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6-Amino-6-deoxy-5,6-di-*N*-(*N*′-octyliminomethylidene)nojirimycin: Synthesis, Biological Evaluation, and Crystal Structure in Complex with Acid β-Glucosidase

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Gaucher disease, the most common lysosomal storage disease,[1–3] is caused by the defective activity of acid β-glucosidase (β-glucocerebrosidase, GlcCerase; EC 3.2.1.45), resulting in accumulation of glucosylceramide (GlcCer). The main treatment for Gaucher disease is enzyme replacement therapy (ERT), in which defective GlcCerase is supplemented by active enzyme, administered to patients intravenously usually every two weeks. Two sources of recombinant GlcCerase are available, namely Cerezyme®, recombinant GlcCerase expressed in Chinese hamster ovary cells, and recombinant GlcCerase expressed in transgenic carrot cells (prGCD).[4] The crystal structures of GlcCerase expressed from these two sources have been determined,[4,5] demonstrating that GlcCerase consists of a $(\beta/\alpha)_8$ (TIM) barrel containing the catalytic residues, and two additional noncatalytic domains.

GlcCerase structures to which small molecules are bound either covalently[6,7] or noncovalently[8,9] have also been solved. Among these are the structures of prGCD bound to two iminosugar glycomimetic inhibitors[10] with *N*-alkylpiperidine structure, namely *N*-butyl-1-deoxynojirimycin (NB-DNJ; Zavesca™) and *N*-nonyl-1-deoxynojirimycin (NN-DNJ). NB-DNJ is used in a new treatment modality known as substrate reduction therapy, in which partial inhibition of glycosphingolipid synthesis results in a decreased accumulation of glycosphingolipids, including GlcCer.[11,12] However, these compounds also stabilize the structure of GlcCerase,[13] thus rendering them suitable for a third treatment paradigm, namely chaperone therapy, in which active-site-directed inhibitors stabilize mutant forms of lysosomal enzymes, such as GlcCerase, as they pass through the secretory pathway.[14]

NB-DNJ and NN-DNJ lack a pseudoanomeric substituent that could mimic the glycosidic linkage of the natural substrates (Figure 1). Not surprisingly, they can simultaneously inhibit several glycosidases, including the human acid α- and β-glucosidases, and this represents a serious problem for clinical applications.[15] Although a hydroxy group might act as universal surrogate for the C–O anomeric linkage of glycosides, the low stability of reducing iminosugars, such as the natural alkaloid nojirimycin (NJ), prevents their therapeutic use. It has been recently shown that iminosugar analogues with an endocyclic pseudoamide-type nitrogen, such as (thio)urea, (thio)carbamate, or iso(thio)urea, with substantial sp^2 character represent a new generation of reducing glycosidase inhibitors (sp^2 iminosugars) with unprecedented stability and tunable anomeric selectivity.[16] Thus, the *N*′-octyl(cyclic isourea)nojirimycin fused hybrid glucomimetic **1** (Figure 1) proved to be a potent and very selective β-glucosidase inhibitor in assays against a panel of commercial glycosidases.[17,18] Further studies have shown that **1** exhibits full selectivity for acid β-glucosidase among human lysosomal glycosidases ($IC_{50} = 5.6\ \mu M$; Table 1), and is therefore a promising candidate as an active-site-specific chaperone (ASSC) for the treatment of Gaucher disease.[19]

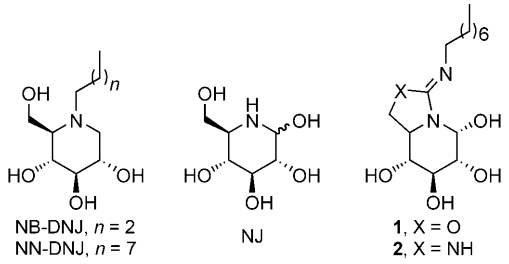

Figure 1. Structures of *N*-butyl- and *N*-nonyl-1-deoxynojirimycin (NB- and NN-DNJ, respectively), 1-deoxynojirimycin (NJ), and *N*′-octyl(cyclic isourea)- and *N*′-octyl(cyclic guanidine)-nojirimycin (**1** and **2**).

The structural basis for the β selectivity of **1** remains unclear. Formally, **1** can be considered as a bicyclic hybrid of NN-DNJ and NJ, combining the presence of the long alkyl chain and a hemiaminal center. However, whereas NJ is present in aqueous

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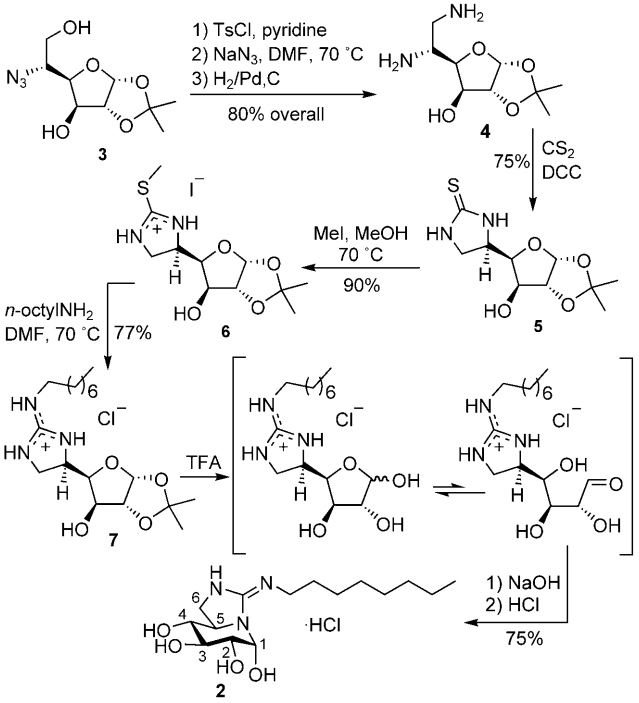
Table 1. Inhibitory activities of compounds **1** and **2** against commercial enzymes.

Enzyme	K_i [μM] 1	2
α-glucosidase (baker's yeast)	168	NI[a]
β-glucosidase (almond), pH 5.5	3.2	27
β-glucosidase (almond), pH 7.3	1.9	0.42
α-galactosidase (green coffee bean)	NI	NI
β-glucosidase/β-galactosidase (bovine liver)[b]	2.7	35
trehalase (pig kidney)	182	40
α-L-fucosidase (pig kidney)	NI	260
naringinase (*Penicillium decumbens*)	–	0.18
β-galactosidase (*E. coli*)	NI	NI
isomaltase (baker's yeast)	–	NI
α-mannosidase (jack bean)	NI	NI
amyloglucosidase (*Aspergillus niger*)	NI	NI
acid α-glucosidase (human, lysosomal)	NI[c]	NI[c]
acid β-glucosidase (human, lysosomal)	5.6[c]	4.0[c]

[a] No inhibition detected at an inhibitor concentration of 2 mM. [b] K_i measurements were carried out using *o*-nitrophenyl-β-D-galactopyranoside as substrate. [c] IC_{50} values [μM]; measurements were carried out at pH 4.5 using the corresponding 4-methylumbelliferone-conjugated α- or β-D-glucopyranoside (1 mM) as substrate.[19]

solution as a mixture of the β (major) and α anomers, the "mismatching" α anomer, with the pseudoanomeric OH group in the axial orientation, is the only form observed for **1** in aqueous solution. On the other hand, strong inhibition by iminosugars is partially ascribed to electrostatic interactions involving the protonated ammonium form,[20] while protonation at the endocyclic nitrogen in **1** is much less favorable. We reasoned that increasing basicity by replacing the isourea segment in **1** with a guanidine functionality should reinforce coulombic interactions at the active site of β-glucosidase and, simultaneously, weaken the anomeric effect by withdrawing electron density at the endocyclic nitrogen, thereby facilitating the "matching" equatorial orientation of the anomeric oxygen. To validate this hypothesis and examine its potential for the design of tighter binding β-glucosidase inhibitors as ASSC candidates, the preparation of 6-amino-6-deoxy-5,6-di-*N*-(*N*′-octyliminomethylidene)nojirimycin (**2**), formally an *N*′-octyl(cyclic guanidine)-nojirimycin fused hybrid, was envisioned. Herein we report the synthesis and biological evaluation of compound **2** as well as the crystal structure of its complex with GlcCerase. The data are discussed in light of previous data for the binding of NN-DNJ.

The synthesis of **2** started from the known 5-azido-5-deoxy-1,2-*O*-isopropylidene-α-D-glucofuranose **3**, available in five steps from commercial D-glucurono-γ-lactone.[21] Introduction of a second azido group at the primary position C6, via the corresponding *p*-toluenesulfonyl ester and subsequent reduction of the resulting diazide, afforded the *vic*-diamine **4**, which was transformed into the 5,6-(cyclic thiourea) **5** by thiocarbonylation with carbon disulfide/dicyclohexylcarbodiimide.[22] Reaction of **5** with methyl iodide in methanol at 70 °C provided the corresponding *S*-methyl isothiouronium salt **6**. Further nucleophilic displacement of the methylthio group by *n*-octylamine provided the *N*-octyl(cyclic guanidine)-D-glucofuranose derivative **7** in 77% yield, which was characterized as the corresponding hydrochloride. Trifluoroacetic acid promoted hydrolysis of the acetal protecting group in **7** led to a mixture of the reducing α- and β-glucofuranose pseudo-*C*-nucleosides, which underwent spontaneous rearrangement to the target bicyclic guanidine sp^2 iminosugar **2** upon treatment with sodium hydroxide (Scheme 1).



Scheme 1. Synthesis of the (cyclic guanidine)-nojirimycin derivative **2**. The dominant α anomer, with the corresponding numbering system, is shown.

Compound **2**, characterized as the corresponding guanidinium chloride salt, was stable in aqueous solution at neutral, acidic, or basic pH (from pH 3 to pH 9), which is significantly different from that encountered in classical reducing nojirimycin derivatives. The basicity of the guanidine group ($pK = 11.9$) warrants full protonation at pH values around neutrality. NMR spectra supported the bicyclic structure, giving evidence for the α anomer as the dominant species in equilibrium with a small proportion of the β anomer (α/β ratio 1:20). The vicinal proton–proton coupling constants around the six-membered ring were in agreement with the $^4C_1$ chair conformation, with the pseudoanomeric group of the major α anomer in axial position, fitting the anomeric effect. In the minor β anomer, this scenario implies an equatorial orientation for the hydroxy group at C1, which is in agreement with the expected attenuation of the anomeric effect in **2** as compared with **1**.

The inhibitory activity of **2** was evaluated against a panel of commercial enzymes, including α-glucosidase (yeast), trehalase (pig kidney), β-glucosidase (almond), β-glucosidase/β-galactosidase (bovine liver, cytosolic), β-galactosidase (*E. coli*), α-galactosidase (green coffee bean), α-mannosidase (jack bean), α-L-fucosidase (pig kidney), isomaltase (baker's yeast) and naringi-

nase (*Penicilium decumbens*, β-glucosidase/β-rhamnosidase activity). The results demonstrate a high β- versus α-glucosidase selectivity (except for trehalase), with K_i values in the low-micromolar to nanomolar range among the β-glucosidase isoenzymes (Table 1). The inhibitory potency for almond β-glucosidase increased by two orders of magnitude on going from pH 5.5 ($K_i = 27$ μM) to pH 7.3 ($K_i = 0.42$ μM). This result points to the free base, which is probably protonated by the catalytic acid, being a more active species than the guanidinium form. The observed trend is analogous to that previously encountered for the cyclic isourea analogue **1**.[18] Most interestingly, compound **2** behaved as a potent and selective competitive inhibitor of human acid β-glucosidase ($IC_{50} = 4$ μM, wild-type), in the same range as NN-DNJ ($IC_{50} = 1$ μM),[13] but with a much higher selectivity (no inhibition for human lysosomal α-glucosidase detected for **2** versus $IC_{50} = 1.5$ μM for NN-DNJ).[23,24]

To investigate the molecular basis of β-glucosidase inhibition by the reducing (cyclic guanidine)-nojirimycin hybrid glycomimetic **2**, X-ray structural studies in the bound state were undertaken. Crystals of prGCD in complex with **2** were obtained in the $P2_1$ space group (Table 2), with two protein molecules in the asymmetric unit. The packing and unit cell dimensions were similar to the previously published crystal structures of prGCD (PDB IDs: 2V3E, 2V3D, and 2V3F). Superimposition of the native prGCD–**2** complex structure (Figure 2) on that of prGCD (PDB ID: 2V3F) revealed an RMSD of 0.2 Å, demonstrating that GlcCerase does not undergo a global structural change upon binding of the inhibitor. Difference electron density maps revealed the presence of the polyhydroxylated bicyclic core in the active site. However, even with concomitant improvement of the phases, the electron density of the aliphatic tail could not be detected, and therefore we did not model it in the structure.

Table 2. Data processing and refining parameters for prGCD in complex with **2**.

data collection beamline (ESRF)	ID23h2
resolution [Å]	20.0–2.3 (2.38–2.30)
space group	$P2_1$
Unit cell parameters	
a, *b*, *c* [Å]	68.3, 96.8, 83.2
α/γ [°]	90
β [°]	104.3
R_{sym} [%]	13.2 (49.7)
mean $I/\sigma I$	7.9 (1.6)
completeness [%]	99.3 (93.8)
redundancy	3.4 (2.3)
resolution (refinement) [Å]	19.7–2.3
no. unique reflections	44184
R_{work}	13.5
R_{free}	19.4
RMSD bonds [Å]	0.019
RMSD angles [°]	1.732
Number of refined atoms	
protein	7743
carbohydrates	98
solvent and ions	803
ligands	28
Ramachandran outliers [%]	0.2
PDB ID	2WCG

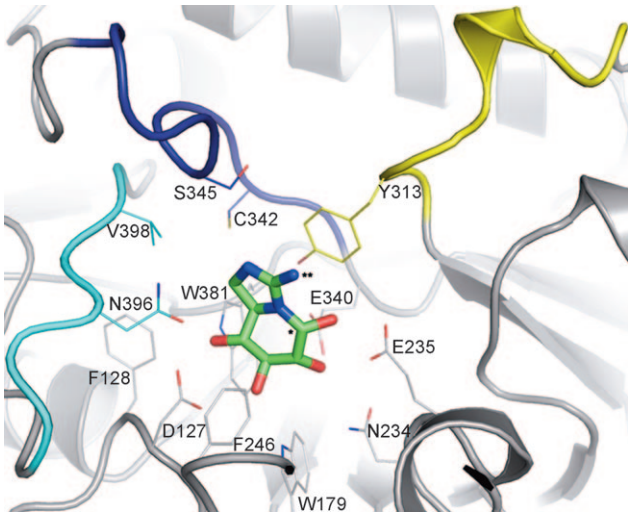


Figure 2. Binding of *N*′-octyl(cyclic guanidine)-nojirimycin **2** in the prGCD active site. The aliphatic tail of **2** was not detected in the electron density, and is therefore not shown. Compound **2** is shown in green, residues 341–349 (loop 1) are shown in dark blue, residues 393–399 (loop 2) are in cyan, and residues 312–319 (loop 3) are in yellow. Active site residues within 4 Å of the inhibitor are shown as sticks. *Indicates the chiral center that corresponds to the anomeric carbon atom in GlcCer. **Indicates the N-bound aliphatic center, which is not visible in the electron density.

The results show that **2** is bound at the active site (Figure 2) similarly to NB-DNJ (Figure 3) and NN-DNJ,[8] which places the endocyclic nitrogen atom at the position of the anomeric carbon in a native glycoside. The inability to detect electron density for the aliphatic tail of **2** is probably related to its high degree of flexibility. However, the geometric orientation of this inhibitor is similar to that observed in the structures of the NB-

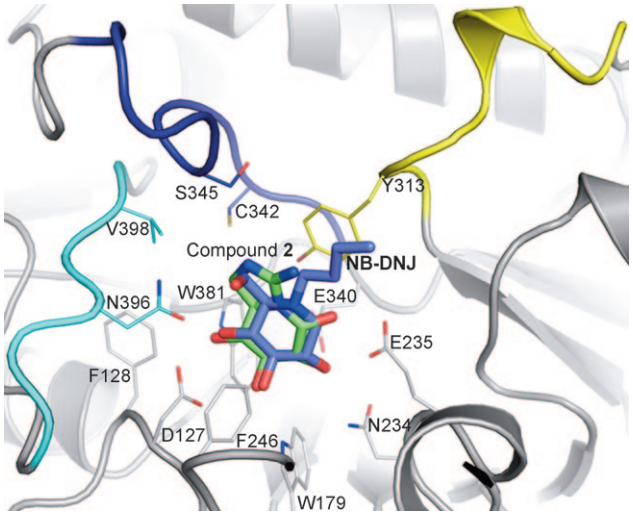


Figure 3. Comparison of the active site regions of the complexes of NB-DNJ and **2** with prGCD. Compound **2** is shown in green and NB-DNJ in blue. Residues 341–349 (loop 1) are shown in dark blue, residues 393–399 (loop 2) are in cyan, and residues 312–319 (loop 3) are in yellow. Active site residues within 4 Å of the active site inhibitors are shown as sticks.

DNJ and NN-DNJ–GlcCerase complexes,[8] suggesting that the aliphatic tail would be oriented toward the entrance of the active site.

The orientation within the active site of the competitive inhibitors is largely determined by hydrogen bond interactions.[8] As in NB-DNJ and NN-DNJ, the hydroxy groups equivalent to the all-equatorial triol system OH-2, OH-3, and OH-4 on the six-membered ring moiety in **2** show stereochemical complementarity in terms of their orientations to the glucose moiety of the natural substrate GlcCer. These hydroxy groups are thought to be responsible for the configurational specificity of the inhibitors. In **2**, a hydroxy group is located on a carbon atom adjacent to the nitrogen in the six-membered ring that corresponds to the anomeric carbon in GlcCer (C1). Surprisingly, this pseudoanomeric hydroxy group is accommodated in the active site of prGCD in the "wrong" α configuration. Hemiaminals are known to mutarotate in solution,[25] with glycosidases selecting out the tighter binding anomer according to their anomeric specificity.[26] The structure of β-glucosidase from *Thermotoga maritima* (*Tm*GH1) in complex with an sp^2 iminosugar glucomimetic having an axially oriented pseudoanomeric oxygen atom intramolecularly anchored in the α configuration was recently solved.[27] This enzyme belongs to the same clan GH-A as human acid β-glucosidase. In the present case, however, it is the enzyme that selects the α-configured inhibitor even when mutarotation is allowed. The reason seems to be that the chair conformation of the six-membered ring in **2** is significantly distorted toward the ^4E conformation upon binding, in a similar manner to the piperidine ring of NB-DNJ or NN-DNJ in their corresponding complexes with prGCD.[8] In this situation, the α pseudoanomeric hydroxy group adopts a pseudoequatorial orientation, resulting in the generation of a new hydrogen bond with the acid/base catalytic residue E235 (Figure 4).

The question remains, however, of why **2** binds to human acid β-glucosidase with such a remarkable anomeric selectivity relative to NN-DNJ. It has been pointed out that inhibitors with suitable configurational and conformational features are able to adjust to the stereochemical and electronic requirements of various glycosidases by adopting more than one orientation.[28] The presence or absence of an anomeric OH group that can adopt either the α or β configuration does seem not to impose a serious restriction on adaptation to the active site of α- or β-glucosidases, nor does the presence of the long alkyl chain appear to influence selectivity, as NN-DNJ inhibits lysosomal α- and β-glucosidase with almost identical potency. In the case of **2**, however, the presence of the five-membered ring imposes a certain restriction to the orientation of the N′ substituent, which is well suited to fit in the hydrophobic pocket lying in the vicinity of the active site of GlCerase; however, this probably results in unfavorable interactions in the case of the α-glucosidase. This is in agreement with the fact that sp^2 iminosugars with bicyclic nojirimycin-type structure that lack exocyclic substituents do serve as α-glucosidase inhibitors.[22,29]

In summary, the data presented herein illustrate the potential of (cyclic guanidine)-nojirimycin fused hybrids as a new series of sp^2 iminosugar glycosidase inhibitors. Compound **2**, the first representative of this family, was designed as a basic reducing analogue of NN-DNJ. The presence of the *N*′-octyl(cyclic guanidine) segment imparts stability to the hemiaminal center, favoring the α anomer in solution. In apparent contradiction, compound **2** behaves as a potent and very selective inhibitor of β-glucosidases, as previously observed for related sp^2 iminosugars, notably with a total specificity for lysosomal acid β-glucosidase. Our findings warrant further investigation of the pharmacokinetic properties of **2** in terms of its potential chaperoning effect on mutant forms of GlcCerase. Moreover, the structural studies of binding at the molecular level, combined with a very efficient and relatively straightforward synthetic route that could be used in a combinatorial fashion, should lead to the discovery of more potent inhibitors for acid β-glucosidase and other therapeutically relevant enzymes.

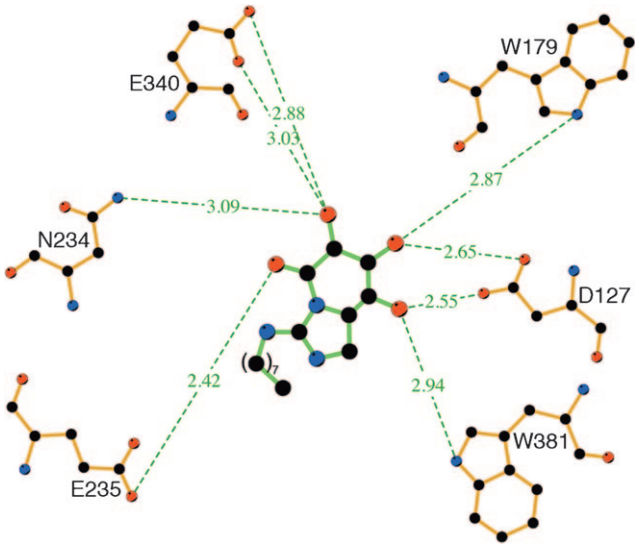


Figure 4. Hydrogen bonding of compound **2** in the active site of prGCD.

Experimental Section

Materials and methods: 5-azido-5-deoxy-1,2-*O*-isopropylidene-α-D-glucofuranose (**3**) was prepared from commercial D-glucofuranurono-6,3-lactone in five steps, as reported.[21,22] 5,6-Diamino-5,6-dideoxy-1,2-*O*-isopropylidene-α-D-glucofuranose (**4**) was obtained by following the procedure previously reported,[22] except for the reduction of the diazide intermediate, which was performed by catalytic hydrogenation with Pd/C and H_2 at atmospheric pressure. Isothiocyanation of **4** with CS_2 and dicyclohexylcarbodiimide (DCC) was used to prepare the key cyclic thiourea precursor **5**.[22] The commercial glycosidases α-glucosidase (from yeast), β-glucosidase (from almonds), β-glucosidase/β-galactosidase (from bovine liver, cytosolic), α-galactosidase (from green coffee beans), isomaltase (from yeast), trehalase (from pig kidney), amyloglucosidase (from *Aspergillus niger*), α-mannosidase (from jack bean), β-galactosidase (from *E. coli*), naringinase (from *Penicillium decumbens*) used in the inhibition studies, as well as the corresponding *o*- and *p*-nitrophenyl glycoside substrates were purchased from Sigma Chemical Co.; prGCD was produced as described.[4]

Reagents and solvents were purchased from commercial sources and used without further purification. Optical rotations were measured at 22 °C in 1-cm or 1-dm tubes. ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75.5 MHz, respectively. 2D COSY and HMQC experiments were carried out to assist in signal assignment. TLC was performed with E. Merck precoated TLC plates, silica gel 30 F_{245}, with visualization by UV light and by charring with 10% H_2SO_4 or 0.2% *w/v* cerium(IV) sulfate/5% ammonium molybdate in 2 M H_2SO_4 or 0.1% ninhydrin in EtOH. Column chromatography was carried out with silica gel 60 (E. Merck, 230–400 mesh). 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 matrixes and the positive ions were separated and accelerated over a potential of 7 keV. NaI was added as cationizing agent. Microanalyses were performed by the Instituto de Investigaciones Químicas (Seville, Spain).

5,6-Diamino-5,6-dideoxy-1,2-*O*-isopropylidene-5,6-di-*N*-(*S*-methylthioxomethylidene)-α-D-glucofuranose iodide (isothiouronium salt 6): A solution of the cyclic thiourea **5** (335 mg, 1.29 mmol) and MeI (0.40 mL, 5 equiv) in MeOH (12 mL) was heated at reflux (70 °C) for 2 h and concentrated. The resulting residue was purified by column chromatography using CH_2Cl_2/MeOH/H_2O (80:10:1→60:10:1) as eluent. Yield: 467 mg (90%); R_f = 0.53 (CH_2Cl_2/MeOH 40:10:1); $[\alpha]_D^{22} = -69.5$ (*c* = 0.7, MeOH); ^{1}H NMR (300 MHz, CD_3OD): δ = 5.97 (d, $J_{1,2}$ = 3.6 Hz, 1H; H-1), 4.62 (ddd, $J_{5,6b}$ = 11.3 Hz, $J_{5,6a}$ = 7.8 Hz, $J_{4,5}$ = 3.5 Hz, 1H; H-5), 4.53 (d, 1H; H-2), 4.35 (t, $J_{3,4}$ = 3.5 Hz, 1H; H-4), 4.21 (d, 1H; H-3), 4.07 (dd, $J_{6a,6b}$ = 11.3 Hz, 1H; H-6a), 3.99 (t, 1H; H-6b), 2.68 (s, 3H; SMe), 1.48, 1.33 (2s, 6H; CMe_2); ^{13}C NMR (75.5 MHz, CD_3OD): δ = 172.1 (SCN), 113.1 (*C*Me$_2$), 106.6 (C1), 86.8 (C2), 81.8 (C4), 75.5 (C3), 59.2 (C5), 48.6 (C6), 27.2, 26.4 (C*Me*$_2$), 14.0 (SMe); IR (KBr): $\tilde{\nu}_{max}$ = 3389, 2924, 1540, 1384, 1216, 1075 cm^{-1}; FABMS: *m/z* (%): 275 (100) $[M-I]^+$; elemental analysis calcd (%) for $C_{11}H_{19}IN_2O_4S$: C 32.84, H 4.76, N 6.96; found: C 32.53, H 4.78, N 6.72.

5,6-Diamino-5,6-dideoxy-1,2-*O*-isopropylidene-5,6-di-*N*-(*N*′-octyliminomethylidene)-α-D-glucofuranose hydrochloride (guanidinium salt 7): A solution of **6** (200 mg, 0.73 mmol) and *n*-octylamine (1.5 equiv) in DMF (15 mL) was heated at 70 °C under Ar for 18 h and concentrated. The resulting residue was purified by column chromatography using CH_2Cl_2/MeOH/H_2O (90:10:1→70:10:1) as eluent. Yield: 200 mg (77%); R_f = 0.41 (CH_2Cl_2/MeOH/H_2O 70:10:1); $[\alpha]_D^{22} = -10.3$ (*c* = 1.0, CH_2Cl_2); ^{1}H NMR (300 MHz, CD_3OD): δ = 5.94 (d, $J_{1,2}$ = 3.6 Hz, 1H; H-1), 4.52 (d, 1H; H-2), 4.33 (m, 1H; H-5), 4.25 (dd, $J_{4,5}$ = 4.8 Hz, 1H; $J_{3,4}$ = 3.0 Hz, H-4), 4.18 (d, 1H; H-3), 3.82 (dd, $J_{6a,6b}$ = 10.0 Hz, $J_{5,6a}$ = 6.9 Hz, 1H; H-6a), 3.77 (t, $J_{5,6b}$ = 10.0 Hz, 1H; H-6b), 3.21 (t, $^3J_{H,H}$ = 7.1 Hz, 2H; CH_2N), 1.61 (m, 2H; C*H*$_2$CH$_2$N), 1.48, 1.32 (2s, 6H; CMe_2), 1.36 (m, 10H; CH_2), 0.92 (t, $^3J_{H,H}$ = 7.0 Hz, 3H; CH_3); ^{13}C NMR (75.5 MHz, CD_3OD): δ = 160.3 (CN), 113.0 (*C*Me$_2$), 106.6 (C1), 86.9 (C2), 82.5 (C4), 75.5 (C3), 55.6 (C5), 46.1 (C6), 43.9 (CH_2N), 32.9, 30.3, 30.2, 30.1 (CH_2), 27.5, 27.2 (C*Me*$_2$), 26.4 (CH_2), 23.7 (*C*H$_2$CH$_3$), 14.4 (CH_3); IR (KBr): $\tilde{\nu}_{max}$ = 3207, 2928, 1673, 1592, 1466, 1375, 1216, 1074 cm^{-1}; FABMS: *m/z* (%): 356 (100) $[M-Cl]^+$; elemental analysis calcd (%) for $C_{18}H_{34}ClN_3O_4$: C 55.16, H 8.74, N 10.72; found: C 55.05, H 8.97, N 10.65.

6-Amino-6-deoxy-5,6-di-*N*-(*N*′-octyliminomethylidene)nojirimycin hydrochloride (guanidium salt 2): A solution of **7** (0.40 mmol) in 90% TFA/H_2O (1.7 mL) was stirred at 0 °C for 1 h, concentrated under reduced pressure, co-evaporated several times with H_2O, treated with 0.1 N NaOH until pH 8 was reached, and subjected to column chromatography using $CH_3CN/H_2O/NH_4OH$ (4:1:1) as eluent. The guanidine was transformed into the corresponding hydrochloride salt by freeze-drying from a solution of hydrochloric acid (pH 5). Yield: 111 mg (75%); R_f = 0.28 ($CH_3CN/H_2O/NH_4OH$ 4:1:1); $[\alpha]_D^{22} = +6.6$ (*c* = 0.97, H_2O); ^{1}H NMR (300 MHz, D_2O): δ = 5.31 (d, $J_{1,2}$ = 3.8 Hz, 1H; H-1), 3.85 (m, 1H; H-5), 3.75 (t, $J_{6a,6b} = J_{5,6a}$ = 9.7 Hz, 1H; H-6a), 3.64 (t, $J_{2,3} = J_{3,4}$ = 9.7 Hz, 1H; H-3), 3.49 (dd, 1H; H-2), 3.42 (t, $J_{4,5}$ = 9.7 Hz, 1H; H-4), 3.39 (t, $J_{5,6b}$ = 9.7 Hz, 1H; H-6b), 3.21 (t, $^3J_{H,H}$ = 7.1 Hz, 2H; CH_2N), 1.50 (m, 2H; C*H*$_2$CH$_2$N), 1.23 (m, 10H; CH_2), 0.79 (t, $^3J_{H,H}$ = 6.9 Hz, 3H; CH_3); ^{13}C NMR (75.5 MHz, D_2O): δ = 156.6 (CN), 74.7 (C1), 73.5 (C4), 72.6 (C3), 71.8 (C2), 56.3 (C5), 46.3 (C6), 43.0 (CH_2N), 31.0, 28.3, 28.2, 25.8 (CH_2), 22.0 (*C*H$_2$CH$_3$), 13.4 (CH_3); FABMS: *m/z* (%): 316 (100) $[M-Cl]^+$; elemental analysis calcd (%) for $C_{15}H_{32}ClN_3O_5$: C 48.71, H 8.72, N 11.36; found: C 48.58, H 8.61, N 11.23.

General procedure for inhibition assays 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 and β-galactosidase from *E. coli*) or *p*-nitrophenyl α- or β-D-glycopyranoside or α,α′-trehalose (for trehalase), in the presence of the guanidine derivative **2**. Each assay was performed in phosphate buffer at the optimal pH for each enzyme. The K_M values for the various glycosidases used in the tests and the corresponding working pH values are as follows: α-glucosidase (yeast), K_M = 0.35 mM (pH 6.8); isomaltase (yeast), K_M = 1.0 mM (pH 6.8); β-glucosidase (almonds), K_M = 3.5 mM (pH 7.3); β-glucosidase/β-galactosidase (bovine liver), K_M = 2.0 mM (pH 7.3); β-galactosidase (*E. coli*), K_M = 0.12 mM (pH 7.3); α-galactosidase (coffee beans), K_M = 2.0 mM (pH 6.8); trehalase (pig kidney), K_M = 4.0 mM (pH 6.2); α-L-fucosidase (pig kidney), K_M = 0.2 mM (pH 6.8); naringinase (*Penicilium decumbens*), K_M = 2.7 mM (pH 6.8); α-mannosidase (jack bean), K_M = 2.0 mM (pH 5.5). 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. After the mixture was incubated for 10–30 min at 37 or 55 °C, the reaction was quenched by the addition of 1 M Na_2CO_3 or a solution of Glc-Trinder (Sigma, for trehalase). The absorbance of the resulting mixture was determined at 405 or 505 nm. The K_i values and enzyme inhibition modes were determined from the slopes of Lineweaver–Burk plots and double reciprocal analysis.

Crystallization and X-ray data collection: prGCD was diluted in crystallization buffer (10 mM citric acid/sodium citrate buffer pH 5.5, 7% (*v/v*) EtOH and 0.02% (*w/v*) NaN_3), washed three times, and concentrated to 4–5 mg mL^{-1} using a Centricon® device with a 30-kDa cutoff filter. Compound **2** was dissolved in H_2O to make a stock solution of 0.1 M, and subsequently added to prGCD to a final concentration of 13 mM. prGCD was co-crystallized with **2** using the micro-batch technique under Al's oil (1:1 *v/v* silicone and paraffin oils). Protein together with the inhibitor and crystallization solutions were dispensed into hydrophobic Vapor Batch crystallization plates under oil, such that the final solution of each crystallization drop contained 50% protein mixture and 50% crystallization solutions. The crystallization solution contained 0.2 M $(NH_4)_2SO_4^{2-}$, 0.1 M Tris pH 6.5, and 25% (*w/v*) PEG 3350. Crystals were cryo-protected prior to cryo-cooling in liquid N_2 with a mixture of 80% crystallization solution and 20% (*v/v*) ethylene glycol solutions. Diffraction was measured on the ID23h2 beamline at the ESRF synchrotron (Grenoble, France). Images were indexed with the HKL2000 software package and scaled with SCALA.[15] The structure was solved using the molecular replacement method and refined with Refmac5[16] (Table 1). An initial model of the crystal structure of prGCD[4] (PDB ID: 2V3F) was used as a starting model. Model manipulation and water editing was performed using Coot graphics software.[17] Images were created with PyMol (http://www.

pymol.org) and LIGPLOT.[18] Structures and structure factors were deposited in the Protein Data Bank (PDB ID: 2WCG).

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