Glycosidase inhibition by ring-modified castanospermine analogues: tackling enzyme selectivity by inhibitor tailoring

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Received 6th April 2009, Accepted 9th April 2009
First published as an Advance Article on the web 22nd May 2009
DOI: 10.1039/b906968b

Synthesis of a panel of iso(thio)urea-type ring-modified castanospermine analogues bearing a freely mutarotating pseudoanomeric hydroxyl group results in tight-binding β-glucosidase inhibitors with unusual binding signatures; the presence of an *N*-octyl substituent imparts a remarkable anomeric selectivity, promoting strong binding of the appropriate β-anomer by the β-glucosidase.

Introduction

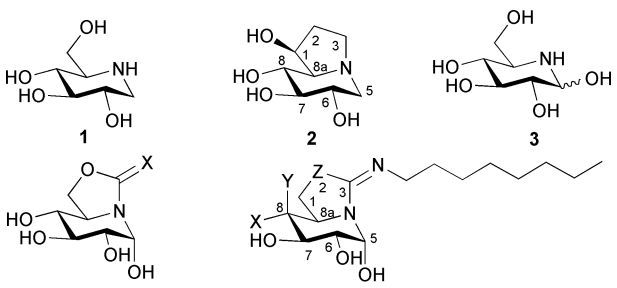
Sugar mimics bearing endocyclic nitrogen atoms (iminosugars and azasugars), such as deoxynojirimycin **1** and castanospermine **2**, have been shown to behave as potent glycosidase inhibitors, targeting enzymes involved in a battery of different biological processes. Consequently, they can play a role in treating a number of diseases including diabetes, viral infections and lysosomal storage disorders.[1] Their remarkable activity has been ascribed, in some cases, to their ability to mimic, in their protonated ammonium form, the positive charge of the oxacarbenium-like transition state.[2–4] Neither **1** nor **2**, however, possess a substituent at the carbon atom analogous to the anomeric position in the natural glucoside substrates. This means there is reduced selectivity between α- and β-glucosidases which is potentially a drawback for clinical applications.

Whilst the derivative of **1** with a hydroxyl group at the anomeric position, the natural product nojirimycin **3**, is chemically and configurationally unstable in solution,[5] synthetic routes have been successful at creating stable reducing castanospermine analogues, such as **4** and **5**, that have the pseudoanomeric hydroxyl group 'axially anchored'[6,7] equating to the configuration of natural α-glucosides. This unusual feature is ascribed to an efficient contribution to the anomeric effect involving the sp^2 orbital hosting the lone pair on the nitrogen atom in the ground state of pseudoamide functionality present in these compounds (that we have termed 'sp^2-iminosugars', by analogy to compounds where the endocyclic oxygen atom has been replaced by a nitrogen atom with substantial sp^2 character, typically a pseudoamide-type nitrogen; see ref. 8 and 9 for examples of recent syntheses of similar compounds). Interestingly, compounds **4** and **5** behaved as potent and selective inhibitors of yeast α-glucosidase, with α/β anomeric selectivity of at least 300-fold, which is much higher than observed with compounds **1–3**,[10–19] Table 1. This is in agreement with the stereocomplementarity of the α-configured hydroxyl group with the key active site residues in an α-glucosidase.

Table 1 K_i values (μM) for compounds **1–5** against selected glycosidases[a]

Enzyme	1	2	3	4[6,7]	5[6,7]
α-glucosidase (baker's yeast)	25[12]	>1500[16]	6.3[18]	2.2	40
β-glucosidase (almond)	29[13]	1.5[16]	0.9[18]	n.i.[b]	15000
β-gluco/β-galactosidase (bovine liver)	210[14]	40[16]	42[19]	n.i.	n.i.
β-glucosidase (*Thermotoga maritima*)	9.0[15]	2.0[17]	n.d.[c]	n.d.	n.d.

[a] Inhibition is competitive in all cases. [b] n.i., no inhibition was observed (at 2 mM inhibitor). [c] n.d., not determined.



Inhibition studies of similar compounds with modifications at the exocyclic heteroatom in the five-membered ring, such as the *n*-butyl (**6**) and *n*-octyl (**7**) cyclic isourea derivatives, led to a dramatic shift in α/β glucosidase selectivity. Compounds **6** and **7** bind to the almond β-glucosidase two and 50 times tighter (K_i values of 30 μM and 3.2 μM, respectively) than with yeast α-glucosidase, respectively.[20,21] Compound **7** also behaves as a good inhibitor of human lysosomal β-glucosidase (β-glucocerebrosidase; IC_{50} of 3.8 μM),[22] and may hold promise as an active site specific chaperone for the treatment of Gaucher disease.[23]

The increase in the glucosidase inhibitory potency of glucosidases upon incorporation of long aliphatic substituents onto (hetero)cyclic frameworks is well documented, such as observed previously in piperidine-,[14,24–26] cyclohexene-[27,28] glycosylamine-[29]

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and imidazole-functionalized inhibitors.[30] A similar scenario has been reported for inhibitors of other glucose-processing enzymes such as glycogen phosphorylase.[31,32] Nevertheless, the β-anomeric selectivity of compounds **6** and **7** for β-glucosidases, despite the synthesis directing a pseudoaxial hydroxyl substituent at the (pseudo)anomeric position (α-configuration), is counter-intuitive and warranted further investigation on their mechanism of action. Here we report the preparation of a series of cyclic iso(thio)urea castanospermine analogues including **7** and the new derivatives **8–10**, as well as their evaluation against several commercial glycosidases. Further kinetic, thermodynamic and X-ray studies with a β-glucosidase from *Thermotoga maritima* (*Tm*GH1),[17] which belongs to family 1 and clan GH-A in the CAZy classification,[33] revealed an unusual role for entropic contributions to binding, providing a new template for glycosidase inhibitor design.

Results and discussion

Synthesis

Our synthetic strategy relies on the ability of furanose pseudo-*C*-nucleosides (II) to rearrange into indolizidine derivatives (I) through the open chain *aldehydo* form (Fig. 1). The configurational pattern of the final bicyclic inhibitor depends on the configuration of the monosaccharide precursor (D-*gluco* or D-*galacto* configuration).

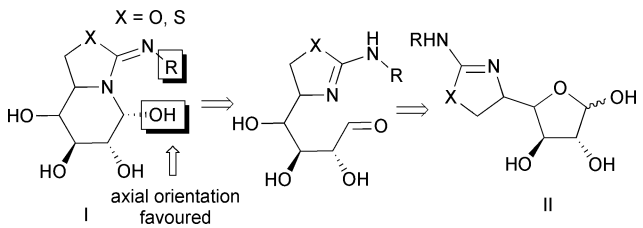
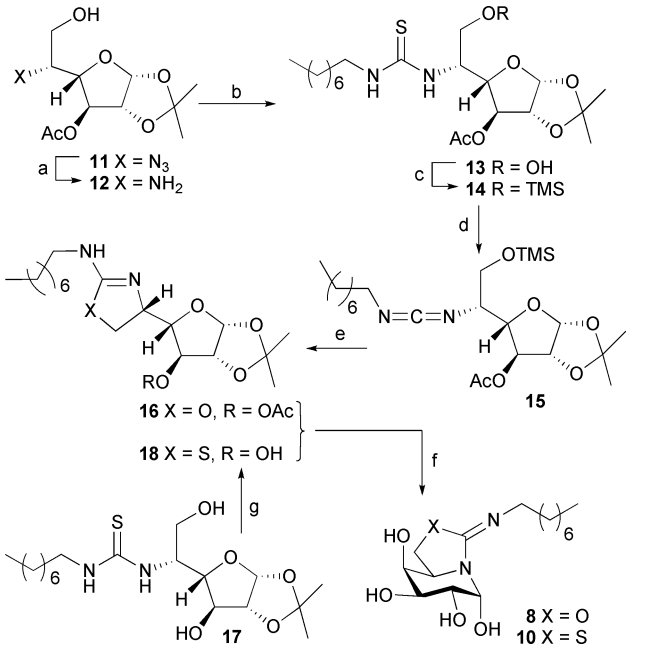


Fig. 1 Retrosynthesis of reducing 2-oxa(thia)-3-iminoindolizidine derivatives (I) from aldohexofuranose-derived 2-iminooxa(thia)zoline precursors (II).

The above strategy is particularly well-adapted to generate molecular diversity, allowing not only modifications of the hydroxylation profile, but also the introduction of different substituents, which could prove extremely important for optimizing inhibitor properties in the future. Thus, the 2-oxa-3-imino-8-*epi*-castanospermine analogue **8**, which possesses hydroxyl substituent groups with a configurational pattern analogous to that of α-D-galactose, was obtained following a similar synthetic approach to that described for **7** (Scheme 1).[21] The starting material 3-*O*-acetyl-5-azido-5-deoxy-1,2-*O*-isopropylidene-D-galactofuranose (**11**), available from commercial α-D-galactose,[34] was hydrogenated and the resulting amine **12** was coupled with *N*-octyl isothiocyanate, which afforded the thiourea adduct **13** (for a review on sugar thioureas see ref. 35). Trimethylsilylation of the primary alcohol function (→**14**) and desulfuration with HgO provided the corresponding carbodiimide **15** (for recent references on the synthesis of sugar carbodiimides from sugar thioureas see refs. 36–38). Further removal of the silyl ether group with TBAF proceeded with spontaneous intramolecular nucleophilic addition of the generated hydroxyl group to the vicinal heterocumulene functionality, to give the (4*S*)-4-(L-threofuranose-4′-yl)-2-octylamino-2-oxazoline pseudo-*C*-nucleoside derivative **16**. Conventional deacetylation, hydrolysis of the acetal protecting group with TFA–water and neutralization with Amberlite IRA 68 (OH^-) afforded the target indolizidine **8**.



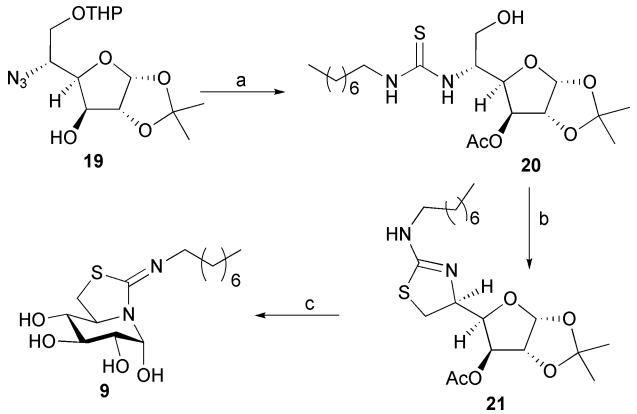
Scheme 1 Synthesis of the cyclic iso(thio)urea 8-*epi*-(+)-castanospermine analogues **8** and **10**. a) H_2, Pd/C, MeOH, r.t., 1 h, 98%; b) RNCS, py, Et_3N, r.t., 18 h, 80%; c) TMSCl, HMDS, py, r.t., 2 h, 90%; d) HgO, CH_2Cl_2–H_2O, r.t., 45 min, 80%; e) TBAF, THF, 0 °C, 25 min, 90%; f) 1. NaOMe, MeOH, CO_2. 2. 90% TFA–H_2O, 15 min. 3. Amberlite IRA 68 (OH^-), 90–95% (global); g) MsCl, pyridine, –20 °C, 7 h, 60%.

For the preparation of the 3-imino-2-thia-(+)-castanospermine **9** and its epimer **10**, an original synthetic strategy involving nucleophilic displacement of a transient 6-*O*-methanesulfonyl ester by the thiocarbonyl sulfur atom in the corresponding *N′*-octylthiourea adducts was implemented. Thus, treatment of the D-*galacto*-thiourea **17** (obtained by conventional deacetylation of **16**; Scheme 1) or the D-*gluco*-epimer **20** (prepared from azide **19** by reduction, coupling with octyl isothiocyanate, acetylation and final hydrolysis of the tetrahydropyranyl group; Scheme 2) with methanesulfonyl chloride, afforded the corresponding 2-aminothiazolines **18** and **20**. Removal of the hydroxyl protecting groups promoted the furanose→thiaindolizidine rearrangement to give the target compound with D-*gluco* **9** (Scheme 2) and D-*galacto* configuration **10** (Scheme 1).

Compounds **7–10** exhibited the presence of both diastereomers at the pseudoanomeric position, but with a large preference (>85%) for α- over the β-anomer in D_2O solution. The high field shift of the pseudoanomeric carbon (C5) resonance (74.9–76.7 ppm) confirmed the aminoketalic bicyclic structure, whereas the vicinal $^3J_{H,H}$ values around the piperidine ring unambiguously pointed to the 5*R* configuration for the new stereocentre, with the pseudoanomeric hydroxyl group in an axial position.

Glycosidase inhibitory activity

The inhibition by **7–10** towards a panel of glycosidases is shown in Table 2.



Scheme 2 Synthesis of the D-*gluco*-thiazoline **9**. a) 1. H_2, 10% Pd/C, MeOH, r.t., 1 h. 2. $(CH_2)_7CH_3NCS$, Et_3N, pyridine, r.t., 18 h. 3. (1:1) Ac_2O-Py, −15 °C, 5 h. 4. TsOH, (1:1) CH_2Cl_2–MeOH, r.t., 2 h, 52% (global); b) MsCl, pyridine, −20 °C → 10 °C, 7 h, 83%; c) 1. NaOMe, MeOH, r.t., 30 min., solid CO_2. 2. 90% TFA-H_2O, r.t., 30 min. 3. Amberlite IRA 68 (OH^-), 90%.

Table 2 K_i values (μM) for **7–10** against a panel of glycosidases[a]

Enzyme	7	8	9	10
α-glucosidase (baker's yeast)	168	n.i.[b]	n.i.	n.i.
β-glucosidase (almond), pH 5.5	3.2	0.4	1.0	2.5
β-glucosidase (almond), pH 7.3	1.9	0.019	0.76	0.023
β-glycosidase (bovine liver)[c]	2.7	0.052	3.7	0.042
trehalase (pig kidney)	182	n.i.	13.4	n.i.
α-L-fucosidase (pig kidney), pH 6.8	200	n.i.	18	11
β-glucosidase (*T. maritima*)	35.2	0.83	9.1	0.26

[a] Inhibition was competitive. No inhibition was observed for any compound (at 2 mM) on jack bean α-mannosidase, *Aspergillus niger* glucoamylase, green coffee bean α-galactosidase or yeast isomaltase. [b] n.i., no inhibition was observed (at 2 mM inhibitor). [c] Assayed using D-*galacto*-configured substrate.

In line with previous results,[21] compounds **7–10** showed either no or very poor inhibition of the α-glucosidases tested (yeast α-glucosidase, yeast isomaltase and *Aspergillus niger* amyloglucosidase). Compounds **7** and **9**, however, which are both D-*gluco*-configured, are reasonable inhibitors of pig kidney trehalase, and **7**, **9** and **10** also inhibit a pig kidney α-L-fucosidase. The iminooxa(thia)zolidines **7–10** were low micromolar or nanomolar inhibitors of the family 1 β-glucosidases from almond, bovine liver (cytosolic) and *T. maritima.*

Comparison of the inhibition data shows that the D-*galacto*-configured compounds **8** and **10** tend to be more potent against each of the three β-glucosidases tested than the D-*gluco*-configured counterparts. Family 1 β-glucosidases are generally not very selective regarding the configuration at C4. The mammalian cytosolic enzyme, for instance, is known to hydrolyse β-D-glucose- and β-D-galactose-containing substrates with comparable efficiencies.[39] Accordingly, iminosugars with hydroxylation patterns of stereochemical complementarity to D-galactose are frequently moderate to good inhibitors of family 1 β-glucosidases.[10,11,40] In the case of *Tm*GH1, **8** and **10** have 30–40-fold higher potency than **7** and **9**, respectively, which differs from the reported higher activity on D-glucoside rather than D-galactoside substrates[41] and a previous comparison of D-*gluco*- *vs.* D-*galacto*-derived hydroximo-1,5-lactams, where the former was around two-fold more potent.[42]

The data also suggest that the iminothiazolidine compounds **9** and **10** tend to have similar or slightly higher potency than the iminooxazolidine equivalents (**7** and **8**, respectively). This is in sharp contrast to that observed for the inhibition of α-glucosidases by castanospermine-type sp^2-iminosugars lacking the 2-alkylamino substituent, *e.g.* **4** and **5**. In this series, the endocyclic oxygen atom (O2 in the indolizidine skeleton) was found to be critical for potent inhibition. The loss of affinity for yeast α-glucosidase on going from **7** to **9** is consistent with this observation. Moreover, **4** and **5** exhibited a reverse selectivity among the α-glucosidase isoenzymes as compared with the natural alkaloid **2** (see Table 1 for yeast α-glucosidase and ref. 7 for other α-glucosidases). This was ascribed to the rigidity of the bicyclic framework, which imposes a distinct conformation about the linkage equivalent to C5–C6 in monosaccharides; going from **2** to **4** or **5** is equivalent to a ~120° rotation (from a conformation close to *gauche-gauche* to the *gauche-trans* conformation; for the nomenclature used to denote the staggered rotamers about the C5–C6 bond in hexopyranoses see ref. 43) about this bond. In the case of the β-glucosidases tested, while the *gauche-gauche* orientation at this region (i.e., the oxygen atom equivalent to O6 in hexoses in a *gauche* arrangement with respect to the piperidine carbon atom C8, as in **2**) results in potent inhibition, anchoring the *gauche-trans* conformation (i.e., C2 and C8 in anti-disposition, as in **4** and **5**) abolishes the inhibitory activity (Table 1). The current data shows, however, that the loss of affinity associated with this conformational change can be overcome by incorporation of the 2-octylamino substituent (**7–10**, Table 2). The presumable hydrophobic nature of the interactions at work in this region might explain the stronger inhibition of the less polar 2-thia (**9** and **10**) as compared with the 2-oxa derivatives (**7** and **8**).

It is also interesting to note that **7–10** are more potent at neutral (pH 7.3) rather than acidic pH (pH 5.5) against the almond β-glucosidase, as has also been observed for isofagomine and deoxynojirimycin on this enzyme[13] and other family 1 β-glucosidases.[15,44] The pK_a values for **7–10** have been potentiometrically determined to be in the range 7.3 ± 0.7. This implies that they are fully protonated at pH 5.5, whereas only ~50% of the inhibitor is in the protonated form at pH 7.3. It is unclear why the compounds bind tighter at higher pH, but may reflect the preference for a doubly deprotonated catalytic apparatus as observed with other charged inhibitors.[45,46]

Structural studies

The observed specificity of the ring-modified castanospermine analogues **7–10** for enzymes hydrolyzing β-configured substrates, in spite of the apparent mismatching of configuration at the pseudoanomeric centre in aqueous media, prompted us to carry out a structural study to visualize them in complex with an enzyme. The β-glucosidase *Tm*GH1 served this purpose well as it is amenable to X-ray crystallography studies and has previously been reported in complex with several other inhibitors, which would allow comparison.[42] The structure of *Tm*GH1 in complex with **7**, **8** and **9** were solved using data to 1.85, 2.00 and 1.70 Å, respectively (Table 3; complexes with **10** afforded 'twinned' crystals that were not amenable to structural investigation). In each case, electron

Table 3 Data processing and refinement statistics for *Tm*GH1 in complex with **7**, **8** and **9**

	*Tm*GH1 in complex with **7**	*Tm*GH1 in complex with **8**	*Tm*GH1 in complex with **9**
Data collection	ESRF, ID14-1	CuKα	ESRF, ID14-4
Resolution (outer shell), Å	20-1.85 (1.92-1.85)	25-2.00 (2.07-2.00)	30-1.70 (1.6-1.70)
Space group	$P2_1$	$P2_1$	$P2_1$
R_{merge} (outer shell)	0.09 (0.48)	0.11 (0.36)	0.10 (0.28)
Mean I/σI (outer shell)	13.5 (2.7)	6.5 (2.4)	5.3 (2.9)
Completeness (outer shell), %	100 (100)	97 (91)	94 (76)
Multiplicity (outer shell)	3.9 (3.9)	3.1 (3.1)	3.6 (2.3)
No. unique reflections	152735	112653	184204
R_{cryst}	0.15	0.18	0.17
R_{free}	0.20	0.25	0.21
RMSD bonds (Å)	0.016	0.014	0.015
RMSD angles (°)	1.45	1.45	1.51
RMSD chiral volume (Å^3)	0.106	0.098	0.106
PDB code	2WBG	2WC3	2WC4

density revealed a single molecule of the inhibitor in the –1 subsite in the active site of each molecule in the asymmetric unit (Fig. 2).

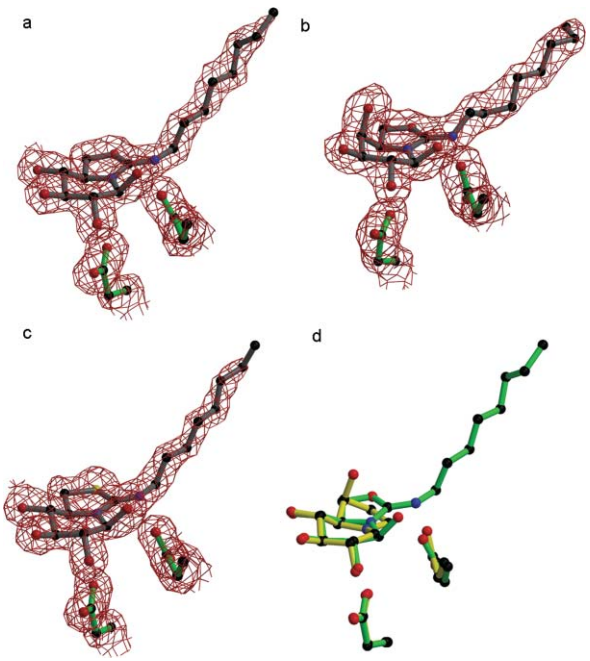


Fig. 2 Ball-and-stick representation of *Tm*GH1 in complex with (a) **7**, (b) **8** and (c) **9**. In each case the residue below the inhibitor is the nucleophile (Glu351) and the residue to the right is the acid/base (Glu166). Observed electron density for the maximum likelihood weighted $2F_{ob} - F_{calc}$ map is contoured at 1σ. (d) Overlap between *Tm*GH1 in complex with **7** (green) and in complex with **2** (yellow). Figures were drawn using BOBSCRIPT.[47]

The piperidine ring of **7–9** was found in a distorted (towards ^4E) chair conformation in which the carbon atom equivalent to C4 in a monosaccharide was further out of the definition plane than C1. Somewhat to our surprise, however, the structures of the complexes showed the unequivocal presence of a pseudoequatorial hydroxyl group at the pseudoanomeric position, that is, the β-anomer of the compounds was bound. NMR spectra had shown that although the α-anomer is the predominant form of each unbound inhibitor in D_2O solution, small amounts (10–15%) of the β-anomer are present. The anomeric ratio was essentially unmodified in the crystallization buffer. It would thus appear that the enzyme binds the thermodynamically less favoured β-anomer from a freely mutarotating mixture. Hemiaminals are known to mutarotate in solution,[48] with glycosidases presumably selecting the tighter-binding anomer (as in ref. 49).

The majority of the interactions made between *Tm*GH1 and each of the inhibitors are identical. The interactions between *Tm*GH1 and the hydroxyl groups equivalent to the positions at C2, C3 and C4 (using glycoside numbering) have been detailed previously,[15] and are shown on Fig. 3. There are some differences in the interactions made by the hydroxyl group at C4 between the D-*gluco*-configured **7** and **9** and the D-*galacto*-configured **8**; in **8**, the hydroxyl group hydrogen bonds with Trp406 instead of Trp398, and both oxygen atoms of Glu405 instead of just Oε1 as seen with **7** and **9**. The interactions at the C4 position mirror those with the D-*gluco*- and D-*galacto*- configured hydroximolactams in complex with *Tm*GH1.[42] It is therefore surprising that with the hydroximolactam compounds the D-*galacto*-configured inhibitor has around two-fold lower potency than the D-*gluco*- counterpart, whereas here **8** and **10** are 35–40-fold more potent than **7** and **9**, respectively. The hydroxyl group in both epimeric forms makes

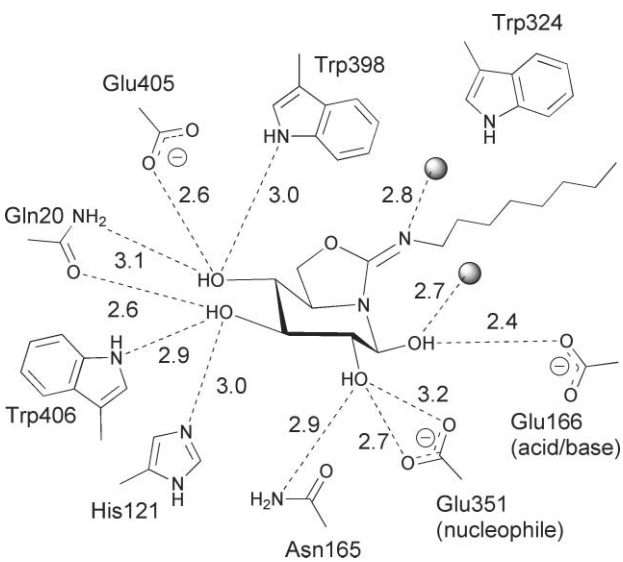


Fig. 3 Interactions between *Tm*GH1 and **7**. Hydrogen bonds are represented by dashed lines and water molecules by grey spheres. Distances are shown in Å.

three hydrogen bonds with active site residues, and therefore the structural analysis makes it difficult to rationalise what makes these D-*galacto*-configured inhibitors so much better. It is possible the lack of the C6 (glycoside numbering) hydroxyl group disrupts intramolecular hydrogen bonding[50] that was present in the hydroximolactam compounds, which makes the binding of the D-*galacto* compounds advantageous, or may otherwise be attributed to differences in the solvation-desolvation processes of the two epimeric forms.[51]

The exocyclic nitrogen atom interacts with a water molecule, and the equatorial hydroxyl group at the pseudoanomeric carbon interacts with Glu166, the acid/base residue, and with a water molecule. The octyl chain makes hydrophobic interactions with Trp324; the electron density is well ordered in this region, unlike another ligand possessing a similar hydrophobic substituent,[52] suggesting that a productive interaction is made on this occasion.

Superposition of *Tm*GH1 in complex with **2** with the complexes of **7**, **8** and **9** shows that the bicyclic indolizidine core lies in virtually the same position (Fig. 2d),[17] despite the absence of the hydroxyl group in the position equivalent to C6 in a glycoside in the new derivatives. The lack of this hydroxyl group means a hydrogen bond is missing with Glu405 when compared to the majority of other inhibitors studied.[42] Neither the oxygen/sulfur atom in the oxa(thia)zolidine ring, nor the exocyclic nitrogen, interact with the enzyme; the observed increase in inhibitory potency on going from the 2-aminooxazoline (**7** and **8**) to the 2-aminothiazoline derivatives (**9** and **10**) is likely to be related to differences in solvation-desolvation processes.

The origin of the β- *versus* α-glucosidase selectivity is less clear, but the fact that compounds which lack the octyl chain (**4** and **5**) are good α-glucosidase inhibitors suggests that this substituent plays a key role as a discriminating element. Overlapping the active site of α-glycosidases with *Tm*GH1 shows that some residues are in close proximity to the alkyl chain, and one may merely speculate that perhaps steric clashes contribute to the lack of binding with α-glycosidases.

Thermodynamic studies

In order to confirm whether *Tm*GH1 inhibition was through selection of the thermodynamically least favoured form of a rapidly interconverting mixture, binding was additionally assessed using isothermal titration calorimetry, at 25 °C and pH 5.8. Compounds **7–10** were tested, but unfortunately the heats of interactions were too small to analyse for all compounds other than **9**. The dissociation constant for **9** with *Tm*GH1 was 5.1 μM, which agreed well with the kinetically determined K_i of 9 μM. Of particular importance is the ITC-derived stoichiometry (based upon the molar ratio of ligand and protein at the inflection of the titration curve) of ~1 (Fig. 4). This shows that inhibition is not derived from a minor contaminant species (which would have yielded a non-unity stoichiometry given that this is calculated from the inflection point of the ITC curve of heat per injection *versus* calculated molar ratio), but from a species obtained from the favoured β-anomer, freely accessible on the timescale taken to return to equilibration during each injection throughout the titration (for a review on ITC see ref. 53). Of additional, and unusual, note is that **9** binds with an unfavourable heat of enthalpy ($\Delta H = +3.3$ kcal/mol) but a large favourable entropy of binding ($T\Delta S = +10.5$ kcal/mol). This entropically-driven binding signature is unique amongst the >20 inhibitors now studied with *Tm*GH1,[42] strongly implicating desolvation of the protein surface in the vicinity of the *n*-octyl chain upon complex formation in the binding thermodynamics.

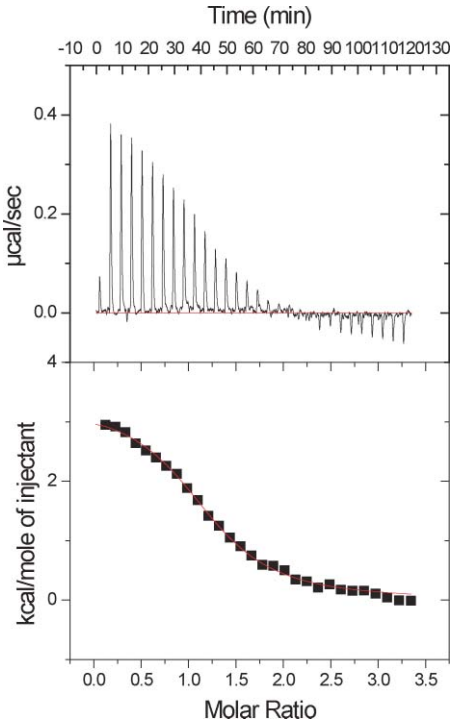


Fig. 4 Isothermal titration calorimetry data for the binding of **9** to *Tm*GH1 (at pH 5.8, 25 °C).

Conclusions

In summary, our results demonstrate the strong potential of the 'sp^2-iminosugar' concept, in combination with molecular diversity-oriented strategies, to finely tune the potency of indolizidine-type inhibitors. The incorporation of an exocyclic octyl substituent in the castanospermine skeleton results in a sharp shift of the selectivity from α- to β-glucosidases, with inhibition potencies down to the nanomolar range. A more detailed kinetic, thermodynamic and structural analysis with a β-glucosidase (*Tm*GH1) demonstrated the presence of the β-anomer, the minor species in the unbound state, in the active site of the enzyme. Calorimetry indicates an unusual unfavourable enthalpy of binding for at least one of the compounds, which is more than compensated by a highly favourable entropic term. This has not been seen for any other inhibitors with *Tm*GH1, or, to our knowledge, with any other glycosidase inhibitor (the binding of isofagomine to the almond β-glucosidase was reported to be entropically driven when determined using van't Hoff methods,[44] but more reliable calorimetry methods showed binding to be derived from enthalpy[15]). The current data elucidate the true mode of binding for this family of ring-modified castanospermine analogues, resolving a conundrum concerning the anomeric specificities in a way we believe can now instruct the development and synthesis of more potent and selective inhibitors in the future.

Experimental

General

Optical rotations were measured with a JASCO DIP-370 digital polarimeter, using a sodium lamp ($\lambda = 589$ nm) at 20 °C. All NMR experiments were performed at 300 (75.5) and 500 (125.7) MHz using Bruker DMX300 and DRX500 spectrometers equipped with a Z-gradient unit for pulsed-field gradient spectroscopy. 1-D TOCSY as well as 2-D COSY and HMQC experiments were carried out to assist in signal assignment. In the FABMS spectra, the primary beam consisted of Xe atoms with a maximum energy of 8 keV. The samples were dissolved in *m*-nitrobenzyl alcohol or thioglycerol as the matrices and the positive ions were separated and accelerated over a potential of 7 keV. NaI was added as cationizing agent. In the CIMS spectra, isobutane was used as the reactive gas (500 mA, 8 kV). Thin-layer chromatography was performed on E. Merck precoated TLC plates, silica gel 30F-245, with visualization by UV light and by charring with 10% H_2SO_4 or 0.2% w/v cerium(IV) sulphate–5% ammonium molybdate in 2 M H_2SO_4 or 0.1% ninhydrin in EtOH. Column chromatography was performed on Kieselgel (E. Merck 230–400 mesh). Elemental analyses were performed at the Servicio de Microanalisis del Instituto de Investigaciones Químicas de Sevilla, Spain. Compounds **7** and **19** were prepared from commercial 6,3-D-glucuronolactone following the procedure already reported.[7,54] 3-*O*-Acetyl-5-azido-5-deoxy-1,2-*O*-isopropylidene-α-D-galactofuranose (**12**) was obtained from D-galactose using the reported route.[34]

3-*O*-Acetyl-5-amino-5-deoxy-1,2-*O*-isopropylidene-α-D-galactofuranose (12). A solution of 3-*O*-acetyl-5-azido-5-deoxy-1,2-*O*-isopropylidene-α-D-galactofuranose[34] (**11**; 230 mg, 0.80 mmol) in MeOH (4.5 mL) was hydrogenated at atmospheric pressure for 1 h using 10% Pd/C (80 mg) as catalyst. The suspension was filtered through Celite and concentrated. Yield: 205 mg (98%); $[\alpha]_D$ −1.4 (*c* 1.0, MeOH); R_f 0.30 (45:5:3 EtOAc-EtOH-H_2O); IR (KBr) ν_{max} 3404, 2991, 2928, 1744, 1379, 1236, 1053 cm^{-1}; ^1H NMR (300 MHz, CD_3OD): δ 6.02 (d, 1 H, $J_{1,2}$ 3.9 Hz, H-1), 5.14 (s, 1 H, H-3), 4.75 (d, 1 H, H-2), 4.12 (dd, 1 H, $J_{4,5}$ 9.9 Hz, H-4), 3.85 (dd, 1 H, $J_{6a,6b}$ 11.7 Hz, $J_{5,6a}$ 3.9 Hz, H-6a), 3.66 (dd, 1 H, $J_{5,6b}$ 5.7 Hz, H-6b), 3.43 (ddd, 1 H, H-5), 2.00 (s, 3 H, MeCO), 1.50, 1.32 (2 s, 6 H, CMe_2); ^{13}C NMR (75.5 MHz, CD_3OD): δ 171.6 (CO), 113.8 (CMe_2), 107.5 (C-1), 85.6 (C-4), 85.1 (C-2), 78.7 (C-3), 60.9 (C-6), 55.5 (C-5), 26.7, 25.7 (CMe_2), 20.7 (*Me*CO); *m/z* (FAB) 284 ([M + Na]$^+$), 262 ([M + H]$^+$). HRFABMS: *m/z* 262.128620; calcd. for $C_{11}H_{20}NO_6$: 262.129063.

3-*O*-Acetyl-5-deoxy-1,2-*O*-isopropylidene-5-(*N*′-octylthioureido)-α-D-galactofuranose (13). A solution of 3-*O*-acetyl-5-amino-5-deoxy-1,2-*O*-isopropylidene-α-D galactofuranose **12**[34] (130 mg, 0.5 mmol) in pyridine (5 mL), Et_3N (0.5 mL, 3.6 mmol) and octyl isothiocyanate (0.6 mmol) were added and the mixture was stirred at room temperature for 18 h. Then, the solvent was removed under reduced pressure and the resulting residue coevaporated several times with toluene and purified by column chromatography using 1:2 → 2:1 EtOAc-petroleum ether as eluent, to afford the thioureido derivative **13**. Yield: 173 mg (80%); $[\alpha]_D$ −45.9 (*c* 1.0, CH_2Cl_2); R_f 0.25 (3:1 EtOAc-petroleum ether); UV (CH_2Cl_2) 251 nm (ε_{mM} 16.0); IR (KBr) ν_{max} 3396, 2920, 1744, 1633, 1553, 1379, 1236, 997 cm^{-1}; ^{1}HNMR (500 MHz, $CDCl_3$, 313 K): δ 6.70 (bs, 1 H, NH), 6.50 (bs, 1 H, NH′), 5.89 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 5.09 (dd, 1 H, $J_{3,4}$ 2.5 Hz, $J_{2,3}$ 0.5 Hz, H-3), 4.63 (d, 1 H, H-2), 4.31 (m, 1 H, H-5), 4.17 (dd, 1 H, $J_{4,5}$ 7.0 Hz, H-4), 3.91 (dd, 1 H, $J_{6a,6b}$ 12.0 Hz, $J_{5,6a}$ 4.0 Hz, H-6a), 3.82 (dd, 1 H, $J_{5,6b}$ 2.5 Hz, H-6b), 3.43 (m, 2 H, CH_2N), 3.19 (m, 1 H, OH), 2.07 (s, 3 H, MeCO), 1.55 (m, 2 H, CH_2CH_2N), 1.56, 1.30 (2 s, 6 H, CMe_2), 1.24 (m, 10 H, CH_2), 0.84 (t, 3 H, $^3J_{H,H}$ 7.0 Hz, CH_3CH_2); ^{13}C NMR (125.7 MHz, $CDCl_3$, 313 K): δ 182.4 (CS), 170.6 (CO), 113.4 (CMe_2), 105.5 (C-1), 84.6 (C-2, C-4), 77.3 (C-3), 63.1 (C-6), 56.6 (C-5), 45.1 (CH_2N), 31.8, 29.3, 29.2, 28.9, 26.9 (CH_2), 26.8, 25.8 (CMe_2), 22.6 (CH_3CH_2), 20.8 (*Me*CO), 14.1 (CH_3CH_2); *m/z* (CI) 433 ([M + H]$^+$). (Found: C, 55.61; H, 8.18; N, 6.48. $C_{20}H_{36}N_2O_6S$ requires C, 55.33; H, 8.39; N, 6.48%).

3-*O*-Acetyl-5-deoxy-1,2-*O*-isopropylidene-5-(*N*′-octylthioureido)-6-*O*-trimethylsilyl-α-D-galactofuranose (14). To a solution of the thioureido derivative **13** (0.58 mmol) in pyridine (5.5 mL) at room temperature, trimethylsilyl chloride (0.94 mL) and hexamethyldisilazane (1.85 mL) were added and the reaction mixture was stirred for 2 h. The solvents were removed, and the residue was extracted with petroleum ether, concentrated, and purified by column chromatography using 1:2 → 1:2 EtOAc-petroleum ether as eluent to afford the silyl derivative **14**. Yield: 263 mg (90%); $[\alpha]_D$ −23.7 (*c* 1.0, CH_2Cl_2); R_f 0.49 (1:2 EtOAc-petroleum ether); UV (CH_2Cl_2) 251 nm (ε_{mM} 14.7); IR (KBr) ν_{max} 3349, 2928, 1752, 1545, 1371, 1228, 1108 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$, 313 K): δ 6.70 (bs, 1 H, NH), 6.19 (bs, 1 H, NH′), 5.85 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 5.11 (d, 1 H, $J_{3,4}$ 2.0 Hz, H-3), 4.58 (d, 1 H, H-2), 4.16 (m, 2 H, H-4, H-5), 3.79 (m, 1 H, H-6a), 3.73 (dd, 1 H, $J_{6a,6b}$ 10.5 Hz, $J_{5,6b}$ 5.0 Hz, H-6b), 3.43 (m, 2 H, CH_2N), 2.07 (s, 3 H, MeCO), 1.58, 1.32 (2 s, 6 H, CMe_2), 1.26 (m, 10 H, 5 CH_2), 0.86 (t, 3 H, $^3J_{H,H}$ 7.0 Hz, CH_3CH_2), 0.13 (s, 9 H, $SiMe_3$); ^{13}C NMR (125.7 MHz, $CDCl_3$, 313 K): δ 183.0 (CS), 169.8 (CO), 113.6 (CMe_2), 105.2 (C-1), 85.0 (C-2, C-4), 76.7 (C-3), 62.5 (C-6), 56.5 (C-5), 45.3 (CH_2N), 31.7, 29.2, 29.1, 28.9 (4 CH_2), 26.9 (CH_2), 27.1, 25.9 (CMe_2), 22.6 (CH_3CH_2), 20.7 (*Me*CO), 13.9 (CH_3CH_2), –0.56 ($SiMe_3$); *m/z* (FAB) 505 ([M + H]$^+$). (Found: C, 54.63; H, 8.59; N, 5.49. $C_{23}H_{44}N_2O_6SSi$ requires C, 54.73; H, 8.79; N, 5.55%).

3-*O*-Acetyl-5-deoxy-1,2-*O*-isopropylidene-5-(*N*′-octylcarbodiimido)-6-*O*-trimethylsilyl-α-D-galactofuranose (15). To a solution of the corresponding thiourea **14** (0.47 mmol) in CH_2Cl_2-H_2O (1:1, 12.0 mL), HgO was added (1.36 mmol). The reaction mixture was vigorously stirred at room temperature for 45 min, diluted with CH_2Cl_2, and the organic phase was separated. The aqueous phase was extracted several times with CH_2Cl_2, and the combined extracts were dried ($MgSO_4$), and concentrated. The resulting residue was purified by column chromatography using 1:3 EtOAc-petroleum as eluent to afford the carbodiimide derivative **15** as an amorphous solid. Yield: 177 mg (80%); $[\alpha]_D$ −56.7 (*c* 1.0, CH_2Cl_2); R_f 0.69 (1:3 EtOAc-petroleum ether); IR (KBr) ν_{max} 2928, 2131, 1750, 1377, 1231, 1094 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 5.86 (d, 1 H, $J_{1,2}$ 4.0 Hz, H-1), 5.26 (d, 1 H, $J_{3,4}$ 3.2 Hz, H-3), 4.56 (d, 1 H, H-2), 3.94 (dd, 1 H, $J_{4,5}$ 6.7 Hz, H-4), 3.73 (dd, 1 H, $J_{6a,6b}$ 9.5 Hz, $J_{5,6a}$ 5.3 Hz, H-6a), 3.68 (m, 1 H, H-5), 3.64 (dd, 1 H, $J_{5,6b}$ 5.6 Hz, H-6b), 3.23 (m, 2 H, CH_2N), 2.05 (s, 3 H, MeCO), 1.53 (m, 2 H, CH_2CH_2N), 1.59, 1.32 (2 s, 6 H, CMe_2), 1.27 (m, 10 H, CH_2), 0.87 (t, 3 H, $J_{H,H}$ 7.0 Hz, CH_3), 0.11 (s, 9 H, $SiMe_3$); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 169.7 (CO), 140.6 (NCN), 113.4 (CMe_2), 105.1 (C-1), 85.3 (C-2), 83.9 (C-4), 77.4 (C-3), 63.7 (C-6),

59.5 (C-6), 46.7 (CH_2N), 31.8, 31.0, 29.2, 26.8 (5 CH_2), 27.0, 26.4 (CMe_2), 22.6 (CH_2CH_3), 20.7 ($MeCO$), 14.0 (CH_3), –0.63 ($SiMe_3$); *m/z* (FAB) 471 ([M + H]$^+$), 455 ([M – Me]$^+$). (Found: C, 58.74; H, 8.70; N, 5.86. $C_{23}H_{42}N_2O_6Si$ requires C, 58.69; H, 8.99; N, 5.95%).

(4*S*)-4-[(4′*S*)-3′-*O*-Acetyl-1′,2′-*O*-isopropylidene-β-L-threofuranose-4′-yl]-2-octylamino-2-oxazoline (16). To a solution of the carbodiimido derivative **15** (0.34 mmol) in THF (6.3 mL) at 0 °C under Ar, TBAF (1 M in THF, 0.35 mL) was added. The solution was stirred for 25 min at 0 °C, then diluted with Et_2O (6 mL), washed with water, dried ($MgSO_4$), filtered, and concentrated. The resulting residue was purified by column chromatography using 50:1 → 10:1 CH_2Cl_2-MeOH as eluent to obtain **16**. Yield: 122 mg (90%); $[\alpha]_D$ –24.3 (*c* 1.0, CH_2Cl_2); R_f 0.30 (20:1 CH_2Cl_2-MeOH); IR (KBr) ν_{max} 3452, 2928, 1744, 1458, 1379, 1236, 1029 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.88 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 4.86 (d, 1 H, $J_{3,4}$ 3.0 Hz, H-3), 4.73 (m, 2 H, H-6a, H-6b), 4.62 (d, 1 H, H-2), 4.55 (m, 1 H, H-5), 3.97 (dd, 1 H, $J_{4,5}$ 7.0 Hz, H-4), 3.24 (t, 2 H, $^3J_{H,H}$ 7.5 Hz, CH_2N), 2.09 (s, 3 H, MeCO), 1.55 (m, 2 H, CH_2CH_2N), 1.50, 1.30 (2 s, 6 H, CMe_2), 1.25 (m, 10 H, CH_2), 0.85 (t, 3 H, $^3J_{H,H}$ 7.0 Hz, CH_3); ^{13}C NMR (125.7 MHz, $CDCl_3$): δ 170.4 (CO), 161.9 (CN), 114.0 (CMe_2), 105.3 (C-1), 85.1 (C-4), 84.4 (C-2), 76.1 (C-3), 71.9 (C-6), 57.8 (C-5), 43.1 (CH_2N), 31.7, 29.7, 29.1, 29.0, 26.5 (CH_2), 27.1, 26.0 (CMe_2), 22.6 (CH_2CH_3), 20.7 ($MeCO$), 14.0 (CH_3); *m/z* (FAB) 399 ([M + H]$^+$). HRFABMS: *m/z* 399.247458; calcd. for: 399.249512. (Found: C, 59.86; H, 9.00; N, 6.96. $C_{20}H_{35}N_2O_6$ requires C, 60.28; H, 8.60; N, 7.03%).

(5*R*,6*R*,7*S*,8*S*,8a*R*)-5,6,7,8-Tetrahydroxy-3-octylimino-2-oxaindolizidine (8). To a solution of the corresponding 2-amino-2-oxazoline precursor **16** (0.28 mmol) in dry MeOH (2 mL), methanolic NaOMe (1 M, 0.1 eq per mol of acetate) was added. The reaction mixture was stirred at room temperature for 30 min, then neutralized with solid CO_2, and concentrated. The residue was treated with TFA-H_2O (9:1, 2 mL) for 15 min, concentrated under reduced pressure, coevaporated several times with water, neutralized with Amberlite IRA-68 (OH^-) ion-exchange resin, and subjected to column chromatography using 10:1 MeCN-H_2O as eluent to obtain the isourea **8**. Yield: 79 mg (90%); $[\alpha]_D$ –9.8 (*c* 0.5, H_2O); R_f 0.44 (10:1:1 MeCN-H_2O-NH_4OH); ^{1}H NMR (500 MHz, D_2O): δ 5.52 (d, 1 H, $J_{5,6}$ 4.0 Hz, H-5), 4.83 (t, 1 H, $J_{8a,1a}$ $J_{1a,1b}$ 8.5 Hz, H-1a), 4.71 (dd, 1 H, $J_{8a,1b}$ 8.5 Hz, H-1b), 4.50 (t, 1 H, H-8a), 4.02 (m, 1 H, H-8), 3.87 (dd, 1 H, $J_{6,7}$ 10.0 Hz, $J_{7,8}$ 2.5 Hz, H-7), 3.78 (dd, 1 H, H-6), 3.30 (t, 2 H, $^3J_{H,H}$ 6.5 Hz, CH_2N), 1.52 (m, 2 H, CH_2CH_2N), 1.21 (m, 10 H, CH_2), 0.78 (t, 3 H, $^3J_{H,H}$ 6.5 Hz, CH_3); ^{13}C NMR (125.7 MHz, D_2O): δ 158.5 (CN), 74.9 (C-5), 70.6 (C-1), 68.8 (C-7), 68.1 (C-8), 67.4 (C-6), 56.2 (C-8a), 43.0 (CH_2N), 31.1, 28.5, 28.3, 28.2, 25.7 (CH_2), 22.0 (CH_2CH_3), 13.4 (CH_3): *m/z* (FAB) 317 ([M + H]$^+$). (Found: C, 56.78; H, 8.56; N, 8.76. $C_{15}H_{28}N_2O_5$ requires C, 56.94; H, 8.92; N, 8.85%).

3-*O*-Acetyl-5-deoxy-1,2-*O*-isopropylidene-5-(*N*′-octylthioureido)-α-D-glucofuranose (20). A solution of 5-azido-5-deoxy-1,2-*O*-isopropylidene-6-*O*-tetrahydropyranyl-α-D-glucofuranose[54] (**19**; 300 mg, 0.91 mmol) in MeOH (5 mL) was hydrogenated at atmospheric pressure for 1 h using 10% Pd/C (90 mg) as catalyst. The suspension was filtered through Celite and concentrated. The resulting residue was dissolved in pyridine (6 mL), Et_3N (0.71 mL, 5.1 mmol) and the corresponding isothiocyanate (1.1 mmol) was added and the mixture was stirred at room temperature for 18 h. Then, the reaction mixture was cooled to –15 °C, Ac_2O (3 mL) was added dropwise, and the mixture was further stirred for 5 h. After conventional work up, the crude acetate was dissolved in CH_2Cl_2-MeOH (1:1, 12 mL) and *p*-toluenesulfonic acid (50 mg, 0.26 mmol) was added. The reaction mixture was stirred for 2 h at room temperature, and then diluted with CH_2Cl_2 (8 mL), washed with saturated aqueous $NaHCO_3$ (2 × 8 mL), dried ($MgSO_4$), and concentrated. The resulting residue was purified by column chromatography using 1:1 EtOAc-petroleum ether as eluent to afford the thioureido derivative **20**. Yield: 205 mg (52%); $[\alpha]_D$ –26.7 (*c* 1.0, CH_2Cl_2); R_f 0.58 (2:1 EtOAc-petroleum ether); UV (CH_2Cl_2) 251 nm (ε_{mM} 11.5); IR (KBr) ν_{max} 3358, 2929, 1753, 1647, 1541, 1364, 1212, 1076 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.24 (bs, 2 H, NH), 5.94 (d, 1 H, $J_{1,2}$ 3.7 Hz, H-1), 5.32 (d, 1 H, $J_{3,4}$ 2.9 Hz, H-3), 4.84 (m, 1 H, H-5), 4.52 (d, 1 H, H-2), 4.49 (dd, 1 H, $J_{4,5}$ 7.0 Hz, H-4), 3.99 (dd, 1 H, $J_{6a,6b}$ 11.3 Hz, $J_{5,6a}$ 2.9 Hz, H-6a), 3.84 (dd, 1 H, $J_{5,6b}$ 3.0 Hz, H-6b), 3.33 (m, 2 H, CH_2N), 2.16 (s, 3 H, MeCO), 1.59 (m, 2 H, CH_2CH_2N), 1.53, 1.32 (2 s, 6 H, CMe_2), 1.29 (m, 10 H, CH_2), 0.90 (t, 3 H, $^3J_{H,H}$ 7.0 Hz, CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 182.0 (CS), 169.9 (CO), 112.3 (CMe_2), 104.7 (C-1), 83.4 (C-2), 78.5 (C-4), 75.7 (C-3), 62.6 (C-6), 53.8 (C-5), 44.2 (CH_2N), 31.7, 29.2, 29.1, 28.8, 26.9 (CH_2), 26.6, 26.1 (CMe_2), 22.6 (CH_2CH_3), 21.4 ($MeCO$), 14.0 (CH_3); *m/z* (FAB) 455 ([M + Na]$^+$), 433 ([M + H]$^+$). (Found: C, 55.37; H, 8.45; N, 6.42. $C_{20}H_{36}N_2O_6S$ requires C, 55.53; H, 8.39; N, 6.48%).

(4*R*)-4-[(4′*R*)-3′-*O*-Acetyl-1′,2′-*O*-isopropylidene-β-L-threofuranose-4′-yl]-2-octylamino-2-thiazoline (21). To a solution of the corresponding thioureido derivative **20** (0.86 mmol) in anhydrous pyridine (23 mL) at –20 °C under Ar, methanesulfonic chloride (80 μL, 1.03 mmol, 1.2 eq) was added. The reaction mixture was stirred for 24 h and allowed to warm to 20 °C. Then, ice-water (30 mL) was added and the solution was extracted with CH_2Cl_2 (3× 25 mL). The combined extracts were washed with iced saturated aqueous $NaHCO_3$ (20 mL), dried ($MgSO_4$), and concentrated. The resulting residue was purified by column chromatography using 1:4 acetone-cyclohexane as eluent. Yield: 296 mg (83%); $[\alpha]_D$ –52.5 (*c* 1.0, CH_2Cl_2); R_f 0.43 (1:2 acetone-cyclohexane, 2 elutions); IR (KBr) ν_{max} 3383, 2928, 1749, 1624, 1511, 1373, 1227, 1078 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.91 (d, 1 H, $J_{1,2}$ 3.7 Hz, H-1), 5.26 (d, 1 H, $J_{3,4}$ 3.0 Hz, H-3), 4.52 (d, 1 H, H-2), 4.44 (m, 1 H, H-5), 4.16 (dd, 1 H, $J_{4,5}$ 9.2 Hz, H-4), 3.49 (dd, 1 H, $J_{6a,6b}$ 11.0 Hz, $J_{5,6a}$ 7.1 Hz, H-6a), 3.42 (dd, 1 H, $J_{5,6b}$ 5.2 Hz, H-6b), 3.22 (t, 2 H, $^3J_{H,H}$ 7.1 Hz, CH_2N), 2.12 (s, 3 H, MeCO), 1.52 (m, 2 H, CH_2CH_2N), 1.52, 1.31 (2 s, 6 H, CMe_2), 1.28 (m, 10 H, CH_2), 0.89 (t, 3 H, $^3J_{H,H}$ 6.9 Hz, CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 169.8 (CO), 162.5 (CN), 112.1 (CMe_2), 104.9 (C-1), 83.5 (C-2), 80.0 (C-4), 76.5 (C-3), 70.0 (C-5), 45.1 (CH_2N), 37.6 (C-6), 31.8, 29.8, 29.3, 29.2, 26.8 (CH_2), 26.7, 26.2 (CMe_2), 22.6 (CH_2CH_3), 21.0 ($MeCO$), 14.1 (CH_3); *m/z* (FAB) 415 ([M + H]$^+$). (Found: C, 57.84; H, 7.98; N, 6.67. $C_{20}H_{34}N_2O_5S$ requires C, 57.94; H, 8.27; N, 6.76%).

(5*R*,6*R*,7*S*,8*R*,8a*R*)-5,6,7,8-Tetrahydroxy-3-octylimino-2-thiaindolizidine (9). To a solution of the corresponding 2-amino-2-thiazoline precursor **21** (0.39 mmol) in dry MeOH (3.5 mL), methanolic NaOMe (1 M, 0.1 equiv per mol of acetate) was added. The reaction mixture was stirred at room temperature for 30 min, neutralized with solid CO_2, and concentrated. The resulting residue was treated with TFA-H_2O (9:1, 2.4 mL) for 30 min, concentrated under reduced pressure, coevaporated several

times with water, neutralized with Amberlite IRA-68 (OH^-) ion-exchange resin, and subjected to column chromatography using 60:10:1 → 40:10:1 CH_2Cl_2-MeOH-H_2O as eluent to obtain the corresponding isothiourea **9**. Yield: 117 mg (90%); $[\alpha]_D$ −24.5 (*c* 0.5, MeOH); R_f 0.49 (40:10:1 CH_2Cl_2-MeOH-H_2O); ^{1}H NMR (300 MHz, D_2O): δ 5.50 (d, 1 H, $J_{5,6}$ 3.7 Hz, H-5), 4.20 (m, 1 H, H-8a), 3.66 (m, 2 H, H-7, H-1a), 3.52 (dd, 1 H, $J_{6,7}$ 10.0 Hz, H-6), 3.45 (t, 1 H, $J_{7,8} = J_{8,8a}$ 9.5 Hz, H-8), 3.32 (m, 3 H, H-1b, CH_2N), 1.56 (m, 2 H, C$H_2$$CH_2N$), 1.18 (m, 10 H, CH_2), 0.74 (t, 3 H, $^3J_{H,H}$ 6.9 Hz, CH_3); ^{13}C NMR (75.5 MHz, D_2O): δ 162.5 (CN), 76.6 (C-5), 73.5 (C-8), 72.0 (C-7), 70.9 (C-6), 63.6 (C-8a), 49.4 (CH_2N), 31.5 (C-1), 31.3, 28.6, 28.5, 28.3, 26.0 (CH_2), 22.3 (*C*H_2CH_3), 13.7 (CH_3); *m/z* (FAB) 355 ([M + Na]$^+$), 333 ([M + H]$^+$); (Found: C, 53.94; H, 8.42; N, 8.29. $C_{15}H_{28}N_2O_4S$ requires C, 54.19; H, 8.49; N, 8.43%).

5-Deoxy-1,2-*O*-isopropylidene-5-(*N*′-octylthioureido)-α-D-galactofuranose (17). A solution of 5-azido-5-deoxy-1,2-*O*-isopropylidene-α-D-galactofuranose[34] (350 mg, 1.43 mmol) in MeOH (7 mL) was hydrogenated at atmospheric pressure for 1 h using 10% Pd/C (123 mg) as catalyst. The suspension was filtered through Celite and concentrated. The resulting residue was dissolved in pyridine (8 mL), Et_3N (0.96 mL, 6.9 mmol, 5.6 eq) and the corresponding isothiocyanate (1.5 mmol, 1.2 eq) was added and the mixture was stirred at room temperature for 18 h. Then, the solvent was removed under reduced pressure and the resulting residue coevaporated several times with toluene and purified by column chromatography using 1:2 acetone-cyclohexane as eluent to afford the thioureido derivative **17**. Yield: 391 mg (70%); $[\alpha]_D$ +15.9 (*c* 1.0, CH_2Cl_2); R_f 0.34 (1:2 acetone-cyclohexane, 2 elutions); UV (CH_2Cl_2) 250 nm (ε_{mM} 11.3); IR (KBr) ν_{max} 3353, 2927, 1556, 1377, 1212, 1064 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$, 313 K): δ 6.58, 6.47 (2 bs, 2 H, NH), 5.79 (d, 1 H, $J_{1,2}$ 4.5 Hz, H-1), 4.73 (m, 1 H, H-5), 4.66 (dd, 1 H, $J_{2,3}$ 3.0 Hz, H-2), 4.23 (dd, 1 H, $J_{3,4}$ 6.9 Hz, H-3), 3.98 (dd, 1 H, $J_{6a,6b}$ 11.7 Hz, $J_{5,6a}$ 3.2 Hz, H-6a), 3.95 (m, 1 H, H-4), 3.83 (dd, 1 H, $J_{5,6b}$ 3.4 Hz, H-6b), 3.38 (m, 2 H, CH_2N), 1.62 (m, 2 H, C$H_2$$CH_2N$), 1.52, 1.40 (2 s, 6 H, CMe_2), 1.30 (m, 10 H, CH_2), 0.91 (t, 3 H, $^3J_{H,H}$ 7.0 Hz, CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$, 313 K): δ 182.0 (CS), 114.0 (*C*Me_2), 104.5 (C-1), 86.6 (C-2), 77.2 (C-4), 75.9 (C-3), 64.1 (C-6), 54.7 (C-5), 44.1 (CH_2N), 31.7, 29.1, 29.0, 28.8, 26.8 (CH_2), 27.8, 27.1 (C*Me*$_2$), 22.6 (*C*H_2CH_3), 14.0 (CH_3); *m/z* (FAB) 413 ([M + Na]$^+$), 391 ([M + H]$^+$); (Found: C, 55.26; H, 8.67; N, 7.10. $C_{18}H_{34}N_2O_5S$ requires C, 55.36; H, 8.78; N, 7.17%).

(4*S*)-4-[(4′*S*)-1′,2′-*O*-Isopropylidene-β-L-threofuranose-4′-yl]-2-octylamino-2-thiazoline (18). To a solution of the corresponding thioureido derivative **17** (0.91 mmol) in anhydrous pyridine (24 mL) at −20 °C under Ar, methanesulfonic chloride (85 μL, 1.09 mmol, 1.2 eq) was added. The reaction mixture was stirred for 7 h and allowed to warm to 10 °C. Then, ice-water (30 mL) was added and the solution was extracted with CH_2Cl_2 (3 × 25 mL). The combined extracts were washed with iced saturated aqueous $NaHCO_3$ (20 mL), dried ($MgSO_4$), and concentrated. The resulting residue was purified by column chromatography using 70:10:1 CH_2Cl_2-MeOH-H_2O to obtain the isothiourea **18**. Yield: 203 mg (60%); $[\alpha]_D$ −11.2 (*c* 0.98, CH_2Cl_2); R_f 0.44 (70:10:1 CH_2Cl_2-MeOH-H_2O); IR (KBr) ν_{max} 3355, 2927, 1618, 1374, 1215, 1065 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.81 (d, 1 H, $J_{1,2}$ 3.9 Hz, H-1), 4.57 (m, 2 H, H-2, H-5), 4.29 (dd, 1 H, $J_{3,4}$ 5.5 Hz, $J_{2,3}$ 1.4 Hz, H-3), 4.03 (t, 1 H, $J_{4,5}$ 5.5 Hz, H-4), 3.80 (bs, 1 H, NH), 3.39 (dd, 1 H, $J_{6a,6b}$ 10.9 Hz, $J_{5,6a}$ 8.1 Hz, H-6a), 3.33 (dd, 1 H, $J_{5,6b}$ 8.0 Hz, H-6b), 3.22 (t, 2 H, $^3J_{H,H}$ 7.1 Hz, CH_2N), 1.55, 1.37 (2 s, 6 H, CMe_2), 1.52 (m, 2 H, C$H_2$$CH_2N$), 1.29 (m, 10 H, CH_2), 0.89 (t, 3 H, $^3J_{H,H}$ 6.9 Hz, CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 164.9 (CN), 114.2 (*C*Me_2), 104.8 (C-1), 88.4 (C-2), 86.7 (C-4), 75.3 (C-3), 71.0 (C-5), 46.3 (CH_2N), 35.2 (C-6), 32.2, 30.4, 29.7, 29.6 (CH_2), 28.1, 27.3 (C*Me*$_2$), 27.2 (CH_2), 23.0 (*C*H_2CH_3), 14.5 (CH_3); *m/z* (FAB) 395 ([M + Na]$^+$), 373 ([M + H]$^+$); (Found: C, 57.94; H, 8.67; N, 7.47. $C_{18}H_{32}N_2O_4S$ requires C, 58.03; H, 8.66; N, 7.52%).

(5*R*,6*R*,7*S*,8*S*,8a*R*)-5,6,7,8-Tetrahydroxy-3-octylimino-2-thia-indolizidine (10). The corresponding 2-amino-2-thiazoline precursor **18** (0.31 mmol) was treated with TFA-H_2O (9:1, 1.8 mL) for 15 min, concentrated under reduced pressure, coevaporated several times with water, neutralized with Amberlite IRA-68 (OH^-) ion-exchange resin, and subjected to column chromatography using 60:10:1 → 40:10:1 CH_2Cl_2-MeOH-H_2O as eluent to obtain the corresponding isothiourea **10**. Yield: 98 mg (95%); $[\alpha]_D$ −14.3 (*c* 0.6, MeOH); R_f 0.49 (40:10:1 CH_2Cl_2-MeOH-H_2O); ^{1}H NMR (300 MHz, DMSO-d_6): δ 5.44 (d, 1 H, $J_{5,6}$ 3.4 Hz, H-5), 4.67 (bt, $J_{8a,1a} = J_{8a,1b}$ 8.5 Hz, 1 H, H-8a), 4.03 (bt, 1 H, $J_{1a,1b}$ 8.5 Hz, H-1a), 3.69 (bs, 1 H, H-8), 3.64 (bd, 1 H, $J_{6,7}$ 9.6 Hz, H-7), 3.52 (dd, 1 H, H-6), 3.25 (m, 1 H, H-1b), 3.09 (m, 2 H, CH_2N), 1.49 (m, 2 H, C$H_2$$CH_2N$), 1.24 (m, 10 H, CH_2), 0.84 (t, 3 H, $^3J_{H,H}$ 6.9 Hz, CH_3); ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 170.9 (CN), 76.7 (C-5), 70.0 (C-7), 69.4 (C-8), 68.3 (C-6), 60.2 (C-8a), 52.6 (CH_2N), 31.7, 30.5, 29.2, 29.1 (CH_2), 27.4 (C-1), 22.5 (*C*H_2CH_3), 14.4 (CH_3); *m/z* (FAB) 355 ([M + Na]$^+$), 333 ([M + H]$^+$); Found: C, 53.83; H, 8.39; N, 8.30. $C_{15}H_{28}N_2O_4S$ requires C, 54.19; H, 8.49; N, 8.43%).

Inhibition assays against commercial enzymes. K_i values were determined by spectrophotometrically measuring the residual hydrolytic activities of the glycosidases against the respective *o*- (for β-glucosidase/β-galactosidase from bovine liver) or *p*-nitrophenyl α- or β-D-glycopyranoside (for other glycosidases) or α,α′-trehalose (for trehalase) in the presence of compounds **7–10**. Each assay was performed in phosphate buffer or phosphate-citrate buffer (for α-mannosidase and amyloglucosidase) at the optimal pH for the enzyme. The reactions were initiated by addition of enzyme to a solution of the substrate in the absence or presence of various concentrations of inhibitor. The mixture was incubated for 10–30 min at 37 °C or 55 °C (for amyloglucosidase) and the reaction was quenched by addition of 1 M Na_2CO_3 or a solution of GLC-Trinder (Sigma, for trehalase). Reaction times were appropriate to obtain 10–20% conversion of the substrate in order to achieve linear rates. The absorbance of the resulting mixture was determined at 405 nm or 505 nm (for trehalase). The K_i value and enzyme inhibition mode were determined from the slope of Lineweaver-Burk plots and double reciprocal analysis.

Kinetic studies with *Tm*GH1. Kinetic studies were conducted by monitoring the change in UV/visible absorbance with a Cintra 10 spectrophotometer equipped with a Thermocell peltier power supply. Experiments were performed at 37 °C, in 100 mM sodium citrate buffer, pH 5.8. Assays contained 15 μM 2,4-dinitrophenyl β-D-glucopyranoside as substrate and 1 mg mL^{-1} bovine serum albumin, in a total volume of 1 mL. Experiments were performed in the absence and presence of compounds **7–10**, which were at

concentrations between 5 and 90 μM for **7**, 0.25 and 3 μM for **8**, 2 and 30 μM for **9** and 0.05 and 2 μM for **10**; *Tm*GH1 was present at a concentration of between 4 and 13 nM. The reaction was initiated by addition of substrate after incubation of *Tm*GH1 with the inhibitor for 20 minutes, which prevented any complications from slow onset inhibition. Rates were monitored for 400 seconds. The fractional decrease in v_i/v_0 for each inhibitor was calculated using the equation $v_i/v_0 = 1 + [I]/K_i$, and the mean K_i value taken.

ITC experiments with *Tm*GH1. Isothermal titration calorimetry was performed on a VC calorimeter (Microcal, Northampton, MA) at 25 °C. *Tm*GH1 was dialysed into 100 mM sodium citrate buffer, pH 5.8, and diluted to a final concentration of 34 μM. Compound **9** was diluted in the same buffer to a final concentration of 0.5 mM. All samples were centrifuged and degassed prior to use. Titrations were performed by injecting 10 μL aliquots of each inhibitor into *Tm*GH1. Data were corrected for heats of dilution by subtracting the excess heat at a high molar ratio of inhibitor to enzyme. The enthalpy (ΔH°_a) and association constant (K_a) were determined from fitting to a bimolecular model with Microcal ORIGIN software. The Gibbs free energy (ΔG°_a) and entropy ($T\Delta S^\circ_a$) were calculated using the equation $\Delta G^\circ_a = -RT\ln K_a = \Delta H^\circ_a - T\Delta S^\circ_a$.

Crystallization and X-ray data collection of *Tm*GH1. *Tm*GH1, at 10 mg mL^{-1}, was crystallised in the presence of a minute amount of solid **7**, **8** or **9** using the same conditions as described previously.[15] The crystal was cryoprotected in a solution containing the mother liquor with the addition of 25% ethylene glycol, and flash frozen in liquid nitrogen. Data for *Tm*GH1 in complex with **7** and **9** were collected at the European Synchrotron Radiation Facility on beamlines ID14-1 and ID14-4, respectively. Data for *Tm*GH1 in complex with **8** was collected on an in-house CuK_α rotating anode source, which consists of an Raxis IV++ imaging plate detector mounted on a Rigaku Micromax 007HF generator equipped with Osmic multilayer optics. Data were integrated and scaled with the HKL2000 suite,[55] and all other crystallographic computing used the CCP4 suite of programs.[56] The differences in space group and/or unit cell dimensions meant molecular replacement using AMoRe[57] was required to solve the structures, using the protein atoms of PDB entry 1OD0. Manual corrections to the models were done using COOT,[58] which were interspersed with cycles of least square refinement in REFMAC.[59] The coordinates and structure factors have been deposited with the PDB with the codes 2WBG (complex with **7**), 2WC3 (complex with **8**) and 2WC4 (complex with **9**).

Acknowledgements

The Biotechnology and Biological Sciences Research Council of the UK, the Spanish Ministerio de Ciencia e Innovación (contract numbers CTQ2006-15515-C02-01/BQU and CTQ2007-61180/PPQ) and the Junta de Andalucía are thanked for funding. G.J.D. is a Royal Society/Wolfson Research Merit Award recipient, T.M.G. is a Sir Henry Wellcome postdoctoral fellow and a Michael Smith for Health Research trainee award holder, and M.A.-M. is a FPI doctoral fellow holder.

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