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Fleetamine (3-*O*-α-D-glucopyranosyl-swainsonine): the synthesis of a hypothetical inhibitor of *endo*-α-mannosidase

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ABSTRACT

3-*O*-α-D-Glucopyranosyl-swainsonine was originally proposed[17] as a potential inhibitor of the mammalian enzyme *endo*-α-mannosidase, but its synthesis has not been reported. Herein we report the total synthesis of this enigmatic compound, utilizing a halide-ion catalysed glycosylation of a swainsonine lactam with a glucosyl iodide donor as the key step. The resulting inhibitor was evaluated as an inhibitor of human *endo*-α-mannosidase, and as a ligand for bacterial orthologs from *Bacteroides thetaiotaomicron* and *Bacteroides xylanisolvens*, including active-centre variants, although no evidence for binding or inhibition was observed. The surprising lack of binding was rationalized by using structural alignment with an *endo*-α-mannosidase inhibitor complex, which identified deleterious interactions with the swainsonine piperidine ring and an essential active site residue.

1. Introduction

The majority of mammalian proteins are modified with N-linked glycans, which help to control protein folding and stability, physiological properties and cellular communication.[1–3] N-linked glycan biosynthesis occurs within the secretory pathway, and commences with the co-translational installation of a pre-formed 14-mer oligosaccharide in the endoplasmic reticulum (Fig. 1).[2] The glycan structures are trimmed by the *exo*-acting α-glucosidases I and II that sequentially remove single glucose residues. These glucosidases can be inhibited by sugar-shaped heterocycles of the D-*gluco* configuration such as deoxynojirimycin **1** (DNJ) and castanospermine **2** (CST) (Fig. 2).[4] Deglucosylated N-linked glycans are substrates for *exo*-acting mannosidases in the endoplasmic reticulum and Golgi apparatus, which ultimately trim these glycans to a core hexasaccharide, prior to their elaboration to hybrid and complex-type N-glycans. The *exo*-mannosidases of the ER and Golgi are inhibited by D-mannose-mimicking nitrogenous heterocycles, such as deoxymannojirimycin **3** (DMJ; inhibitor of ER mannosidase I) and swainsonine **4** (inhibitor of Golgi mannosidase II).[4]

endo-α-Mannosidase (classified into the carbohydrate-active enzymes family GH99;[5,6] www.cazy.org) is a glycoside hydrolase of the Golgi apparatus that uniquely cleaves mannoside linkages internally, within the first branch of an N-glycan chain (Fig. 1).[7] Cells in which glucosidase I or II has been inhibited or subjected to genetic knockout retain 40–80% normal N-glycan processing function as a result of *endo*-α-mannosidase.[8–10] Similarly, during a CST-blockade of α-glucosidase I and II, HepG2 cells produced N-linked glycans with a normal structure owing to the rescue by *endo*-α-mannosidase, as demonstrated by the release of Glc_{1-3}-Man oligosaccharides.[11] Specific inhibitors of *endo*-α-mannosidase have been developed by modifying the *exo*-mannosidase specific inhibitors, DMJ **1** and isofagomine[12,13] with an *endo*-α-mannosidase targeting α-1,3-linked glucosyl residue.[14–16] Thus, α-glucosyl-1,3-deoxymannojirimycin **5** (GlcDMJ) has been widely used as a specific *endo*-α-mannosidase inhibitor.[15] Recently, we reported the development of α-glucosyl-1,3-isofagomine **6** (GlcIFG), which is a 40-fold stronger binding ligand of *Bacteroides thetaiotaomicron* GH99 *endo*-α-mannosidase (*Bt*GH99) compared to GlcDMJ **5**.[16] Ternary complexes of GlcDMJ **5** or GlcIFG **6** (in the −2 and −1 subsites) and α-1,2-mannobiose (in the +1 and +2 subsites) with *Bacteroides xylanisolvens* GH99 *endo*-α-mannosidase (*Bx*GH99) allowed the active site residues to be defined and was suggestive of an enzyme mechanism involving a 1,2-anhydro sugar intermediate.[16]

A previous report in this journal proposed the structure of a new potential inhibitor of *endo*-α-mannosidase, based on the glu-

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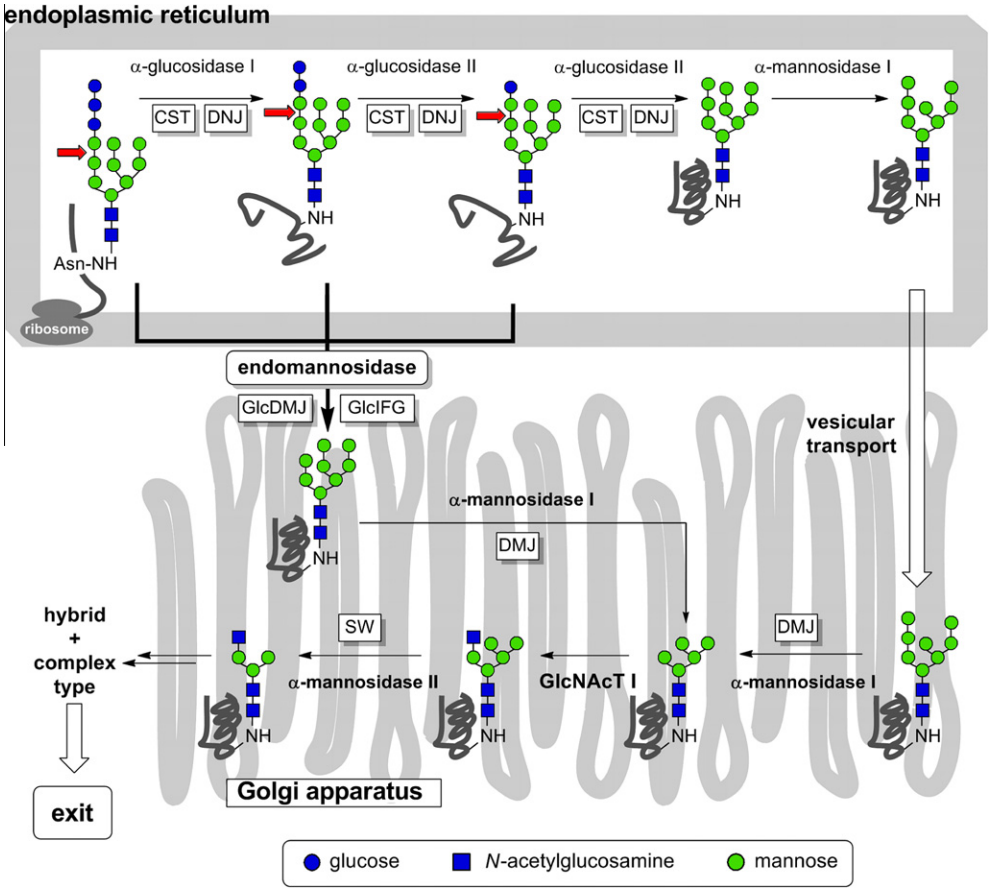

Figure 1. Summary of the major steps of the N-glycan trimming in the endoplasmic reticulum and Golgi apparatus. Red block arrows indicate the sites of *endo*-α-mannosidase cleavage. Boxed abbreviations represent inhibitors for the indicated steps: DNJ = deoxynojirimycin **1**, CST = castanospermine **2**, DMJ = deoxymannojirimycin **3**, SW = swainsonine **4**, GlcDMJ = α-D-glucopyranosyl-1,3-deoxymannojirimycin **5**, GlcIFG = α-D-glucopyranosyl-1,3-isofagomine **6**.

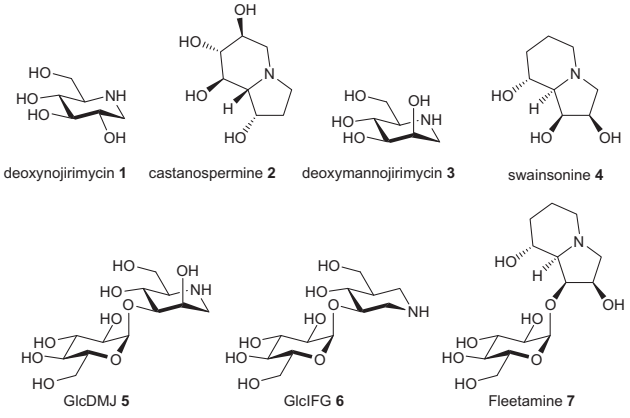

Figure 2. Structures of various glycosidase inhibitors.

cosylation of swainsonine, namely 3-*O*-α-D-glucopyranosyl-swainsonine **7** (Fleetamine).[†17] These authors noted that whereas DMJ is a strong inhibitor of ER mannosidase I and a weak inhibitor of mannosidase II, swainsonine possessed a reciprocal specificity.[17] Thus, it is unclear whether swainsonine or DMJ might represent the preferred cores for elaboration to an α-1,3-glucosylated analogue for *endo*-α-mannosidase inhibition. However, to the best of our knowledge, no synthesis of Fleetamine has been reported.[23] Herein we report the first synthesis of Fleetamine and its evaluation as an inhibitor of bacterial and mammalian *endo*-α-mannosidases.

2. Results and discussion

Our approach to Fleetamine required the identification of an efficient route to a selectively-protected swainsonine derivative. Whilst dozens of syntheses of swainsonine have been reported,[24,25] only a handful of these are sufficiently practical enough to allow the synthesis of adequate quantities to enable elaboration to the glycoside. Additionally, the tertiary amine of swainsonine is basic and may interfere with reagents used for glycosylation. With these concerns in mind, we identified the work of Pearson et al. as a practical route to swainsonine, which proceeds via a δ-lactam intermediate that appeared to constitute a promising starting point for our synthesis.[26,27] Accordingly, following Pearson's route, we synthesized gram quantities of the advanced δ-lactam **8** in eight steps from D-ribose.[27] Benzylation of alcohol **8** afforded monobenzyl ether **9**, which upon exposure to TsOH in methanol afforded diol **10**. Identification of suitable protecting groups for this derivative was problematic. After some experimentation, we ultimately elected to selectively protect the *cis*-diol of **10** as a monobenzyl ether. Alternative approaches involving monosilylation (with *tert*-butylchlorodiphenylsilane) occurred with the desired regioselectivity; however the resultant alcohol was a refractory glycosyl

[†] We have coined the name Fleetamine for this compound based on the original proposal of this structure by Fleet et al.,[17] and in recognition of his seminal contributions to the development of glycosidase inhibitors,[18] including the first total synthesis of (−)-swainsonine.[19] To describe this compound we use an informal numbering system that highlights the structural similarity of swainsonine to mannose and is consistent with the binding orientation of swainsonine in co-complexes with various glycoside hydrolases.[20–22] The numbering used in the Section 4 reflects the formal numbering used for indolizidines.[4]

acceptor. Treatment of **10** with NaH and benzyl bromide in DMF afforded dibenzyl ether **12**, accompanied by tribenzyl ether **11**, which could be recycled (hydrogenolysis, then isopropylidenation to afford **8**). Evidence for the structure of the dibenzyl ether was obtained by detailed ^1H–^1H COSY analysis of the acetate derivative **13** (Fig. 3).

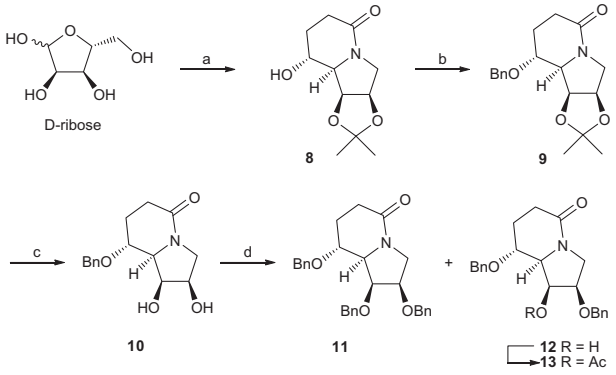

Figure 3. Reagents and conditions: (a) eight steps;[27] (b) BnBr, NaH, Bu_4NI, DMF, 86%; (c) TsOH, MeOH, 87%; (d) BnBr, NaH, Bu_4NI, DMF, 29% dibenzyl ether **12**, 35% tribenzyl ether **11**; (f) Ac_2O, pyridine, quantitative.

The next step required the challenging α-glucosylation of the hindered 3-hydroxy group of **12**, which is located on the *endo*-face, adjacent to a bridgehead, and flanked by a *cis*-vicinal benzyl ether. After testing several glucosylation methods, we obtained some success with Lemieux's halide-ion catalysed glycosylation,[28] using glycosyl iodide **14**.[29] Thus, treatment of **12** with iodide **14** (prepared from the corresponding anomeric acetate by treatment with iodotrimethylsilane)[30] afforded the target glycoside **15** in low yield (27%; unreacted **12** was recovered in 51%) but in sufficient amounts to proceed (Fig. 4).

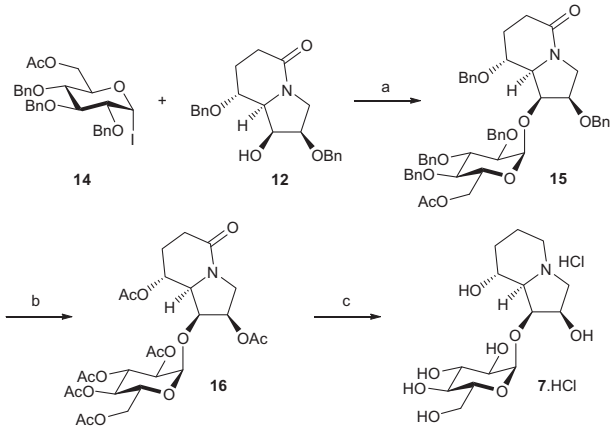

Figure 4. Reagents and conditions: (a) Bu_4NI, 2,4,6-tri-*tert*-butylpyrimidine, toluene, 4d, 27%; (b) (i) H_2, $Pd(OH)_2$-C, MeOH/EtOAc/AcOH; (ii) Ac_2O, pyr, 77% over two steps; (c) (i) $BH_3.Me_2S$, THF; (ii) NaOMe, MeOH then HCl, 67% over two steps.

Deprotection of disaccharide **15** required considerable experimentation to determine the optimum order and choice of reagents. Unexpectedly, the free base of Fleetamine proved to be unstable. Ultimately, a sequence involving hydrogenolysis of disaccharide lactam **15**, followed by acetylation to afford hexaacetate **16**, and then borane dimethyl sulfide reduction, deacetylation and immediate conversion to the hydrochloride afforded Fleetamine·HCl **7**·HCl.

The inhibitory activity of Fleetamine was explored using human recombinant *endo*-α-mannosidase with a fluorescent $Glc_3Man_7GlcNAc_2$ substrate.[31,32] However, at concentrations of up to 1.0 mM, no inhibition was noted. Fleetamine was also evaluated as a ligand for wild-type *Bt*GH99 using isothermal titration calorimetry (ITC). Using ITC, no binding was observed to either the wild-type or E154A variant (vide infra) *Bt*GH99. Attempts at complex formation using both soaking and co-crystallization of Fleetamine with *Bx*GH99 at ligand concentrations of up to 50 mM also failed to reveal any binding.

Whilst the rationale for adding an *endo*-α-mannosidase-targeting α-1,3-linked glucose to traditional *exo*-mannosidase inhibitors appears sound in principle, and is effective in converting DMJ **3** and isofagomine into *endo*-α-mannosidase inhibitors, our results highlight the limitations of this approach. In order to gain insight into the poor inhibitory properties of Fleetamine **7**, we performed a structural alignment using the macromolecular model building software COOT.[33] A single swainsonine molecule **4** was positioned over the deoxymannojirimycin moiety within the previously solved *Bx*GH99–GlcDMJ co-complex (PDB entry 4AD3)[16] using Least Squares Fitting of matching atom positions between the two molecules. Within the solved enzyme–inhibitor complex, the O4 and O6 atoms of DMJ appear at appropriate H-bonding distances from the Oε1 and Oε2 atoms of glutamate 156 (E156, equivalent to E154 of *Bt*GH99), an interaction required for catalysis as shown by site-directed mutagenesis studies.[16] The structural alignment of swainsonine **4** highlights a likely breakdown of this important interaction and a rationale for the apparent poor binding of **7** to *endo*-α-mannosidase. The O1 atom of swainsonine appears approximately 0.4 Å closer to Oε1 (as calculated by COOT), whilst there is no equivalent of the H-bonding of E156 and the O6 position of GlcDMJ. Carbon 7 of swainsonine approaches the side chain of E156 to within a distance of approximately 2.2 Å, which whilst perhaps being insufficient to be judged as a serious steric clash, this close interaction coupled with a loss of hydrogen-bonding explains the poor inhibitory properties observed for **7**. In order to probe whether removal of the equivalent residue in *Bt*GH99 could reinstate inhibitor binding, we generated the E154A *Bt*GH99 mutant, which was designed to create additional space in the active site. The ITC of Fleetamine and E154A *Bt*GH99 still failed to display any evidence of binding. It thus appears that even with the extra space created within the −1 subsite in this mutant, the bulky nature of the swainsonine moiety present within Fleetamine may prevent access into the enzyme active site (Fig. 5).

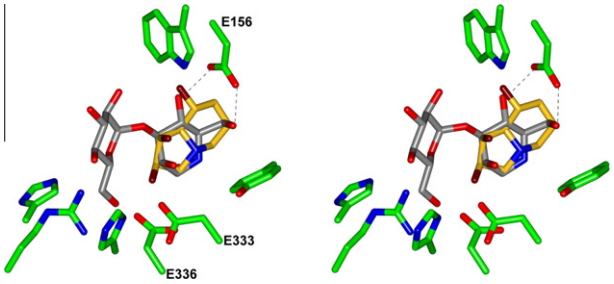

Figure 5. Superposition of swainsonine **4** with GlcDMJ **5** in a complex with *Bx*GH99 (PDB ID: 4AD3)[16] in a divergent stereoview prepared using CCP4Mg.[34] Shown is a Least Squares Fit (LSQ) alignment of swainsonine with the deoxymannojirimycin fragment of **5** prepared using COOT.[35] GlcDMJ is shown as a disaccharide with carbons in grey and oxygen atoms in red. Swainsonine is shown with carbon atoms in yellow and oxygens in dark red. Amino acid residues required for catalysis/binding are labelled; H-bonding interactions between E156 and GlcDMJ are illustrated as a dashed line.

3. Conclusion

Herein we have reported the synthesis of Fleetamine **7**, a putative inhibitor of *endo*-α-mannosidase that has remained an enigma since its original proposal by Fleet et al.,[17] Despite the enticing

rationale for its proposal as an inhibitor of *endo*-α-mannosidase,[17] we found no evidence that it acts as an inhibitor of either the human or bacterial forms of this enzyme. Fleet has suggested that the transition state for *exo*-acting α-mannosidase cleavage may in some cases be better inhibited by pyranose mimics, and in others by furanose mimics, in which cases swainsonine provides more effective inhibition than DMJ.[4,17] In this context, the transition states of the bacterial and mammalian *endo*-α-mannosidases can be considered to be 'pyranose-like' given that GlcIFG and GlcDMJ are better inhibitors than Fleetamine.

4. Experimental

4.1. General

All reactions were performed under a nitrogen atmosphere and glassware was flame-dried prior to use when necessary. Dichloromethane and THF were dried over alumina according to the method reported by Pangborn et al.,[36] Reactions were monitored by TLC analysis (pre-coated silica gel 60 F_{254} plates, 250 μm layer thickness on aluminium) and visualized with a 254 nm UV light and/or by staining with a CAM solution (5 g of cerium sulfate, 25 g of ammonium molybdate, 50 mL of concd H_2SO_4 and 450 mL of H_2O). Flash chromatography was performed according to the method of Still et al.[37] ^{1}H NMR spectra were obtained at 400 or 500 MHz in $CDCl_3$ or otherwise as noted. Chemical shifts are reported in parts per million with the residual solvent peak used as an internal standard. Multiplicity is denoted by: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, br s = broad singlet, dd = doublet of doublet, dt = doublet of triplet, dq = doublet of quartet, tt = triplet of triplet. ^{13}C NMR spectra were measured at 100 or 125 MHz using a proton-decoupled pulse sequence with a delay of 3 s. IR spectra were obtained on a Perkin–Elmer Spectrum One FTIR spectrometer with a zinc selenide/diamond Universal ATR sampling accessory as a thin film.

4.1.1. (3a*R*,9*R*,9a*R*,9b*S*)-9-Benzyloxy-2,2-dimethylhexahydro-[1,3]dioxolo[4,5-*a*]indolizin-6(9b*H*)-one 9

NaH (60% w/w, 44.7 mg, 1.12 mmol), BnBr (222 μL, 1.86 mmol) and Bu_4NI (27.5 mg, 0.0745 mmol) were added to a solution of alcohol **8**[27] (169 mg, 0.745 mmol) in DMF (15 mL) at 0 °C and the resultant mixture was warmed to rt and stirred for 19 h. The reaction mixture was diluted with EtOAc (15 mL), quenched with water (5 mL) and the aqueous layer extracted with EtOAc (2 × 25 mL). The combined organic extracts were washed with water (3 × 20 mL) and brine (20 mL), dried ($MgSO_4$) and concentrated under reduced pressure to give the crude product. Flash chromatography (2% MeOH/EtOAc) gave benzyl ether **9** (204 mg, 86%) as a colourless solid. $[\alpha]_D^{23} = -21.2$ (*c* 1.62, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.37–7.28 (m, 5H, Ph), 4.74 (m, 1H), 4.70 (m, 1H), 4.65 (ABq, *J* = 12.0 Hz, 2H, C*H*$_2$Ph), 4.18 (d, *J* = 14.0 Hz, 1H), 3.97 (ddd, *J* = 11.0, 7.5, 3.5 Hz, 1H), 3.38 (dd, *J* = 7.5, 4.0 Hz, 1H), 3.05 (dd, *J* = 13.5, 5.0 Hz, 1H), 2.48 (dt, *J* = 18.0, 5.0 Hz, 1H), 2.32 (ddd, *J* = 17.0, 11.5, 5.5 Hz, 1H), 2.15 (m, 1H), 1.85 (m, 1H), 1.38 (s, 3H, CH_3), 1.32 (s, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$): δ 168.7 (C=O), 138.3, 128.5, 127.9, 127.8 (Ph), 111.9 (*C*Me$_2$), 80.0, 77.6, 72.0, 71.7, 65.4, 50.6, 29.4, 26.8, 26.6, 24.9. HR-ESIMS *m/z* 318.16998 $[M+H]^+$, calcd 318.16998 for $C_{18}H_{24}NO_4$. FTIR: *ν* 2961, 2913, 2852, 2233, 1599, 1490, 1452, 1442, 1377, 1358, 1323, 1112, 1069, 1025, 911, 829, 754, 690 cm^{-1}.

4.1.2. (1*S*,2*R*,8*R*,8a*S*)-8-Benzyloxy-1,2-dihydroxyhexahydroindolizin-5(1*H*)-one 10

At first, *p*-TsOH (450 mg, 2.61 mmol) was added to acetal **9** (393 mg, 1.24 mmol) in MeOH (15 mL) and the reaction mixture was heated at reflux. After 21 h, additional *p*-TsOH (50 mg) was added, and the resulting mixture was heated at reflux for 5 h. The reaction was cooled to rt, then Et_3N (1 mL) was added and the mixture stirred at rt for 15 min. Removal of the volatiles under reduced pressure gave a crude residue, which was purified by flash chromatography (10% MeOH/EtOAc) to give **10** (295 mg, 87%) as a white fluffy material. $[\alpha]_D^{24} = -30.9$ (*c* 1.1, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.39–7.30 (m, 5H, Ph), 4.64 (ABq, *J* = 12.0 Hz, 2H, C*H*$_2$Ph), 4.34 (m, 1H, H2), 4.20 (m, 1H, H1), 3.86 (ddd, *J* = 11.0, 9.0, 4.0 Hz, 1H, H8), 3.69 (ddd, *J* = 12.0, 8.0, 3.0 Hz, 1H, H3), 3.45 (d, *J* = 8.5 Hz, 1H, H8a), 3.34 (dd, *J* = 12.0, 10.0 Hz, 1H, H3), 2.50 (m, 1H, H6), 2.32 (m, 1H, H6), 2.14 (m, 1H, H7), 1.76 (m, 1H, H7). ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.4 (C=O), 138.3, 128.6, 128.0, 127.8 (Ph), 71.6, 71.2, 71.0, 69.9, 65.6, 49.5, 29.5, 26.5. FTIR: *ν* 2961, 2913, 2852, 2233, 1599, 1490, 1452, 1442, 1377, 1358, 1323, 1112, 1069, 1025, 911, 829, 754, 690 cm^{-1}. HR-ESIMS *m/z* 278.13867 $[M+H]^+$, calcd 278.13868 for $C_{15}H_{20}NO_4$.

4.1.3. (1*S*,2*R*,8*R*,8a*R*)-1,2,8-Tris(benzyloxy)hexahydroindolizin-5(1*H*)-one 11 and (1*S*,2*R*,8*R*,8a*S*)-2,8-bis(benzyloxy)-1-hydroxy-hexahydroindolizin-5(1*H*)-one 12

NaH (60% w/w, 57.8 mg, 1.45 mmol), BnBr (107 μL, 0.903 mmol) and Bu_4NI (44.5 mg, 0.120 mmol) were added to a solution of diol **10** (334 mg, 0.836 mmol) in THF (25 mL) at 0 °C and the reaction stirred at 4 °C for 18 h, and then at rt for 30 min. The reaction mixture was diluted with EtOAc (25 mL), quenched with water (1 mL) and the aqueous layer extracted with EtOAc (2 × 25 mL). The combined organic extracts were washed with water (2 × 20 mL) and brine (20 mL), dried ($MgSO_4$) and concentrated under reduced pressure to give a crude residue. Flash chromatography (65% EtOAc/toluene) gave tribenzyl ether **11** (191 mg, 35%), followed by dibenzyl ether **12** (130 mg, 29%), both as yellow oils. The column was eluted with 40% MeOH/H_2O and the concentrated filtrate purified by flash chromatography (EtOAc then 4–25% MeOH/EtOAc) to give unreacted diol **10** (96.4 mg). Data for tribenzyl ether **11**: ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.37–7.24 (m, 10H, Ph), 4.97 (d, *J* = 11.5 Hz, 1H), 4.59 (dd, *J* = 11.5, 1.5 Hz, 2H), 4.40 (d, *J* = 11.5 Hz, 1H), 4.22 (t, *J* = 3.0 Hz, 1H), 4.10 (ddd, *J* = 9.5, 8.0, 1.5 Hz, 1H), 3.93 (ddd, *J* = 10.5, 8.5, 4.0 Hz, 1H), 3.77 (dd, *J* = 11.5, 8.0 Hz, 1H), 3.56 (dd, *J* = 10.5, 10.0 Hz, 1H), 3.49 (dd, *J* = 8.5, 3.0 Hz, 1H), 2.51 (ddd, *J* = 18.0, 6.5, 2.5 Hz, 1H), 2.35 (ddd, *J* = 18.0, 11.5, 7.0 Hz, 1H), 2.24 (m, 1H), 1.73 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 168.7 (C=O), 138.5, 138.1, 137.7, 128.7, 128.6, 128.1, 128.00, 127.98, 127.81, 127.76, 127.6 (Ph), 78.3, 76.3, 73.7, 72.4, 71.7, 71.1, 64.9, 47.4, 29.6, 26.3. Data for dibenzyl ether **12**: $[\alpha]_D^{24} = -20.2$ (*c* 0.845, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.40–7.29 (m, 10H, Ph), 4.66 (ABq, *J* = 11.5 Hz, 2H), 4.61 (s, 2H), 4.32 (dd, *J* = 4.0, 3.0 Hz, 1H), 4.11 (td, *J* = 8.5, 4.0 Hz, 1H), 3.95 (ddd, *J* = 11.5, 8.5, 4.0 Hz, 1H), 3.72 (dd, *J* = 10.0, 8.5 Hz, 1H), 3.50 (dd, *J* = 12.0, 8.5 Hz, 1H), 3.42 (dd, *J* = 8.5, 2.5 Hz, 1H), 2.51 (ddd, *J* = 18.0, 6.5, 2.5 Hz, 1H), 2.33 (ddd, *J* = 18.0, 12.0, 6.5 Hz, 1H), 2.19 (m, 1H), 1.76 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 168.8 (C=O), 138.4, 137.0, 128.8, 128.6, 128.5, 128.04, 127.97, 127.9 (Ph), 76.5, 72.5, 71.8, 71.4, 69.1, 65.7, 47.7, 29.7, 26.9. FTIR: *ν* 3349, 2931, 1739, 1620, 1454, 1096, 1068, 737, 698 cm^{-1}. HR-ESIMS *m/z* 368.18569 $[M+H]^+$, calcd 368.18563 for $C_{22}H_{26}NO_4$.

4.1.4. (1*S*,2*R*,8*R*,8a*R*)-2,8-Bis(benzyloxy)-1-acetoxy-hexahydroindolizin-5(1*H*)-one 13

A solution of alcohol **12** (3.2 mg, 8.7 μmol) in Ac_2O (400 μL) and pyridine (400 μL) was stirred at rt for 4 h, and then concentrated under reduced pressure to give crude acetate **13** (3.5 mg). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.38–7.28 (m, 10H, Ph), 5.70 (dd, *J* = 3.5, 2.5 Hz, 1H, H1), 4.55 (ABq, *J* = 11.5 Hz, 2H, C*H*$_2$Ph), 4.54 (ABq, *J* = 11.5 Hz, 2H, C*H*$_2$Ph), 4.10 (td, *J* = 9.0, 3.5 Hz, 1H, H2),

3.72 (dd, J = 12.0, 8.5 Hz, 1H, H3), 3.67–3.61 (m, 2H, H8 and H8a), 3.41 (dd, J = 12.0, 9.5 Hz, 1H, H3), 2.55 (ddd, J = 18.0, 6.5, 2.5 Hz, 1H, H6), 2.36 (ddd, J = 18.0, 11.5, 6.5 Hz, 1H, H6), 2.30 (m, 1H, H7), 1.98 (s, 3H, OAc), 1.74 (m, 1H, H7). Assignments were confirmed by COSY analysis.

4.1.5. (1*S*,2*R*,8*R*,8a*R*)-2,8-Bis(benzyloxy)-1-(6-*O*-acetyl-2,3,4-tri-*O*-benzyl-α-D-glucopyranosyloxy)-hexahydroindolizin-5(1*H*)-one 15

A solution of alcohol **12** (77.3 mg, 0.210 mmol) in dry toluene (2 mL) was added to a mixture of flame-dried powdered 4 Å molecular sieves (80 mg), Bu_4NI (427 mg, 1.16 mmol) and 2,4,6-tri-*tert*-butylpyrimidine (144 mg, 0.578 mmol) after which the suspension was stirred at rt for 10 min. Iodide **14** [prepared by the treatment of the corresponding anomeric acetate (557 mg, 1.04 mmol) in CH_2Cl_2 (12 mL) with TMSI (658 μL, 4.63 mmol) at 0 °C][30] in toluene (3 mL) was added via cannula and the mixture was stirred at 55 °C for 4 d. The reaction was quenched by the addition of water (1 mL), stirred at rt for 1 h and filtered through Celite. The aqueous phase was extracted with EtOAc (5 mL) and the combined organic extracts washed with satd aq $NaHCO_3$ (5 mL) and brine (5 mL), dried ($MgSO_4$) and concentrated under reduced pressure to give a crude residue. Flash chromatography (50–85% EtOAc/petrol) gave glycoside **15** (47.0 mg, 27%) as a yellow oil, in addition to unreacted alcohol **12** (39.7 mg). $[\alpha]_D^{22} = +24.2$ (c 0.21, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.36–7.24 (m, 23H, Ph), 7.16–7.14 (m, 2H, Ph), 5.07 (d, J = 11.0 Hz, 1H), 5.02 (d, $J_{1,2}$ = 3.5 Hz, 1H, H1′), 4.96 (ABq, J = 11.0 Hz, 2H), 4.84 (d, J = 11.0 Hz, 1H), 4.57 (d, J = 11.5 Hz, 1H), 4.55 (s, 2H), 4.52 (d, J = 10.5 Hz, 1H), 4.48 (dt, J = 10.5, 2.5 Hz, 1H, H2), 4.35 (dd, J = 3.5, 2.5 Hz, 1H, H1), 4.18 (ddd, J = 11.0, 9.0, 4.0 Hz, 1H), 4.14–4.10 (m, 2H), 4.06 (t, J = 11.0 Hz, 1H), 4.00 (ddd, J = 10.0, 8.5, 3.5 Hz, 1H), 3.96 (dd, J = 2.5, 1.0 Hz, 2H), 3.76 (dd, J = 11.5, 8.0 Hz, 1H), 3.58 (dd, J = 10.0, 4.0 Hz, 1H), 3.53 (dt, J = 10.0, 9.0 Hz, 2H), 3.41 (dd, J = 8.5, 2.0 Hz, 1H), 2.41 (ddd, J = 18.0, 6.0, 2.0 Hz, 1H, H6), 2.17 (ddd, J = 18.0, 12.0, 6.5 Hz, 1H, H6), 1.99 (s, 3H, Ac), 1.89 (m, 1H, H7), 1.50 (m, 1H, H7). ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.8, 168.9 (2C, C=O), 138.5, 138.2, 138.1, 137.2, 128.7, 128.62, 128.60, 128.56, 128.54, 128.21, 128.18, 127.99, 127.95, 127.89, 127.78, 127.6. 127.1 (Ph), 99.0 (C1′), 82.4, 80.6, 77.8, 77.4, 76.4, 75.9, 75.7, 75.0, 72.3, 70.2, 69.1, 68.9, 65.1, 62.8, 47.4, 29.4, 25.4, 21.0. FTIR: ν 3379, 2925, 1739, 1645, 1454, 1364, 1236, 1072, 1028, 738, 698 cm^{-1}. HR-ESIMS m/z 864.37169 [M+Na]$^+$, calcd 864.37182 for $C_{51}H_{55}NNaO_{10}$.

4.1.6. (1*S*,2*R*,8*R*,8a*R*)-2,8-Bis(acetoxy)-1-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyloxy)-hexahydroindolizin-5(1*H*)-one 16

A mixture of $Pd(OH)_2$ on carbon (10%, 255 mg), pentabenzyl ether **15** (51.0 mg, 60.6 μmol) in MeOH (5 mL), EtOAc (1.5 mL) and AcOH (0.5 mL) in a thick-walled flask was shaken under 70 psi of H_2 for 21 h. The flask was evacuated and the contents filtered through Celite. The filtrate was concentrated and the residue dried twice azeotropically using toluene to give the crude product, which was dissolved in pyridine (3.5 mL) and Ac_2O (3.5 mL) and heated at 50 °C for 15 h. The reaction mixture was concentrated to give a crude residue, which was purified by flash chromatography (100% EtOAc) to give hexaacetate **16** (28.0 mg, 77%) as a clear oil. $[\alpha]_D^{23} = +46.7$ (c 0.50, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.36 (dd, J = 10.0, 10.0 Hz, 1H), 5.19–5.12 (m, 2H), 5.11–5.07 (m, 2H), 4.86 (d, J = 3.5 Hz, 1H), 4.47 (dd, J = 3.5, 3.0 Hz, 1H), 4.33 (ddd, J = 10.0, 3.5, 2.5 Hz, 1H), 4.25 (dd, J = 12.5, 4.0 Hz, 1H), 4.06 (dd, J = 12.5, 2.0 Hz, 1H), 3.91 (dd, J = 12.0, 8.5 Hz, 1H), 3.71 (dd, J = 7.0, 2.5 Hz, 1H), 3.54 (dd, J = 12.0, 10.0 Hz, 1H), 2.53 (dt, J = 17.5, 6.0 Hz, 1H), 2.43 (ddd, J = 17.5, 9.0, 6.0 Hz, 1H), 2.22 (m, 1H), 2.11 (s, 3H), 2.10 (s, 3H), 2.09 (s, 3H), 2.05 (s, 3H), 2.03 (s, 3H), 1.99 (s, 3H), 1.89 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.7, 170.3, 170.1, 170.0, 169.8, 169.6, 168.9 (7C, C=O), 97.5 (C1′), 70.8, 70.0, 69.8 (2C), 68.3, 66.7, 64.0, 61.6, 46.3, 28.9, 26.5, 21.1, 20.9, 20.8, 20.76, 20.73, 20.72. FTIR: ν 1747, 1647, 1454, 1228, 1043 cm^{-1}. HR-ESIMS m/z 624.18978 [M+Na]$^+$, calcd 624.18989 for $C_{26}H_{35}NNaO_{15}$.

4.1.7. (1*S*,2*R*,8*R*,8a*R*)-2,8-Bis(hydroxy)-1-(α-D-glucopyranosyloxy)-octahydroindolizine (Fleetamine·HCl) 7·HCl

4.1.7.1. (1*S*,2*R*,8*R*,8a*R*)-2,8-Bis(acetoxy)-1-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyloxy)-octahydroindolizine. $BH_3{\cdot}SMe_2$ (10 M, 12 μL, 120 μmol) was added to a solution of lactam **16** (14.0 mg, 23.3 μmol) in THF (1.5 mL) at 0 °C and the solution was stirred at 0 °C for 30 min and then at rt for 1.5 h. The reaction mixture was then cooled to 0 °C and quenched carefully with EtOH (0.5 mL). The solvent was removed under reduced pressure and the residue dissolved in EtOH (1.5 mL) and then heated at reflux for 2 h. The solution was cooled to rt and concentrated under reduced pressure to give a crude residue of the title compound (14.9 mg), which was used without purification in the next step. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.56 (t, J = 12.0 Hz, 1H), 5.22 (td, J = 7.2, 2.4 Hz, 1H), 5.10 (dd, J = 10.4, 4.0 Hz, 1H), 5.03 (app t, J = 10.0 Hz, 1H), 4.82 (d, J = 3.6 Hz, 1H), 4.76 (dd, J = 10.0, 4.4 Hz, 1H), 4.43 (dd, J = 6.8, 4.0 Hz, 1H), 4.27 (ddd, J = 12.8, 4.8, 2.0 Hz, 1H), 4.17 (dd, J = 12.4, 4.8 Hz, 1H), 4.09 (dd, J = 12.4, 2.0 Hz, 1H), 3.12 (dd, J = 11.2, 2.4 Hz, 1H), 3.04–3.02 (m, J = 11.0 Hz, 1H), 2.57 (dd, J = 10.8, 7.6 Hz, 1H), 2.45–2.39 (m, 1H), 2.17 (s, 3H), 2.14 (s, 3H), 2.08 (s, 3H), 2.00 (s, 3H), 1.99 (s, 3H), 1.97 (s, 3H), 1.92 (td, J = 11.2, 3.6 Hz, 1H), 1.11 (dq, J = 4.8 Hz, 1H). HR-ESIMS m/z 588.22851 [M+H]$^+$, calcd 588.22868 for $C_{26}H_{38}NO_{14}$.

4.1.7.2. (1*S*,2*R*,8*R*,8a*R*)-2,8-Bis(hydroxy)-1-(α-D-glucopyranosyloxy)-octahydroindolizine (Fleetamine·HCl) 7·HCl. At first, NaOMe (0.5 M in MeOH, 45.0 μL, 22.5 μmol) was added to a solution of the hexaacetate amine (13.0 mg, 22.1 μmol) in dry MeOH (1.5 mL) and the reaction was stirred at rt for 2 h. The pH was adjusted to 4 by adding 0.1 M HCl in MeOH (0.5 mL). The solution was concentrated under reduced pressure to give a residue, which was purified by flash chromatography (100% MeOH) to afford Fleetamine·HCl **7**·HCl (5.5 mg, 67%) as a white solid. $[\alpha]_D^{22} = +44.5$ (c 0.28, MeOH). ^{1}H NMR (400 MHz, CD_3OD): δ 5.14 (d, J = 4.0 Hz, 1H), 4.35–4.28 (m, 2H), 3.96 (ddd, J = 10.0, 5.6, 2.4 Hz, 1H), 3.88–3.83 (m, 1H), 3.79 (dd, J = 12.0, 2.4 Hz, 1H), 3.74 (t, J = 7.2 Hz, 1H), 3.66 (dd, J = 12.0, 5.6 Hz, 1H), 3.37 (dd, J = 9.6, 3.6 Hz, 1H), 3.35 (s, 1H), 2.99–2.95 (m, 2H), 2.55 (dd, J = 10.4, 7.6 Hz, 1H), 2.06–2.03 (m, 1H), 1.96 (dd, J = 11.6, 3.2 Hz, 1H), 1.91 (dd, J = 9.2, 3.6 Hz, 1H), 1.74–1.61 (m, 2H), 1.18 (dq, J = 4.8 Hz, 1H). FTIR: ν 1747, 1647, 1454, 1228, 1043 cm^{-1}. HR-ESIMS m/z 336.1659 [M+H]$^+$, calcd 336.1658 for $C_{14}H_{26}NO_8$.

4.2. Binding studies

The ability of Fleetamine to bind bacterial *endo*-α-mannosidases was assessed through isothermal titration calorimetry (ITC) with both wild-type and an E154A variant of *Bt*GH99, and through attempted complex formation by soaking and co-crystallization with wild-type *Bx*GH99. Inhibition studies were performed using $Glc_3Man_7GlcNAc_2$ as the substrate with recombinant human *endo*-α-mannosidase.

4.2.1. ITC

The ligand affinity of *Bt*GH99 wild-type and *Bt*GH99 E154A for Fleetamine **7** was studied by ITC using an iTC_{200} calorimeter (MicroCal). Assays were carried out at 25 °C with Fleetamine (5 mM) titrated into the ITC cell containing 315 μM (wild-type) and 429 μM

(E154A *Bt*GH99) enzyme. Data were analysed using the Origin 7 software package (MicroCal).

4.2.2. Crystallization and data collection

*Bx*GH99 was crystallized in both native form and as attempted protein–ligand co-complexes from 0.1 M sodium acetate pH 4.9, 20% w/v PEG 2000 MME (mono-methyl ether), 1.6% poly-γ-glutamic acid (low molecular weight). Soaking of native protein crystals was conducted by the addition of approximately 50 mM ligand over time periods ranging from 30 min to 12 h, prior to cryo-protection. All crystals were cryo-protected by the stepwise addition of ethylene glycol to a final concentration of 20% v/v. Diffraction data were collected at York using a Rigaku RUH3R rotating anode X-ray generator.

4.2.3. Enzyme inhibition assay

Glucosylated substrate ($Glc_3Man_7GlcNAc_2$) was obtained from tissue-cultured CHO cells treated with *N*-butyl-deoxynojirimycin (NB-DNJ) as described previously.[32] An aliquot was labelled with anthranilic acid and analysed by normal phase (NP)-HPLC. The amount of fluorescence under the curve was determined and converted into molar amounts using an experimentally-derived conversion factor. Oligosaccharide substrate, 5 pmole, was dried and resuspended in 10 μL (0.01 μg protein) of human recombinant *endo*-α-mannosidase enzyme,[38] in 0.1 M sodium/MES buffer, pH 6.5. Following incubation at 37 °C for 45 min in the presence or absence of an inhibitor, the reaction was stopped with glacial AcOH (4 μL), diluted with water and an equal volume of acetonitrile added. An aliquot was analysed by NP-HPLC to separate the reaction products and the amount of hydrolysis quantified using peak area analysis.

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