A 1-acetamido derivative of 6-*epi*-valienamine: an inhibitor of a diverse group of β-*N*-acetylglucosaminidases

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The synthesis of an analogue of 6-*epi*-valienamine bearing an acetamido group and its characterisation as an inhibitor of β-*N*-acetylglucosaminidases are described. The compound is a good inhibitor of both human *O*-GlcNAcase and human β-hexosaminidase, as well as two bacterial β-*N*-acetylglucosaminidases. A 3-D structure of the complex of *Bacteroides thetaiotaomicron Bt*GH84 with the inhibitor shows the unsaturated ring is surprisingly distorted away from its favoured solution phase conformation and reveals potential for improved inhibitor potency.

Introduction

2-Acetamido-2-deoxy-β-D-glycopyranosides are naturally occurring glycosides found within many of the glycoconjugates and oligosaccharides that are present in an extremely wide variety of organisms, ranging from microbes to humans. Unsurprisingly, given their abundance in nature, there are several classes of enzymes comprising both hydrolases and lyases that have evolved to cleave glycosidic linkages involving this saccharide residue. Glycoside hydrolases are particularly abundant and have been classified into many different families on the basis of structural and primary sequence similarities.† Members of each family have similar three-dimensional structures and use similar catalytic mechanisms. Interestingly though, just two main enzyme-catalyzed hydrolytic mechanisms are known for cleaving the β-glycosidic linkage of 2-acetamido-2-deoxy-β-D-glycopyranosides. Examples of these two mechanistic classes include the *exo*-β-*N*-acetylglucosaminidases from family 3, and those from families 20 and 84 of the glycoside hydrolases, all of which are functionally related in that they catalyze the release of terminal 2-acetamido-2-deoxy-β-D-glycopyranose residues from glycoconjugates.[1–4] *exo*-β-*N*-Acetylglucosaminidases from family 3 use a catalytic mechanism involving the formation and breakdown of a covalent glycosyl–enzyme intermediate.[4] The enzymes from families 20[1,2] and, more recently, 84[5–7] of the glycoside hydrolases have been shown to differ in that they use a catalytic mechanism involving assistance from the 2-acetamido group of the substrate.

As a result of the biological importance of these β-*N*-acetylglucosaminidases, the design of small molecule inhibitors[2,5,8–11] has received considerable attention.[10] 2′-Methyl-α-D-glucopyrano-[2,1-*d*]-Δ2′-thiazoline (NAG-thiazoline) **1** (Fig. 1) has been found to be a potent inhibitor of family 20 hexosaminidases[2,3] and, more recently, family 84 *O*-GlcNAcases.[5] Its poor inhibitory potency toward family 3 is consistent with this family of enzymes. *O*-(2-Acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate (PUGNAc) **2**,[12] 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 **3**,[13] and the triazoles, all prepared by Vasella *et al.*, have all been shown to potently inhibit family 3 β-*N*-acetylglucosaminidases,[14] family 20 human β-hexosaminidases[9,13] and family 84 *O*-GlcNAcases.[6,15,16,17] Some imino- and azasugars, as well as being good inhibitors of glucosidases, have also been found to be inhibitors of β-*N*-acetylglucosaminidases. The *N*-acetylamino analogues **4** and **5** of 1-deoxynojirimycin and isofagomine, respectively, are potent inhibitors of family 20 β-hexosaminidases.[18,19]

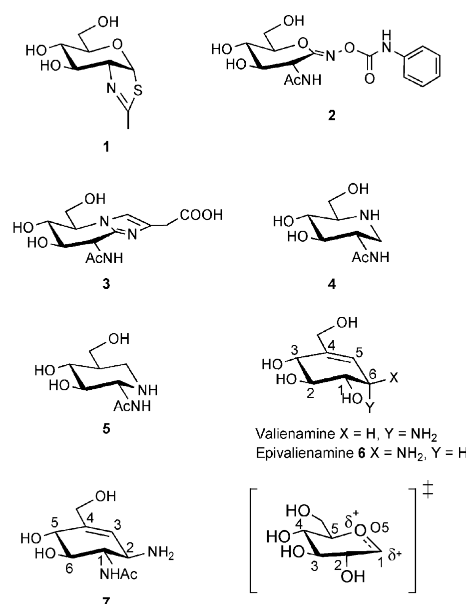

Fig. 1 Compounds referred to in this study, as well as one of the likely transition states ($^4H_3$) for an α-glucosidase.

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† For the classification of glycoside hydrolases, see the CAZY Database (available at http://afmb.cnrs-mrs.fr/CAZY/).

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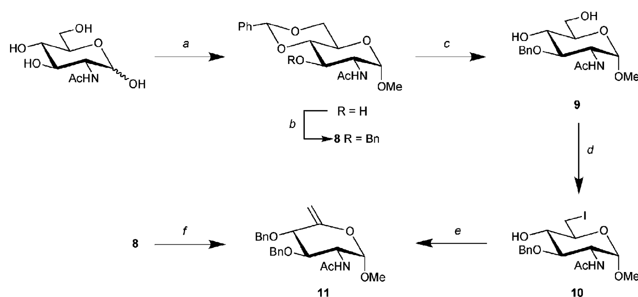
To expand the repertoire of inhibitors of β-*N*-acetylglucosaminidases, we thought to modify the known inhibitor, *epi*-valienamine **6**, which is the C-6 epimer of valienamine.[20] Valienamine is an essential core unit in many kinds of pseudo-oligosaccharidic α-glucosidase inhibitors, such as acarbose,[21] acarviosin,[21] adiposin-2[22] and validoxylamine A.[23] The valienamine core itself is also an inhibitor of some α-glucosidases.[19] A rationale for the potency of valienamine against α-glucosidases is that it is thought to mimic both the charge and the ring distortion of the transition state. The $^1H_2$ ($^2H_3$ conformation using the numbering as according to D-glucopyranose) conformation‡ exhibited by valienamine resembles in some aspects both the $^4H_3$ and $^{2,5}B$ conformations for the D-glucopyranose transition state, found for related hydrolases,[24,25] which are the two likely transition state conformations for α-glucosidases (Fig. 1). The potency of valienamine stems from the crucially important double bond. "Dihydro" acarbose, a compound where the double bond has been saturated, is only a modestly good glucoamylase inhibitor (low micromolar K_i) compared to the very potent (picomolar K_i) acarbose which retains the double bond.[26,27]

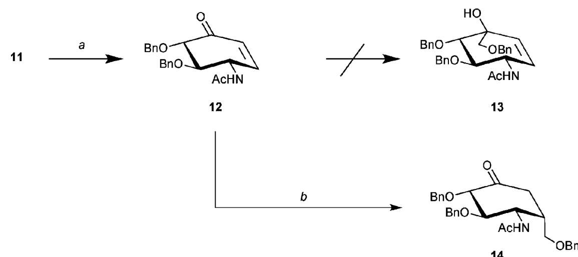
On the other hand, little is known about the inhibitory potency of 6-*epi*-valienamine **6**.[28] Some *N*-alkyl analogues have been synthesised and have been found to be potent β-D-glucocerebrosidase inhibitors,[29] and one, *N*-octyl-6-*epi*-valienamine, has potential as a therapeutic for Gaucher disease.[30] We felt that owing to the success of 6-*epi*-valienamine analogues as potent inhibitors of β-glucocerebrosidases, an analogue of 6-*epi*-valienamine, where the hydroxyl group at C-1 was replaced with an acetamido group, as in **7**, could yield a useful inhibitor scaffold for β-*N*-acetylglucosaminidases. We therefore engaged in a rational synthesis of such a putative 6-*epi*-valienamine-based inhibitor.

Results and discussion

The methyl glycoside **8** was efficiently prepared in three steps from *N*-acetyl-D-glucosamine (Scheme 1).[31] Removal of the benzylidene ring gave the diol **9**. Selective iodination of the liberated primary hydroxyl group *via* a tosylate intermediate furnished the iodide **10**. Following benzylation of the free hydroxyl group of **10**, elimination of hydrogen iodide afforded the alkene **11**.[32] The alkene **11** could also be prepared *via* a reductive ring-opening of the benzylidene ring of **8**, followed by sequential iodination and elimination (Scheme 1). Treatment of the alkene **11** with mercury(II) trifluoroacetate under Ferrier reaction conditions[33] smoothly provided the presumed intermediate carbocycle (not shown), which was dehydrated to form the enone **12** (Scheme 2). Addition of the required exocyclic carbon to **12** is well precedented and we chose to use benzyloxymethylmagnesium chloride to facilitate the conversion. Interestingly, performing the Grignard reaction using **12** did not proceed to give the desired alcohol **13** but resulted in a Michael addition to give a ketone, assigned the structure **14**. We propose that the amide moiety is first deprotonated, directing the Grignard reagent to the wrong carbon



Scheme 1 (a) i. HCl, MeOH, ii. $PhCH(OMe)_2$, camphor-10-sulfonic acid, DMF; (b) BnBr, NaH, THF; (c) AcOH–H_2O (4 : 1); (d) i. TsCl, pyridine, ii. NaI, DMF; (e) NaH, BnBr, DMF; (f) i. $Et_3N{\cdot}BH_3$, $AlCl_3$, CH_2Cl_2, ii. I_2, PPh_3, imidazole, PhMe, iii. NaH, BnBr, DMF.



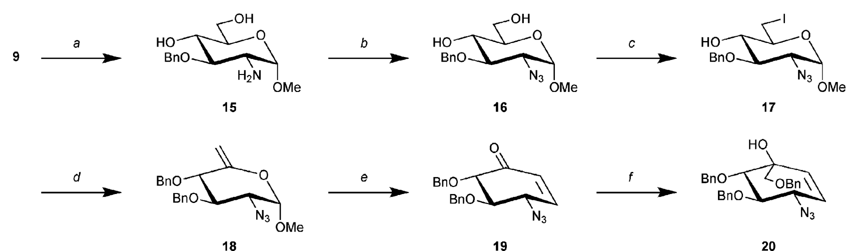
Scheme 2 (a) i. $Hg(CF_3COO)_2$, acetone–H_2O (4 : 1), ii. MsCl, Et_3N, CH_2Cl_2; (b) Mg, $BnOCH_2Cl$, $HgCl_2$, THF.

and resulting in its axial addition. To circumvent this problem associated with the presence of the 2-acetamido moiety, we envisaged that a 2-azido group could instead be used.

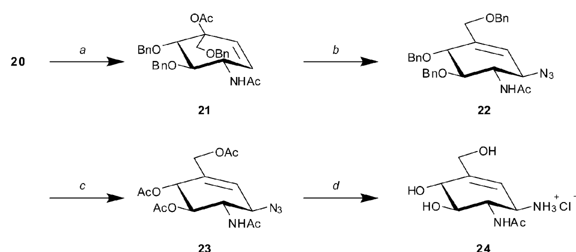
Returning to the diol **9**, we obtained **15** after using hydrazine hydrate; treatment of **15** with trifluoromethanesulfonyl azide then afforded the azide **16** in good yield (Scheme 3). Iodination of **16** gave **17**, which upon benzylation and elimination furnished the alkene **18** in good yield. Alkene **18** was then treated with mercury(II) trifluoroacetate to provide smoothly the intermediate carbocycle, which was dehydrated to form the enone **19**. Gratifyingly, as we had predicted, treatment of the azide **19** with benzyloxymethylmagnesium chloride gave the desired alkene **20**. Continuing on, alkene **20** was next treated with triphenylphosphine to reduce the azide to the corresponding amine, which was acetylated *in situ* to provide **21** (Scheme 4). Treatment of **21** with sodium azide and pure tetrakis(triphenylphosphine)palladium(0)[34] yielded the azide **22**. At this point, the required exchange of protecting groups went smoothly to give **23**. The azide **23** was then subjected to triphenylphosphine to give the presumed intermediate amine which was treated *in situ* with *tert*-butyl dicarbonate to furnish the Boc-protected amine (not shown). Deacetylation followed by removal of the protecting group gave the desired compound **7** as its hydrochloride salt **24**.

We evaluated the inhibitor **7** against family 3 β-*N*-acetylglucosaminidase, NagZ from *Vibrio cholerae* (K_i = 50 μM) and the family 20 human placental β-hexosaminidase (K_i = 34 μM). Also, family 84 human *O*-GlcNAcase (K_i = 6.2 μM) and a close bacterial homologue *Bt*GH84[6] (K_i = 26 μM) were assayed with this inhibitor. It is interesting that despite the close structural relationship of 6-*epi*-valienamine **6** and the 1-acetamido analogue **24**, compound **24** is clearly much more potent. Indeed, Ogawa *et al.* saw no inhibition of β-glucocerebrosidase with 6-*epi*-valienamine

‡ The ring conformations referred to in this paper are based on the IUPAC naming system for each individual compound. Since valienamine and its analogues discussed here bind in a manner that mimics the pyranose ring of the substrate, a more intuitive (although incorrect) numbering system using the glucopyranose ring system is given in brackets for convenient comparison.



Scheme 3 (a) $N_2H_4{\cdot}H_2O$, H_2O; (b) TfN_3, $CuSO_4{\cdot}5H_2O$, MeOH, CH_2Cl_2; (c) i. TsCl, pyridine, ii. NaI, DMF; (d) NaH, BnBr, DMF; (e) i. $Hg(CF_3COO)_2$, acetone–H_2O (4 : 1), ii. MsCl, Et_3N, CH_2Cl_2; (f) i. Mg, $BnOCH_2Cl$, $HgCl_2$, THF.



Scheme 4 (a) i. PPh_3, THF, H_2O, ii. Ac_2O, DMAP, pyridine; (b) $Pd(PPh_3)_4$, NaN_3, THF, H_2O; (c) i. $FeCl_3$, CH_2Cl_2, ii. Ac_2O, DMAP, pyridine; (d) i. PPh_3, THF, H_2O, ii. Boc_2O, KOH, MeOH, iii. 6 M HCl, MeOH.

6,[29] which differs from the results described here using the 1-acetamido analogue. It is difficult to ascertain the basis for these differences.

Therefore, to gain a more detailed understanding of the molecular basis for these apparent differences, we evaluated the interactions between **7** and a family 84 β-*N*-acetylglucosaminidase. The 3-D structure of *Bt*GH84 (whose active centre residues are invariant with the human enzyme) was solved in complex with **7** by X-ray crystallography at a resolution of approximately 2 Å. To our knowledge this is the first time that a crystal structure involving any 6-*epi*-valienamine analogue has been reported. The 3-D structure revealed clear and unambiguous electron density for **7** bound in the "−1" subsite of *Bt*GH84 (Fig. 2). The acetamide **7** makes similar interactions to those previously observed for thiazoline complexes of the enzyme: notably a tight network of hydrogen bonds (O6 to NZ of Lys166 and the main chain carbonyl of Gly135, O5 and O in the hydroxymethyl group to Asp344 and the acetyl NH and carbonyl group to OD2 of Asp242 and ND2 of Asn339). Surprisingly, the unsaturated ring of **7** is distorted away from its favoured solution phase $^{1}H_{6}$ ($^{2}H_{3}$) conformation toward a $^{2}S_{6}$ ($^{1}S_{3}$) conformation in which the amine moiety is pseudo-axial and makes a 2.7 Å hydrogen bond to the catalytic acid, Asp243. Such a conformation does not resemble the predicted transition state geometry but rather mimics those observed for the Michaelis complexes of retaining enzymes active on β-glycosides of the *gluco*-series (for example, ref. 35,36) and reflects in-line geometry for nucleophilic substitution at the anomeric centre: the O–C2–N2 (O–C1–N1) angle is 167°. Such distortion appears critical for establishing a powerful electrostatic interaction between the amine **7** and Asp 243.

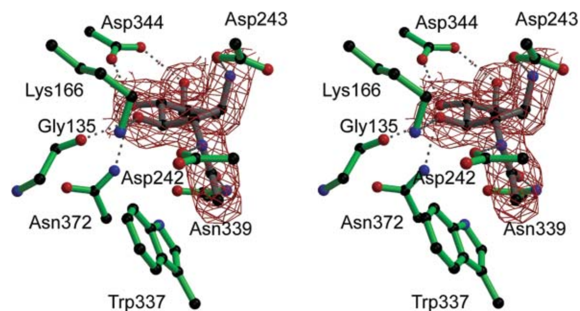


Fig. 2 Observed electron density for **7** binding to *Bt*GH84 shown in divergent (wall-eyed) stereo; **7** is shown in grey and with observed $2F_{obs} - F_{calc}$ electron density at 0.4 electrons $Å^{-3}$. Interacting amino-acids discussed in the text are shown and are coloured green.

Conclusions

We have devised a route that enables the rapid synthesis of an inhibitor of β-*N*-acetylglucosaminidases from a diverse range of glycoside hydrolase families. Also, this inhibitor scaffold has the potential to be used to create more potent and potentially selective inhibitors by elaborating the functional groups present within the inhibitor. The structural analysis of *Bt*GH84 in complex with **7** given here should facilitate this development. Notably, although partial distortion away from the expected half-chair must come with a significant energetic penalty, the additional electrostatic interaction this permits clearly mitigates these costs. Furthermore, the pseudo-axial C2–N2 geometry suggests that augmentation of the inhibitor at the amine position should generate even more powerful inhibitors in which aglycone binding energy may be captured. Such inhibitors could be useful molecules for studying the cellular role of *O*-GlcNAc since they may have useful pharmacological properties.

Experimental

General

Experimental procedures have been given previously.[37] Elemental analyses of all synthesized compounds used in enzyme assays were performed at the Simon Fraser University.

Methyl 2-acetamido-3-*O*-benzyl-2,6-dideoxy-6-iodo-α-D-glucopyranoside 10. (i) The acetal **8** (18.5 g) was heated in AcOH–H_2O (4 : 1, 200 mL) (70 °C, 1 h). The solution was concentrated to leave a powder that was washed with toluene, followed by hexanes, to afford a colourless powder (15.5 g). This material, presumably methyl 2-acetamido-3-*O*-benzyl-2-deoxy-α-D-glucopyranoside **9**,[31] was used in the next step without any further purification.

(ii) 4-Toluenesulfonyl chloride (7.6 g, 40 mmol) was added to the material from (i) (6.5 g, 20 mmol) and Et_3N (10 mL) in CH_2Cl_2 (125 mL) at 0 °C and the solution was kept at room temperature (3 h) before being quenched with H_2O. A usual workup (CH_2Cl_2) yielded a yellow residue. Sodium iodide (15 g, 0.10 mol) was added to the residue in *N*,*N*-dimethylformamide (DMF, 100 mL) and the solution was stirred at 100 °C (3 h). Concentration of the reaction mixture followed by a usual workup (CH_2Cl_2) gave a brown residue that was subjected to flash chromatography (EtOAc–petrol, 1 : 1) to return the iodide **10** (5.8 g, 67%) as a colourless solid. R_f 0.5 (EtOAc–hexane, 7 : 3); mp 150–152 °C; $[\alpha]_D^{20}$ +74.2; δ_H (600 MHz) 7.37–7.28 (m, 5H, Ph), 5.59 (d, 1H, $J_{2,NH}$ 9.5 Hz, NH), 4.70, 4.67 (ABq, 2H, *J* 11.7 Hz, CH_2Ph), 4.65 (d, 1H, $J_{1,2}$ 3.7 Hz, H1), 4.27 (ddd, 1H, $J_{2,3}$ 10.4 Hz, H2), 3.56 (dd, 1H, $J_{5,6}$ 2.4 Hz, $J_{6,6}$ 10.7 Hz, H6), 3.55 (dd, 1H, $J_{3,4}$ 8.7 Hz, H3), 3.48 (dd, 1H, $J_{4,5}$ 9.3 Hz, H4), 3.43–3.40 (m, 1H, H5), 3.40 (s, 3H, OCH_3), 3.30 (dd, 1H, $J_{5,6}$ 7.1 Hz, H6), 1.92 (s, 3H, $COCH_3$); δ_C (150.9 MHz) 169.94 (*C*OCH$_3$), 138.05–127.98 (Ph), 98.66 (C1), 79.90, 73.91, 70.53, 52.01 (4C, C2, C3, C4, C5), 73.92 (*C*H$_2$Ph), 55.30 (OCH_3), 23.34 (CO*C*H$_3$), 6.73 (C6); *m*/*z* (FAB) 436.0600, (M + H)$^+$ requires 436.0621.

Methyl 2-acetamido-3,4-di-*O*-benzyl-2,6-dideoxy-α-D-xylohex-5-enoside 11. Sodium hydride (60% dispersion in mineral oil, 500 mg, 12.5 mmol) was added to **10** (1.0 g, 2.3 mmol) and BnBr (0.30 mL, 2.5 mmol) in DMF (20 mL) and the mixture was stirred (1 h). The reaction was monitored by ^{1}H NMR spectroscopy before being quenched with MeOH, concentrated and subjected to a usual workup (EtOAc), followed by flash chromatography (EtOAc–hexane, 1 : 1), to return **11** (700 mg, 77%) as a colourless solid. R_f 0.25 (EtOAc–hexane, 1 : 1); mp 148–150 °C; $[\alpha]_D^{20}$ +67.9; δ_H (600 MHz) 7.38–7.26 (m, 10H, Ph), 5.44 (d, 1H, $J_{2,NH}$ 9.3 Hz, NH), 4.85–4.84, 4.74–4.73 (2 m, 2H, H6, H6), 4.84, 4.63 (ABq, 2H, *J* 11.9 Hz, CH_2Ph), 4.78, 4.71 (ABq, 2H, *J* 11.2 Hz, CH_2Ph), 4.74 (d, 1H, $J_{1,2}$ 3.3 Hz, H1), 4.33 (ddd, 1H, $J_{2,3}$ 9.2 Hz, H2), 4.00 (ddd, 1H, $J_{3,4}$ 8.4 Hz, $J_{4,6} \approx J_{4,6}$ 1.5 Hz, H4), 3.64 (dd, 1H, H3), 3.40 (s, 3H, OCH_3), 1.84 (s, 3H, $COCH_3$); δ_C (150.9 MHz) 169.79 (*C*OCH$_3$), 153.67 (C5), 138.28–127.79 (Ph), 99.50 (C1), 97.57 (C6), 79.62, 78.50, 51.69 (3C, C2, C3, C4), 74.34, 73.83 (2C, *C*H$_2$Ph), 55.53 (OCH_3), 23.35 (CO*C*H$_3$); *m*/*z* (FAB) 398.1956, (M + H)$^+$ requires 398.1967.

***N*-[(1*S*,5*S*,6*R*)-5,6-Dibenzyloxy-4-oxocyclohex-2-en-1-yl]acetamide 12.** Mercury(II) trifluoroacetate (5.0 mg, 17 μmol) was added to **11** (200 mg, 0.5 mmol) in dioxane–aqueous H_2SO_4 (5 mM) (2 : 1, 8 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 leave a colourless powder that was dissolved in CH_2Cl_2 (10 mL) and treated with methanesulfonyl chloride (MsCl, 0.20 mL, 2.6 mmol) and Et_3N (0.70 mL, 5.3 mmol). The mixture was quenched with H_2O, followed by a usual workup (CH_2Cl_2) to afford a brown residue that was subjected to flash chromatography (EtOAc–petrol, 7 : 3) to furnish **12** (70 mg, 38%) as flakes. R_f 0.5 (EtOAc); mp 164–166 °C; $[\alpha]_D^{20}$ +118.5; δ_H (600 MHz) 7.40–7.27 (m, 10H, Ph), 6.72 (dd, 1H, $J_{2,3}$ 10.2 Hz, $J_{1,2}$ 3.3 Hz, H2), 6.06 (dd, 1H, $J_{1,3}$ 2.1 Hz, H3), 5.58 (d, 1H, $J_{1,NH}$ 7.8 Hz, NH), 4.82–4.77 (m, 1H, H1), 4.92, 4.66 (ABq, 2H, *J* 11.4 Hz, CH_2Ph), 4.79, 4.66 (ABq, 2H, *J* 11.7 Hz, CH_2Ph), 4.03 (d, 1H, $J_{5,6}$ 7.9 Hz, H5), 3.80 (dd, 1H, $J_{1,6}$ 7.7 Hz, H6), 1.83 (s, 3H, $COCH_3$); δ_C (150.9 MHz) 196.07 (C4), 169.67 (*C*OCH$_3$), 146.90 (C2), 137.57–127.99 (Ph), 82.31, 78.93, 49.52 (3C, C1, C5, C6), 73.82, 73.57 (2C, *C*H$_2$Ph), 23.15 (CO*C*H$_3$); *m*/*z* (FAB) 366.1696, (M + H)$^+$ requires 366.1705.

***N*-[(1*S*,2*R*,3*S*,5*S*)-2,3-Dibenzyloxy-6-benzyloxymethyl-4-oxocyclohex-1-yl]acetamide 14.** Magnesium (180 mg, 7.4 mmol), benzyl chloromethyl ether (0.90 mL, 6.7 mmol) and $HgCl_2$ (25 mg) in THF (10 mL) were stirred at 0 °C (1.5 h). The enone **12** (100 mg, 0.30 mmol) in THF (5 mL) was added to the mixture at −78 °C and the mixture was stirred (2 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–petrol, 3 : 2) to yield **14** (90 mg, 67%) as colourless plates. R_f 0.35 (EtOAc–hexane, 7 : 3); mp 146–148 °C; $[\alpha]_D^{20}$ +14.3; δ_H (600 MHz) 7.35–7.23 (m, 15H, Ph), 6.12 (d, 1H, $J_{1,NH}$ 8.3 Hz, NH), 4.71, 4.49 (ABq, 2H, *J* 11.2 Hz, CH_2Ph), 4.65, 4.61 (ABq, 2H, *J* 11.9 Hz, CH_2Ph), 4.52–4.47 (m, 1H, H1), 4.47, 4.36 (ABq, 2H, *J* 11.8 Hz, CH_2Ph), 3.95 (d, 1H, $J_{2,3}$ 5.6 Hz, H3), 3.90 (dd, 1H, $J_{1,2}$ 5.9 Hz, H2), 3.47 (dd, 1H, $J_{7,7}$ 9.7 Hz, $J_{6,7}$ 5.3 Hz, H7), 3.40 (dd, 1H, $J_{6,7}$ 5.6 Hz, H7), 2.69–2.64 (m, 1H, H6), 2.55–2.52 (m, 2H, H5, H5), 1.75 (s, 3H, $COCH_3$); δ_C (150.9 MHz) 205.94 (C4), 169.68 (*C*OCH$_3$), 137.88–127.49 (Ph), 84.07, 78.82, 49.51 (3C, C1, C2, C3), 73.20, 73.03, 72.69, 70.61 (4C, C7, *C*H$_2$Ph), 38.46 (C5), 36.86 (C6), 23.16 (CO*C*H$_3$); *m*/*z* (FAB) 488.2441, (M + H)$^+$ requires 488.2437

Methyl 2-amino-3-*O*-benzyl-2-deoxy-α-D-glucopyranoside 15. The amide **9** (13.0 g) was dissolved in $N_2H_4 \cdot H_2O$ (200 mL) and the solution was refluxed (30 h). The solution was concentrated, followed by flash chromatography (MeOH–$CHCl_3$, 1 : 19), to yield **15** (10.0 g, 88%) as a gum. R_f 0.7 (MeOH–$CHCl_3$, 1 : 4); $[\alpha]_D^{20}$ +62.2; δ_H (600 MHz) 7.33–7.25 (m, 5H, Ph), 4.91, 4.72 (ABq, 2H, *J* 11.7 Hz, CH_2Ph), 4.67 (d, 1H, $J_{1,2}$ 3.6 Hz, H1), 3.77 (dd, 1H, $J_{6,6}$ 11.9 Hz, $J_{5,6}$ 3.8 Hz, H6), 3.73 (dd, 1H, $J_{5,6}$ 3.1 Hz, H6), 3.60–3.52 (m, 2H, H4, H5), 3.40 (dd, 1H, $J_{2,3}$ 9.9 Hz, $J_{3,4}$ 8.5 Hz, H3), 3.33 (s, 3H, OCH_3), 2.69 (dd, 1H, H2); δ_C (150.9 MHz) 138.58–127.77 (Ph), 100.34 (C1), 83.57, 71.66, 71.11 (3C, C3, C4, C5), 75.14 (*C*H$_2$Ph), 61.83 (C6), 55.26, 55.03 (2C, C2, OCH_3); *m*/*z* (FAB) 284.1497, (M + H)$^+$ requires 284.1498.

Methyl 2-azido-3-*O*-benzyl-2-deoxy-α-D-glucopyranoside 16. A freshly prepared solution of trifluoromethanesulfonyl azide (TfN_3) in CH_2Cl_2 (0.50 M, 100 mL, 50 mmol)[38] was added dropwise to **15** (8.8 g, 31 mmol), 4-(dimethylamino)pyridine (DMAP, 4.6 g, 38 mmol) and $CuSO_4 \cdot 5H_2O$ (200 mg) in MeOH (100 mL) and the solution was stirred at room temperature (3 h). Toluene (50 mL) was added to the solution before being concentrated to approximately 50 mL. Flash chromatography (EtOAc–petrol, 1 : 1) yielded **16** (9.5 g, 99%) as a colourless gum. R_f 0.6 (EtOAc–hexane, 7 : 3); $[\alpha]_D^{20}$ +95.5; δ_H (600 MHz) 7.41–7.31 (m, 5H, Ph), 4.79 (d, 1H, $J_{1,2}$ 3.5 Hz, H1), 4.96, 4.74 (ABq, 2H, *J* 11.3 Hz, CH_2Ph), 3.83–3.76, 3.66–3.60 (2 m, 5H, H3, H4, H5, H6, H6), 3.43 (s, 3H, OCH_3), 3.34 (dd, 1H, $J_{2,3}$ 10.1 Hz, H2); δ_C (150.9 MHz) 137.86–128.01 (Ph), 98.79 (C1), 80.09, 71.00, 70.82, 63.16 (4C, C2, C3, C4, C5), 75.01 (*C*H$_2$Ph), 62.11 (C6), 55.22 (OCH_3); *m*/*z* (FAB) 308.1236, (M + H)$^+$ requires 308.1246.

Methyl 2-azido-3-*O*-benzyl-2,6-dideoxy-6-iodo-α-D-glucopyranoside 17. 4-Toluenesulfonyl chloride (8.0 g, 42 mmol) was added to **16** (9.5 g, 31 mmol) in pyridine–CH_2Cl_2 (1 : 3, 100 mL) at 0 °C. The solution was stirred at room temperature (3 h) before being quenched with MeOH. A usual workup ($CHCl_3$) gave a

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yellow residue. Sodium iodide (22.0 g, 147 mmol) was added to the residue in DMF (100 mL) and the solution was stirred at 100 °C (2 h). Concentration of the reaction mixture followed by a usual workup ($CHCl_3$) gave a brown residue that was subjected to flash chromatography (EtOAc–petrol, 3 : 17) to yield **17** (10.5 g, 82%) as an oil. R_f 0.6 (EtOAc–hexane, 7 : 3); $[\alpha]_D^{20}$ +62.5; δ_H (600 MHz) 7.40–7.33 (m, 5H, Ph), 4.97, 4.67 (ABq, 2H, *J* 11.3 Hz, C*H*$_2$Ph), 4.82 (d, 1H, $J_{1,2}$ 3.6 Hz, H1), 3.78 (dd, 1H, $J_{2,3}$ 10.1 Hz, $J_{3,4}$ 8.3 Hz, H3), 3.53 (dd, 1H, $J_{6,6}$ 10.7 Hz, $J_{5,6}$ 2.3 Hz, H6), 3.45 (s, 3H, OCH_3), 3.44–3.37 (m, 2H, H4, H5), 3.37 (dd, 1H, H2), 3.30 (dd, 1H, $J_{5,6}$ 6.4 Hz, H6), 2.23 (d, 1H, $J_{4,OH}$ 3.0 Hz, OH); δ_C (150.9 MHz) 137.72–128.11 (Ph), 98.74 (C1), 79.75, 74.18, 70.02, 63.27 (4C, C2, C3, C4, C5), 75.07 (*C*H$_2$Ph), 55.55 (OCH_3), 6.65 (C6); *m/z* (FAB) 420.0445, (M + H)$^+$ requires 420.0420.

Methyl 2-azido-3,4-di-*O*-benzyl-2,6-dideoxy-α-D-xylohex-5-enoside 18. Sodium hydride (60% dispersion in mineral oil, 4.5 g, 110 mmol) was added to BnBr (5.0 mL, 42 mmol) and **17** (9.5 g, 23 mmol) in DMF (100 mL) and the mixture was stirred (6 h). The reaction was monitored by ^{1}H NMR spectroscopy before being quenched with MeOH, concentrated and subjected to a usual workup (CH_2Cl_2). Flash chromatography of the residue (EtOAc–petrol, 1 : 19) then returned **18** (6.6 g, 76%) as an oil that solidified on standing. R_f 0.5 (EtOAc–hexane, 1 : 4); $[\alpha]_D^{20}$ +62.5; δ_H (600 MHz) 7.40–7.28 (m, 10H, Ph), 4.94–4.93 (m, 1H, H6), 4.94, 4.83 (ABq, 2H, *J* 10.7 Hz, C*H*$_2$Ph), 4.84 (d, 1H, $J_{1,2}$ 3.5 Hz, H1), 4.82, 4.77 (ABq, 2H, *J* 11.2 Hz, C*H*$_2$Ph), 4.80–4.78, 4.00–3.95, 3.53–3.50 (3 m, 4H, H2, H3, H4, H6), 3.47 (s, 3H, OCH_3); δ_C (150.9 MHz) 153.16 (C5), 137.72–127.79 (Ph), 99.26 (C1), 97.54 (C6), 80.14, 79.29, 63.13 (3C, C2, C3, C4), 75.52, 74.40 (2C, *C*H$_2$Ph), 55.48 (OCH_3); *m/z* (FAB) 382.1747, (M + H)$^+$ requires 382.1767.

(4*S*,5*R*,6*S*)-4-Azido-5,6-dibenzyloxy-cyclohex-2-enone 19. Mercury(II) trifluoroacetate (60 mg, 0.10 mmol) was added to **18** (700 mg, 1.80 mmol) in acetone–H_2O (4 : 1, 10 mL) and the suspension was stirred (12 h). Volatile solvents were removed and the resulting aqueous mixture was subjected to a usual workup (EtOAc) to give a colourless gum. Methanesulfonyl chloride (0.50 mL, 6.4 mmol) and Et_3N (1.1 mL, 7.9 mmol) were added to the gum in CH_2Cl_2 (20 mL) at 0 °C. The solution was then left at room temperature (2 h), followed by a usual workup (CH_2Cl_2), to leave a gum that was subjected to flash chromatography (EtOAc–petrol, 1 : 9) to return **19** (395 mg, 64%) as a gum. R_f 0.8 (EtOAc–hexane, 2 : 3); $[\alpha]_D^{20}$ +7.8; δ_H (500 MHz) 7.45–7.30 (m, 10H, Ph), 6.64 (dd, 1H, $J_{2,3}$ 10.3 Hz, $J_{2,4}$ 2.2 Hz, H2), 6.10 (dd, 1H, $J_{3,4}$ 2.6 Hz, H3), 5.11, 4.74 (ABq, 2H, *J* 11.3 Hz, C*H*$_2$Ph), 4.98, 4.80 (ABq, 2H, *J* 10.6 Hz, C*H*$_2$Ph), 4.37 (ddd, 1H, $J_{4,5}$ 8.7 Hz, H4), 4.11 (d, 1H, $J_{5,6}$ 10.5 Hz, H6); δ_C (125.8 MHz) 196.78 (C1), 144.94 (C3), 137.42–127.97 (C2, Ph), 84.17, 83.39, 62.86 (3C, C4, C5, C6), 75.66, 74.62 (2C, *C*H$_2$Ph); *m/z* (FAB) 350.1533, (M + H)$^+$ requires 350.1505.

(1*R*,4*S*,5*R*,6*S*)-4-Azido-5,6-dibenzyloxy-1-benzyloxymethyl-cyclohex-2-en-1-ol 20. Magnesium (105 mg, 4.30 mmol), benzyl chloromethyl ether (0.50 mL, 3.7 mmol) and $HgCl_2$ (14 mg) in dry THF (5 mL) were stirred at 0 °C (1.5 h). The enone **19** (250 mg, 0.70 mmol) in THF (5 mL) was added at −78 °C and the mixture was stirred (2 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–toluene, 1 : 19) to yield **20** (260 mg, 76%) as an oil. R_f 0.4 (EtOAc–toluene, 1 : 9); $[\alpha]_D^{20}$ +10.0; ν_{max} (film)/cm^{-1} 2100 (N_3); δ_H (600 MHz) 7.38–7.28 (m, 15H, Ph), 5.79 (dd, 1H, $J_{2,3}$ 10.2 Hz, $J_{2,4}$ 2.4 Hz, H2), 5.58 (dd, 1H, $J_{3,4}$ 2.4 Hz, H3), 4.85, 4.82 (ABq, 2H, *J* 10.5 Hz, C*H*$_2$Ph), 4.84, 4.73 (ABq, 2H, *J* 10.7 Hz, C*H*$_2$Ph), 4.61, 4.57 (ABq, 2H, *J* 11.0 Hz, C*H*$_2$Ph), 4.06 (ddd, 1H, $J_{4,5}$ 6.9 Hz, H4), 3.83 (dd, 1H, $J_{5,6}$ 9.8 Hz, H5), 3.79 (d, 1H, H6), 3.74, 3.62 (ABq, 2H, *J* 9.2 Hz, H7, H7); δ_C (150.9 MHz) 138.39–124.34 (C2, C3, Ph), 83.51, 80.56, 63.08 (3C, C4, C5, C6), 75.50, 75.06, 73.70, 73.45 (4C, C7, *C*H$_2$Ph), 75.38 (C1); *m/z* (FAB) 472.2232, (M + H)$^+$ requires 472.2236.

(1*R*,4*S*,5*R*,6*S*)-4-Acetamido-5,6-dibenzyloxy-1-benzyloxymethyl-cyclohex-2-en-1-yl acetate 21. Triphenylphosphine (70 mg, 0.30 mmol) was added to **20** (100 mg, 0.20 mmol) in THF–H_2O (3 : 1, 4 mL) and the solution was heated at 50 °C (4 h). The reaction was subjected to a usual workup (EtOAc) to afford a yellow gum. Acetic anhydride (0.20 mL, 2.0 mmol) and DMAP (5 mg) were then added to the yellow gum in pyridine (5 mL) and the solution was heated at 60 °C (3 h) before being quenched with MeOH, followed by a usual workup (EtOAc), to leave a gum that was subjected to flash chromatography (EtOAc–petrol, 1 : 1) to yield **21** (90 mg, 80%) as a colourless gum. R_f 0.5 (EtOAc–hexane, 7 : 3); $[\alpha]_D^{20}$ +75.3; δ_H (600 MHz) 7.36–7.25 (m, 15H, Ph), 5.90 (dd, 1H, $J_{2,3}$ 10.3 Hz, $J_{2,4}$ 2.3 Hz, H2), 5.62 (dd, 1H, $J_{3,4}$ 3.2 Hz, H3), 4.96 (d, 1H, $J_{4,NH}$ 8.9 Hz, NH), 4.83, 4.73 (ABq, 2H, *J* 11.3 Hz, C*H*$_2$Ph), 4.74, 4.62 (ABq, 2H, *J* 12.0 Hz, C*H*$_2$Ph), 4.70–4.65 (m, H4), 4.56, 4.50 (ABq, 2H, *J* 11.4 Hz, C*H*$_2$Ph), 4.40 (d, 1H, $J_{5,6}$ 8.8 Hz, H6), 4.07, 3.78 (ABq, 2H, *J* 9.3 Hz, H7, H7), 3.87 (dd, 1H, $J_{4,5}$ 6.5 Hz, H5), 1.87, 1.56 (2s, 6H, $COCH_3$); δ_C (150.9 MHz) 169.71, 169.08 (2C, *C*OCH$_3$), 138.46–127.63 (Ph), 132.05, 127.20 (C2, C3), 83.36 (C1), 80.86, 79.66, 50.79 (3C, C4, C5, C6), 75.32, 73.78, 73.36, 70.30 (4C, C7, *C*H$_2$Ph), 23.10, 21.98 (2C, CO*C*H$_3$); *m/z* (FAB) 530.2529, (M + H)$^+$ requires 530.2542.

***N*-[(1*S*,2*R*,5*R*,6*R*)-2-Azido-5,6-dibenzyloxy-4-benzyloxymethyl-cyclohex-3-en-1-yl]acetamide 22.** Freshly prepared $Pd(PPh_3)_4$[34] (12 mg, 8.9 μmol) was added to NaN_3 (75 mg, 1.2 mmol) and **21** (100 mg, 0.20 mmol) in deoxygenated THF–H_2O (2 : 1, 3 mL) and the mixture was refluxed (6 h). A usual workup (EtOAc) returned a brown gum that was subjected to flash chromatography (EtOAc–petrol, 3 : 7) to afford an orange solid that was washed with Et_2O to furnish **22** (55 mg, 55%) as a pale yellow powder. R_f 0.6 (EtOAc–hexane, 1 : 1); mp 116–118 °C; $[\alpha]_D^{20}$ −192.2; δ_H (600 MHz) 7.37–7.18 (m, 15H, Ph), 6.47 (d, 1H, $J_{1,NH}$ 8.3 Hz, NH), 5.90 (d, 1H, $J_{2,3}$ 3.8 Hz, H3), 4.75, 4.60 (ABq, 2H, *J* 12.0 Hz, C*H*$_2$Ph), 4.56, 4.54 (ABq, 2H, *J* 11.0 Hz, C*H*$_2$Ph), 4.55, 4.41 (ABq, 2H, *J* 11.7 Hz, C*H*$_2$Ph), 4.47–4.43 (m, 1H, H1), 4.27 (d, 1H, $J_{7,7}$ 12.9 Hz, H7), 4.09 (d, 1H, $J_{5,6}$ 3.3 Hz, H5), 3.98 (d, 1H, H7), 3.89 (dd, 1H, $J_{1,6}$ 4.8 Hz, H6), 3.86 (m, 1H, H2), 1.78 (s, 3H, $COCH_3$); δ_C (150.9 MHz) 169.59 (*C*OCH$_3$), 137.86–127.74 (C4, Ph), 122.16 (C3), 74.10, 72.93, 57.79, 49.43 (4C, C1, C2, C5, C6), 73.57, 72.05, 71.63, 70.38 (4C, C7, *C*H$_2$Ph), 23.26 (CO*C*H$_3$); *m/z* (FAB) 513.2487, (M + H)$^+$ requires 513.2502.

***N*-[(1*S*,2*R*,5*R*,6*R*)-2-Azido-5,6-diacetoxy-4-acetoxymethyl-cyclohex-3-en-1-yl]acetamide 23.** Anhydrous iron(III) chloride (115 mg, 0.70 mmol) was added to **22** (30 mg, 0.06 mmol) in dry CH_2Cl_2 (3 mL) at 0 °C. The mixture was stirred at room

temperature (3 h) before being quenched with H_2O (0.1 mL). The mixture was concentrated and co-evaporated with toluene to leave a brown residue. Acetic anhydride (0.20 mL, 2.0 mmol) and DMAP (5 mg) were then added to the brown residue in pyridine (5 mL) and the solution was heated at 50 °C (1 h) before being quenched with MeOH, followed by a usual workup (CH_2Cl_2), to leave a gum. Flash chromatography (EtOAc–petrol, 3 : 2) yielded pale yellow plates that were washed with Et_2O to return **23** (9.0 mg, 42%) as colourless plates. R_f 0.4 (EtOAc–hexane, 7 : 3); mp 147–149 °C; ν_{max} (film)/cm^{-1} 2095 (N_3); δ_H (600 MHz) 5.85 (s, 1H, H3), 5.83–5.80 (m, 1H, H5), 5.70 (d, 1H, $J_{1,NH}$ 9.4 Hz, NH), 5.17 (dd, 1H, $J_{1,6}$ 11.2 Hz, $J_{5,6}$ 7.8 Hz, H6), 4.68 (d, 1H, $J_{7,7}$ 13.3 Hz, H7), 4.45–4.35 (m, 2H, H1, H7), 4.12–4.08 (m, 1H, H2), 2.08, 2.06, 2.05, 2.00 (4s, 12H, $COCH_3$); δ_C (150.9 MHz) 171.24, 170.22, 170.20, 169.57 (4C, $COCH_3$), 134.17 (C4), 126.34 (C3), 72.05, 69.92, 60.73, 52.81 (4C, C1, C2, C5, C6), 62.41 (C7), 23.12, 20.61, 20.56, 20.50 (4C, $COCH_3$); m/z (FAB) 369.1418, $(M + H)^+$ requires 369.1410.

***N*-[(1*S*,2*R*,5*R*,6*R*)-2-Amino-5,6-dihydroxy-4-hydroxymethyl-cyclohex-3-en-1-yl]acetamide hydrochloride 24.** Triphenylphosphine (20 mg, 76 μmol) was added to **23** (20 mg, 54 μmol) in THF–H_2O (2 : 1, 3 mL) and the solution was heated at 50 °C (5 h). The mixture was concentrated, the residue was dissolved in MeOH (2 mL), followed by the addition of Boc_2O (25 mg, 0.10 mmol) and KOH (20 mg, 0.40 mmol). The solution was stirred at room temperature (1 h) before being concentrated and subjected to flash chromatography (MeOH–EtOAc, 1 : 4) to furnish a gum. This gum was treated with 6 M HCl in MeOH (2 mL) to afford **24** (10 mg, 73%) as a colourless solid. Mp >230 °C; $[\alpha]_D^{20}$ −59.6; δ_H (600 MHz, D_2O) 5.28–5.25 (m, 1H, H3), 4.35–4.32 (m, 1H, H5), 4.30–4.18 (m, 2H, H7, H7), 4.12 (dd, 1H, $J_{1,2}$ 9.8 Hz, $J_{1,6}$ 11.0 Hz, H1), 4.05–4.02 (m, 1H, H2), 3.72 (dd, 1H, $J_{5,6}$ 8.0 Hz, H6), 2.10 (s, 3H, $COCH_3$); δ_C (150.9 MHz, D_2O) 175.62 ($COCH_3$), 143.68 (C4), 117.29 (C3), 73.00, 71.76, 57.83, 52.67 (4C, C1, C2, C5, C6), 60.84 (C7), 22.30 ($COCH_3$); m/z (FAB) 217.1189, $(M - Cl)^+$ requires 217.1188. Anal. calcd for $C_9H_{17}ClN_2O_4$: C, 42.78; H, 6.78. Found: C, 42.52; H, 6.59%.

Kinetic analysis of β-*N*-acetylglucosaminidases

O-GlcNAcase and *Vibrio cholerae* NagZ were overexpressed and purified just prior to use.[5,14] Human placental β-hexosaminidase B was purchased from Sigma (lot 043K3783). Assays involving human β-hexosaminidase and B *Bt*GH84 were carried out in triplicate at 37 °C for 30 minutes by using a stopped assay procedure in which the enzymatic reactions (50 μL) were quenched by the addition of a four-fold excess (200 μL) of quenching buffer (200 mM glycine, pH 10.75). Assays using NagZ and human *O*-GlcNAcase were carried out using continuous based assays in which the enzymatic reactions (80 μL) were conducted at 25 °C (NagZ) and 37 °C (human *O*-GlcNAcase) as the enzymes were considerably more stable at these temperatures. Assays were initiated by the careful addition, *via* pipette, of enzyme, and in all cases the final pH of the resulting quenched solution was greater than 10. Time-dependent assays of NagZ, β-hexosaminidase B, *O*-GlcNAcase and *Bt*GH84 revealed that the enzymes were stable and rates were linear in their respective buffers over the period of the assay: 50 mM NaH_2PO_4, 100 mM NaCl, 0.1% BSA, pH 6.5 for *O*-GlcNAcase and *Bt*GH84, and 50 mM citrate, 100 mM NaCl, 0.1% BSA, pH 4.25 for β-hexosaminidase B. The progress of the reaction at the end of 30 minutes for stopped assays was determined by measuring the amount of 4-nitrophenolate liberated as determined by UV measurements at 400 nm using a 96-well plate (Sarstedt) and 96-well plate (Molecular Devices) reader. Reaction velocities for continuous based assays were determined by monitoring the liberation of 4-nitrophenolate using linear regression of the linear region of the reaction progress curve between the first and third minute. *O*-GlcNAcase and β-hexosaminidase B were used in the inhibition assays at a concentration (μg μL^{-1}) of 0.0406 and 0.012, respectively, using 4-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside as the substrate at a concentration of 0.50 mM. The inhibitor was tested at seven concentrations ranging from three times to one-third K_i. K_i values were determined by linear regression of data from Dixon plots.

Crystallization and structure solution of *Bt*GH84[6]

N-Terminally His_6-tagged *Bt*GH84 was produced *via* isopropyl-1-thio-β-D-galactopyranoside (IPTG) induction (overnight) of pGH84-containing *Escherichia coli* BL21 (DE3; Novagen) cultures grown at 37 °C to an OD_{600} = 0.6. *Bt*GH84 was purified from cell free extracts *via* Ni-affinity chromatography followed by gel filtration. Purified protein was concentrated to 5 mg mL^{-1} and exchanged into 20 mM HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) (pH 7.5) using a 10 kDa cut-off concentrator. *Bt*GH84 crystals were grown using hanging drop vapour diffusion as for the native structure[6] with *N*-[(1*S*,2*R*,5*R*,6*R*)-2-amino-5,6-dihydroxy-4-hydroxymethyl-cyclohex-3-en-1-yl)acetamide hydrochloride **24** included at a concentration of 10 mM. X-Ray data were collected from a single crystal at 100 K at the European Synchrotron Radiation Facility on ID 14–2 with 30% v/v glycerol used as the cryo-protectant. All X-ray data were processed with MOSFLM from the CCP4 suite[39] (Table 1). Structure solution was carried out using the molecular replacement technique as implemented in the CCP4[39] version of MOLREP using *Bt*GH84 (PDB code 2CHO) as the search model. COOT[40] was used to make manual corrections to the model with refinement using REFMAC.[41] Solvent molecules were added using COOT and checked manually. Structural figures were

Table 1 X-Ray data quality and structure refinement statistics for the complex *Bt*GH84 with **24**

*Bt*GH84 with **24**[a]	
Data collection	
Resolution/Å	20–1.95 (2.06–1.95)
R_{merge}	0.086 (0.53)
$I/\sigma I$	8.3 (1.6)
Completeness (%)	97 (94)
Redundancy	2.5 (2.4)
Refinement	
Resolution/Å	20–1.95
R_{work}/R_{free}	0.20 (0.24)
Root mean square deviations	
Bond lengths/Å	0.014
Bond angles/°	1.4

[a] Highest resolution shell in parentheses.

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drawn with MOLSCRIPT[42]/BOBSCRIPT.[43] Coordinates have been deposited with the Protein Data Bank, PDB code 2JIW.

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