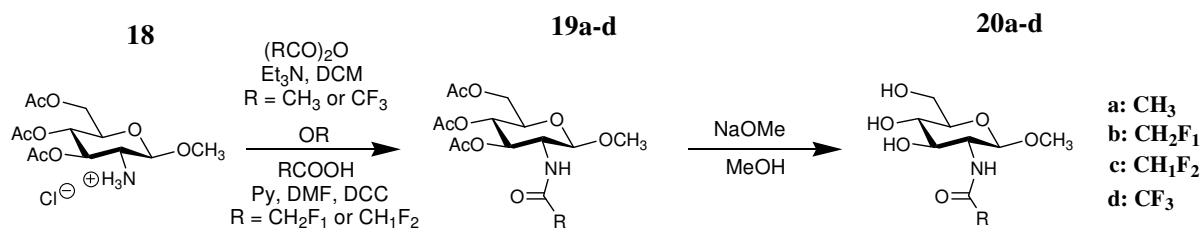*Synthesis of Aryl 2-deoxy-2-N-pentanoyl-β-D-glucopyranosides* (**24a-h**) – Deprotection was carried out under the same conditions as described for compounds 14-17.

Aryl 2-deoxy-2-N-pentanoyl-β-D-glucopyranosides (**24a-h**) – 4-methylumbelliferyl 2-deoxy-2-*N*-pentanoyl-β-D-glucopyranoside was prepared for a previous study (7). Complete ^{1}H NMR assignments for the rest of the compounds are located in Tables 19-20.

Scheme 1

14a-k: $R_6 = CH_3$
15a-k: $R_6 = CH_2F_1$
16a-k: $R_6 = CH_1F_2$
17a-k: $R_6 = CF_3$

a: $R_1=R_4=R_5=H$, $R_2=R_3=NO_2$
b: $R_1=R_4=R_5=H$, $R_2=F$, $R_3=NO_2$
c: $R_1=R_2=R_4=R_5=H$, $R_3=NO_2$
d: $R_1=R_4=R_5=H$, $R_2= OC(O)CHC(CH_3) =R_3$

e: $R_1=R_4=R_5=H$, $R_2=NO2$, $R_3=Cl$
f: $R_1=R_2=R_4=R_5=H$, $R_3=CN$
g: $R_1=R_3=R_4=R_5=H$, $R_2=NO_2$
h: $R_1=R_4=R_5=H$, $R_2=R_3=F$
i: $R_1=R_2=R_4=R_5=H$, $R_3=Cl$
j: $R_1=R_2=R_3=R_4=R_5=H$
k: $R_1=R_2=R_4=R_5=H$, $R_3=OCH_3$

Scheme 2

18 → **19a-d** → **20a-d**

Reagents and conditions:

(RCO)$_2$O, Et$_3$N, DCM, R = CH$_3$ or CF$_3$

OR

RCOOH, Py, DMF, DCC, R = CH$_2$F$_1$ or CH$_1$F$_2$

NaOMe, MeOH

a: CH$_3$
b: CH$_2$F$_1$
c: CH$_1$F$_2$
d: CF$_3$

Scheme 3

a: $R_1=R_4=R_5=H$, $R_2=R_3=NO_2$
b: $R_1=R_4=R_5=H$, $R_2=F$, $R_3=NO_2$
c: $R_1=R_2=R_4=R_5=H$, $R_3=NO_2$
d: $R_1=R_4=R_5=H$, $R_2=OC(O)CHC(CH_3)=R_3$
e: $R_1=R_3=R_4=R_5=H$, $R_2=NO_2$
f: $R_1=R_4=R_5=H$, $R_2=R_3=F$
g: $R_1=R_2=R_4=R_5=H$, $R_3=Cl$
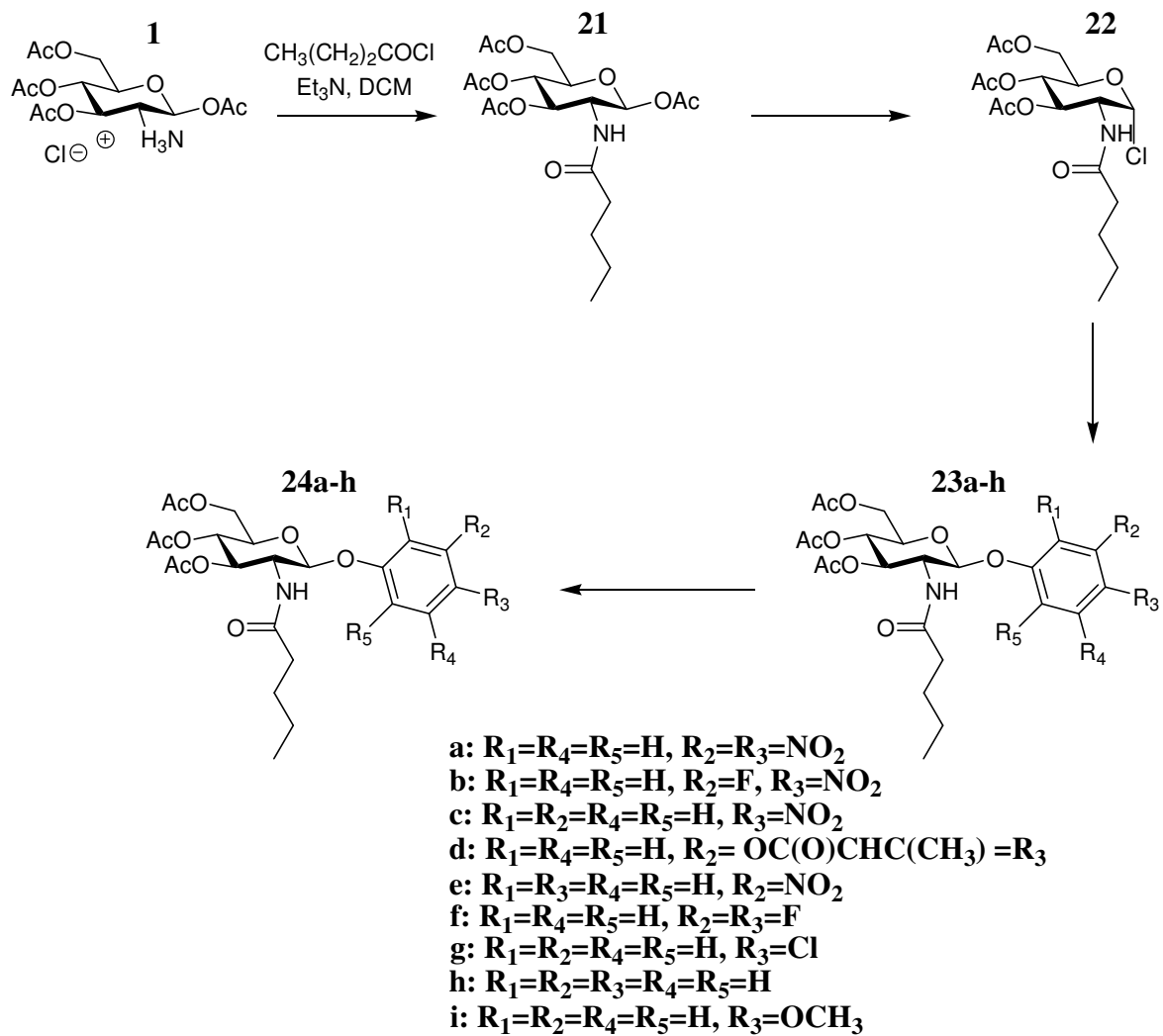h: $R_1=R_2=R_3=R_4=R_5=H$
i: $R_1=R_2=R_4=R_5=H$, $R_3=OCH_3$

Table S1. Values of k_{cat}/K_m (and their standard deviations) measured for the wild-type Human *O*-GlcNAcase-catalyzed cleavage of **Ar**-GlcNAc-**F$_n$**s.

Leaving Group (pK_a)	Wavelength at which reaction was monitored / nm	k_{cat}/K_m in x 10^3 M^{-1} s^{-1}				
		Substitution of 2-*N*-Acetamido group (nucleophile's Taft σ^*)				
		–CH$_3$ (0.0)	–CH$_2$F (0.8)	–CHF$_2$ (2.0)	–CF$_3$ (2.8)	–*n*-Bu (-0.15)
3,4-Dinitrophenol (5.42)	400	19.89 ± 0.13	18.24 ± 0.06	11.93 ± 0.04	0.49 ± 0.09	8.59
3-Fluoro-4-nitrophenol (6.42)	388	12.15 ± 0.05	7.67 ± 0.06	1.67 ± 0.01	0.49 ± 0.09	1.11
4-Nitrophenol (7.14)	400	12.78 ± 0.13	2.95 ± 0.01	0.50 ± 0.03	0.13 ± 0.02	0.197
4-Methylumbelliferone (7.5)	360	10.61 ± 0.11	5.41 ± 0.09	1.14 ± 0.03	0.23 ± 0.03	0.241
4-Chloro-3-nitrophenol (7.8)	330	10.36 ± 0.11	4.81 ± 0.05	1.47 ± 0.01	0.41 ± 0.08	not measured
4-Cyanophenol (8.0)	267	10.27 ± 0.10	4.51 ± 0.05	2.17 ± 0.02	0.85 ± 0.09	not measured
3-Nitrophenol (8.35)	330	8.49 ± 0.13	2.71 ± 0.05	1.96 ± 0.01	1.16 ± 0.12	0.154 ± 0.015
3,4-Difluorophenol (9.1)	275	6.84 ± 0.06	2.07 ± 0.01	0.68 ± 0.06	0.150 ± 0.015	0.098± 0.010
4-Chlorophenol (9.38)	280	6.69 ± 0.12	1.07 ± 0.03	0.49 ± 0.06	0.107 ± 0.011	0.086 ± 0.009
Phenol (9.99)	269	5.82 ± 0.03	0.94 ± 0.01	0.15 ± 0.01	not detected	0.066 ± 0.007
4-Methoxyphenol (10.2)	296	5.04 ± 0.07	0.73 ± 0.02	0.11 ± 0.01	not detected	not measured
Methanol (15.54)	Stopped assay	0.44 ± 0.05	0.088 ± 0.009	not measured	not measured	not measured

Monitoring the *O*-GlcNAc-catalyzed reaction by NMR Spectroscopy:

^{1}H NMR spectroscopy (600 MHz Bruker AMX spectrometer) was used to follow the progress and identify the products of the enzyme-catalyzed reaction in a similar manner as described previously(1). The reaction was carried out in 0.5 ml containing 5 mM pNP-GlcNAc in buffer containing 50 mM sodium phosphate and 100 mM sodium chloride, which had been dissolved in D_2O, evaporated, redissolved in 99.9% D_2O (Cambridge Isotopes), and adjusted to a pD of 7.81. Initiation of the reaction was done by the addition of 50 μl of enzyme, which had been buffer exchanged into the same deuterated buffer. Concentration of enzyme in the reaction was approximately 1 μM. The reaction was placed at 37 °C immediately and placed in the NMR spectrometer at the appropriate time to acquire a spectrum. During acquisition, the temperature of the spectrometer was maintained at 20 °C to optimize the chemical shift of the water signal as well as to slow down mutarotation.

Extrapolation of rates of the chemical step for the hydrolysis of glucosaminides possessing the *N*-acetyl (wild-type) nucleophile:

As indicated in the text, the value of β_{lg} for the hydrolysis of **Ar**-GlcNAc-**F$_0$**s reports primarily on a step that precedes glycosidic bond cleavage. The value of β_{lg} determined for **Ar**-GlcNAc-**F$_1$**s (–0.45) falls between that determined for **Ar**-GlcNAc-**F$_0$**s and those determined for **Ar**-GlcNAc-(**n-Bu/F$_2$/F$_3$**)s. This intermediate value for the **Ar**-GlcNAc-**F$_1$**s is therefore most likely an artefact of a change in kinetically significant step that occurs over the three **Ar**-GlcNAc-**F$_1$**s studied in regimen **I**. Indeed, the rates of hydrolysis of **3,4-dinitrophenyl** GlcNAc-**F$_0$** and **3,4-dinitrophenyl** GlcNAc-**F$_1$** are identical within experimental error implying that a physical step is rate limiting for both substrates.

Furthermore, a previously reported free-energy relationship was found to correlate the rates of hydrolysis glcosaminides possessing the methylumbelliferyl leaving group and a nucleophiles possessing a variety of alkyl substituents (y-axis, **Fig. S1**) with the inhibition constants of a series of thiazolines possessing equivalent alkyl substituents (x-axis, **Fig. S1**).[27] It is apparent that the rates of reaction of substrates possessing R = **Me** or **Et** deviate noticeably from the good linear free energy relationships defined by the other pairs of compounds…

$$\log\,(k_{cat}/K_m) = (1.13 \pm 0.04) \cdot \log\,(1/K_i) - (3.63 \pm 0.23) \qquad (r = 0.998,\ n = 5)$$

The extent of this deviation is estimated as 0.4 log units, these observations being consistent with our analysis that the Brønsted plot observed for **Ar**-GlcNAc-**F$_0$**s reports on some step that precedes the chemical step.
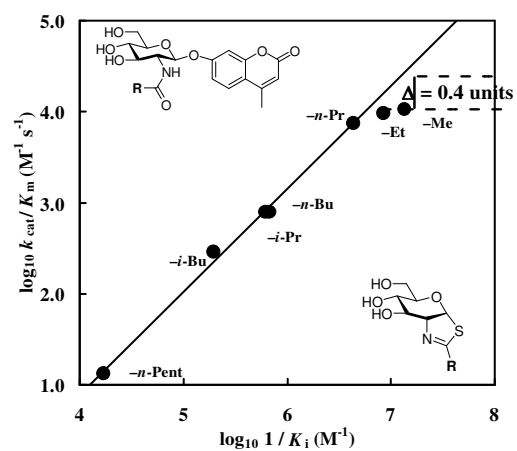
Figure S2: Correlation of log (k_{cat}/K_m) for substrates vs. log ($1/K_i$) for competitive inhibitors for different alkyl groups, **R**.

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