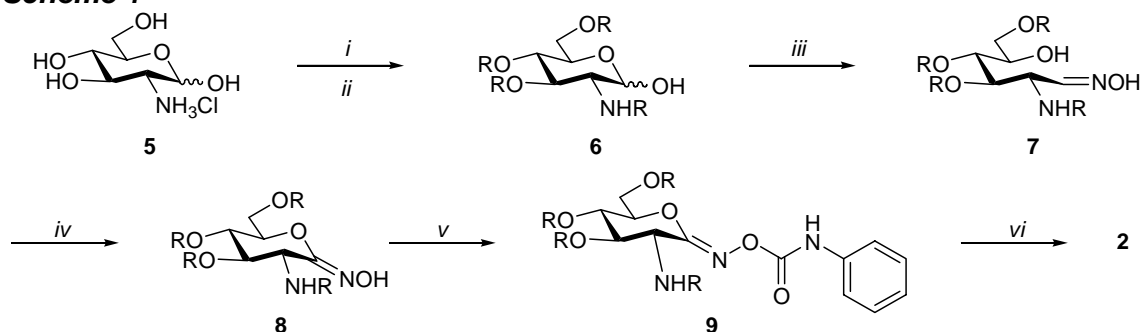


### Supporting Information:

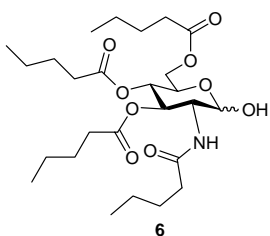
The synthesis of **2** was accomplished via the original pathway developed by Vasella and coworkers (Scheme 1).<sup>14</sup> Exhaustive acylation of glucosamine (**5**) with valeric anhydride and subsequent treatment with ammonium carbonate affected de-acylation at the anomeric site to provide **6** in acceptable yield over two steps. Refluxing **6** with ammonium hydroxide in basic methanol provided for the ring-open oxime **7** which, following treatment with DBU and *N*-chlorosuccinamide in methylene chloride at depressed temperatures, gave the ring-closed oxime **8** in good yield. Addition of phenyl isocyanate in basic THF provided the fully acylated **9** and exposure to ammonia effectively deacetylated the triol moiety to provide **2**. Purification by HPLC provided only the biochemically relevant Z oxime based upon NMR comparison of relevant protons to a series of Z PUGNAc derivatives.

### Scheme 1



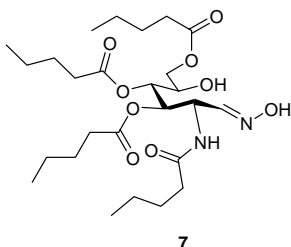
R = COCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>

Reagents and conditions: (i) valeric anhydride, pyridine, cat. DMAP (100%); (ii) (NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub>, MeOH:THF (2:1) (31%); (iii) NH<sub>4</sub>OH HCl, pyridine, MeOH, reflux (74%); (iv) DBU, NCS, CH<sub>2</sub>Cl<sub>2</sub>, -42°C (73%); (v) PhNCO, Et<sub>3</sub>N, THF (57%); (vi) NH<sub>3</sub>, MeOH (82%)

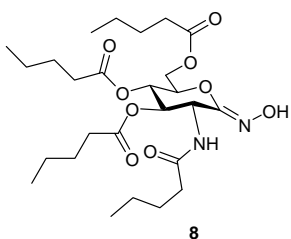


**(3S,4R,5S,6R)-6-hydroxy-5-pentanamido-2-(pentanoyloxymethyl)tetrahydro-2H-pyran-3,4-dipentanoate (6).** To a solution of D-(+)-glucosamine hydrochloride (2.0 g, 9.3 mmol) in pyridine (35 ml) was added valeric anhydride (9.2 ml, 46.5 mmol) and a catalytic amount of DMAP. The mixture was stirred for 24 h, concentrated and purified by column chromatography (EtOAc) to yield a colorless oil representing the per-acylated compound; 5.6 g (100%); <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 6.20 (d, *J* = 3.6Hz, 1H), 5.56 (d, *J* = 8.7Hz, 1H), 5.30-5.18 (m, 2H), 4.51-4.12 (m, 1H), 4.25-4.03 (m, 2H), 4.05-3.32 (m, 1H), 2.45-2.25 (m, 10H), 1.69-1.47 (m, 10H), 1.44-1.24 (m, 10H), 0.98-0.86 (m, 15H); TOFMS *m/z* (M + Na<sup>+</sup>) 622.3563 (calculated for C<sub>31</sub>H<sub>53</sub>NNaO<sub>10</sub><sup>+</sup>) 622.3567. To a solution of this oil (5.02g, 8.4 mmol) in MeOH:THF 2:1 (40 ml) was added excess ammonium carbonate. The mixture stirred for 6 h, concentrated and purified by column

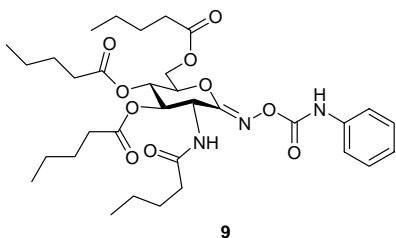
chromatography (3:1 hexane/EtOAc) to yield **6** as a colorless oil; 1.34 g, (31%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  5.77 (d,  $J = 9.0\text{Hz}$ , 1H), 5.36-5.29 (m, 2H), 5.16 (t,  $J = 9.6\text{Hz}$ , 1H), 4.35-4.27 (m, 1H), 4.22-4.16 (m, 3H), 3.17 (bs, 1H), 2.39-2.33 (m, 3H), 2.30-2.23 (m, 4H), 2.14-2.11 (m, 1H), 1.66-1.48 (m, 8H), 1.39-1.25 (m, 8H), 0.96-0.86 (m, 12H); TOFMS  $m/z$  ( $\text{M} + \text{H}^+$ ) 516.3165 (calculated for  $\text{C}_{26}\text{H}_{46}\text{NO}_9^+$ ) 516.3173.



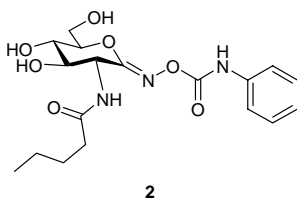
**(3S,4R,5S)-2-hydroxy-6-(hydroxyimino)-5-pentanamidohexane-1,3,4-tripentanoate (7).** To a solution of **6** (1.34 g, 2.6 mmol) in MeOH (25 ml) was added hydroxylamine hydrochloride (271 mg, 3.9 mmol) and pyridine (541  $\mu\text{l}$ , 6.7 mmol). The mixture was heated to reflux for 5 h to give a red/brown solution that was cooled, concentrated and purified by column chromatography (3:1 hexane/EtOAc) to yield **7** as a colorless oil; 1 g, (74%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  7.49-7.46 (m, 1H), 5.52-5.49 (m, 1H), 5.15-5.11 (m, 3H), 4.18-4.02 (m, 4H), 3.85-3.82 (m, 1H), 2.40-2.17 (m, 8H), 1.67-1.50 (m, 8H), 1.43-1.23 (m, 8H), 0.95-0.89 (m, 12H); TOFMS  $m/z$  ( $\text{M} + \text{Na}^+$ ) 553.3121 (calculated for  $\text{C}_{26}\text{H}_{46}\text{N}_2\text{NaO}_9^+$ ) 553.3101.



**(3S,4R,5S)-6-(hydroxyimino)-5-pentanamido-2-(pentanoyloxymethyl)tetrahydro-2H-pyran-3,4-dipentanoate (8).** To a  $-42^\circ\text{C}$  solution of **7** (200 mg, 0.38 mmol) in  $\text{CH}_2\text{Cl}_2$  (10 ml) was added 1,8-diazobicyclo[5.4.0]undec-7-ene (62  $\mu\text{l}$ , 0.42 mmol) over 10 min. The mixture was stirred for 5 min and *N*-chlorosuccinimide (56 mg, 0.42 mmol) was added in three portions over 15 min and the reaction stirred for a 30 min. period. The solution was quenched with  $\text{H}_2\text{O}$ , extracted with EtOAc, dried over  $\text{MgSO}_4$  and concentrated. Purification by column chromatography yielded **8** as a colorless oil; 145 mg, (73%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  6.53 (d,  $J = 8.7\text{Hz}$ , 1H), 5.43 (t,  $J = 9.0\text{Hz}$ , 1H), 5.19-4.99 (m, 2H), 4.38-4.29 (m, 2H), 4.23-4.18 (m, 2H), 2.44-2.11 (m, 8H), 1.68-1.49 (m, 8H), 1.43-1.26 (m, 8H), 0.97-0.87 (m, 12H); TOFMS  $m/z$  ( $\text{M} + \text{H}^+$ ) 529.3126 (calculated for  $\text{C}_{26}\text{H}_{45}\text{N}_2\text{O}_9^+$ ) 529.3125.



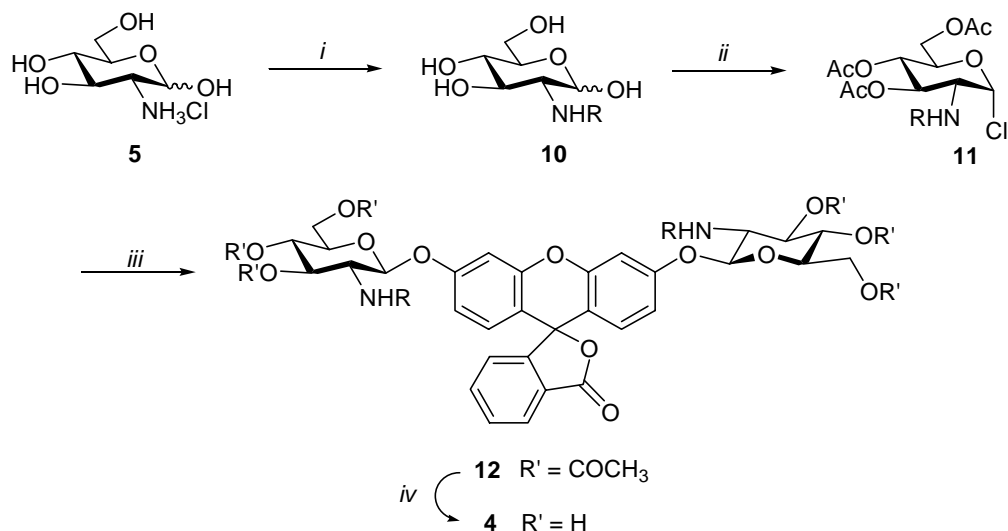
**(3S,4R,5S,Z)-5-pentanamido-2-(pentanoyloxymethyl)-6-(phenylcarbamoyloxyimino)tetrahydro-2H-pyran-3,4-dipentanoate (9).** To a solution of **8** in THF (5 ml) was added phenylisocyanate (59  $\mu$ l, 0.54 mmol) and Et<sub>3</sub>N (191  $\mu$ l, 1.37 mmol). The mixture stirred for 1 h, concentrated and purified by column chromatography (3:2 hexane/EtOAc) to yield **9** as a white solid; 100 mg, (57%); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>)  $\delta$  9.69 (s, 1H), 8.52 (d, *J* = 8.1 Hz, 1H), 7.49-7.43 (m, 2H), 7.33-7.25 (m, 2H), 7.06-7.01 (m, 1H), 5.42-5.26 (2H), 4.85-4.79 (m, 1H), 4.67-4.62 (m, 1H), 4.39-4.34 (m, 1H), 4.23-4.18 (m, 1H), 2.35-2.21 (m, 8H), 1.53-1.44 (m, 8H), 1.32-1.22 (m, 8H), 0.85 (t, *J* = 7.2 Hz, 12H); TOFMS *m/z* (*M* + H<sup>+</sup>) 648.3495 (calculated for C<sub>33</sub>H<sub>50</sub>N<sub>3</sub>O<sub>10</sub><sup>+</sup>) 648.3496.



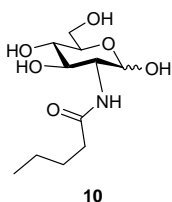
**N-((3S,4R,5S,Z)-4,5-dihydroxy-6-(hydroxymethyl)-2-(phenylcarbamoyloxyimino)tetrahydro-2H-pyran-3-yl)pentanamide (2).** To a solution of **9** (100 mg, 0.15 mmol) in MeOH (10 ml) was treated with NH<sub>3</sub> (excess), stirred for 1 h and concentrated. Purification by HPLC gave **2** as a white solid; 50 mg, (82%) HPLC purification was achieved using a linear gradient of water containing increasing amounts of CH<sub>3</sub>CN (0  $\rightarrow$  15 min, linear gradient from 10% to 90% CH<sub>3</sub>CN at a flow rate of 4 ml/min: 7.0 min); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>)  $\delta$  9.55 (s, 1H), 8.25 (d, *J* = 7.5 Hz, 1H), 7.48 (d, *J* = 8.4 Hz, 2H), 7.29 (t, *J* = 8.4 Hz, 2H), 7.02 (t, *J* = 7.5 Hz, 1H), 5.53-5.47 (m, 2H), 4.91 (t, *J* = 6.0 Hz, 1H), 4.37 (t, *J* = 8.4 Hz, 1H), 3.96-3.51 (m, 5H), 2.14 (t, *J* = 7.2 Hz, 2H), 1.56-1.46 (m, 2H), 1.37-1.23 (m, 2H), 0.85 (t, *J* = 6.9 Hz, 3H); TOFMS *m/z* (*M* + Na<sup>+</sup>) 418.1597 (calculated for C<sub>18</sub>H<sub>25</sub>N<sub>3</sub>NaO<sub>7</sub><sup>+</sup>) 418.1590.

The synthesis of **4** was accomplished in accordance with our published method (Scheme 2).<sup>11</sup> Treatment of glucosamine (**5**) with a single equivalent of valeric anhydride in the presence of sodium methoxide provided pentanamide **10** in acceptable yield. Acetylation of the remaining hydroxyl and insertion of an  $\alpha$ -chloro group to give **11** was done concomitantly via treatment with neat acetyl chloride. A mixture of **11** and fluorescein in the presence of silver oxide in basic acetonitrile over a 48 h period gave the fully protected **12** in a modest yield. Deacetylation was accomplished via treatment with ammonia to provide **4**. HPLC purification was performed prior to biochemical evaluation.

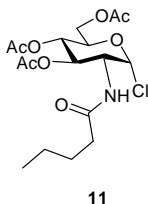
## Scheme 2



Reagents and conditions: (i) valeric anhydride, NaOMe, MeOH (55%); (ii) acetyl chloride (23%); (iii) fluorescein, Ag<sub>2</sub>O, pyridine, CH<sub>3</sub>CN, 40°C, 2 days (39%); (iv) NH<sub>3</sub>, MeOH, -10°C (71%)

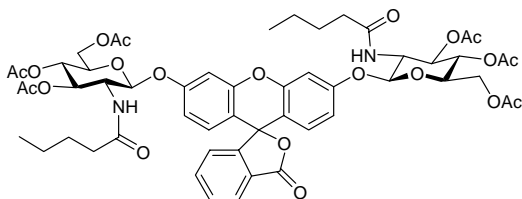


**N-((2R,3S,4R,5S)-2,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)pentanamide (10)**. To a solution of glucosamine hydrochloride (15 g, 84 mmol) in MeOH (120 ml) at 0 °C was added NaOMe (84 ml, 84 mmol) and the mixture stirred for a 30 min. period. Valeric anhydride (19.8 ml, 100.6 mmol) was added, the mixture stirred for 24 h and concentrated to yield **10** as a colorless oil; 12 g, (55%); <sup>1</sup>H NMR (MeOH-*d*<sub>4</sub>) δ 5.11 (d, *J* = 3.6Hz, 1H), 4.60 (d, *J* = 8.4Hz, 1H), 3.90-3.59 (m, 4H), 3.48-3.34 (m, 5H), 2.29-2.24 (m, 2H), 1.68-1.56 (m, 2H), 1.46-1.32 (m, 2H), 0.94 (t, *J* = 7.2Hz, 3H); TOFMS *m/z* (M + Na<sup>+</sup>) 286.1241 (calculated for C<sub>11</sub>H<sub>21</sub>NNaO<sub>6</sub><sup>+</sup>) 286.1267.



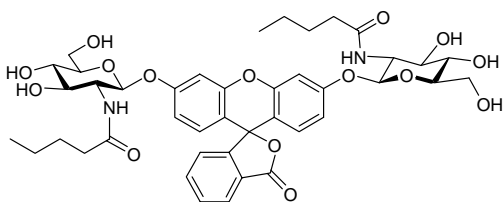
**(3S,4R,5S,6R)-2-(acetoxymethyl)-6-chloro-5-pentanamidotetrahydro-2H-pyran-3,4-diacetate (11)**. A neat solution of **10** (6.5 g, 25 mmol) and acetyl chloride (50 ml) was stirred for an 18 h period. The resulting pink solution was concentrated and purified by

column chromatography (2:1 hexane/EtOAc) to yield **11** as a white solid; 4.4 g, (23%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  6.19 (d,  $J = 3.6\text{Hz}$ , 1H), 5.84 (d,  $J = 8.1\text{Hz}$ , 1H), 5.37-5.19 (m, 2H), 4.59-4.51 (m, 1H), 4.33-4.25 (m, 2H), 4.16-4.11 (m, 1H), 2.22-2.05 (m, 2H), 2.11 (s, 3H), 2.06 (s, 3H), 2.05 (s, 3H), 1.62-1.52 (m, 2H), 1.38-1.24 (m, 2H), 0.91 (t,  $J = 7.2\text{Hz}$ , 3H); TOFMS  $m/z$  ( $\text{M} + \text{Na}^+$ ) 430.1242 (calculated for  $\text{C}_{17}\text{H}_{26}\text{ClNNaO}_8^+$ ) 430.1245



12

**(2R,3S,6S)-2-(acetoxymethyl)-6-(3'-((2S,3S,4R,5S)-4,5-diacetoxy-6-(acetoxymethyl)-3-pentanamidotetrahydro-2H-pyran-2-yloxy)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-6'-yloxy)-5-pentanamidotetrahydro-2H-pyran-3,4-diacetate (12).** To a solution of **11** (2 g, 4.9 mmol) in  $\text{CH}_3\text{CN}$  (100 ml) was added fluorescein (408 mg, 1.23 mmol) and the mixture allowed to stir for a 5 min. period. To this mixture was added silver oxide (855 mg, 3.69 mmol) and pyridine (3 drops) and the mixture was heated to  $40^\circ\text{C}$  for 2 days. The orange/brown reaction solution was filtered through celite, concentrated and purified by column chromatography (EtOAc) to yield **12** as a pale yellow solid; 2 g, (39%) HPLC purification was achieved using a linear gradient of water containing increasing amounts of  $\text{CH}_3\text{CN}$  (0  $\rightarrow$  12 min, linear gradient from 30% to 90%  $\text{CH}_3\text{CN}$  at a flow rate of 4 ml/min: 9.0 min);  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ )  $\delta$  8.08-7.99 (m, 2H), 7.82-7.69 (m, 2H), 7.31-7.25 (m, 1H), 7.07-7.06 (m, 2H), 6.71-6.69 (m, 3H), 6.59-6.58 (m, 2H), 5.43-5.537 (m, 2H), 5.22 (t,  $J = 10.5\text{Hz}$ , 2H), 4.91 (t,  $J = 9.3\text{Hz}$ , 2H), 4.22-3.99 (m, 7H), 2.06-1.98 (m, 5H), 2.04 (s, 6H), 2.01 (s, 6H), 1.93 (s, 6H), 1.42-1.37 (m, 4H), 1.23-1.13 (m, 4H), 0.77 (t,  $J = 7.2\text{Hz}$ , 6H); TOFMS  $m/z$  ( $\text{M} + \text{H}^+ + \text{C}_{20}\text{H}_{12}\text{O}_5$ ) 1407.4640 (calculated for  $\text{C}_{54}\text{H}_{63}\text{N}_2\text{O}_{21}^+ + \text{C}_{20}\text{H}_{12}\text{O}_5$ ) 1407.4608.



4

**N-((2S,3S,4R,5S)-2-(3'-((2S,5S,6R)-4,5-dihydroxy-6-(hydroxymethyl)-3-pentanamidotetrahydro-2H-pyran-2-yloxy)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-6'-yloxy)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)pentanamide (4).** To a solution of **12** (2 g, 1.9 mmol) in MeOH (10 ml) at  $-10^\circ\text{C}$  was added an excess of  $\text{NH}_3$  and the reaction mixture was allowed to warm to room temperature over a 3 h period and concentrated. Purification by column chromatography (EtOAc) and HPLC yielded **4** as a white solid; 1.1 g, (71%) HPLC purification was achieved using a linear gradient of water containing increasing amounts of  $\text{CH}_3\text{CN}$  (0

→12 min, linear gradient from 30% to 90% CH<sub>3</sub>CN at a flow rate of 4 ml/min: 4.6 min); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 8.02 (d, *J* = 3.9Hz, 1H), 7.81-7.78 (m, 1H), 7.75-7.72 (m, 3H), 7.27 (d, *J* = 3.8Hz, 1H), 6.95-6.92 (m, 2H), 6.72-6.69 (m, 4H), 5.15-5.07 (m, 6H), 4.67-4.64 (m, 2H), 3.73-3.70 (m, 4H), 3.53-3.48 (m, 2H), 3.37-3.35 (m, 3H), 3.21-3.17 (m, 3H), 2.06-2.03 (m, 4H), 1.46-1.44 (m, 4H), 1.24-1.12 (m, 4H), 0.80-0.76 (m, 6H); TOFMS *m/z* (M + Na<sup>+</sup>) 845.3134 (calculated for C<sub>42</sub>H<sub>50</sub>N<sub>2</sub>NaO<sub>15</sub><sup>+</sup>) 845.3109.

### **Expression and purification of recombinant *O*-GlcNAcase**

Cultures of TOP 10 cells containing pBAD/HisA human *O*-GlcNAcase clone expression vector were grown overnight in Luria-Bertani (LB) broth (Digene) supplemented with ampicillin (50 µg/ml) at 37 °C. Induction of *O*-GlcNAcases was initiated by adding arabinose solution (0.02%) when the OD<sub>600</sub> of cultures reached at around 0.5. The cultures were further grown for 4 hours at 30 °C at 200 rpm. The cells were harvested by centrifugation at 4000 × *g* for 15 min in a Sorvall® RC 5C Plus (DuPont) centrifuge. The pellet was subjected to freeze-thaw and suspended in 1/50 of the original volume in 0.1 mg/ml lysozyme, 20 mM Tris-HCl, pH 8.0, 1 mM DTT, 0.1% Triton X-100, an EDTA-free protease inhibitor cocktail tablet (1 tablet/10ml). The suspension was incubated at room temperature for 5 min and the lysate was sonicated on ice (4 × 10 s, setting 3, Misonix Ultrasonic Processor). The supernatant obtained after centrifugation at 20,000 × *g* for 15 min. Expressed His<sub>6</sub>-tagged *O*-GlcNAcase in the supernatant was purified on His-Trap HP column (Amersham Biosciences) using the slightly modified manufacture's protocol. Fractions (1ml) collected throughout the separation were assayed for protein (absorbance at 280 nm) and were further assayed for *O*-GlcNAcase activity according to the protocol described below. Fractions showing enzyme activity were pooled and concentrated using a Centricon 100 microconcentrator (Amicon). Pierce BCA protein assay reagent was used to estimate protein concentration. Purity of *O*-GlcNAcase was confirmed by Ponceau S staining of the membrane (Sigma) and Western Blot analysis using anti His-Tag antibody (Abcam).

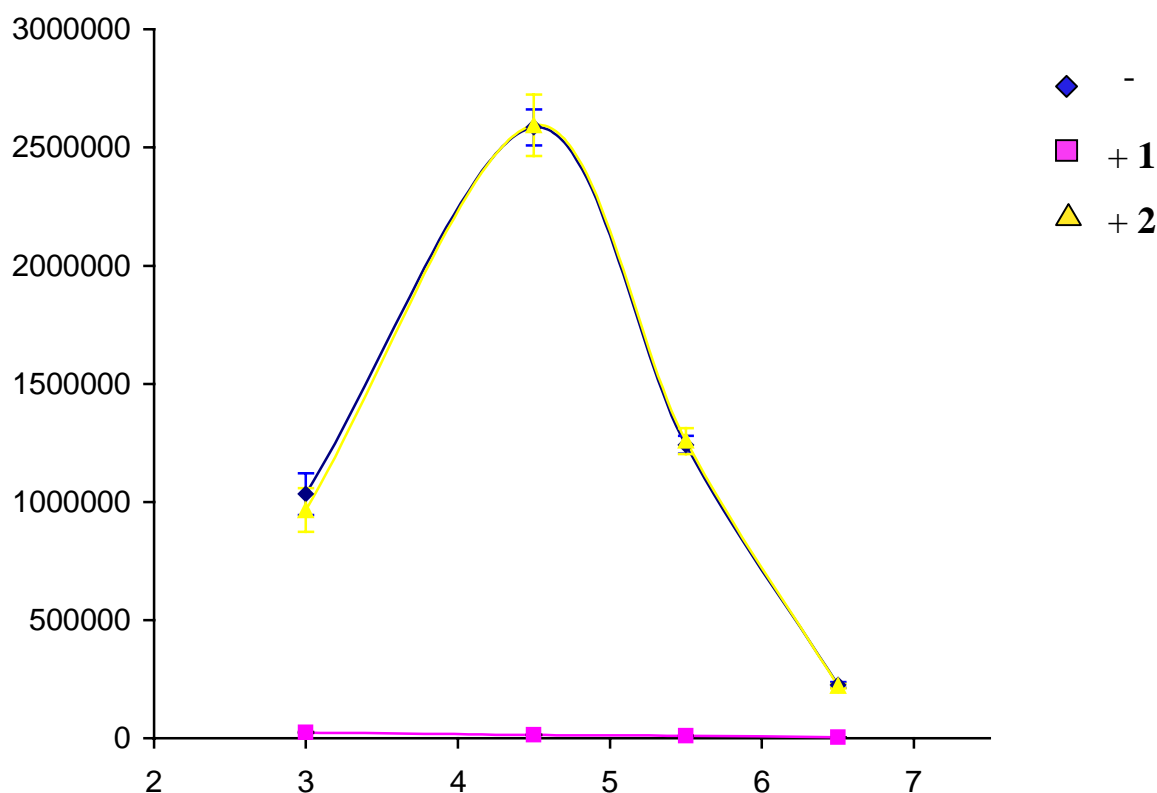
### ***O*-GlcNAcase's enzymatic assay**

*O*-GlcNAcase assays were performed in 100 µL of 0.1M citrate/phosphate buffer, pH 6.5, 50 µM of the fluorogenic substrate **3** and with 5 µL (0.69 µg) of purified recombinant *O*-GlcNAcase in the presence of 5 µM of inhibitors **1** or **2** and in the absence of the inhibitors at 37 °C for a 30 min. period. The reaction was stopped with addition of 0.9 mL of 0.5 M sodium carbonate. Two hundred microliters of assay solution was transferred into a 96 well plate and fluorescence was measured at the excitation wavelength of 485 nm and at the emission wavelength of 535 nm on the Wallac 1420 fluorometer. Assays were performed in triplicate. Percent of inhibition was calculated by comparing enzyme activity in the absence of the inhibitor with the activity in the presence of the inhibitor. Hexosaminidase A and B assays were performed in the same manner as *O*-GlcNAcase assays described above except 0.1 M citrate/phosphate buffer, pH 4.5 was used as the assay buffer. Hexosaminidase A (3-*N*-acetylglucosaminidase from bovine kidney, E.C. 3.2.1.52) and B (from bovine epididymis, E.C. 3.2.1.52) were purchased from Sigma-Aldrich. Activity exhibited by either hexoamnidase A or B is roughly 1.2-fold greater than the activity exhibited by *O*-GlcNAcase in the absence of any inhibitor at optimal pH. One unit of enzyme is defined as the amount that catalyzes

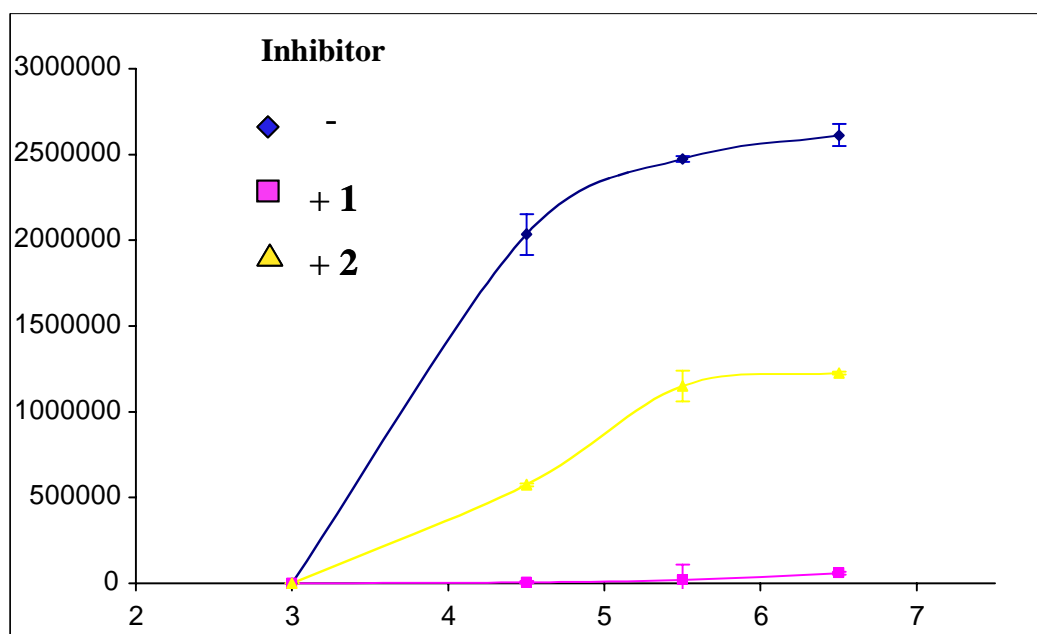
the hydrolysis of 1  $\mu$ mole of FDGlcNAc to 1 mmole of FMGlcNAc and 1  $\mu$ mole of *N*-acetyl-D-glucosamine per minute at 37°C and at optimal pH. To establish that the observed selectivity is not pH dependent a range of responses was established at pH 3.0, 4.5, 5.5 and 6.5 for both **1** and **2** (Figure s1 and s2) and the results clearly show that the inhibitory potential of both **1** and **2** is not pH dependent.

#### Analysis of fluorogenic substrate **4**.

Various stock solutions of **4** were prepared by serial dilutions in 10.5% DMSO aqueous solutions. Enzyme assays were performed as described above over 45 minute incubation times.



**Figure s1.** The activity of lysosomal hexosaminidase A at varying pH in the absence of any inhibitor (blue diamond) or in the presence of PUGNAc (**1**, pink square) and pentanamide PUGNAc derivative (**2**, yellow triangle).



**Figure s2.** The activity of O-GlcNAcase at varying pH in the absence of any inhibitor (blue diamond) or in the presence of PUGNAc (**1**, pink square) and pentanamide PUGNAc derivative (**2**, yellow triangle).



