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The synthesis and biological evaluation of some carbocyclic analogues of PUGNAc

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ABSTRACT

The synthesis of some analogues of *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino *N*-phenylcarbamate, PUGNAc, an inhibitor of β-*N*-acetylglucosaminidases, is described. The analogues were tested against a range of β-*N*-acetylglucosaminidases to establish any biological activity. As well, the analogues were tested as inhibitors of a uridine diphosphate-*N*-acetyl-D-glucosamine: polypeptidyl transferase, OGT, a critical protein involved in the post-translational modification of nuclear and cytosolic proteins by *N*-acetyl-D-glucosamine.

1. Introduction

The post-translational modification of nuclear and cytoplasmic proteins with 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc), to generate 2-acetamido-2-deoxy-β-D-glucopyranosides, is found in a wide variety of organisms, ranging from microbes to humans.[1] This monosaccharide, attached to serine or threonine residues (*O*-GlcNAc), is found on many cellular proteins having a wide range of vital functions, including those involved in transcription,[2–5] proteosomal degradation[6] and cellular signalling;[7] *O*-GlcNAc is also found on many structural proteins.[8–10] It has also been observed that the *O*-GlcNAc modification is dynamic, with cycles of addition and removal occurring several times during the lifetime of a protein.[11] A reciprocal relationship has also been noted between global levels of cellular *O*-GlcNAc and O-phosphorylation,[12] and residues that bear the *O*-GlcNAc modification are, at least in some cases, also known to be phosphorylated.[13,14] Not surprisingly, owing to the abundance of *O*-GlcNAc on intracellular proteins, this post-translational modification has been implicated in the etiology of several disease states. For example, recent studies have suggested that elevation of *O*-GlcNAc levels in adipocytes[7,15] as well as on TORC2 (Crtc2), the transducer of regulated cyclic adenosine monophosphate response element-binding protein[16] are involved in the development of insulin resistance and gluconeogenesis, respectively.

To maintain the appropriate levels of cellular *O*-GlcNAc, two different carbohydrate-processing enzymes are critical. The enzyme catalyzing the installation of *O*-GlcNAc is a known glycosyltransferase termed OGT (E.C. 2.4.1.94);[†,17,18] this enzyme has been found to be essential at the single cell level.[19] The enzyme responsible for catalyzing the cleavage of *O*-GlcNAc from modified proteins and returning them to their unmodified state is the glycoside hydrolase known as *O*-GlcNAcase (E.C. 3.2.1.52)[20,21] (Fig. 1). As a result of the biological importance of these two enzymes, the design of small molecules to inhibit their activity is receiving considerable attention. Inhibitors for *O*-GlcNAcase are known[22–27] but in the case of OGT, very few inhibitors are currently available to study the physiological role of this enzyme in terms of the *O*-GlcNAc post-translational modification. Alloxan **1** (Fig. 2) inhibits OGT with an IC_{50} of 100 μM,[28] but this inhibitor causes several other cellular effects limiting its use. Walker and co-workers have developed a high-throughput screen of small molecule libraries[29] and identified compounds **2**–**4**, all of which have moderate inhibitory potency (IC_{50} ~50 μM) towards OGT.[29] Hanover and co-workers recently developed a C-linked compound **5** that mimics the donor-sugar substrate of OGT, UDP-GlcNAc **6**. Unfortunately, **5** was found to be a poor inhibitor of the enzyme.[30]

On the other hand, more success has been realized with inhibitors of *O*-GlcNAcase. 2′-Methyl-α-D-glucopyrano-[2,1-*d*]-Δ2′-thiazoline (NAG-thiazoline) **7** (Fig. 3) has been found to be a potent

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† OGT is more correctly known as uridine diphosphate-*N*-acetyl-D-glucosamine: polypeptidyl transferase.

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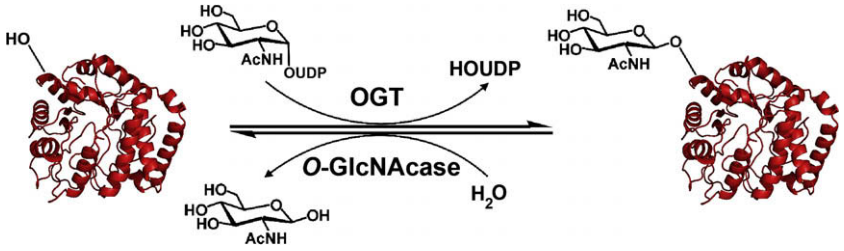


Figure 1. The *O*-GlcNAc modification of intracellular proteins is dynamic. OGT catalyzes the attachment of *N*-acetyl-D-glucosamine (GlcNAc) to serine/threonine residues in target proteins, and *O*-GlcNAcase catalyzes the hydrolysis.

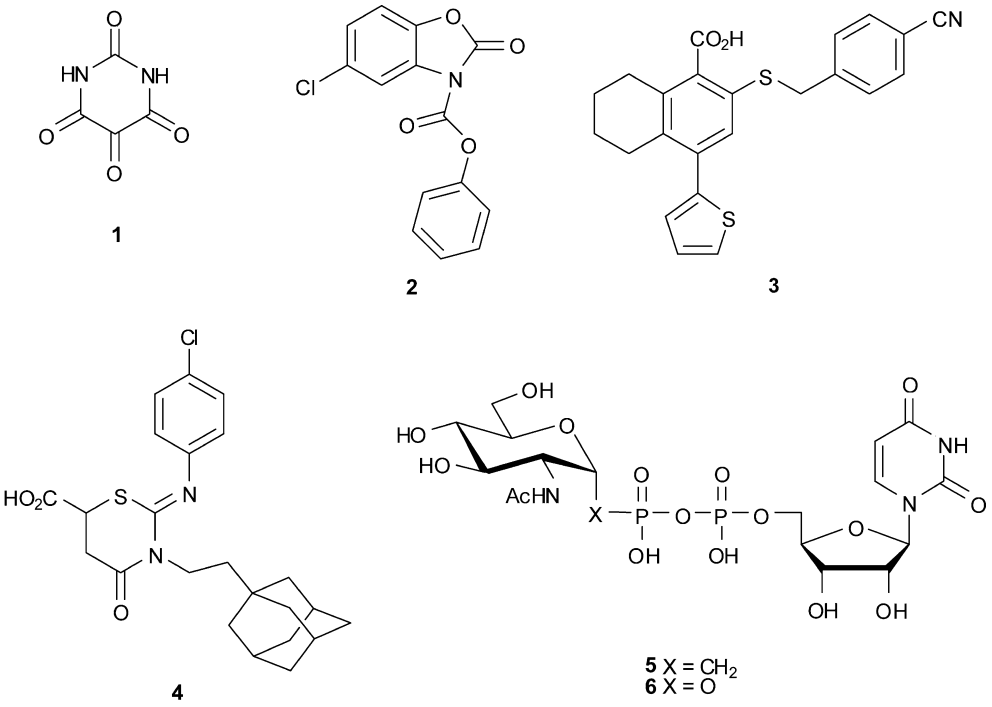


Figure 2. Some previously reported inhibitors of OGT.

inhibitor of *O*-GlcNAcase,[22] with analogues of this compound, such as **8**–**10**, showing selectivity for *O*-GlcNAcase over other mechanistically similar enzymes found in humans.[22,27] *O*-(2-Acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate (PUGNAc) **11**,[31] the tetrahydroimidazopyridines, with the most important being 8-acetamido-5,6,7,8-tetrahydro-6,7-dihydroxy-5-(hydroxymethyl)imidazo[1,2-*a*]pyridine-2-acetic acid, *gluco*-nagstatin **12**,[32] prepared by Vasella et al., as well as GlcNAcstatin **13**[26] prepared by the van Aalten group, have also been shown to be potent inhibitors of *O*-GlcNAcase.[20,22,23,26] Three structural features of PUGNAc are associated with its binding affinity to *O*-GlcNAcase. Firstly, its sp^2 hybridized carbon is thought to mimic some part of the transition state of the enzyme-catalyzed reaction.[‡] Secondly, the phenyl ring is thought to bestow significant binding energy owing to the fact that 2-acetamido-2-deoxy-D-glucohydroximino-1,5-lactone (LOGNAc) **14**[31] is a significantly weaker inhibitor.[48] Finally, the oxime nitrogen is involved in favourable hydrogen bonding to the catalytic acid residue in the active site.[§ 35]

Recently, we prepared compound **15**[36] based on the known glycoside hydrolase inhibitor, epivalienamine, the C-6 epimer of valienamine,[37] and found it had good potency against *O*-GlcNAcase (K_i = 6 μM). As a result of this work, and with the aid of a co-crystal structure of **15** in complex with a bacterial homologue of *O*-GlcNAcase,[36] we thought to increase the potency of **15** by mimicking certain elements of PUGNAc. As well, we felt that some of these compounds might be effective inhibitors of OGT because they share some structural elements common to the donor-sugar substrate as well as potentially mimicking the oxocarbenium ion-like transition state, that glycosyltransferases are thought to stabilize.[38] Thus, the compounds of interest to us were the carbamate **16**, the ethyl ester **17** and the acid **18**.

2. Results and discussion

The alkene **19**, prepared previously from 2-acetamido-2-deoxy-D-glucopyranose,[36] was subjected to a Ferrier transformation[39] to give the alcohol **20** in 53% yield (Scheme 1). A somewhat ambitious Grignard reaction was conducted on the alcohol **20**, using excess reagent, to furnish the diol **21**, which was subsequently oxidised to the ketone **22** with the Dess–Martin periodinane.[40] At this stage it was decided to investigate the possibility of eliminating water

[‡] Recently, a report suggested that PUGNAc may be a poor transition state analogue.[33]

[§] As the three-dimensional crystal structure of a true eukaryotic *O*-GlcNAcase is yet to be determined, this piece of data was obtained from a sequence-related bacterial homologue.[35]

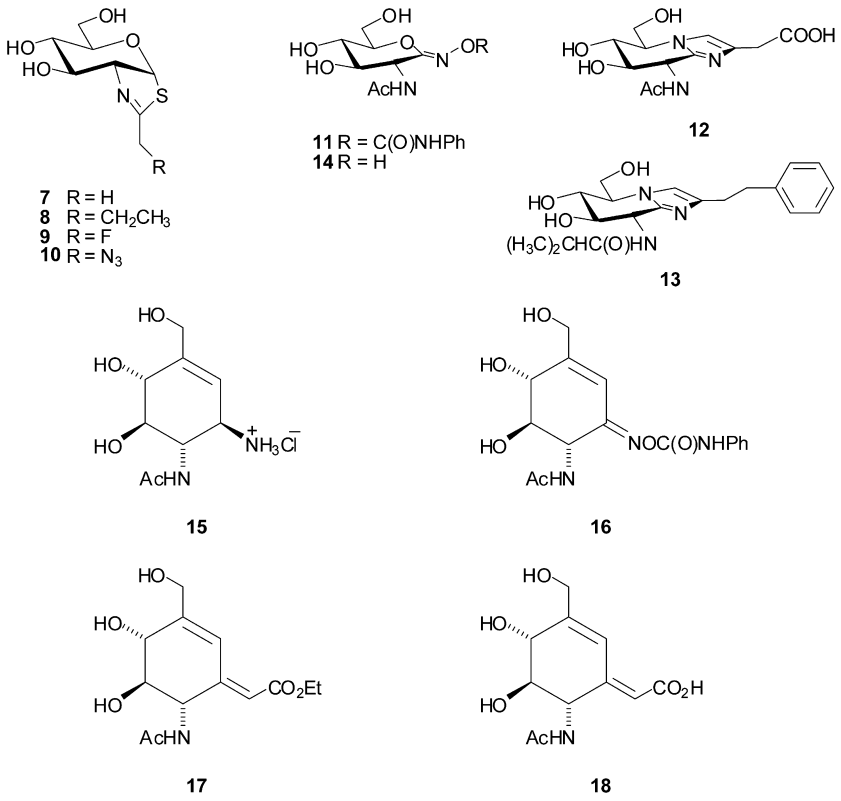


Figure 3. Some previously reported inhibitors of *O*-GlcNAcase, and compounds prepared in this study.

from across C4 and C5 of **22**, a critical procedure in the overall synthesis of the desired targets.

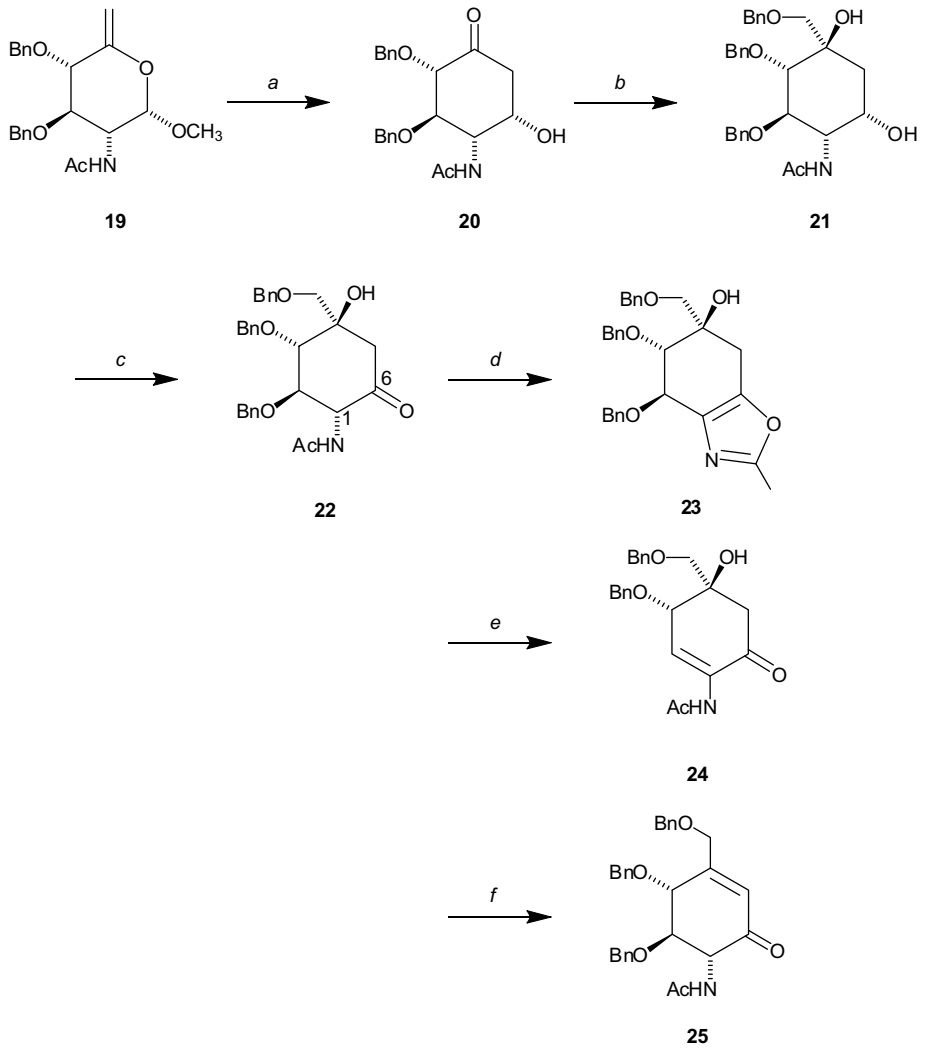
Initial attempts at the proposed dehydration involved treating **22** with trifluoromethanesulfonic anhydride and pyridine; however, only the oxazole **23** was produced. In a second attempt, the ketone **22** was treated with chloroform saturated with hydrogen chloride; however, only the enone **24** was isolated. As a last attempt, treatment of the ketone **22** with sulfuryl chloride at 0 °C resulted in the elimination of water to give the desired enone **25** in 69% yield. Having now established that dehydration across C4 and C5 was possible, we proceeded with the synthetic route. The ketone **22** was converted into the oxime **26** in 86% yield (Scheme 2); the assignment of stereochemistry of this molecule was based on previous work by Abell and co-workers.[41] Treatment of the oxime **26** with phenyl isocyanate furnished the phenylcarbamate **27**, which was dehydrated to afford the alkene **28**. Removal of the benzyl ethers was effected using ferric chloride.[42] Acetylation to give the triacetate **29** and subsequent deacetylation aided isolation of the desired carbamate **16**. Attention was then turned to the ester **17** and the acid **18**.

With the ketone **22** in hand, the alkene **30** was prepared using a Wittig olefination involving ethyl (triphenylphosphoranylidene)acetate,[43] with the stereochemistry once again tentatively assigned (Scheme 3). It has been shown that in order to minimize dipole–dipole interactions during the Wittig olefination, ylides containing stabilizing electron-withdrawing substituents, such as esters, react with gluco- and galacto-configured substrates to give predominately *Z*-isomers while manno-configured substrates result in *E*-isomers.[44,45] With this in mind, an *E* configuration was assigned for the alkene **30** in order to avoid repulsion between the ester and acetamido moieties.[¶] The diene **31** was prepared from the alkene **30** via the established dehydration protocol. Removal of the benzyl ethers from **31**, followed by acetylation, afforded the triacetate **32**. Subsequent deacetylation of **32** with sodium ethoxide (in order to avoid transesterification of the ethyl ester) yielded the triol **17**. On the other hand, to gain access to the acid **18**, the sodium ethoxide was replaced with sodium hydroxide to afford **18** in good yield.

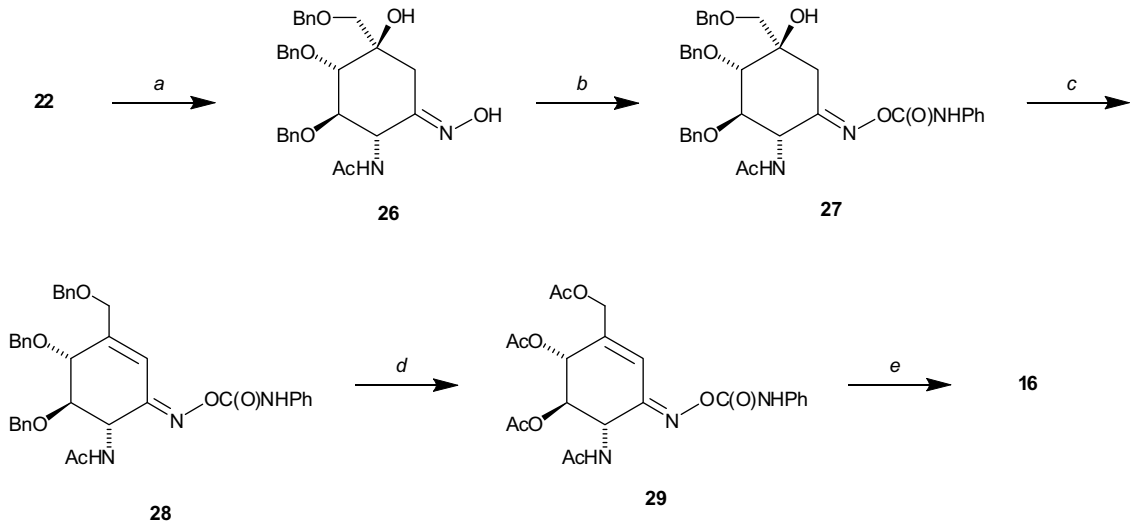
At this point, we evaluated the prepared compounds **16**, **17** and **18** against *O*-GlcNAcase. Unfortunately, none of the compounds inhibited the enzyme significantly (Table 1). Thus, it was decided to evaluate the compounds against other known β-*N*-acetylglucosaminidases. The enzymes chosen for evaluation were *Bt*GH84,[34] which is a close bacterial homologue of *O*-GlcNAcase; human placental β-hexosaminidase, which is an enzyme that has a catalytic mechanism similar to that of *O*-GlcNAcase but acts on various glycosphingolipids; and finally NagZ, a β-*N*-acetylglucosaminidase involved in the peptidoglycan recycling pathway used by Gram-negative bacteria. Again, no significant inhibition was observed with any of these enzymes, with the exception of NagZ, which showed high micromolar inhibition with the carbamate **16** and the acid **18** (Table 1). These results seem to lend credence to what is observed in the co-crystal structural of **15** with *Bt*GH84:[36] the important structural motif of **15**, and potentially the reason for its good potency against *O*-GlcNAcase, is the amino group, in that it establishes powerful electrostatic interactions with an active site residue. Any changes that remove this interaction (e.g., incorporating a phenylcarbamate moiety to mimic PUGNAc) seem to reduce greatly the affinity of the resultant molecule.

We next evaluated the three synthetic compounds as inhibitors of OGT, using recombinant OGT[18] and monitoring the *O*-GlcNAc modification of recombinantly expressed nuclear pore protein, p62, via Western blot.[8] To confirm that the p62 glycosylation assay was viable, various controls were performed (Fig. 4). The results clearly indicate that only in the presence of all of the reagents was p62 *O*-GlcNAc modified, and that the assay appeared approx-

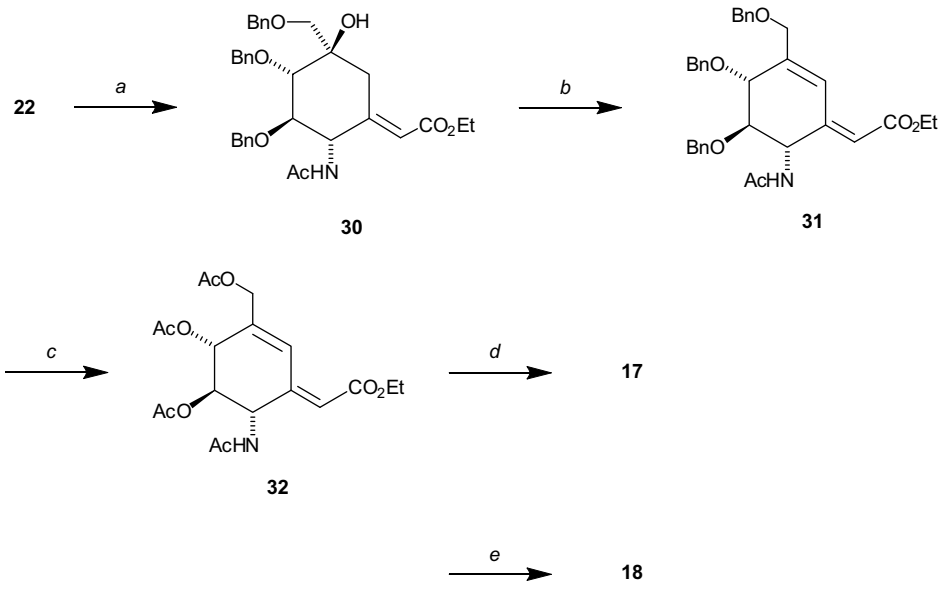
[¶] Note the priority changes between the pyranose and cyclohexane ring.



Scheme 1. Reagents and conditions: (a) $HgSO_4$, H_2SO_4, dioxane, H_2O, 53%; (b) Mg, $BnOCH_2Cl$, $HgCl_2$, THF, 53%; (c) Dess–Martin periodinane, CH_2Cl_2, 90%; (d) Tf_2O, pyridine, CH_2Cl_2, 58%; (e) HCl, $CHCl_3$, 89%; (f) SO_2Cl_2, pyridine, CH_2Cl_2, 69%.



Scheme 2. Reagents and conditions: (a) $NH_2OH{\cdot}HCl$, NaOAc, CH_3OH, 86%; (b) PhNCO, Et_3N, THF, 69%; (c) SO_2Cl_2, pyridine, CH_2Cl_2, 86%; (d) (i) $FeCl_3$, CH_2Cl_2; (ii) Ac_2O, 48% over two steps; (e) $NaOCH_3$, CH_3OH, 95%.



Scheme 3. Reagents and conditions: (a) Ph_3PCHCO_2Et, CH_2Cl_2, 78%; (b) SO_2Cl_2, pyridine, CH_2Cl_2, 67%; (c) (i) $FeCl_3$, CH_2Cl_2; (ii) Ac_2O, 81% over two steps (d) NaOEt, EtOH, 79%; (e) NaOH, EtOH, H_2O, 75%.

Table 1
Inhibition constants determined for **16**, **17** and **18** against *O*-GlcNAcase and other β-*N*-acetylglucosaminidases

Enzyme	Compound K_i (mM)		
	16	**17**	**18**
Human *O*-GlcNAcase	>5	>5	>5
Bacterial *O*-GlcNAcase	>5	>5	>5
β-Hexosaminidase	>2.2	>5	>5
NagZ	0.95	3.1	0.75

imately linear over the 5h reaction time. We next turned our attention to observing the effect of **15**, **16**, **17** and **18** on OGT activity. Co-incubation of p62 with UDP-GlcNAc, OGT, and each of the synthetic compounds (1 μM–1 mM concentrations) failed to show any detectable differences in the intensity of the band corresponding to glycosylated p62 when compared to the control (Fig. 5).

3. Conclusion

We have presented an extension of some of the chemistry of the known inhibitor of *O*-GlcNAcase, **15**, in the synthesis of the molecules **16**–**18**. Based on the kinetic results obtained against *O*-GlcNAcase with the synthetic molecules, it seems that modification of the inhibitor by incorporating an sp^2 hybridized carbon (as found in PUGNAc) results in poorer inhibition of the enzyme. As well, removal of the amine functionality from the epivalienamine scaffold also results in poor binding of the compounds.

4. Experimental

4.1. General methods

^1H and ^{13}C NMR spectra were obtained on a BrukerAV600 (600 MHz for ^1H and 150.9 MHz for ^{13}C) spectrometer. Unless stated otherwise, deuterated chloroform ($CDCl_3$) was used as the solvent with $CHCl_3$ (δ_H 7.26) or $CDCl_3$ (δ_C 77.0) being employed as internal standards. NMR spectra run in D_2O used internal CH_3OH (δ_H 3.34, δ_C 49.0) as the standard. Melting points were determined on a Reichert hot stage melting point apparatus. Optical rotations were performed with a Perkin–Elmer 141 Polarimeter in a microcell (1 mL, 10 cm path length) in $CHCl_3$ at room temperature, unless stated otherwise. Mass spectra were recorded with a VG-Autospec spectrometer using the fast atom bombardment (FAB)

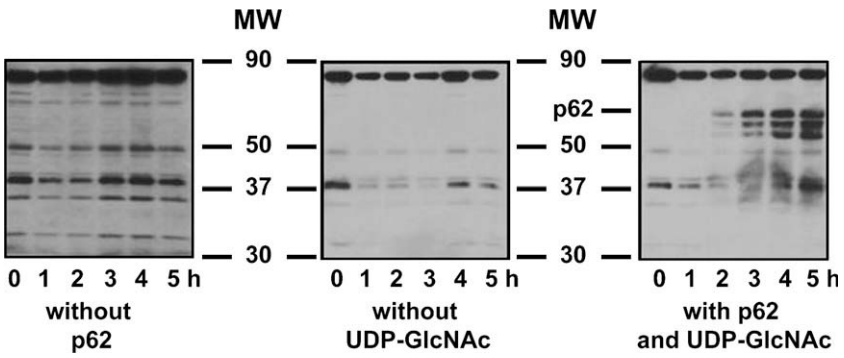


Figure 4. Western blot analysis showing assay validation. Controls to establish the *O*-GlcNAc modification assay of recombinant nuclear pore protein p62 by OGT monitored using Western blot with CTD110.6 as the primary antibody to detect *O*-GlcNAc modified proteins. From left to right; glycosylation assay performed without p62, glycosylation assay performed without UDP-GlcNAc, and glycosylation assay with all reagents to establish the approximate linearity of the assay. Bands at positions corresponding to proteins other than p62 stem from lack of specificity of CTD110.6 binding when used with the unoptimized conditions for this assay.

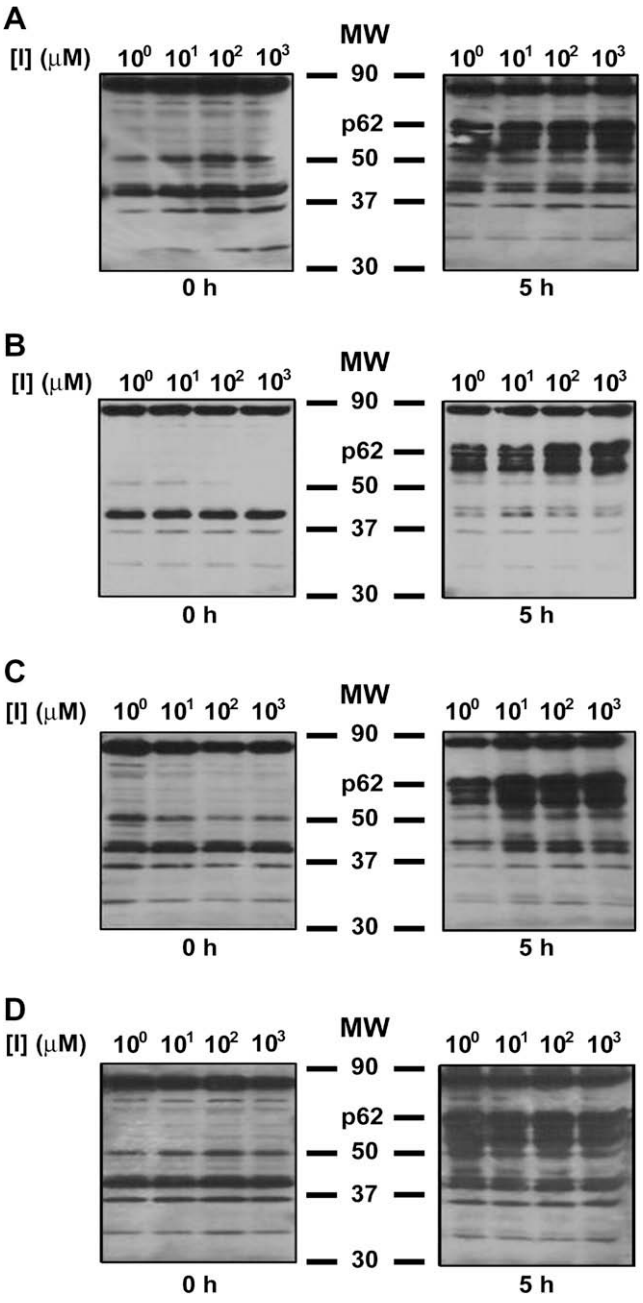


Figure 5. Inhibition assay of OGT versus **15**, **16**, **17** and **18**. The extent of the *O*-GlcNAc modification of p62 was monitored at 0 and 5 h with various concentrations (1 μM, 10 μM, 100 μM and 1 mM) of (A) **15**, (B) **16**, (C) **17** and (D) **18**. The *O*-GlcNAc modification of p62 was monitored using Western blot with CTD110.6 as the primary antibody to monitor *O*-GlcNAc modification of p62 in this assay. Bands at positions corresponding to proteins other than p62 stem from lack of specificity of CTD110.6 binding when used with the unoptimized conditions for this assay.

technique, with 3-nitrobenzyl alcohol as a matrix, unless otherwise stated. Elemental analyses of all synthesized compounds used in enzyme assays were performed at the Simon Fraser University. Flash chromatography was performed on BDH silica gel or Geduran Silica Gel 60 with the specified solvents. Thin-layer chromatography (TLC) was effected on Merck Silica Gel 60 F254 aluminum-backed plates that were stained by heating (>200 °C) with 5% sulfuric acid in EtOH. Percentage yields for chemical reactions as described are quoted only for those compounds that were purified by recrystallization or by column chromatography, and the purity was assessed by TLC or ^{1}H NMR spectroscopy. All solvents except DMF and MeCN were distilled before use and dried according to the methods of Burfield and Smithers.[46] 'Usual workup' refers to dilution with water, repeated extraction into an organic solvent, sequential washing of the combined extracts with hydrochloric acid (1 M, where appropriate), saturated aqueous sodium bicarbonate and brine solutions, followed by drying over anhydrous magnesium sulfate, filtration and evaporation of the solvent by means of a rotary evaporator at reduced pressure. All buffer salts used in this study were obtained from Bioshop. Milli-Q (18.2 MΩ cm^{-1}) water was used to prepare all buffers. β-Hexosaminidase (lot 043K3783) and 4-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside were purchased from Sigma. The mouse anti-*O*-GlcNAc monoclonal IgM antibody (mAb CTD110.6) was purchased from Covance.

4.2. *N*-[(1*S*,2*R*,3*S*,6*S*)-2,3-Dibenzyloxy-6-hydroxy-4-oxocyclohexyl]acetamide (20)

Mercury(II) sulfate (200 mg, 0.70 mmol) was added to **19** (3.6 g, 12 mmol) in dioxane–aqueous H_2SO_4 (5 mM) (2:1, 120 mL), and the mixture was heated at 80 °C (3 h). The mixture was cooled and subjected to a usual workup (CH_2Cl_2) to yield a colourless powder that was washed with Et_2O to afford **20** (2.4 g, 53%); mp 226–228 °C; $[\alpha]_D$ −50.0 (*c* 1.0, CH_3OH); ^{1}H NMR (600 MHz, CD_3OD) 1.95 (s, 3H, $COCH_3$), 2.47 (dd, 1H, $J_{5,5}$ = 14.2, $J_{5,6}$ = 3.5 Hz, H-5), 2.85 (dd, 1H, $J_{5,6}$ = 2.5 Hz, H-5), 3.87 (dd, 1H, $J_{1,2}$ = 10.0, $J_{2,3}$ = 9.4 Hz, H-2), 4.07–4.11 (m, 1H, H-6), 4.32 (d, 1H, H-3), 4.36–4.40 (m, 1H, H-1), 4.51, 4.88 (ABq, 2H, *J* = 11.4 Hz, C*H*$_2$Ph), 4.61, 4.80 (ABq, 2H, *J* = 11.1 Hz, C*H*$_2$Ph), 7.23–7.32 (m, 10H, Ph), 7.39 (d, 1H, $J_{1,NH}$ = 7.2 Hz, N–H); ^{13}C NMR (150.9 MHz, CD_3OD) 21.24 ($COCH_3$), 45.12 (C-5), 54.42, 67.17, 80.15, 86.44 (C-1, C-2, C-3, C-6), 73.17, 74.32 (2C, *C*H$_2$Ph), 127.00–138.58 (Ph), 171.78 (*C*OCH$_3$), 205.55 (C-4); HR-MS *m/z* (FAB) 384.1819; $[M+H]^+$ requires 384.1811.

4.3. *N*-[(1*S*,2*R*,3*S*,4*R*,6*S*)-2,3-Benzyloxy-4-benzyloxymethyl-4,6-dihydroxycyclohexyl]acetamide (21)

Magnesium (500 mg, 21 mmol), benzyl chloromethyl ether (2.5 mL, 16 mmol) and $HgCl_2$ (70 mg, 0.30 mmol) in THF (25 mL) were stirred at 0 °C (1.5 h). The ketone **20** (300 mg, 0.8 mmol) in THF (20 mL) was added at −78 °C, and the mixture was stirred at 0 °C (1 h) before being quenched with saturated $NaHCO_3$ solution. The suspension was filtered through Celite, followed by a usual workup (EtOAc), to give a pale yellow oil that was subjected to flash chromatography (EtOAc) to yield **21** as colourless needles (210 mg, 53%); mp 110–112 °C; $[\alpha]_D$ +29.3 (*c* 1.0, $CDCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) 1.87 (s, 3H, $COCH_3$), 1.97 (dd, 1H, $J_{5,5}$ = 15.3, $J_{5,6}$ = 3.1 Hz, H-5), 2.02 (dd, 1H $J_{5,6}$ = 3.0 Hz, H-5), 3.21, 3.44 (Abq, 2H, $J_{7,7}$ = 8.7 Hz, H-7), 3.82 (d, 1H, $J_{2,3}$ = 9.3 Hz, H-3), 3.84 (dd, 1H, $J_{1,2}$ = 10.3 Hz, H-2), 3.93–3.95 (m, 1H, H-6), 4.08–4.12 (m, 1 H, H-1), 4.42, 4.47 (ABq, 2H, *J* = 11.9 Hz, C*H*$_2$Ph), 4.55, 4.89 (ABq, 2H, *J* = 10.8, C*H*$_2$Ph), 4.68, 4.83 (ABq, 2H, *J* = 11.4 Hz, C*H*$_2$Ph), 6.18 (d, 1H, $J_{1,NH}$ = 9.3 Hz, N–H), 7.22–7.35 (m, 15H, Ph); ^{13}C NMR (150.9 MHz, $CDCl_3$) 23.23 ($COCH_3$), 35.15 (C-5), 54.71, 69.98, 78.85, 81.50 (C-1, C-2, C-3, C-6), 72.99, 73.08, 75.24, 75.39 (4C, C-7, *C*H$_2$Ph), 76.65 (C-4), 127.47–138.25 (Ph), 169.95 (*C*OCH$_3$); HR-MS *m/z* (FAB) 506.2569; $[M+H]^+$ requires 506.2543.

4.4. *N*-[(1*R*,2*R*,3*S*,4*R*)-2,3-Dibenzyloxy-4-benzyloxymethyl-4-hydroxy-6-oxocyclohexyl]acetamide (22)

Dess–Martin periodinane (360 mg, 0.85 mmol) was added to **21** (200 mg, 0.40 mmol) in CH_2Cl_2 (10 mL), and the solution was kept (1 h). The reaction mixture was diluted with CH_2Cl_2, washed with aqueous $Na_2S_2O_3$ and then saturated with aqueous $NaHCO_3$, and then dried over $MgSO_4$. Filtration followed by evaporation returned

a colourless solid that was washed with Et_2O to return **22** as a colourless powder (180 mg, 90%); mp 156–158 °C; $[\alpha]_D$ −9.8 (*c* 1.0, CH_3CN); ^1H NMR (600 MHz, CD_3CN) 1.90 (s, 3H, $COCH_3$), 2.35, 2.95 (ABq, 2H, $J_{5/7,5/7}$ = 14.6 Hz, H-5/7), 3.22, 3.65 (ABq, 2H, $J_{7/5,7/5}$ = 8.8 Hz, H-7/5), 3.84 (dd, 1H, $J_{1,2}$ = 10.3, $J_{2,3}$ = 9.5 Hz, H-2), 4.05 (m, 1H, H-3), 4.50, 4.54 (ABq, 2H, *J* = 12.0 Hz, CH_2Ph), 4.65, 4.75 (ABq, 2H, *J* = 11.0 Hz, CH_2Ph), 4.66, 4.88 (ABq, 2H, *J* = 11.0 Hz, CH_2Ph), 4.73–4.76 (m, 1H, H-1), 6.60 (d, 1H, $J_{1,NH}$ = 9.0 Hz, N–H), 7.25–7.38 (m, 15 H, Ph); ^{13}C NMR (150.9 MHz, CD_3CN) 23.05 ($COCH_3$), 47.83 (C-5), 61.95, 82.68, 83.08 (C-1, C-2, C-3), 73.92, 74.00, 76.04, 76.15 (4C, C-7, CH_2Ph), 75.16 (C-4), 128.55–139.72 (Ph), 170.92 ($COCH_3$), 203.68 (C-6); HR-MS *m/z* (FAB) 504.2423; $[M+H]^+$ requires 504.2386.

4.5. (4*R*,5*S*,6*R*)-4,5-Dibenzyloxy-6-benzyloxymethyl-2-methyl-4,5,6,7-tetrahydrobenzoxazol-6-ol (23)

Trifluoromethanesulfonic anhydride (140 mg, 0.50 mmol) in CH_2Cl_2 (1 mL) was added dropwise to **22** (50 mg, 0.10 mmol) in pyridine–CH_2Cl_2 (5 mL, 1:9) at 0 °C, and the solution was kept (1 h). The reaction mixture was quenched with CH_3OH (2 mL) and subjected to a usual workup (CH_2Cl_2), followed by flash chromatography (EtOAc–petrol, 3:7), to yield **23** as a gum (28 mg, 58%); $[\alpha]_D$ −27.6 (*c* 1.0, CH_3CN); ^1H NMR (600 MHz, CD_3CN) 2.38 (s, 3H, CH_3), 2.65, 3.04 (ABq, 2H, $J_{8,8}$ = 16.7 Hz, H-8), 3.25 (br s, 1H, OH), 3.44, 3.67 (ABq, 2H, $J_{7,7}$ = 9.3 Hz, H-7), 3.86 (d, 1H, $J_{4,5}$ = 5.3 Hz, H-5), 4.49, 4.54 (ABq, 2H, *J* = 12.0 Hz, CH_2Ph), 4.55–4.57 (m, 1H, H-4), 4.67, 4.82 (ABq, 2H, *J* = 11.3 Hz, CH_2Ph), 4.77, 4.95 (ABq, 2H, *J* = 11.6 Hz, CH_2Ph), 7.26–7.38 (m, 15H, Ph); ^{13}C NMR (150.9 MHz, CD_3CN) 14.20 (CH_3), 30.96 (C-7), 72.93, 73.95, 74.48, 75.33 (4C, C-8, CH_2Ph), 75.78, 82.06 (C-4, C-5), 76.25 (C-6), 128.43–139.91 (Ph), 132.92, 146.44 (C-1, C-3), 162.06 (C-2); HR-MS *m/z* (FAB) 486.2267; $[M+H]^+$ requires 486.2280.

4.6. *N*-[(3*S*,4*R*)-3-Benzyloxy-4-benzyloxymethyl-4-hydroxy-6-oxocyclohexenyl]acetamide (24)

Hydrogen chloride gas was bubbled through $CHCl_3$ until the mixture was saturated. One drop of this solution was added to the ketone **22** (50 mg, 0.10 mmol) in $CHCl_3$ (5 mL), and the solution was kept (2 h). Concentration of the mixture followed by flash chromatography ((EtOAc–petrol, 3:7) yielded **24** as an oil (35 mg, 89%); $[\alpha]_D$ +87.2 (*c* 1.0, $CDCl_3$); ^1H NMR (600 MHz) 2.12 (s, 3H, $COCH_3$), 2.69, 2.84 (ABq, 2H, $J_{5/7,5/7}$ = 17.0 Hz, H-5/7), 3.20, 3.56 (ABq, 2H, $J_{7/5,7/5}$ = 9.0 Hz, H-7/5), 4.40, 4.46 (ABq, 2H, *J* = 11.9 Hz, CH_2Ph), 4.55, 4.82 (ABq, 2H, *J* = 11.4 Hz, CH_2Ph), 4.62 (d, 1H, $J_{2,3}$ = 2.8 Hz, H-3), 7.23–7.36 (m, 10H, Ph), 7.68 (d, 1H, H-2), 7.83 (br s, 1H NH); ^{13}C NMR (150.9 MHz) 24.50 ($COCH_3$), 43.56 (C-5), 72.15, 72.72, 73.21 (3C, C-7, CH_2Ph), 73.17 (C-3), 75.30 (C-4), 122.71 (C-2), 127.66–137.51 (Ph), 131.75 (C-1), 168.92 ($COCH_3$), 192.18 (C-6); HR-MS *m/z* (FAB) 396.1841; $[M+H]^+$ requires 396.1811.

4.7. *N*-[(1*R*,5*R*,6*R*)-5,6-Dibenzyloxy-4-benzyloxymethyl-2-oxocyclohex-3-enyl]acetamide (25)

Sulfuryl chloride (15 mg, 0.10 mmol) in CH_2Cl_2 (1 mL) was added dropwise to **22** (30 mg, 0.06 mmol) and pyridine (0.3 mL) in CH_2Cl_2 (5 mL) at 0 °C (1 h). The reaction mixture was diluted with CH_2Cl_2, washed with 2 M citric acid solution and then saturated with aqueous $NaHCO_3$, followed by drying over $MgSO_4$. Filtration followed by evaporation and flash chromatography (EtOAc–petrol, 1:1) yielded **25** as a gum (20 mg, 69%); $[\alpha]_D$ −15.2 (*c* 1.0, CH_3CN); ^1H NMR (300 MHz, CD_3CN) 1.90 (s, 3H, $COCH_3$), 4.00 (dd, 1H, $J_{1,6}$ = 11.1, $J_{5,6}$ = 8.4 Hz, H-6), 4.15–4.35 (m, 2H, H7), 4.50–4.60 (m, 4 H, H-1, H-5, CH_2Ph), 4.68, 4.76 (ABq, 2H, *J* = 11.2 Hz, CH_2Ph), 4.74, 4.88 (ABq, 2H, *J* = 11.0 Hz, CH_2Ph), 6.10–6.15 (m, 1H, H-3), 6.73 (d, 1H, $J_{1,NH}$ = 8.6 Hz, N–H), 7.27–7.40 (m, 15H, Ph); ^{13}C NMR (75.5 MHz, CD_3CN) 23.07 ($COCH_3$), 60.40, 80.73, 84.07 (C-1, C-5, C-6), 69.53 (C-7), 73.43, 75.62, 76.23 (3C, CH_2Ph), 124.23 (C-3), 128.71–139.40 (Ph), 161.34 (C-4), 171.10 ($COCH_3$), 194.69 (C-2), HR-MS *m/z* (FAB) 486.2287; $[M+H]^+$ requires 486.2280.

4.8. *N*-[(*E*)-(1*S*,2*R*,3*S*,4*R*)-2,3-Dibenzyloxy-4-benzyloxymethyl-4-hydroxy-6-(hydroxyamino)cyclohexyl]acetamide (26)

Hydroxylamine hydrochloride (20 mg, 0.30 mmol) was added to **22** (45 mg, 0.10 mmol) and $NaOAc{\cdot}3H_2O$ (60 mg, 0.40 mmol) in DMF–MeOH (5 mL, 1:19), and the solution was kept overnight. The mixture was concentrated and subjected to a usual workup (CH_2Cl_2), followed by flash chromatography (EtOAc–petrol, 4:1), to afford a colourless residue that was crystallized to yield **26** as a colourless powder (40 mg, 86%); mp 128–130 °C (Et_2O); $[\alpha]_D$ +3.7 (*c* 1.0, CH_3CN); ^1H NMR (600 MHz, CD_3CN) 1.88 (s, 3H, $COCH_3$), 2.18, 3.26 (ABq, 2H, $J_{5/7,5/7}$ = 15.1 Hz, H-5/7), 3.05 (br s, 1H, OH), 3.30, 3.59 (ABq, 2H, $J_{7/5,7/5}$ = 8.9 Hz, H-7/5), 3.74 (dd, 1H, $J_{1,2}$ = 9.3, $J_{2,3}$ = 8.8 Hz, H-2), 3.77 (d, 1H, H-3), 4.48, 4.53 (ABq, 2H, *J* = 12.0 Hz, CH_2Ph), 4.60, 4.83 (ABq, 2H, *J* = 11.1 Hz, CH_2Ph), 4.67, 4.74 (ABq, 2 H, *J* = 11.0 Hz, CH_2Ph), 4.67–4.72 (m, 1H, H-1), 6.63 (d, 1H, $J_{1,NH}$ = 9.5 Hz, N–H), 7.27–7.37 (m, 15H, Ph), 8.80 (br s, 1H, NOH); ^{13}C NMR (150.9 MHz, CD_3CN) 23.30 ($COCH_3$), 31.17 (C-5), 54.69, 82.69, 83.54 (C-1, C-2, C-3), 73.93, 74.45, 75.93, 75.98 (4C, C-7, CH_2Ph), 74.89 (C-4), 128.49–139.84 (Ph), 154.36 (C-6), 170.54 ($COCH_3$); HR-MS *m/z* (FAB) 519.2503; $[M+H]^+$ requires 519.2495.

4.9. *O*-[(2*S*,3*R*,4*S*,5*R*)-2-Acetamido-3,4-dibenzyloxy-5-benzyloxymethyl-5-hydroxycyclohexylidene]amino *N*-Phenylcarbamate (27)

Phenyl isocyanate (0.20 mL, 1.7 mmol) was added to **26** (100 mg, 0.20 mmol) and Et_3N (0.5 mL) in THF (10 mL), and the solution was kept (3 h). Concentration of the mixture followed by flash chromatography (EtOAc–petrol, 2:3 to 1:1) gave **27** as a colourless powder (85 mg, 69%); mp 187–189 °C; $[\alpha]_D$ +23.3 (*c* 1.0, CH_3CN); ^1H NMR (600 MHz, CD_3CN) 1.94 (s, 3H, $COCH_3$), 2.45 (dd, 1H, $J_{7,7}$ = 14.9, $J_{7,OH}$ = 1.5 Hz, H-7), 3.24 (d, 1H, OH), 3.28, 3.60 (ABq, 2H, $J_{6,6}$ = 8.9 Hz, H-6), 3.34 (d, 1H, H-7), 3.80–3.84 (m, 2H, H-3, H-4), 4.48, 4.53 (ABq, 2H, *J* = 12.0 Hz, CH_2Ph), 4.57, 4.82 (ABq, 2H, *J* = 11.0 Hz, CH_2Ph), 4.66, 4.75 (ABq, 2H, *J* = 11.0 Hz, CH_2Ph), 4.86–4.91 (m, 1H, H-2), 6.87 (d, 1H, $J_{2,NH}$ = 9.4 Hz, N–H), 7.08–7.48 (m, 20H, Ph), 8.39 (s, 1H, N–H); ^{13}C NMR (150.9 MHz, CD_3CN) 23.35 ($COCH_3$), 33.78 (C-6), 54.99, 82.47, 83.57 (C-2, C-3, C-4), 73.98, 74.09, 76.16, 76.30 (4C, C-7, CH_2Ph), 75.17 (C-5), 120.64–139.63 (Ph), 153.01 (C-1), 162.14 (CNHPh), 170.01 ($COCH_3$); HR-MS *m/z* (FAB) 638.2841; $[M+H]^+$ requires 638.2866.

4.10. *O*-[(4*R*,5*R*,6*S*)-6-Acetamido-4,5-dibenzyloxy-3-benzyloxymethylcyclohex-2-enylidene]amino *N*-Phenylcarbamate (28)

Sulfuryl chloride (25 mg, 0.20 mmol) in CH_2Cl_2 (1 mL) was added dropwise to **27** (55 mg, 0.10 mmol) and pyridine (0.5 mL) in CH_2Cl_2 (6 mL) at −70 °C, and the mixture was kept (1 h). The reaction mixture was diluted with CH_2Cl_2, washed with 2 M citric acid and then saturated with aqueous $NaHCO_3$ solution, followed by drying over $MgSO_4$. Filtration followed by evaporation and flash chromatography (EtOAc–petrol, 2:3) afforded a powder that was washed with EtOAc–Et_2O (1:1) to leave **28** as a colourless powder (46 mg, 86%); mp 182–184 °C; $[\alpha]_D$ −52.4 (*c* 1.0, CH_3CN); ^1H NMR (600 MHz, CD_3CN) 1.85 (s, 3H, $COCH_3$), 4.02 (dd, 1H, $J_{4,5}$ = 6.4,

$J_{5,6}$ = 4.6 Hz, H-5), 4.17–4.23 (m, 2H, H-7), 4.24 (d, 1H, H-6), 4.51, 4.55 (ABq, 2H, J = 11.8 Hz, CH_2Ph), 4.59, 4.70 (ABq, 2H, J = 11.1 Hz, CH_2Ph), 4.66, 4.70 (ABq, 2H, J = 11.4 Hz, CH_2Ph), 5.03 (d, 1H, H-4), 6.93 (s, 1H, H-2), 7.08–7.50 (m, 20H, Ph); ^{13}C NMR (150.9 MHz) 23.33 (CO*C*H$_3$), 50.67, 76.71, 77.89 (C-4, C-5, C-6), 70.80, 73.63, 74.20, 75.01 (4C, C-7, *C*H$_2$Ph), 113.70 (C-2), 120.84–139.25 (Ph), 151.28, 153.29, 156.45 (C-1, C-3, *C*NHPh), 171.51 (*C*OCH$_3$); HR-MS *m/z* (FAB) 620.2731; [M+H]$^+$ requires 620.2761.

4.11. *O*-[(4*R*,5*R*,6*S*)-6-Acetamido-4,5-diacetoxy-3-acetoxymethylcyclohex-2-enylidene]amino *N*-Phenylcarbamate (29)

Anhydrous ferric chloride (300 mg, 1.9 mmol) was added to **28** (110 mg, 0.20 mmol) in dry CH_2Cl_2 at 0 °C. The mixture was stirred at room temperature (2 h) before being treated with Ac_2O (1.0 mL). The mixture was kept (0.5 h) before being quenched with saturated $NaHCO_3$ solution, followed by a usual workup (CH_2Cl_2), to leave a solid. Flash chromatography (EtOAc–petrol, 3:2) yielded a powder that was washed with EtOAc–Et_2O (1:1) to furnish **29** as a colourless powder (40 mg, 48%); mp 166–168 °C, $[\alpha]_D$ −52.6 (*c* 1.0, CH_3CN); ^{1}H NMR (600 MHz, CD_3CN) 1.95, 2.00, 2.05, 2.07 (4s, 12H, COCH_3), 4.66–4.70 (m, 1H, H-7), 4.76–4.80 (m, 1H, H-7), 5.12 (dd, 1H, $J_{5,6}$ = 9.7, $J_{6,NH}$ = 8.5 Hz, H-6), 5.28 (dd, 1H, $J_{4,5}$ = 7.2 Hz, H-5), 5.82–5.85 (m, 1H, H-4), 6.75 (d, 1H, N–H), 6.99–7.01 (m, 1H, H-2), 7.11–7.52 (m, 5H, Ph), 8.38 (s, 1H, N–H); ^{13}C NMR (150.9 MHz, CD_3CN) 20.87, 20.91, 23.07 (CO*C*H$_3$), 50.60, 70.18, 72.76 (C-4, C-5, C-6), 63.44 (C-7), 116.28 (C-2), 120.61, 125.01, 130.01, 138.68 (Ph), 146.42, 152.35, 155.04 (C-1, C-3, *C*NHPh), 170.79, 170.82, 170.92, 170.99 (4C, *C*OCH$_3$); HR-MS *m/z* (FAB) 476.1664; [M+H]$^+$ requires 476.1669.

4.12. *O*-[(4*R*,5*R*,6*S*)-6-Acetamido-4,5-dihydroxy-3-hydroxymethylcyclohex-2-enylidene]amino *N*-Phenylcarbamate (16)

A small piece of Na was added to CH_3OH (2 mL) and the resulting solution was added to **29** (20 mg, 0.05 mmol) and the solution was kept at room temperature (1 h). Concentration of the mixture followed by flash chromatography (CH_3OH–EtOAc, 1:9) afforded **16** as a colourless powder (14 mg, 95%); mp 170–172 °C; ^{1}H NMR (600 MHz, D_2O) 2.10 (s, 3H, COCH_3), 3.76 (dd, 1H, $J_{5,6}$ = 10.8, $J_{4,5}$ = 8.0 Hz, H-5), 4.34–4.45 (m, 3H, H-4, H-7), 4.82 (d, 1H, H-6), 6.85 (br s, 1H, H-2), 7.16–7.42 (m, 5H, Ph); ^{13}C NMR (150.9 MHz, D_2O) 23.55 (CO*C*H$_3$), 54.06, 73.26, 75.94 (C-4, C-5, C-6), 62.52 (C-7), 111.90 (C-2), 122.02, 126.35, 130.72, 138.13 (Ph), 155.81, 157.35, 158.71 (C-1, C-3, *C*NHPh), 175.93 (*C*OCH$_3$); HR-MS *m/z* (FAB) 350.1364; [M+H]$^+$ requires 350.1352. Anal. Calcd for $C_{16}H_{19}N_3O_6$: C, 55.01; H, 5.48. Found: C, 55.22; H, 5.38.

4.13. (*E*)-{Ethyl [(2′*S*,3′*R*,4′*S*,5′*R*)-2′-acetamido-3′,4′-dibenzyloxy-5′-benzyloxymethyl-5′-hydroxycyclohexylidene]acetate} (30)

Ethyl (triphenylphosphoranylidene)acetate (300 mg, 0.90 mmol) was added to **22** (90 mg, 0.20 mmol) in CH_2Cl_2, and the solution was kept (3 h). The reaction mixture was diluted with CH_2Cl_2, washed with 1 M HCl and then saturated aqueous $NaHCO_3$ solution followed by drying over $MgSO_4$. Filtration followed by evaporation and flash chromatography (EtOAc–petrol, 2:3) yielded **30** as a powder (80 mg, 78%); mp 179–181 °C; $[\alpha]_D$ +68.5 (*c* 1.0, $CDCl_3$); ^{1}H NMR (600 MHz) 1.23 (t, 3H, J = 7.1 Hz, CH_2CH_3), 1.84 (s, 3H, $COCH_3$), 2.50, 3.85 (ABq, 2 H, $J_{6'/7',6'/7'}$ = 15.0 Hz, H-6′/7′), 3.26, 3.49 (ABq, 2H, $J_{7'/6',7'/6'}$ = 8.7 Hz, H-7′/6′), 3.67 (dd, 1H, $J_{2',3'} \approx J_{3',4'}$ = 9.0 Hz, H-3′), 3.94 (d, 1H, H-4′), 4.05–4.15 (m, 2H, CH_2CH_3), 4.40, 4.50 (ABq, 2H, J = 11.9 Hz, CH_2Ph), 4.51–4.56 (m, 1H, H-2′), 4.60, 4.81 (ABq, 2H, J = 12.5 Hz, CH_2Ph), 4.60, 4.84 (ABq, 2H, J = 10.9 Hz, CH_2Ph), 5.47 (br s, 1H, N–H), 5.77–5.79 (m, 1H, H-2), 7.22–7.38 (m, 15H, Ph); ^{13}C NMR (150.9 MHz) 14.06 (CH_2CH_3), 23.08 (CO*C*H$_3$), 33.98 (C-6′), 56.15, 80.92, 81.55 (C-2′, C-3′, C-4′), 59.96 (*C*H$_2$CH$_3$), 73.27 (C-7′), 73.40, 74.36, 75.37 (3C, *C*H$_2$Ph), 74.44 (C-5′), 115.99 (C-2), 127.40–138.11 (Ph), 152.92 (C-1′), 166.30 (C-1), 169.91 (*C*OCH$_3$); HR-MS *m/z* (FAB) 574.2843; [M+H]$^+$ requires 574.2805.

4.14. (*E*)-{Ethyl [(4′*R*,5′*R*,6′*S*)-6′-acetamido-4′,5′-dibenzyloxy-3′-benzyloxymethylcyclohex-2′-enylidene]acetate} (31)

Sulfuryl chloride (10 mg, 0.07 mmol) in CH_2Cl_2 (1 mL) was added dropwise to **30** (20 mg, 0.03 mmol) and pyridine (0.2 mL) in CH_2Cl_2 (3 mL) at −70 °C, and the solution was kept (1 h). The reaction mixture was diluted with CH_2Cl_2, washed with 2 M citric acid solution and then saturated aqueous $NaHCO_3$ solution, followed by drying over $MgSO_4$. Filtration followed by evaporation and flash chromatography (EtOAc–petrol, 3:7) afforded **31** as a powder (13 mg, 67%); mp 157–159 °C, $[\alpha]_D$ −31.1 (*c* 1.0, CH_3CN); ^{1}H NMR (600 MHz, CD_3CN) 1.24 (t, 3H, J = 7.1 Hz, CH_2CH_3), 1.84 (s, 3H, $COCH_3$), 3.84 (dd, 1H, $J_{5',6'}$ = 7.5, $J_{4',5'}$ = .4 Hz, H-5′), 4.14 (q, 2H, CH_2CH$_3$) 4.12–4.15 (m, 1H, H-7′), 4.23–4.27 (m, 2H, H-4′,H-7′), 4.47, 4.53 (ABq, 2H, J = 11.7 Hz, CH_2Ph), 4.63, 4.73 (ABq, 2H, J = 11.1 Hz, CH_2Ph), 4.67, 4.69 (ABq, 2H, J = 11.4 Hz, CH_2Ph), 4.83 (ddd, 1H, $J_{6',NH}$ = 9.0, $J_{2',6'}$ = 1.4 Hz, H-6′), 5.77–5.78 (m, 1H, H-2), 6.75 (d, 1H, N–H), 7.28–7.38 (m, 15H, Ph), 7.56–7.57 (m, 1H, H-2′); ^{13}C NMR (150.9 MHz, CD_3CN) 14.50 (CH_2CH_3), 23.29 (CO*C*H$_3$), 52.92, 77.68, 79.15 (C-4′, C-5′, C-6′), 60.92 (*C*H$_2$CH$_3$), 71.09, 73.05, 74.06, 74.69 (4C, C-7′, *C*H$_2$Ph), 116.92 (C-2), 122.05 (C-2′), 128.63–139.41 (Ph), 144.76, 149.78 (C-1′, C-3′), 166.83 (C-1), 170.40 (*C*OCH$_3$); HR-MS *m/z* (FAB) 556.2642; [M+H]$^+$ requires 556.2699.

4.15. (*E*)-{Ethyl [(4′*R*,5′*R*,6′*S*)-6′-acetamido-4′,5′-diacetoxy-3′-acetoxymethylcyclohex-2′-enylidene]acetate} (32)

Anhydrous ferric chloride (300 mg, 1.9 mmol) was added to **31** (100 mg, 0.20 mmol) in dry CH_2Cl_2 at 0 °C. The mixture was stirred at room temperature (2 h) before being treated with Ac_2O (1.0 mL). The mixture was kept (0.5 h) before being quenched with saturated $NaHCO_3$ solution, followed by a usual workup (CH_2Cl_2), to leave a gum. Flash chromatography (EtOAc–petrol, 1:1) yielded **32** as a glass (60 mg, 81%); $[\alpha]_D$ −27.4 (*c* 1.0, CH_3CN); ^{1}H NMR (600 MHz, CD_3CN) 1.26 (t, 3H, J = 7.2 Hz, CH_2CH_3), 1.94, 1.97, 2.02, 2.04 (4s, 12H, $COCH_3$), 4.16 (q, 2H, CH_2CH$_3$), 4.60, 4.74 (ABq, 2H, $J_{7',7'}$ = 14.2 Hz, H-7′), 4.93–4.97 (m, 1H, H-6′), 5.09 (dd, 1H, $J_{5',6'}$ = 10.8, $J_{4',5'}$ = 7.8 Hz, H-5′), 5.77–5.79 (m, 1H, H-2), 5.81–5.84 (m, 1H, H-4′), 6.56 (d, 1H, $J_{6',NH}$ = 9.1 Hz, N–H), 7.65–7.67 (m, 1H, H-2′); ^{13}C NMR (150.9 MHz, CD_3CN) 14.52 (CH_2CH_3), 20.91, 20.94, 23.00 (4C, $COCH_3$), 52.21, 71.18, 73.56 (C-4′, C-5′, C-6′), 61.25, 63.84 (C-7′, *C*H$_2$CH$_3$), 117.04 (C-2), 124.60 (C-2′), 140.09, 148.01 (C-1′, C-3′), 166.57 (C-1), 170.91, 171.04, 171.08, 171.12 (*C*OCH$_3$); HR-MS *m/z* (FAB) 412.1627; [M+H]$^+$ requires 412.1634.

4.16. (*E*)-{Ethyl 2-[(4′*R*,5′*R*,6′*S*)-6′-acetamido-4′,5′-dihydroxy-3′-hydroxymethylcyclohex-2′-enylidene]acetate} (17)

A small piece of Na was added to EtOH (2 mL) and the resulting solution was added to **32** (20 mg, 0.05 mmol) and the solution was kept at room temperature (1 h). Concentration of the mixture followed by flash chromatography (EtOH–EtOAc, 3:17–3:10) afforded **17** as a colourless powder (11 mg, 79%); mp 158–160 °C; ^{1}H NMR (600 MHz, D_2O) 1.30 (t, 3H, J = 7.2 Hz, CH_2CH_3), 2.17 (s, 3H, $COCH_3$), 3.67 (dd, 1H, $J_{5',6'}$ = 11.2, $J_{4',5'}$ = 8.2 Hz, H-5′), 4.20–4.25 (m, 2H,

CH_2CH_3), 4.32–4.40 (m, 2H, H-7′), 4.43–4.45 (m, 1H, H-4′), 4.66 (dd, 1H, $J_{2',6'}$ = 1.8 Hz, H-6′), 5.73–5.74 (m, 1H, H-2), 7.39–7.41 (m, 1H, H-2′); ^{13}C NMR (150.9 MHz, D_2O) 14.06 (CH_2CH_3), 22.66 ($COCH_3$), 54.95, 72.82, 75.05 (C-4′, C-5′, C-6′), 61.86, 62.12 (C-7′, CH_2CH_3), 113.61, 119.59 (C-2, C-2′), 148.84, 149.72 (C-1′, C-3′), 168.93, 175.46 (C-1, $COCH_3$); HR-MS *m/z* (FAB) 286.1305; $[M+H]^+$ requires 286.1291; Anal. Calcd for $C_{13}H_{19}NO_6$: C, 54.73; H, 6.71. Found: C, 54.58; H, 6.58.

4.17. (*E*)-[(4′*R*,5′*R*,6′*S*)-6′-Acetamido-4′,5′-dihydroxy-3′-hydroxymethylcyclohex-2′-enylidene]acetic acid (18)

Sodium hydroxide (50 mg, 1.2 mmol) was added to **32** (30 mg, 0.07 mmol) in EtOH–H_2O (1:1, 2 mL), and the solution was kept (1 h). The reaction mixture was neutralized with AcOH and concentrated to leave a pale yellow residue that was subjected to flash chromatography (MeOH–EtOAc–AcOH, 2:8:2) to yield **18** as a colourless powder (14 mg, 75%); mp 145–147 °C; ^{1}H NMR (600 MHz, D_2O) 2.04 (s, 3H, $COCH_3$), 3.51 (dd, 1H, $J_{5',6'}$ = 10.9, $J_{4',5'}$ = 8.1 Hz, H-5′), 4.13, 4.24 (ABq, 2H, $J_{7',7'}$ = 14.7 Hz, H-7′), 4.30 (d, 1H, H-4′), 4.48 (d, 1H, H-6′), 5.65 (s, 1H, H-2), 6.85 (s, 1H, H-2′); ^{13}C NMR (150.9 MHz, D_2O) 22.63 ($COCH_3$), 54.25, 72.68, 75.22 (C-4′, C-5′, C-6′), 62.09 (C-7′), 121.56, 122.12 (C-2, C-2′), 139.34, 143.19 (C-1′, C-3′), 175.42, 181.58 (C1, $COCH_3$); HR-MS *m/z* (FAB) 257.0895; $[M]^+$ requires 257.0899; Anal. Calcd for $C_{11}H_{15}NO_6$: C, 51.36; H, 5.88. Found: C, 51.43; H, 5.71.

4.18. Kinetic analysis of β-hexosaminidase and *Bt*GH84

All Michaelis–Menten kinetic experiments were conducted using concentrations of one-fifth to five times K_m and incubated at 37 °C for 30 min. All assays were conducted in triplicate by using a stopped-based assay procedure, where the enzymatic reactions (50 μL) were quenched with the addition of a fourfold excess of quenching buffer (200 mM glycine, pH 10.75). Enzyme addition was via pipette (5 μL) and in all cases the final pH of the quenched solution was greater than 10. Buffer systems for β-hexosaminidase and *Bt*GH84 were 50 mM citrate–100 mM NaCl pH 4.5, and 50 mM NaH_2PO_4–100 mM NaCl pH 7.4, respectively; time dependent assays of β-hexosaminidase and *Bt*GH84 showed that the enzymes were stable in their respective buffers over the period of the assays. The reactions, quenched after 30 min, were monitored by determining the extent of liberated 4-nitrophenolate by UV measurements at 400 nm, using a 96-well plate (Stastedt) and 96-well plate reader (Molecular Devices). Human β-hexosaminidase was purchased from Sigma and *Bt*GH84 was overexpressed and purified prior to use.[34] Concentrations of β-hexosaminidase and *Bt*GH84 used in inhibition assays were 0.04 μg μL^{-1} and 0.07 μg μL^{-1}, respectively, using *p*NP-GlcNAc at a concentration of 0.5 mM. All inhibitors were tested at several concentrations, ranging from one-third to three times K_i for those inhibitors for which K_i values were determined.

4.18.1. Kinetic analysis of NagZ and human *O*-GlcNAcase

All Michaelis–Menten kinetic experiments were conducted using concentrations of one-fifth to five times K_m at 37 °C. All assays were conducted in 80 μL quartz microcuvettes. Reactions were initiated by the addition of a small aliquot of enzyme via pipette and continuously monitored, the extent of liberated 4-nitrophenolate ascertained by UV measurements at 400 nm using a Cary 3E UV–VIS spectrometer equipped with a Peltier temperature controller. Reaction velocities were determined by linear regression of the linear region of the reaction progress curve between the first and third minutes. A phosphate buffer (50 mM NaH_2PO_4–100 mM NaCl, pH 7.4) was used with both NagZ and *O*-GlcNAcase, and the enzymes were overexpressed and purified prior to use.[47,48] Concentrations of NagZ and human *O*-GlcNAcase used in the inhibition assays were 0.07 μg μL^{-1}, using *p*NP-GlcNAc at a concentration of 0.5 mM. All inhibitors were tested at several concentrations, ranging from one-third to three times K_i where possible.

4.18.2. Activity of 15, 16, 17, 18 against OGT analyzed by Western blot

An aliquot of OGT[18] (20 μL, 5.9 mg/mL) was combined with recombinant nuclear pore protein p62[8] (40 μL, 2.3 mg/L) in the presence of UDP-GlcNAc (5 μL, 500 μM) in PBS buffer containing $MgCl_2$ (1.25 μL, 12.5 mM), 2-mercaptoethanol (2 μL, 1 mM) and the synthetic compounds **15–18** (various concentrations), and the mixture was incubated at room temperature; total volume of the assay was 100 μL. A small aliquot (10 μL) was removed and diluted with SDS/PAGE loading buffer (10 μL) before being heated at 96 °C for 5 min. Aliquots were loaded onto 10% Tris·HCl polyacrylamide gels. After electrophoresis, the samples were electroblotted to nitrocellulose membrane (0.45 μm, Bio-Rad). Transfer was confirmed by visual inspection of the transferred pre-stained markers (Dual Colour Precision Plus Protein Standards, Bio-Rad). The membrane was treated with a blocking buffer, 5% BSA in PBS pH 7.4 containing 0.1% Tween 20, for 1 h at room temperature with rocking. The blocking buffer was decanted and a solution of blocking buffer containing mouse anti-*O*-GlcNAc monoclonal IgM antibody (mAb CTD110.6, Covance) (1:2500) was added and rocked for 1 h. After blocking, the solution was decanted, the membrane was washed two times for 5 min and three times for 15 min with washing buffer (PBS, pH 7.4, containing 0.1% Tween 20). A second antibody, goat anti-mouse-IgG–HRP conjugate (1:10000) in blocking buffer, was added and the membrane rocked for 1 h at room temperature. After blocking, the solution was decanted, and the membrane was washed two times for 5 min and three times for 15 min with washing buffer. Detection of membrane-bound goat anti-mouse IgM–HRP conjugate was accomplished by chemiluminescence using the SuperSignal West Pico chemiluminescent detection kit (Pierce) and film (Kodak Biomax MR).

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Supplementary data

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