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Molecular Basis for β-Glucosidase Inhibition by Ring-Modified Calystegine Analogues

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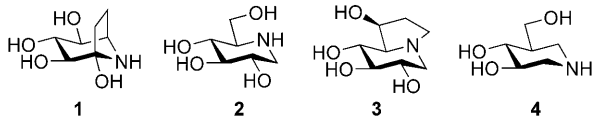
Calystegines, polyhydroxy nortropane alkaloids first isolated from the extracts of *Calystegia sepium*, represent the most recently discovered members of the azasugar[1] and iminosugar glycomimetic families.[2,3] Similar to the well-studied polyhydroxypiperidine, indolizidine, pyrrolidine and pyrrolizidine-type iminocyclitols,[4] calystegines exhibit potent glycoside hydrolase (glycosidase) inhibitory properties. Given the broad range of biological events in which glycosidases are involved, inhibitors of these enzymes have potential in therapies that are targeted at, for example, cancer,[5] viral infections,[6] tuberculosis,[7] diabetes[8] and glycosphingolipid storage disorders.[9]

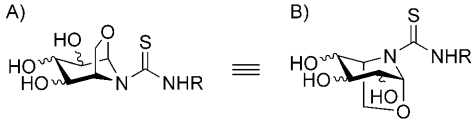
The molecular basis for glycosidase inhibition by the calystegines remain poorly understood. Recent structural and mechanistic studies on a β-glucosidase from *Thermotoga maritima*, *Tm*GH1,[10] which belongs to family 1, clan GH-A, in the CAZy classification,[11] showed unambiguously that calystegine B_2 (**1**; Scheme 1), one of the most powerful representatives, binds at



Scheme 1. Structures of (+)-calystegine B_2 (**1**), 1-deoxynojirimycin (**2**), castanospermine (**3**), isofagomine (**4**).

the active site in an orientation with the endocyclic nitrogen atom at the position of the anomeric carbon in a native glycoside ("1-azasugar" orientation). This scenario differs from that encountered in the case of 1-deoxynojirimycin (**2**) and castanospermine (**3**), which adopt an orientation that places the endocyclic nitrogen atom at the position where O5 is found in the native glycoside substrate ("iminosugar" orientation).[10,12] In this sense, **1** might be regarded as an analogue of the potent β-glucosidase inhibitor isofagomine (**4**).[13]

Within the framework of a project that aims at developing anomeric-specific glycosidase inhibitors for clinical applications, we have devised a new family of ring-modified calystegine analogues in which a methylene group in the pyrrolidine ring is replaced by an oxygen atom, namely 1-deoxy-6-oxa-*N*-(thiocarbamoyl)calystegines (Scheme 2).[14] Interestingly, some



Scheme 2. General structure of 1-deoxy-6-oxa-*N*-(thiocarbamoyl)calystegines.

of these neutral derivatives were shown to be more potent and significantly more selective inhibitors of β-glucosidases than the parent amine-type compound **1**, which is particularly attractive for mechanistic studies and optimisation of biological activity.

The parallels between the structures of **1** and the 1-deoxy-6-oxa-*N*-thiocarbamoyl analogues would suggest a similar "1-azasugar" mode of binding to β-glucosidases (Scheme 2A). Yet, mapping of the structural requirements for potent β-glucosidase inhibition through systematic modifications of the hydroxylation profile did not discard an alternative "iminosugar" orientation of these compounds in the active centre (Scheme 2B).[15] In any case, strong binding and selectivity cannot be rationalised in 1-deoxy-6-oxa-*N*-(thiocarbamoyl)calystegines by the formation of electrostatic interactions as with compounds that can be protonated. In principle, resonance delocalisation of the lone pairs in the thiourea functionality must result in partial positive charge density at the nortropane nitrogen atom, which might more closely resemble the situation at the transition state than the fully protonated amine nitrogen atom in **1**–**4**. To address these questions, the *N*′-butyl and *N*′-octyl derivatives **8** and **9** (Scheme 3), bearing aliphatic pseudoaglyconic substituents, have been prepared and evaluated as glycosidase inhibitors. A structural, thermodynamic and kinetic analysis of their binding with *Tm*GH1 is reported. The data are discussed in light of previous data for the binding of **1**–**4**[10,12] as examples of 1-azasugar (**1** and **4**) and iminosugar (**2** and **3**) inhibitors.

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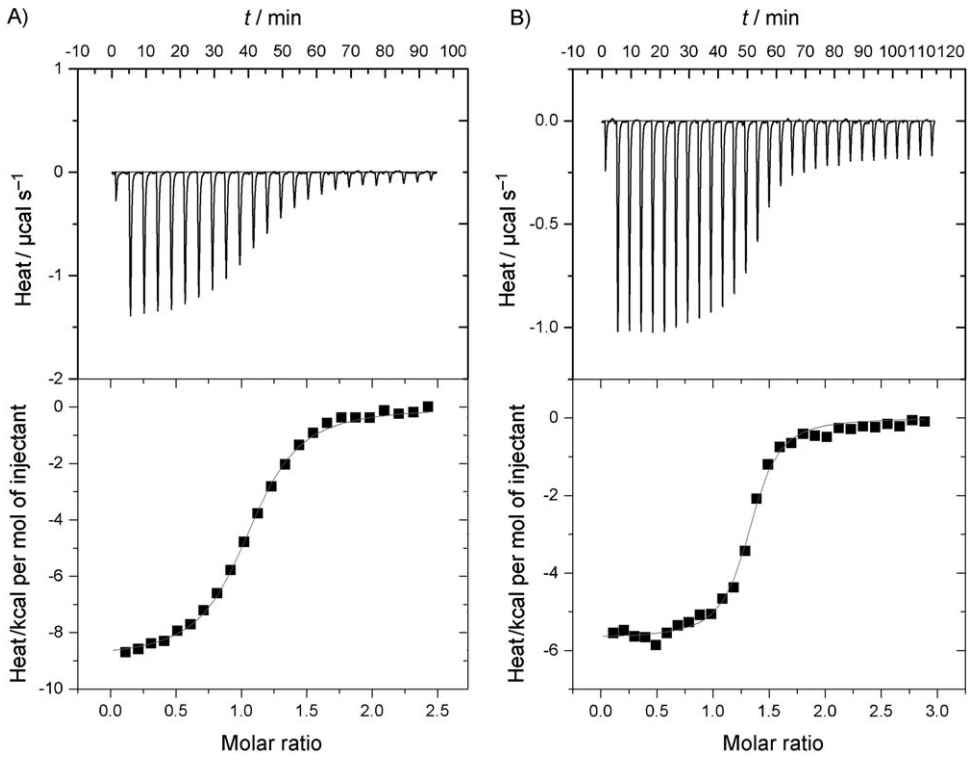
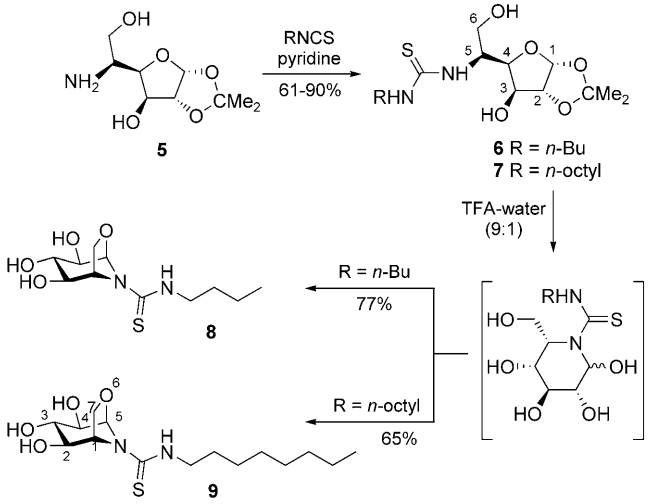


Figure 1. ITC titration data for the binding of *Tm*GH1 (at pH 5.8, 25 °C) with A) **8** and B) **9**.

The four and eight carbon atom substituents at the exocyclic thiourea nitrogen in compounds **8** and **9** were chosen to match the *N*-alkyl substituents in amine-type glycosidase inhibitors that have already proved to be efficient β-glucosidase inhibitors.[16] Their synthesis was accomplished in just two steps from 5-amino-5-deoxy-1,2-*O*-isopropylidene-β-L-idofuranose (**5**)[17] by nucleophilic addition of commercially available *n*-butyl(octyl) isothiocyanate in pyridine (61–90%), and the subsequent deisopropylidenation of the corresponding thiourea adducts **6** and **7** by treatment with 9:1 trifluoroacetic acid/water (65–77%). The latter transformation involves spontaneous nucleophilic addition of the thiourea nitrogen atom, which is located at the furanose C5 position, to the masked aldehyde group of the monosaccharide, followed by in situ intramolecular glycosylation in the transient piperidine derivative to close the bicyclic skeleton (Scheme 3). The simplicity and efficiency of the synthetic scheme illustrates its suitability for combinatorial approaches.

The inhibitory activities of the oxacalystegine B_2 derivatives **8** and **9** were first mapped against a panel of commercial enzymes, including α-glucosidase (yeast), amyloglucosidase (*Aspergillus niger*), trehalase (pig kidney), invertase (yeast) β-glucosidase (almond), β-glucosidase/β-galactosidase (bovine liver, cytosolic), α-galactosidase (green coffee bean) and α-mannosidase (jack bean). The results are shown in Table 1. Neither of the compounds inhibited α-glucosidases. The inhibition potency towards the mammalian β-glucosidase was around 10- to 50-fold higher for **8** and **9** as compared to the natural compound **1** (K_i value of 45 μM).[18] Compounds **8** and **9** were further evaluated as inhibitors of the recombinant human β-glucocerebrosidase (GCase; Imiglucerase, Cerezyme® from Genzyme). The *n*-octyl derivative **9** (K_i = 2.2 μM; IC_{50} = 6.5 μM) was found to be a tenfold better inhibitor for this enzyme than the *n*-butyl derivative **8** (K_i 24.8 μM; IC_{50} value 64 μM),[19] which agrees with the correlation between lipophilicity (chain length) and the inhibitory activity that was observed in other glycomimetic families.[20]

The kinetic determination of the inhibition constants for **8** and **9** with *Tm*GH1, which was carried out at the pH optimum of catalytic activity for the enzyme (pH 5.8) afforded values of 1.1 and 0.28 μM, respectively (compared with 4 μM, 9 μM, 2 μM and 23 nM for **1**–**4**, respectively). Isothermal titration calorimetry (ITC), which was carried out at pH 5.8 and 25 °C (Figure 1), allowed the dissection of the thermodynamic contributions to binding.



Scheme 3. Synthesis of the new 6-oxacalystegine inhibitors **8** and **9**. The numbering system for the nortropane bicyclic system is shown. Note that the anomeric carbon (C1) in the L-idose precursor **6** or **7** becomes C5 in the final compound **8** or **9**.

The dissociation constants (K_d) for **8** and **9** that were determined by calorimetry (1.4 μM and 0.5 μM, respectively) were in good agreement with the K_i values determined by kinetic methods. Despite their similar inhibitory potential and chemical structure, however, the thermodynamic data show significant differences in the enthalpic and entropic contributions to

Table 1. Inhibitory activities (K_i, μM) for compounds **8** and **9**.

Enzyme	**8**	**9**
α-glucosidase (baker yeast)	n.i.[a]	n.i.
β-glucosidase (almond), pH 5.5	501	n.i.
β-glucosidase (almond), pH 7.3	567	n.i.
α-galactosidase (green coffee bean)	n.i.	n.i.
β-glucosidase/β-galactosidase (bovine liver)[b]	3.6	0.85
β-glucosidase/β-galactosidase (bovine liver)[b]	2.2	2.3
trehalase (pig kidney)	n.i.	n.i.
invertase (yeast), pH 6.0	n.i.	n.i.
invertase (yeast), pH 4.5	n.i.	n.i.
α-mannosidase (jack bean)	n.i.	n.i.
amyloglucosidase (*Aspergillus niger*)	n.i.	n.i.
imiglucerase	24.8	2.2
β-glucosidase (*Thermotoga maritima*)	1.1	0.28

[a] n.i., no inhibition detected at 2 mM inhibitor concentration. [b] K_i measurements were carried out by using *o*-nitrophenyl-β-D-glucopyranoside/*o*-nitrophenyl-β-D-galactopyranoside as the substrate, respectively.

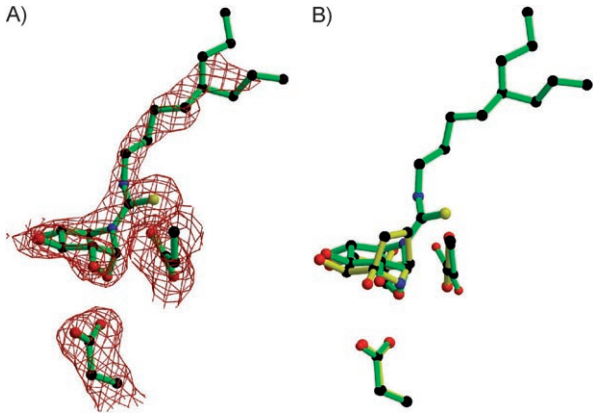


Figure 2. Ball-and-stick representation of A) *Tm*GH1 (the nucleophile Glu351 (bottom) and acid/base Glu166 (right) are shown) in complex with **9**; observed electron density for the maximum likelihood weighted $2F_{obs}-F_{calcd}$ is contoured at 1σ, and B) overlap of *Tm*GH1 in complex with **9** (green) and **1** (yellow). The figures were drawn by using BOBSCRIPT.[23] The *n*-octyl chain is shown in two different conformations (each has an occupancy of 0.5), as suggested by the disordered electron density in this region, which likely reflects the inherent flexibility of the chain.

binding. Compound **8** has a large favourable enthalpy of binding ($\Delta H_a^\circ = -9.3$ kcal mol^{-1}), which overcomes a small unfavourable entropic term ($T\Delta S_a^\circ = -1.4$ kcal mol^{-1}). Compound **9**, however, binds with both a favourable enthalpy and entropy ($\Delta H_a^\circ = -5.4$ kcal mol^{-1}; $T\Delta S_a^\circ = +2.9$ kcal mol^{-1}); this suggests that the longer alkyl chain might be involved in displacement of more water molecules in the active site. Both thermodynamic signatures are different to that observed for the parent calystegine B_2 **1**, which has a small enthalpy term that is offset by a large favourable entropic contribution ($\Delta H_a^\circ = -2.9$ kcal mol^{-1}; $T\Delta S_a^\circ = +4.5$ kcal mol^{-1}).[10]

X-ray data for *Tm*GH1 in complex with **9** were collected to a resolution of 1.90 Å (Table 2) and refined to a final R_{cryst} of 0.20 and R_{free} of 0.24.

Table 2. Data processing and refining statistics for *Tm*GH1 in complex with **9**.

Data collection beamline (ESRF)	ID29
resolution (outer shell) [Å]	20–1.90 (1.97–1.90)
space group	$P2_12_12_1$
unit cell parameters	$a = 93.7$ Å
	$b = 94.4$ Å
	$c = 113.0$ Å
	$\alpha = \beta = \gamma = 90^\circ$
R_{merge} (outer shell)	0.056 (0.37)
mean $I/\sigma I$ (outer shell)	25 (5.1)
completeness (outer shell) [%]	99.8 (100.0)
multiplicity (outer shell)	6.0 (6.1)
no. unique reflections	79606
R_{cryst}	0.20
R_{free}	0.24
RMSD bonds [Å]	0.014
RMSD angles [°]	1.37
RMSD chiral volume [Å^3]	0.100
PDB ID	2VRJ

The electron density revealed a molecule of **9** bound in the −1 subsite of both molecules in the asymmetric unit, with the piperidine ring in a chair conformation and the oxamethylene bridge lying below the plane of this ring (Figure 2 A). This situation strongly contrasts with the complex of *Tm*GH1 with **1**, in which the ethylene bridge is above the plane of the six-membered ring, which places the nitrogen atom at the position of the anomeric carbon in a native glycoside (Figure 2 B).[10] Compound **9** binds in an orientation resembling that seen with *Tm*GH1 in complex with **2**, with the nitrogen atom in the region of the endocyclic oxygen (Figure 3 B);[12] however, the pseudoanomeric oxygen (O6 in the nortropane nomenclature; homologous to O1 in a monosaccharide derivative)[21] and the nitrogen atom of **9** are found at least 0.7 Å "higher" in the active site of *Tm*GH1 compared to other previously observed inhibitors such as **1**, **2** and **3**, as well as the trapped 2-fluoro, covalent glycosyl–enzyme intermediate.[12] This situation means that the oxygen atom in the oxazolidine ring of **9** (O6 in Scheme 3) is in a similar position to the endocyclic oxygen in the trapped 2-fluoro complex (O5 in glucose nomenclature).

The binding of **9** with the bridge below the plane of the glycoside ring, as opposed to above, might prevent any unfavourable steric clashes between the oxazolidine ring and Trp398 and Tyr295, which line the bottom of the active site. In addition, the absence of a hydroxymethyl group at the position equivalent to the C5 of glucose (C1 in Scheme 3) means that no interaction is formed with the Oε2 atom of Glu405, which might limit the positional freedom in the other inhibitors that were studied with *Tm*GH1. Despite the difference in binding position, the majority of the interactions with the hydroxyl groups equivalent to those at C2, C3 and C4 of glucose are as described previously.[10,12] The oxygen atom in the oxazolidine ring hydrogen bonds with Tyr295; an interaction between an electronegative atom of a residue at the position of Tyr295 and the endocyclic oxygen of the substrate is a common feature and thought to play a role in transition state stabilisation.[22] The thiourea nitrogen atom might make a weak hydro-

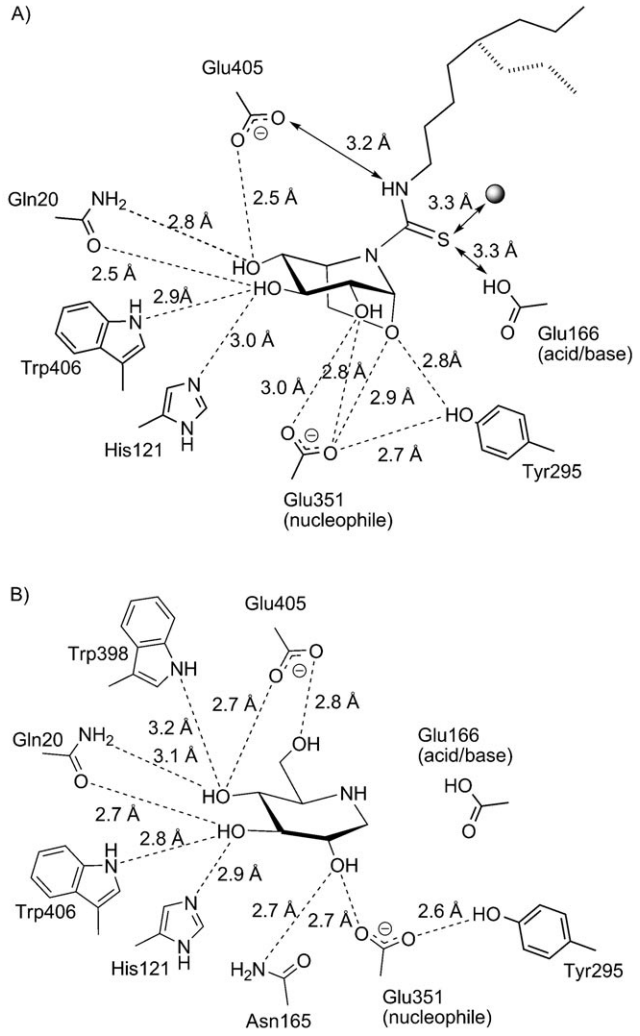


Figure 3. Interactions between *Tm*GH1 and A) **9** and B) **2** The solid and dashed bonds in the octyl chain of **9** correspond to the two different conformations in which it can be modelled.

gen-bond interaction with Glu405 (distance of 3.2 Å), and the thiocarbonyl sulfur atom with Glu166 and a water molecule (with both at a distance of 3.3 Å; Figure 3 A).

The *N*′-(*n*-octyl) chain of **9** when in complex with *Tm*GH1 lays in a channel that is surrounded by a number of hydrophobic amino acid residues in the aglycone subsites; this is analogous to that observed for the *n*-nonyl chain of *N*-(*n*-nonyl)-1-deoxynojirimycin (NN-DNJ) in the complex with recombinant human GCase, an enzyme that belongs to the same clan GH-A.[24] The electron density for the alkyl chain, however, is more disordered than for the 6-oxa-(+)-calystegine B_2 core, and in both molecules in the asymmetric unit it can be built in two conformations. This indicates that there is a certain amount of flexibility in the chain, which is supported by the absence of any interactions with active-site residues that would limit the movement.

The question remains as to why **9** binds to *Tm*GH1 in the "opposite" orientation to **1**. It has already been discussed that inhibitors that possess suitable three-dimensional fit and geometry are able to adjust to the stereochemical as well as electronic requirements of different glycosidases in possibly more than one orientation.[25] Similarly, it can be argued that structural modifications in an inhibitor can alter the complementarity relationships at the active site of a given enzyme by favouring alternative binding modes. In the case of **9** there might be several factors that contribute to this. Compound **1** possesses a hydroxyl group at positions that are equivalent to both C2 and C5 in glucopyranosides (C4 and C1 in the nortropane notation, respectively), which means that it is able to form hydrogen bonds between the hydroxyl group at C5 and Glu405 (although this interaction might be weaker than in "true" gluco-derived compounds in which there would be a hydroxymethyl substituent) and the hydroxyl group at C2 and His121, Asn165 and Glu351. In comparison, **9** has only one of these hydroxyl groups, which, depending on the orientation in which it binds, can take advantage of the hydrogen-bond interactions in only one of these positions. The binding of **9** in the observed orientation means hydrogen bonds can form between the C2 hydroxyl group (C4 in the nortropane notation) and both oxygen atoms of Glu351; previous studies have demonstrated that the hydroxyl group at the C2 position contributes the most energy to transition state stabilisation[26] and, thus, interactions with the hydroxyl group at this position are likely to be more favourable than at the C5 position. In addition, the presence of the alkyl chain at the position equivalent to the endocyclic oxygen in a native glycoside means there is enough space in the active site for it to be accommodated. If, however, it had bound in the opposite orientation, it is possible the alkyl chain would require significant movement of the acid/base residue, Glu166. This is also consistent with the fact that *N*-substitution of compound **4**, in which the alkyl chain was attached to a nitrogen atom at the anomeric position, abolishes inhibitory activity, whilst the identical substitution at the position equivalent to the endocyclic oxygen results in more potent inhibitors.[19a]

It is interesting that **9** has a higher inhibitory potency compared to **8**; **9** possesses an octyl chain, whereas **8** has a butyl chain. The calorimetry data indicates that **9** binds with a larger entropic contribution than **8**, and so it can be surmised that the extra potency for **9** is derived from the displacement of more water molecules in the active site. This has been discussed previously, particularly in relation to the binding of glucoimidazole-derived compounds in complex with *Tm*GH1, where, for example, the addition of a phenethyl substituent produces an extremely potent inhibitor, but the three-dimensional structure reveals no productive interactions are formed between this group and the active site residues.[27]

In summary, the data presented here illustrate the potential of 1-deoxy-6-oxa-*N*-thiocarbamoylcalystegine B_2 derivatives as a new family of glycosidase inhibitors. Analysis of the thermodynamic contributions to binding of **8** and **9**, combined with insights into the active-site interactions with the clan GH-A β-glucosidase *Tm*GH1, unambiguously shows an orientation for **9** with the bridge below the plane of the glycoside ring and with the alkyl substituent projecting into a channel lined with a number of hydrophobic residues. Detailed studies at the mo-

lecular level into the intricacies of binding, combined with a relatively straightforward synthetic route for these 1-deoxy-6-oxa-*N*-thiocarbamoylcalystegine B_2 derivatives, which could be used in a combinatorial fashion, should lead to the discovery of more potent inhibitors.

Experimental Section

Materials: 5-amino-5-deoxy-1,2-*O*-isopropylidene-β-L-idofuranose (**5**) was prepared from commercial D-glucofuranurono 6,3-lactone in four steps, as reported previously.[17,28] Imiglucerase (Cerezyme®; recombinant human β-glucocerebrosidase analogue, M_W 60430 Da) was kindly provided by Genzyme. The glycosidases α-glucosidase (from baker yeast), β-glucosidase (from almond), β-glucosidase/β-galactosidase (from bovine liver, cytosolic), trehalase (from pig kidney), α-galactosidase (from green coffee bean), α-mannosidase (from jack bean), invertase (from yeast) and amyloglucosidase (from *Aspergillus niger*) that were used in the inhibition studies, as well as 4-methylumbelliferyl-β-D-glucoside, α,α′-trehalose, sucrose and the corresponding *o*- and *p*-nitrophenyl glycoside substrates, were purchased from Sigma. *Tm*GH1 was expressed and purified as described previously.[12]

5-(*N*′-Alkylthioureido)-5-deoxy-1,2-*O*-isopropylidene-β-L-idofuranoses (6 and 7): *n*-Butyl or *n*-octyl isothiocyanate (2.29 mmol) and Et_3N (0.05 mL) were added to a solution of **5** (0.5 g, 2.29 mmol) in pyridine (15 mL). The mixture was stirred at room temperature for 18 h, then concentrated and coevaporated several times with toluene under vacuum. The resulting residue was purified by column chromatography by using the eluent that is indicated in each case to give the corresponding thiourea adduct as an amorphous solid.

5-(*N*′-Butylthioureido)-5-deoxy-1,2-*O*-isopropylidene-β-L-idofuranose (6): Eluent: 2:1 EtOAc/petroleum ether; yield: 0.69 g (90%); R_f 0.32 (3:1 EtOAc/petroleum ether); $[\alpha]_D$ −38.0 (*c* 1.0, MeOH); UV (MeOH): 248 nm (ε_{mM} 12.1); IR (KBr): ν_{max} = 3397, 3237, 2951, 1564, 1468, 1375, 1072 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$, 313 K): δ = 6.36 (br s, 1H; NH), 5.93 (d, $J_{1,2}$ = 3.7 Hz, 1H; H1), 4.90 (m, 1H; N′H), 4.50 (d, 1H; H2), 4.32 (d, $J_{3,4}$ = 2.0 Hz, 1H; H3), 4.25 (dd, $J_{4,5}$ = 2.9 Hz, 1H; H4), 3.92 (dd, $J_{6a,6b}$ = 11.1 Hz, $J_{5,6a}$ = 4.4 Hz, 1H; H6a), 3.87 (m, 1H; H5), 3.76 (dd, $J_{5,6b}$ = 5.3 Hz, 1H; H6b), 3.34 (m, 2H; CH_2N), 2.69, 1.63 (br s, 2H; OH), 1.57 (m, 2H; CH_2CH_2N), 1.38 (m, 2H; CH_2CH_3), 1.50, 1.32 (2 s, 6H; CMe_2), 0.94 (t, $^3J_{H,H}$ = 7.4 Hz, 3H; CH_3); ^{13}C NMR (125.7 MHz, $CDCl_3$, 313 K): δ = 181.8 (CS), 111.9 (CMe_2), 104.6 (C1), 84.8 (C2), 80.4 (C4), 75.7 (C3), 64.7 (C6), 54.7 (C5), 44.0 (CH_2N), 30.8 (CH_2CH_2N), 26.7, 26.0 (CMe_2), 19.9 (CH_2CH_3), 14.3 (CH_3); FABMS: *m/z*: 357 (100) $[M+Na]^+$, 335 (50) $[M+H]^+$; elemental analysis calcd (%) for $C_{14}H_{26}N_2O_5S$ (334.43): C 50.28, H 7.84, N 8.37; found: C 50.33, H 7.76, N 8.16.

5-Deoxy-1,2-*O*-isopropylidene-5-(*N*′-octylthioureido)-β-L-idofuranose (7): Eluent: 1:1→2:1 EtOAc/petroleum ether; yield: 0.54 g (61%); R_f 0.32 (3:1 EtOAc/petroleum ether); $[\alpha]_D$ = −84.0 (*c* 1.0, CH_2Cl_2); UV (CH_2Cl_2): 248 nm (ε_{mM} 12.4). IR (KBr): ν_{max} = 3349, 2928, 1555, 1379, 1092 cm^{-1}; ^1H NMR (300 MHz, CD_3OD, 313 K): δ = 5.88 (d, $J_{1,2}$ = 3.7 Hz, 1H; H1), 4.55 (m, 1H; H5), 4.49 (d, 1H; H2), 4.29 (dd, $J_{4,5}$ = 7.7 Hz, $J_{3,4}$ = 2.7 Hz, 1H; H4), 4.12 (d, 1H; H3), 3.74 (dd, $J_{6a,6b}$ = 11.2 Hz, $J_{5,6a}$ = 4.3 Hz, 1H; H6a), 3.70 (dd, $J_{5,6b}$ = 4.6 Hz, 1H; H6b), 3.45 (t, $^3J_{H,H}$ = 7.0 Hz, 2H; CH_2N), 1.56–1.30 (m, 12H; $6CH_2$), 1.45 (s, 3H; *C*Me), 1.28 (s, 3H; *C*Me), 0.89 (t, $^3J_{H,H}$ = 7.4 Hz, 3H; CH_3); ^{13}C NMR (75.5 MHz, CD_3OD, 313 K): δ = 183.7 (CS), 113.8 (CMe_2), 104.9 (C1), 86.0 (C2), 80.2 (C4), 74.8 (C3), 61.7 (C6), 55.2 (C5), 46.6 (CH_2N), 33.9–31.2 (CH_2), 28.0, 27.4 (CMe_2), 24.7 (CH_2CH_3), 15.4 (CH_3); FABMS: *m/z*: 391 (50) $[M+H]^+$; elemental analysis calcd (%) for $C_{18}H_{34}N_2O_5S$ (390.54): C 55.36, H 8.77, N 7.16; found: C 55.21, H 8.58, N 7.05.

(1*S*,2*R*,3*S*,4*R*,5*R*)-*N*-(*N*′-Alkylthiocarbamoyl)-2,3,4-trihydroxy-6-oxa-nortropanes (8 and 9): The corresponding L-idofuranose precursor **6** or **7** (0.5 mmol) was dissolved in a mixture of TFA/H_2O (9:1, 5 mL) and stirred at room temperature for 15 min until the starting material disappeared (TLC). The solvent was removed under vacuum and the residue was coevaporated several times with water. Finally, an aqueous solution of the reaction product was neutralised by treatment with Amberlite IRA 68 (OH^-) ion-exchange resin and freeze-dried. The resulting residue was purified by column chromatography with the eluent that is indicated in each case.

(1*S*,2*R*,3*S*,4*R*,5*R*)-*N*-(*N*′-Butylthiocarbamoyl)-2,3,4-trihydroxy-6-oxa-nortropane (8): Eluent: EtOAc→20:1 EtOAc/EtOH; yield: 106 mg (77%); R_f 0.51 (45:5:3 EtOAc/EtOH/H_2O); $[\alpha]_D$ +97.0 (*c* 1.0, H_2O); UV (MeOH): 248 nm (ε_{mM} 9.4); ^1H NMR (300 MHz, D_2O, 313 K): δ = 5.92 (s, 1H; H5), 4.82 (t, $J_{1,2}$ = $J_{1,7b}$ = 4.7 Hz, 1H; H1), 4.11 (d, $J_{7a,7b}$ = 8.5 Hz, 1H; H7a), 3.82 (dd, 1H; H7b), 3.69 (m, 1H; H2), 3.60 (d, $J_{3,4}$ = 7.9 Hz, 1H; H4), 3.59 (t, $J_{2,3}$ = 7.9 Hz, 1H; H3), 3.52 (t, $^3J_{H,H}$ = 7.4 Hz, 2H; CH_2N), 1.55 (m, 2H; CH_2CH_2N), 1.29 (m, 2H; CH_2CH_3), 0.87 (t, $^3J_{H,H}$ = 7.4 Hz, 3H; CH_3); ^{13}C NMR (125.7 MHz, D_2O, 313 K): δ = 178.5 (CS), 89.6 (C5), 76.3 (C3), 75.0 (C4), 71.8 (C2), 67.3 (C7), 59.5 (C1), 46.6 (CH_2N), 31.6 (CH_2CH_2N), 20.7 (CH_2CH_3), 14.3 (CH_3); FABMS: *m/z*: 299 (80) $[M+Na]^+$; elemental analysis calcd (%) for $C_{11}H_{20}N_2O_4S$ (276.35): C 47.81, H 7.29, N 10.14; found: C 47.67, H 7.02, N 10.02.

(1*S*,2*R*,3*S*,4*R*,5*R*)-2,3,4-Trihydroxy-*N*-(*N*′-octylthiocarbamoyl)-6-oxa-nortropane (9): Eluent: 20:1→6:1 CH_2Cl_2/MeOH; yield: 108 mg (65%); R_f 0.53 (6:1 CH_2Cl_2/MeOH); $[\alpha]_D$ +48.0 (*c* 1.0, H_2O); UV (MeOH): 249 nm (ε_{mM} 12.9); ^1H NMR (300 MHz, CD_3OD, 313 K): δ = 5.81 (s, 1H; H5), 4.85 (t, $J_{1,2}$ = $J_{1,7b}$ = 4.5 Hz, 1H; H1), 4.08 (d, $J_{7a,7b}$ = 8.0 Hz, 1H; H7a), 3.73 (dd, 1H; H7b), 3.64 (m, 1H; H4), 3.57 (m, 2H; CH_2N), 3.53 (m, 2H; H2, H3), 1.61 (m, 2H; CH_2CH_2N), 1.31 (m, 6H; 3 CH_2), 0.89 (t, 3H; $^3J_{H,H}$ = 7.2 Hz, CH_3); ^{13}C NMR (75.5 MHz, CD_3OD, 313 K): δ = 180.0 (CS), 90.2 (C5), 77.4 (C3), 75.8 (C4), 72.4 (C2), 66.8 (C7), 60.0 (C1), 46.5 (CH_2N), 33.0–28.0 (CH_2), 30.0 (CH_2CH_2N), 23.7 (CH_2CH_3), 14.4 (CH_3); FABMS: *m/z*: 355 (80) $[M+Na]^+$; elemental analysis calcd (%) for $C_{15}H_{28}N_2O_4S$ (332.46): C 54.19, H 8.49, N 8.43; found: C 54.22, H 8.28, N 8.32.

General procedure for the inhibition assay against the commercial enzymes: Inhibitory potencies 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), sucrose (for invertase) or α,α′-trehalose (for trehalase) in the presence of 6-oxacalystegine derivative **8** or **9**. 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 the 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 the addition of 1 M Na_2CO_3 or a solution of GLC–Trinder (Sigma, for trehalase and invertase). The absorbance of the resulting mixture was determined at 405 nm or at 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 by using a Sigma Plot program (version 4.14, Jandel Scientific, Corte Madera, CA, USA).

Imiglucerase inhibition assay: Enzyme solutions (0.1 mg mL^{-1} (1.65 nM), 0.25% (*w*/*v*) sodium taurocholate and 0.1% (*v*/*v*) Triton X-100 in McIlvaine buffer, pH 5.2) were incubated at 37 °C without (control) or with either **8** or **9** at a final volume of 40 μL for 30 min. After addition of 60 μL 4-methylumbelliferyl-β-D-glucopyranoside (4 mM, McIlvaine buffer, pH 5.2), the samples were incubated at 37 °C for 10 min. Enzymatic reactions were stopped by the addition of aliquots (150 μL) of glycine/NaOH buffer (100 mM, pH 10.6). The amount of 4-methylumbelliferone produced was determined with a Spectramax Gemini XPS (Molecular Devices Corporation) at 355 nm (excitation) and 460 nm (emission). The IC_{50} values were determined by plotting percent activity versus log [I], by using at least five different inhibitor concentrations. The K_i values and enzyme inhibition modes were determined from the slope of Lineweaver–Burk plots as above. The absence of any significant quenching of the fluorophore signal by **8** or **9** was confirmed by measuring the fluorescence intensity of 4-methylumbelliferyl-β-D-glucopyranoside (4 μM, McIlvaine buffer, pH 5.2) in the presence of increasing concentrations of the calystegine derivatives (from 5 to 10 μM). Results differed by less than 4%.

Kinetic studies with *Tm*GH1: Kinetic studies were conducted by monitoring the change in UV/vis 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 **8** and **9**, which were at concentrations between 0.3 and 7 μM for **8** and 0.05 and 1 μM for **9**; *Tm*GH1 was present at a concentration of 4 nM. The reaction was initiated by addition of substrate after incubation of *Tm*GH1 with the inhibitor for 20 min, which prevented any complications from slow onset inhibition. Rates were monitored for 400 s. 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: 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 36–38 μM. Compounds **8** and **9** were 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 aliquots (10 μL) 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 ($T\Delta S_a^\circ$) were calculated by using the equation $\Delta G_a^\circ = -RT\ln K_a = \Delta H_a^\circ - T\Delta S_a^\circ$.

Crystallisation and X-ray data collection: *Tm*GH1, at 10 mg mL^{-1}, was crystallised in the presence of a minute amount of solid **9** by using the same conditions as described previously.[12] The crystal was cryoprotected in a solution that contained the mother liquor with the addition of 25% ethylene glycol, and flash frozen in liquid nitrogen. Data for *Tm*GH1 in complex with **9** were collected at 100 K at the European Synchrotron Radiation Facility (Grenoble, France), on beamline ID29. Data were integrated and scaled with the HKL2000 suite,[29] and the CCP4 suite of programs was used for all other crystallographic computing.[30] Isomorphism between the native structure (PDB ID code 1OD0) and the complex meant that refinement could commence after rigid body refinement in REFMAC.[31] Manual corrections were done by using COOT,[32] which were interspersed with cycles of least square refinement in REFMAC.[31] The coordinates and structure factors have been deposited with the accession code 2VRJ.

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[1] Although the term "azasugar" is widely used in the literature to refer to glycomimetics where the endocyclic oxygen atom has been replaced by nitrogen, the term is not correct according to the IUPAC–IUBM nomenclature recommendations for carbohydrates; the accepted name is "iminosugar". "Azahexose" actually implies that a carbon atom has been exchanged for a nitrogen atom. See: A. D. McNaught, *Pure Appl. Chem.* **1996**, *68*, 1919–2008.

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