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Polyhydroxylated Bicyclic Isoureas and Guanidines Are Potent Glucocerebrosidase Inhibitors and Nanomolar Enzyme Activity Enhancers in Gaucher Cells

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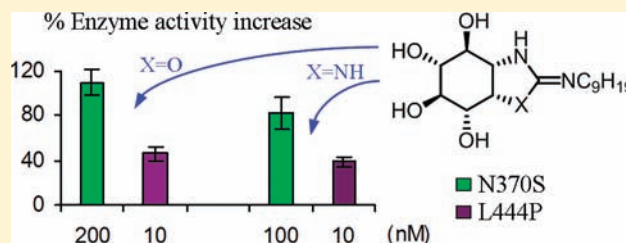
S Supporting Information



ABSTRACT: Four diastereomeric series of *N*-alkylated [6+5] bicyclic isoureas having hydroxyl substituents mimicking glucose hydroxyl groups have been synthesized as potential β-glucocerebrosidase (GCase) inhibitors with the aim of developing pharmacological chaperones for enzyme deficiency in Gaucher disease (GD). The bicyclic compounds differ either by the configuration of the ring fusion carbon atoms or by the nature of the *N*-alkyl substituents. When assayed for effects on GCase activity, the isoureas displayed selective inhibition of GCase with low micromolar to nanomolar IC_{50}'s in isolated enzyme experiments. One of the series of isoureas, a family having a specific cis ring fusion, exhibited strong inhibition of recombinant GCase activity with K_i values in the 2−42 nM range. In addition, the [6+5] bicyclic guanidine derivatives with a substitution pattern analogous to the most active isoureas were also found to be potent inhibitors of GCase with K_i values between 3 and 10 nM. Interestingly, the active bicyclic isoureas and guanidines also behaved as GCase inhibitors in wild-type human fibroblasts at nanomolar concentrations. The potential of these compounds as pharmaceutical chaperones was determined by analyzing their capacity for increasing GCase activity in GD lymphoblasts derived from N370S and L444P variants, two of the most prevalent Gaucher mutations. Six compounds were selected from the different bicyclic isoureas and guanidines obtained that increased GCase activity by 40−110% in N370S and 10−50% in L444P cells at low micromolar to nanomolar concentrations following a 3 day incubation. These results describe a promising series of potent GCase ligands having the cellular properties required for pharmacological chaperones.

INTRODUCTION

Lysosomal storage diseases (LSDs) are a group of genetic disorders due to defects in some aspect of lysosomal biology.[1] Among them, Gaucher disease (GD) is the most prevalent, and it is caused by the reduced activity of the lysosomal enzyme acid β-glucosidase, also known as β-glucocerebrosidase (GCase).[2] This leads to the accumulation of the substrate glucosylceramide (GlcCer) in the lysosomes of macrophages. GlcCer storage results in hepatomegaly, splenomegaly, anemia, bone lesions, and central nervous system (CNS) involvement.[3] Patients without CNS symptoms are classified as type 1, whereas those with CNS symptoms are classified as either type 2 (acute infantile) or type 3 (juvenile or early adult onset). Current treatment approaches[4] for GD include replacement of the defective enzyme with recombinant enzyme[5] (imiglucerase or velaglucerase alfa) or inhibition of GlcCer production.[6] Type 1 disease can be managed with enzyme replacement therapy.[7] However, the treatment is expensive, and the enzyme is unable to cross the blood−brain barrier to alleviate the CNS symptoms of severe disease.[8] A drug that inhibits the production of GlcCer (*N*-butyl-deoxynojirimycin), thus decreasing the substrate load on GCase, has recently become available as a therapy for type 1 patients.[9] However, its efficacy against CNS Gaucher variants is unknown.[10] Since this therapy reduces GlcCer biosynthesis nonselectively, cellular pathways utilizing GlcCer and other metabolites are likely to be adversely affected by the drug, which may explain its side effects.

An approach that has recently attracted much interest for the treatment of GD and other lysosomal storage disorders is enzyme enhancement therapy with pharmacological chaperones.[11,12] These are small molecules that specifically bind to and stabilize the functional form or three-dimensional shape of a misfolded protein in the endoplasmic reticulum (ER) of a cell. When misfolded because

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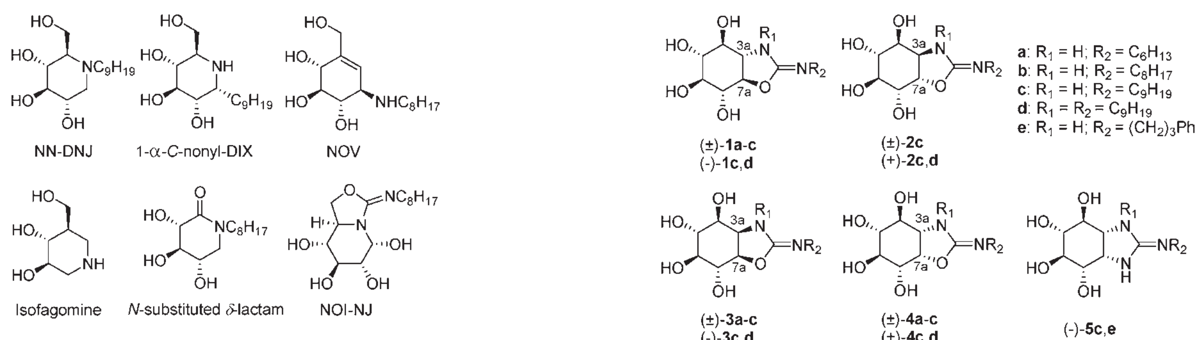


Figure 1. Chemical structures of pharmacological chaperones for GCase.

of a genetic mutation, the enzyme is unable to adopt the correct functional shape. This misfolded protein is recognized by the quality control system in the cell and destroyed, leading to decreased amounts of the enzyme transported from the ER to the lysosome and, as a consequence, a reduction of the effective enzyme activity results. The binding of the chaperone molecule is proposed to help during the protein folding and trafficking from the ER and is distributed to the lysosome in the cell, thereby increasing enzyme activity and cellular function, reducing substrate and stress on cells. Thus, iminosugar-type glycomimetics with GCase inhibitory activity, such as *N*-nonyl-deoxynojirimycin (NN-DNJ),[13] α-1-*C*-octyl-1-deoxynojirimycin (CO-DNJ),[14] α-1-*C*-nonyl-1-deoxynojirimycin (α-1-*C*-nonyl-DIX),[15] valienamine derivatives (NOV),[16] isofagomine derivatives,[17−19] *N*-substituted δ-lactams,[20] and bicyclic nojirimycin analogues with the structure of sp_2 iminosugars (NOI-NJ)[21] have all shown high promise as pharmacological chaperones for the treatment of GD by increasing cellular GCase catalytic activity (Figure 1).

The discovery of selective and potent glycosidase inhibitors has been intensely stimulated by this novel perspective for therapeutic intervention in the LSDs. Important members of this group originate from the structural modification of natural sugars to increase their metabolic stability and recognition by enzymes or other carbohydrate binding proteins.[22] These compounds mimic either enzyme substrates or products or even the enzymatic hydrolysis reaction intermediates.[23]

Several polyhydroxylated bicyclic systems are efficient glycoside hydrolase inhibitors. These include products of natural origin, such as castanospermine,[24] swainsonine,[25] or the calystegins,[26] or compounds of synthetic origin, such as the glycoside-aromatic heterocycle hybrids developed by Vasella[27] or the cyclic amidine sugars described by Mioskowski.[28] To the best of our knowledge, the above sp_2-iminosugar analogues of the nojirimycin, such as the compound NOI-NJ, are the only bicyclic compounds which have shown promise as pharmacological chaperones for the treatment of GD.[21]

Based on bibliographic data,[29−31] improvement of potency and selectivity against GCase was observed when substituents close to the nitrogen atom were preferentially hydrophobic, aliphatic, or aromatic. Accordingly, we now report the synthesis of four families of *N*-nonyl bicyclic isourea derivatives[32] having a relative trans (isoureas **1c** and **2c**) or cis (isoureas **3c** and **4c**) disposition between the C3a and C7a substituents to explore their effects on GCase activity (Figure 2). Since the *N*-nonyl derivative **4c** was found to be a strong inhibitor of GCase, guanidines **5c** and **5e** in enantiomerically pure forms were also synthesized. Furthermore, *N*-hexyl and *N*-octyl derivatives of

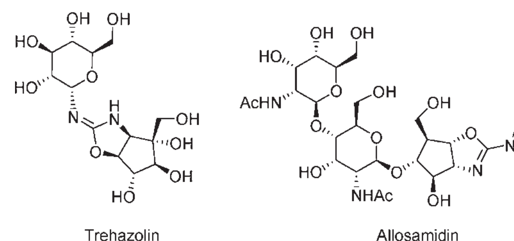


Figure 2. Chemical structures of bicyclic isoureas and guanidines (**1**−**5**) evaluated in this work. The structures of the cyclopentitols trehazolin and allosamidin are also depicted.

series **1**, **3**, and **4** in racemic form and the enantiomerically pure 2,3-disubstituted isoureas (**1d**−**4d**) were also prepared and evaluated.

It is interesting to note the structural relationship of these bicyclic compounds with the α-glucosidase inhibitor trehazolin and the chitinase inhibitor allosamidin (Figure 2),[33] which contain cyclic isourea moieties although in a different structural arrangement to that present in our molecules.

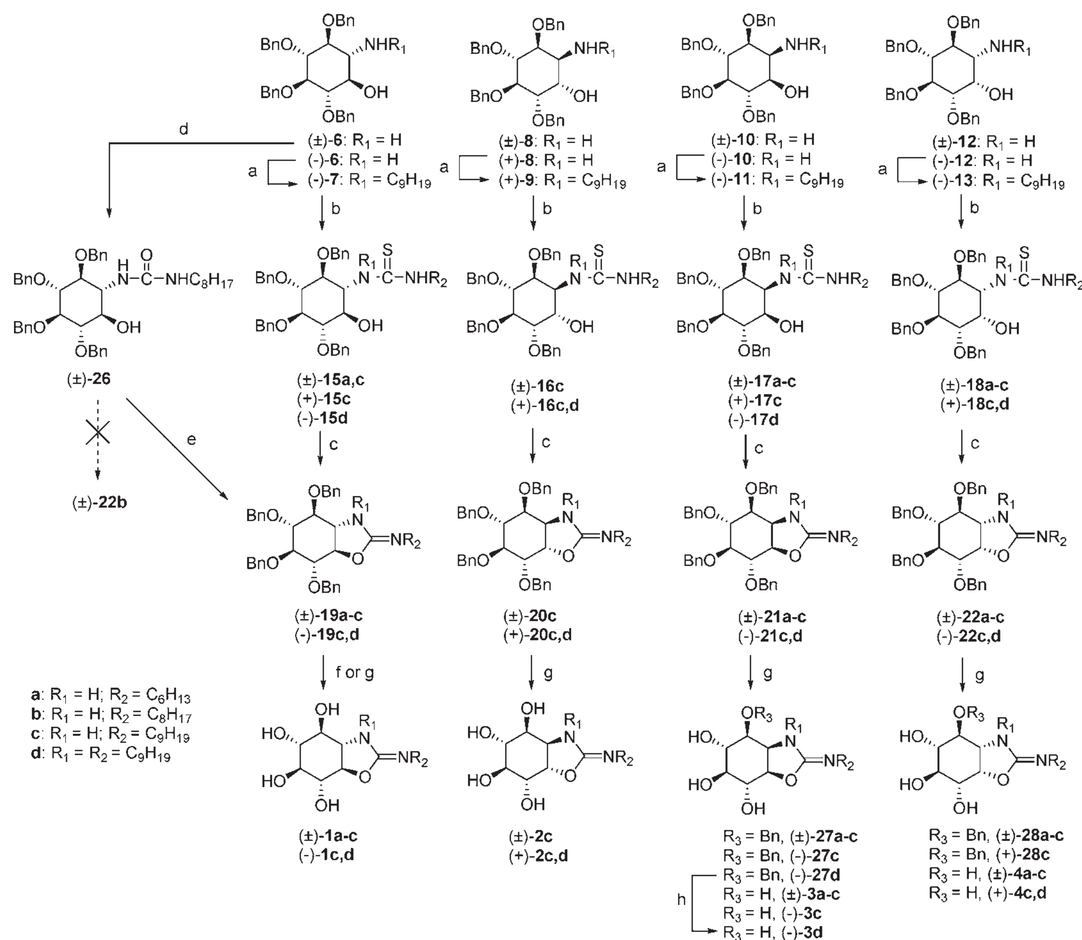
The biological activity of these new bicyclic compounds was determined using recombinant GCase, and the selectivity inhibitory profile of compounds examined for other glycosidases and glucosylceramide synthase (GCS) from cell homogenates. After studying the toxicity of compounds, the inhibitory effect of bicyclic isoureas and guanidines on GCase was examined in wild-type human fibroblasts. Next, the effect of some of the active compounds on the stabilization of recombinant GCase activity after thermal denaturation was determined as a measure for potential pharmacological chaperone activity. Finally, *N*-nonyl derivatives **1c**−**5c** and guanidine **5e** were further investigated as pharmacological chaperones in human lymphoblasts derived from Gaucher patients homozygous for N370S (non-neuronophatic form) or L444P (neuronophatic form) variants, which are the two most common mutations associated with GD.[34]

■ RESULTS

Synthesis. Bicyclic isoureas and guanidines **1**−**5** were obtained by coupling the amino alcohols **6**−**13** or diamine **14** with an isothiocyanate and subsequent cyclization of the resulting thioureas using an excess of yellow mercury oxide (II) provided the bicyclic isourea and guanidine analogues. The final products **1**−**5** were obtained after the *O*-benzyl deprotection by hydrogenolysis or by reaction with BCl_3, following reported protocols.[35,36]

The *N*-nonyl protected aminocyclitols **7**,[29] **9**, **11**, and **13** were obtained by reductive amination of nonanal with the corresponding protected aminocyclitol **6**,[37] **8**,[37,38] **10**,[39] and **12**,[35,37] which were obtained from conduritol B epoxide according to literature procedures. The above protected aminocyclitols (**6**−**13**) were

Scheme 1. Synthesis of Bicyclic Isoureas 1−4 from Amino Alcohols 6, 8, 10, and 12[a]



[a] Reagents and conditions: (a) $C_8H_{17}CHO$, $NaBH_3CN$, AcOH, MeOH; (b) RNCS, THF, 45 °C; (c) HgO, toluene, 115 °C; (d) $C_8H_{17}NCO$, CH_2Cl_2; (e) Tf_2O, pyridine, CH_2Cl_2, −40 °C; (f) $Pd(OH)_2$, EtOH, H_2 (2 atm), 60 °C; (g) BCl_3, CH_2Cl_2, −78 °C; and (h) Pd/C, MeOH, H_2 (2 atm).

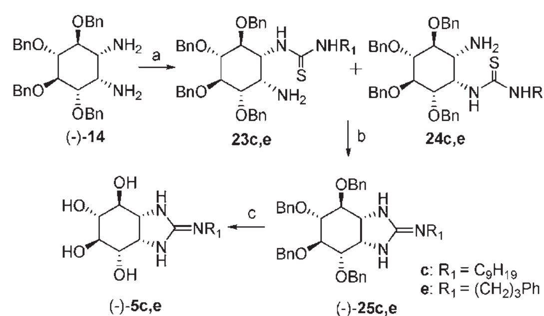
coupled with commercial *N*-alkyl isothiocyanates, which afforded the thiourea adducts **15**−**18** (Scheme 1) in a reaction that proceeds with total chemoselectivity in the presence of unprotected hydroxyl group.[40]

Treatment of the resulting thioureas with an excess of yellow mercury oxide (II) afforded the corresponding bicyclic isoureas **19**−**22** (Scheme 1).

Similarly, reaction of diamine **14**[41] with 1 equiv of nonyl or 3-phenylpropyl isothiocyanate gave a diastereoisomeric mixture of the thioureas **23** and **24**, which were easily cyclized with yellow mercury oxide (II) to give bicyclic guanidine analogues **25c**, **e** (Scheme 2).

It has been reported[42] the reaction of *N*-alkyl urea compounds with a vicinal hydroxyl group with triflic anhydride in the presence of pyridine afforded the corresponding 2-aminooxazoline with concomitant inversion of stereochemistry at the center bearing the secondary hydroxyl group through the intramolecular S_N2 displacement of a transient triflate by vicinal carbonyl group. We initially sought to apply this procedure for the synthesis of bicyclic isoureas **22a**−**c** from amino alcohol **6**. Thus urea **26** was obtained by reaction of amino alcohol **6** with octyl isocyanate. However, the treatment of the above urea under similar reaction conditions as previously reported[42] led to the bicyclic isourea **19b** with retention of stereochemistry at the carbon bearing the secondary hydroxyl group (Scheme 1).[43]

Scheme 2. Synthesis of Bicyclic Guanidines 5c, e from Diamine 14[a]



[a] Reagents and conditions: (a) $C_9H_{19}NCS$ or $Ph(CH_2)_3NCS$, THF, 45 °C; (b) HgO, toluene, 115 °C; and (c) BCl_3, CH_2Cl_2, −78 °C.

The final step of the synthesis would require the removal of all *O*-benzyl protective groups in isoureas **19a**−**c**. This was accomplished by hydrogenolysis to give the desired bicyclic compounds **1a**−**c** in good yields (Scheme 1). However, hydrogenolysis of **21** and **22** afforded complex mixtures. Gratifyingly, alternative benzyl removal could be efficiently achieved with BCl_3 at low temperature (Schemes 1 and 2).[37] It is noteworthy that monobenzylated compounds **27a**−**d** and **28a**−**c** were obtained as a byproduct,

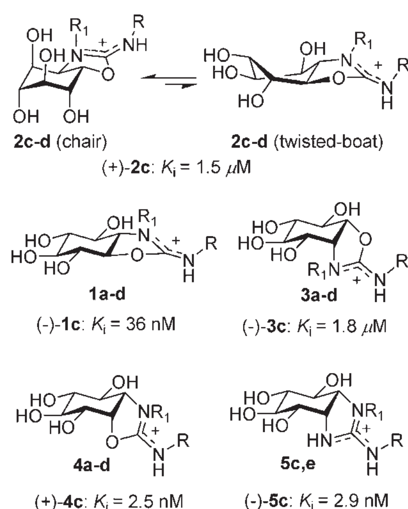


Figure 3. Observed (NMR) chair conformations of the studied compounds and inhibition constants (K_i) of the *N*-nonyl derivatives (R_1=H and R=C_9H_{19}) for the inhibition of imiglucerase.

which could be easily separated by column chromatography.[44] Surprisingly, the BCl_3 *O*-benzyl deprotection in isourea **21d** led exclusively to monobenzylated compound **27d**, which was transformed into the final compound **3d** after removing the remaining *O*-benzyl protecting group by hydrogenolysis (see Scheme 1).

All the newly synthesized compounds were fully characterized, being their corresponding ^{1}H and ^{13}C NMR signals assigned with the help of 2D NMR experiments (see Supporting Information). The knowledge of the solution structure of the final compounds (**1**−**5**) could be important for the rationalization of the biological results (see below). From the NMR analysis (see Figure S1, Supporting Information), we concluded that all the derivatives are protonated at neutral or slightly acidic pH, which are the conditions for the biological tests. Besides, we unambiguously determined that compounds **1a**−**d**, **3a**−**d**, **4a**−**d**, and **5c,e** have their cyclohexane ring in a chairlike conformation with the hydroxyl groups at positions 4−7 in equatorial (see Figures 3 and S2, Supporting Information). Thus, the fused cyclic isourea/guanidine must adopt a trans diequatorial disposition for **1a**−**d**, and a cis disposition for **3a**−**d**, **4a**−**d**, and **5c,e** (see Figures 3 and S2, Supporting Information). The cis arrangement of the fused heterocycle locates one of the heteroatoms in axial (N for **3a**−**d**, O for **4a**−**d**, and N for **5c,e**) and the other one in equatorial (O for **3a**−**d**, N for **4a**−**d**, and N for **5c,e**). In the special case of the **2a**−**d** family, the configuration of the isourea cycle forced a chair-like geometry with all the OH groups in axial, although an all-equatorial twisted-boat conformer should be energetically accessible (for further NMR and modeling details, see Supporting Information).

BIOLOGICAL RESULTS

Inhibition of Imiglucerase and GCase in Wild-Type Human Fibroblasts by Bicyclic Isoureas and Guanidines. As pointed out above, we seek to develop enzymatic inhibitors with potential as pharmacological chaperones for GCase. One of the first conditions is that compound binds the protein with high affinity. Most of pharmacological chaperones are competitive inhibitors of their target enzyme.[45] Therefore all final compounds were first evaluated as inhibitors of recombinant GCase (imiglucerase, Cerezyme) at lysosomal pH (5.2). Moreover, bicyclic compounds **1**−**5** were also tested at lumenal ER pH (7.0) to determine a possible change in the affinity for the enzyme with pH changes and to obtain information on the possible affinity for the protein at the different cellular pH values relevant for chaperone effects. The inhibitory activities of all compounds against imiglucerase are summarized in Table 1.

Compounds **1**−**5** inhibited the enzyme activity at both neutral and acidic pH, as shown in Table 1. Remarkably, the potency for inhibiting imiglucerase activity was increased by more than three-fold when measured at pH 7.0 compared to pH 5.2 for several compounds.[46] A similar dependence of the inhibitory activity from the pH has been previously reported for other compounds, including ambroxol, which showed enzyme enhancement on human fibroblasts with various GCase mutations.[47,48] As expected, compounds with one of the hydroxyl group protected as a benzyl ether (**27** and **28**) were less potent inhibitors of imiglucerase than the corresponding bicyclic compounds with all free hydroxyl groups (**3** and **4**), but they still showed inhibition with K_i's in the nanomolar range for (±)-**27c** and **28b**−**c**.

The *N*-nonyl derivatives **1c**, **3c**, and **4c** were found to be better inhibitors for this enzyme than the corresponding *N*-hexyl and *N*-octyl derivatives in each series, a fact that is in agreement with the correlation between lipophilicity and the inhibitory activity that was observed in other glycomimetic families in this enzyme.[29,30] Remarkably, the simple extension of the *N*-alkyl chain length, from octyl to nonyl, resulted in more than a three-fold increase in enzyme activity. In contrast, the introduction of second alkyl chain at the endocyclic nitrogen greatly decreased the inhibitory activity (IC_{50} values around 10 μM at pH 5.2 for compounds **1d**, **3d**, and **4d** and 160 μM for **2d**).

All tested compounds were found competitive inhibitors of imiglucerase, as illustrated in Figure 4 for (+)-**4c** (see also Figures S4−S8, Supporting Information). It is worth mentioning the low K_i values found for isoureas **4a**−**c** and guanidines **5c,e** that were in the 2−42 nM range.

In spite of the good correlation between lipophilicity and K_i values, the configuration of the ring junction carbon atoms bearing the oxygen and nitrogen atoms of the fused heterocycle had a decisive effect on the inhibition of imiglucerase (see Figure 3). Compounds **2c** and **3a**−**c** exhibited K_i values between 0.4 and 11 μM, whereas **1b**−**c**, **4a**−**c**, and **5c,e** were effective inhibitors of recombinant GCase with K_i values in the mid- to low-nanomolar range.

An interesting point is the hydroxyl matching between the series obtained and that present in the β-gluco configuration of GlcCer substrate. Except for compounds **2c**−**d**, all the other series have equatorial hydroxyl substituents in the cyclohexane ring that can match exactly the 2, 3, and 4-OH substituents present in glucose. As pointed out above, the series **2** showed a chair conformation with all axial hydroxyl arrangement according to NMR experiments performed with these compounds and therefore a very poor match with hydroxyl groups in glucose results (see Figures 3 and S2, Supporting Information). However, when the compounds of this series were tested, compounds (±)-**2c** or (+)-**2c** have been found to be low micromolar inhibitors of the enzyme, and although are less potent than **1**, **4**, and **5** series, a similar activity to **3c** resulted, in spite of the fact of the very different orientation of the hydroxyl groups. It is known that GCase has a high selectivity for lipidic β-gluco

Table 1. Inhibitory Activity of Compounds against Imiglucerase and GCase Inhibition in Wild-Type (wt) Human Fibroblasts[a]

	imiglucerase			
	IC_{50} (μM)			
compound	pH 7.0	pH 5.2	K_i (μM)[b]	% GCase inhibition (in wt human fibroblasts)[c]
(±)-**1a**	1.6	6.4	3.4	4
(±)-**1b**	0.08	0.60	0.23	67
(±)-**1c**	0.04	0.22	0.07	72
(−)-**1c**	0.02	0.12	0.04	87
(−)-**1d**	5.6	11.1	12.5	24[d]
(±)-**2c**	0.60	2.9	2.4	34
(+)-**2c**	0.32	1.9	1.5	33
(+)-**2d**	56.7	160	ND[e]	40[d]
(±)-**3a**	4.4	12.9	11.1	0
(±)-**3b**	0.54	2.5	1.7	2
(±)-**3c**	0.19	0.71	0.44	53
(−)-**3c**	1.7	6.0	1.8	46
(−)-**3d**	1.2	10.4	4.8	63[d]
(±)-**4a**	0.04	0.10	0.04	77
(±)-**4b**	0.01	0.02	0.007	97
(±)-**4c**	0.008	0.009	0.002	99
(+)-**4c**	0.005	0.008	0.002	100
(+)-**4d**	2.4	6.8	2.8	17[d]
(−)-**5c**	0.004	0.01	0.003	99
(−)-**5e**	0.01	0.04	0.01	100
(±)-**27a**	ND	64.4	25.9	ND
(±)-**27b**	ND	6.4	3.6	ND
(±)-**27c**	ND	1.3	0.53	ND
(−)-**27c**	ND	17.3	8.8	ND
(−)-**27d**	ND	157	ND	ND
(±)-**28a**	ND	3.9	1.3	ND
(±)-**28b**	ND	0.74	0.13	ND
(±)-**28c**	ND	1.7	0.47	ND
(+)-**28c**	ND	0.35	0.18	ND
NN-DNJ	0.30	0.66[f]	0.30[f]	ND

[a] Typical results differed by less than 6%. See Supporting Information, Tables S2 and S5. [b] The inhibition was competitive in all cases (determined at pH 5.2). [c] Incubation for 24 h at 50 μM inhibitor and 5 mM substrate in wt human fibroblast. [d] Toxic at 50 μM. Incubation for 24 h at 5 μM inhibitor and 5 mM substrate in wt human fibroblast. [e] ND: not determined. [f] See ref 15.

substrates, so it is not easy to understand why these compounds are competitive inhibitors of the enzyme. If the all axial OH chair would be the binding conformation, then this would probably preclude the recognition of the inhibitor by the enzyme, and no activity would be expected. We suspected that probably a conformation other than the chair was the binding conformation of (+)-**2c** compound, which should match the gluco OH tridimensional arrangement and also must be energetically accessible from the chair conformation. A calculation performed with a model showed that a twist conformation is found approximately 1.5 kcal mol^{-1} above the chair conformation and when compared to a β-glucose chair showed a reasonable match (see Figure S3, Supporting Information). Moreover, the barrier between these conformations is about 7 kcal mol^{-1} and is the same order (or even lower) to that found in cyclohexanes for chair to chair conformational transitions through a twist intermediate. Although there is no evidence of this conformation as actually being the binding conformation for (+)-**2c**, this would explain why this molecule, having a structure apparently different from a β-glucoside, is an enzyme inhibitor for GCase. The higher value of K_i for this compound would be in agreement with a high-energy conformer binding.

Apart from the hydroxyl substituents, it is also interesting to note that the more active series (compounds **1**, **4**, and **5**) have the endocyclic nitrogen atom of the fused heterocycle in an equatorial arrangement matching the β-glycoside anomeric oxygen present in the substrate GlcCer. The oxygen by nitrogen replacement is commonly found in other aminocyclitol inhibitors.[29,36,49] The reasons for the increase in potency in isoureas **4a**−**c**, which have a cis ring fusion due to the axial configuration of the isourea oxygen atom, over diastereomeric **1a**−**c** having a trans ring fusion and an equatorial oxygen are presently unclear and will be addressed in future structural studies. However, a better recognition by the enzyme for the cis compounds due to selective interactions with active site amino acids is hypothesized.

The data also indicate that isourea **4c** and guanidine **5c** displayed similar GCase inhibitory potencies, suggesting that

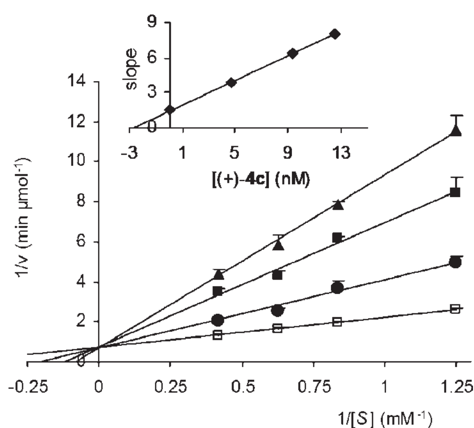


Figure 4. Type of inhibition of imiglucerase by (+)-**4c**. Double reciprocal plot of imiglucerase incubated at different concentrations of substrate and compound (+)-**4c** [empty square (□): 0 nM, circle (●): 5 nM, solid square (■): 9 nM, triangle (▲): 12 nM). Regression lines arise from data obtained in two different experiments with duplicates.

the replacement of isourea oxygen by an NH group is not essentially affecting the binding of these compounds to the enzyme. Interestingly, guanidine **5e** having an *N*-3-phenylpropyl substituent was a potent competitive inhibitor of GCase with a K_i value of 10 nM. This result would indicate that nitrogen substituents other than linear alkyl can be tolerated without affecting the inhibition of the enzyme to a large extent. It is noteworthy that the racemic isourea (±)-**3c** was found to be more active than the enantiopure (−)-**3c**, this would point toward a better activity for the enantiomer of (−)-**3c**, especially at neutral pH. However, the activity in cellular assays was similar for (±)-**3c** and (−)-**3c**.

These results show that these compounds are potent GCase inhibitors in isolated enzyme assays. To further progress in their activity profiling, the cellular GCase inhibition of bicyclic isoureas and guanidines **1**−**5** was also studied in wild-type human fibroblasts after 24 h of incubation at 50 μM. Cytotoxicity assays were done previously to the enzyme cellular assay in wild-type human fibroblasts to ensure that the measured activity was not affected by the possible toxicity of the compounds. Since fibroblasts incubated with compounds **1d**−**4d** at 50 μM displayed some signs of cytotoxicity, these isoureas were tested at 5 μM, well below the CC_{50} determined for these compounds (see Table S4, Supporting Information). However, the rest of compounds were not cytotoxic at concentrations up to 300 μM. The GCase inhibition in wild-type human fibroblasts at above concentrations is shown in Table 1. In general, a good correlation was observed between the chain length in each series and the inhibition in cell culture as well as between K_i values against imiglucerase and lysosomal β-glucosidase inhibition. The *N*-nonyl derivatives (+)-**2c** and (−)-**3c** showed moderate inhibition (33 and 46%, respectively) at 50 μM, whereas isoureas (−)-**1c**, (+)-**4c**, and guanidines (−)-**5c,e** showed more than 87% of GCase inhibition at the same concentration. Surprisingly, isoureas **1d**, **2d**, and **4d**, which exhibited moderate inhibitory activities against imiglucerase, showed between 17 and 40% of inhibition at 5 μM, whereas **3d** displayed an inhibition of 63% at the same concentration.

The high potency of isoureas **1b**−**c**, **3c**, **4a**−**c**, and guanidines **5c,e** required analysis at lower concentrations (see Figure S9, Supporting Information). Interestingly, some of them also behaved as GCase inhibitors at nanomolar to low-micromolar concentrations, as shown in Figure 5. These results show that the

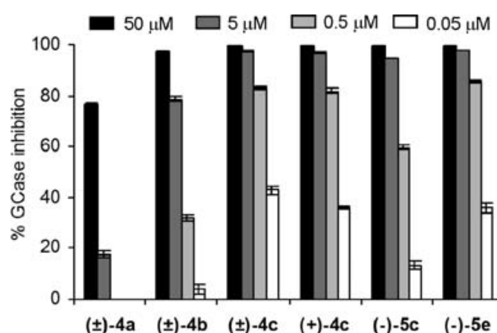


Figure 5. GCase inhibition of **4a**−**c** and **5c,e** in wild-type human fibroblasts after 24 h incubation time at the indicated inhibitor concentrations.

compounds are powerful GCase inhibitors in live cells, reflecting good membrane permeability and cellular stability properties to inhibit the cellular enzyme.

The selectivity of the bicyclic inhibitors for GCase was evaluated using a panel of commercial glucosidases and galactosidases (see Table S3, Supporting Information). Some compounds showed evidence of inhibitory activity on α-glucosidase from baker's yeast and on β-galactosidase from bovine liver at concentrations similar to those required for GCase inhibition. However, with regard to other possible glycosidase cellular targets of these compounds, no essential inhibitory activity was found for lysosomal β-galactosidase or α-glucosidase enzymes at concentrations of active compound where GCase was almost totally inhibited (see Table S6, Supporting Information), indicative of a high selectivity for the GCase enzyme.

Stabilization of Recombinant GCase (imiglucerase) under Thermal Denaturation Conditions. One of the main features of pharmacological chaperones is the thermodynamic stabilization they provide when bound to the protein, preventing it from aggregating and keeping it in a foldable state until it is properly folded and exported from the ER. Resistance to thermal denaturation in the presence of a potential pharmacological chaperone is a method used to assess the stabilization effect.[13,29,47,49] In this context, the effect of *N*-nonyl derivatives **1c**−**5c**, *N*-3-phenylpropyl guanidine **5e**, and 2,3-disubstituted isoureas **1d** and **4d** on the stabilization of recombinant GCase activity after thermal denaturation at 48 °C was determined in the presence and in the absence of increasing concentrations of compounds at different incubation times (see Figures S10 and S11, Supporting Information) as a measure for potential pharmacological chaperone activity. For comparative purposes, NN-DNJ was also assayed under the same experimental conditions.

The stabilization ratio values found for each compound at 5 μM (for **1c**, **4c**, and **5c,e**), 10 μM (for **1d**, **2c**, **3c**, and **4d**) or 50 μM for NN-DNJ and 60 min incubation time are summarized in Table 2.

Interestingly, bicyclic compounds **1c**, **4c**, **5c**, and **5e**, which were effective inhibitors of recombinant GCase with K_i values in the low-nanomolar range, showed stabilization ratios between 9 and 27 at 5 μM and 60 min incubation time. Compounds **1d** and **4d**, which displayed relatively weak imiglucerase inhibition, showed an enzyme stabilization profile comparable to or even superior to that of NN-DNJ, with stabilization ratios around 10 at 50 μM after 1 h of incubation at 48 °C (see Figure S10, Supporting Information). Collectively, these results reflect a positive effect of the compounds on enzyme stability and provide

Table 2. Enzyme Stabilization Ratio of Imiglucerase after Thermal Denaturation at 48 °C and Maximum Observed Increase in GCase Activity Using Pharmacological Chaperones and NN-DNJ

compound	stabilization ratio[a] (imiglucerase)	N370S GCase activity increase[b]	L444P GCase activity increase[b]
(−)-**1c**	20[c]	1.4 ± 0.1 (1 μM)	1.1 ± 0.1 (1 μM)
(−)-**1d**	1.7	ND[d]	ND
(+)-**2c**	13	1.7 ± 0.2 (10 μM)	1.3 ± 0.1 (1 μM)
(±)-**3c**	11	1.7 ± 0.1 (5 μM)	ND
(−)-**3c**	5	1.8 ± 0.1 (10 μM)	1.4 ± 0.1 (0.1 μM)
(±)-**4c**	ND	1.7 ± 0.1 (0.2 μM)	ND
(+)-**4c**	23[c]	2.1 ± 0.1 (0.2 μM)	1.5 ± 0.1 (0.01 μM)
(+)-**4d**	1.5	ND	ND
(−)-**5c**	27[c]	1.8 ± 0.1 (0.1 μM)	1.4 ± 0.1 (0.01 μM)
(−)-**5e**	9[c]	1.9 ± 0.1 (0.05 μM)	1.4 ± 0.1 (0.01 μM)
NN-DNJ	7.6[e]	1.4 ± 0.1 (5 μM)	no activity

[a] Defined as the ratio of relative enzymatic activities (inhibitor vs control) at a given inhibitor concentration and incubation time. Tabulated values for 10 μM inhibitor and 60 min incubation time. [b] N370S and L444P lymphoblasts from Gaucher patients were incubated with test compounds for 3 days before being used for enzyme assay. Data in parentheses correspond to the concentration of the tested compound. Experiments were performed in triplicate, and the mean ± SD is shown. The relative activity was obtained by normalizing the activity corresponding to each compound concentration tested to the activity of untreated cells. [c] Tabulated values for 5 μM inhibitor and 60 min incubation time. [d] ND: not determined. [e] Tabulated values for 50 μM inhibitor and 60 min incubation time.

support for the evaluation of these bicyclic derivatives as pharmacological chaperones for GD.

Activity of Bicyclic Isoureas and Guanidines on L444P and N370S GCase Gaucher Cells. The effects of bicyclic compounds **1c**−**5c** and **5e** in enantiomerically pure form on human GCase activity were further evaluated in human lymphoblasts derived from Gaucher patients homozygous for N370S[50] or L444P mutations at different concentrations.[51] The iminosugar NN-DNJ, which is a known chaperone for N370S GCase, but not L444P GCase, was used as a control.[13] The fold increase in enzyme activity and the pharmacological chaperone concentration which maximizes GCase activity after 3 days of incubation are summarized in Table 2. The results obtained with the compounds at other concentrations are given in the Supporting Information (Figures S12 and S13).

A good correlation was found between GCase inhibition in wild-type human fibroblast or recombinant GCase and concentration of maximal N370S or L444P GCase activity, with the exception of isourea (−)-**1c**. In the analysis of N370S GCase activation, the above isourea, which had a high affinity and enzyme thermal stabilization properties, only showed a 40% increase in enzyme activity at 1 μM. In contrast, the rest of compounds showed more than a 1.7-fold increase over a range of concentrations. Treatment with 10 μM of isoureas **2c** and **3c** caused 1.7- and 1.8-fold increase in the GCase activity of N370S cell line, respectively, whereas bicyclic compounds **4c**, **5c**, and **5e** maximally increased the activity between 1.7- and 2.1-fold at nanomolar concentrations (between 50 nM and 200 nM). Using similar conditions, NN-DNJ gave a maximal enhancement of 1.4-fold at 5 μM. It is worth mentioning that the enantiomerically pure compounds **4c**, **5c**, and **5e** led to an approximate 40% increase in N370S GCase activity at a very low concentration of 10 nM (see Figure 6A).

An additional 5 day time-course analysis of chaperone activities was carried out at 0.1 μM for enantiomerically pure compounds **4c**, **5c**, and **5e** and the iminosugar NN-DNJ. N370S lymphoblasts were cultured in the presence of compounds for 5 days, and GCase activity was measured every day in lysed cells, as described in the Supporting Information. As shown in Figure 6B, the GCase activity increased in a time-dependent manner in the presence of compounds, reached a peak on days 3−4, then decreased slightly.

The effects of enantiomerically pure compounds **1c**−**5c** and **5e** on L444P GCase activity were measured using GD type 2 patient-derived lymphoblasts cell line after incubation with above compounds for 3 days. Compound **1c** only slightly increased (10% at 1 μM) GCase activity, which probably is not significant, and therefore, it can be concluded that this compound is not promising as a pharmacological chaperone for this mutation. Under similar conditions, NN-DNJ was found to be completely inactive as reported previously in the L444P enzyme.[13] However, **2c**−**5c** and **5e** showed a moderate increase in GCase activity (1.3- to 1.5-fold) at low concentrations, as shown in Table 2. Treatment with 1 μM of isourea **2c** or 0.1 μM of **3c** caused 1.3- and 1.4-fold increase in the GCase activity of L444P cell line, respectively. Furthermore, isoureas **4c**−**5c** and guanidine **5e** maximally increased the activity between 40 and 50% at 10 nM, in agreement with the high affinity for GCase enzyme in both isolated protein and cellular assays.

DISCUSSION

Pharmacological chaperones for the treatment of lysosomal diseases and singularly for GD have been an object of intense interest[12] as a new therapy for these disorders.[52] The bicyclic isoureas and guanidines obtained have found to be potent inhibitors of GCase in vitro. Moreover, these compounds maintain a high cellular inhibitory activity at concentrations similar to those found effective in isolated enzyme. This would indicate a good cellular penetration and stability of the compounds. One of the possible problems associated to the chaperone therapy is how to manage the opposing role of a molecule acting both as inhibitor and activator for an enzyme. This is usually faced by administration of the compound at subinhibitory concentrations, a question that could be relatively simple in cultured cells, but it is not evident in vivo. In any case, the use of low concentrations of chaperones is highly desirable due to the increase in its selectivity for GCase and expected lower side effects. Therefore the compounds here described, with activity on the nanomolar range

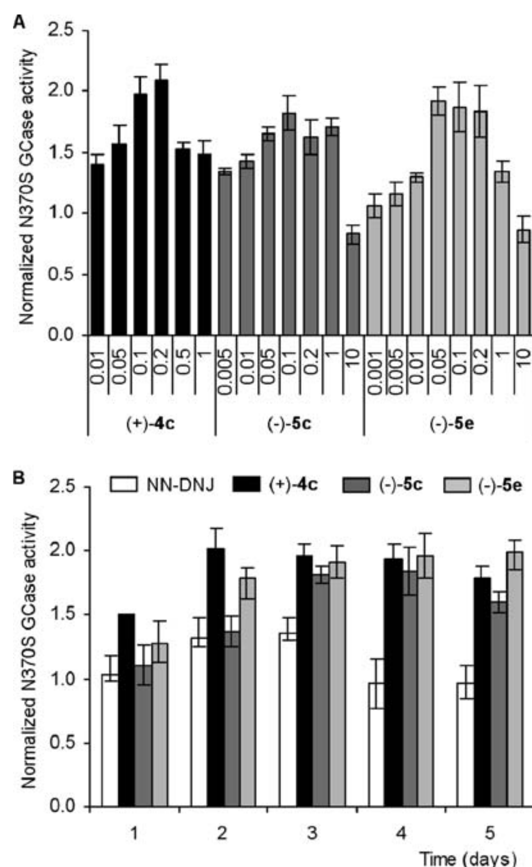


Figure 6. The effects of compounds (+)-**4c**, (−)-**5c**, and (−)-**5e** on GCase activity in mutant N370S lymphoblasts. Experiments were performed in triplicate, and each bar represents the mean ± SD. Enzyme activity is normalized to untreated cells, assigned a relative activity of 1. (A) Cells were cultured for 3 days in the absence or presence of increasing concentrations (μM) of compounds before GCase activity was measured. (B) Lymphoblasts were cultured for 5 days with NN-DNJ, (+)-**4c**, (−)-**5c**, and (−)-**5e** at 0.1 μM and cells were analyzed each day for enzyme activity.

in cultured cells are among the best candidates for pharmacological chaperones for GD.

The assay of the bicyclic isoureas and guanidines on L444P GCase, which characterizes a more severe disease phenotype than N370S, results in a moderate increase of enzymatic activity (1.3- to 1.5-fold) at nanomolar to micromolar concentrations. The effect of the compounds on L444P cell assays is a second remarkable feature, since this mutation is highly reluctant to chaperone enhancement. Several reports describe the use of pharmacological chaperones for different GCase mutations that, however, failed in L444P cell assays.[13,16,17,21,47,53] This lack of activity has been attributed to the distant location of the mutation site from the enzyme catalytic center. To our knowledge, only α-1-*C*-octyl-1-deoxynojirimycin,[14] isofagomine,[19] and ambroxol[48] have been reported to increase activity in L444P Gaucher cells. However, these compounds must be used at relatively high concentrations to significantly increase GCase activity. In a recent study on the use of the isofagomine as a pharmacological chaperone for Gaucher L444P mutation,[19] it has been reported the failure of obtaining clinically meaningful improvements. The assay included tests on animal models and a phase 2 randomized, open-label clinical study to assess the safety, tolerability, and efficacy of isofagomine in adult patients with type 1 GD. The lack of efficacy could be related to the low activity of isofagomine in intact cell GCase, which requires 3 orders of magnitude higher concentrations of compound to increase the GCase activity in cells compared with the concentration needed to inhibit GCase in vitro.[18] This would reflect a low membrane permeability, a limited ER access, or a poor cellular stability of isofagomine. In the case of the active bicyclic isoureas and guanidines, we have found that nanomolar concentrations of compound produce enzyme activity enhancements on L444P and N370S mutations. It is clear that, in addition to their high enzyme affinities, these compounds reflect an advantageous cell permeability, distribution, and metabolism as required for pharmacological chaperones.

Finally, a comment is warranted on the exact mechanism of these compounds and other compounds designed as pharmacological chaperones. With the data currently available for our compounds and most of the literature reports, which are based on the enzyme activity enhancement measurements, the use of the chaperone term is probably inexact, and enzyme activator or enhancer would be more accurate. Rigorously, a pharmacological chaperone would bind to the mutant protein assisting to its folding process and/or stabilize it against denaturation and degradation in the cellular environment. This can promote the correct subcellular localization of the mutant enzyme increasing its cellular activity and preventing the accumulation of substrate. These subjects have not been addressed in this work. Another point of interest questions whether the activity enhancement due to compound treatment is high enough for clinical improvement or is too low to prevent disease progression. Although this cannot be fully answered now, there is an interesting study in the literature that demonstrates in a model of GD that there is a relatively low threshold activity—about 11−15% of the wild-type enzyme—that leads to substrate accumulation and hence to the conditions for disease development.[54] Therefore, it seems that even relatively small relative increases in GCase activity can be important in Gaucher treatment specially for those variants insensitive to current therapies, such as L444P. Research addressed to answer these questions is beyond this study and will be subject of future work.

CONCLUSIONS

Several diastereomeric bicyclic isoureas (**1**−**4**) and guanidine analogues **5** have been synthesized as potential GCase pharmacological chaperones. The final compounds have been tested as GCase inhibitors using isolated enzyme (imiglucerase) and wild-type human fibroblasts. Among them, compounds **4b**,**c**, **5c**, and **5e** were found to be potent inhibitors of recombinant GCase with K_i values between 2 and 10 nM. In spite of the good correlation between lipophilicity of the *N*-alkyl chain derivatives in each series and GCase inhibition, the configurations of the oxygen and nitrogen atoms of the fused heterocycle are of utmost importance for bicyclic inhibitors. In contrast, isoureas bearing an extra alkyl chain at endocyclic nitrogen (**1d**−**4d**) were markedly less potent inhibitors. The most active compounds were found to stabilize recombinant GCase under denaturing conditions and inhibit GCase enzyme in human fibroblasts at nanomolar concentrations. Furthermore, the effects of *N*-nonyl derivatives **1c**−**5c** and guanidine **5e** on human GCase activities were evaluated in lymphoblasts derived from Gaucher patients for N370S or L444P mutations. Among them, compounds **4c**, **5c**, and **5e** are able to increase the activity of N370S GCase

between 1.8- and 2-fold at 100 nM. Moreover, the above compounds maximally increase the GCase activity of L444P cell line by about 40% at 10 nM. Further optimization and study of these compounds on other mutations and in animal models to lead to clinical candidates is of relevance. In addition, the structural details of the binding of these inhibitors are also of evident interest. Work along these lines is currently in progress.

EXPERIMENTAL SECTION

For general methods, see the Supporting Information.

General Procedure for the Preparation of Thioureas: Synthesis of (+)-18c as a Representative Example. To a solution of the amino alcohol (−)-**12** (111 mg, 0.21 mmol) in THF (6 mL) was added 87 μL of nonyl isothiocyanate (0.42 mmol). The resulting mixture was stirred at 45 °C for 18 h, and the solvent was removed under reduced pressure to give a yellow oil, which was purified by column chromatography using a mixture of hexane/EtOAc (4:1) to afford 141 mg (0.19 mmol, 95%) of (+)-**18c**. $[\alpha]_D^{25}$ + 18.6 (*c* 0.6, $CHCl_3$); IR (film): υ = 3305, 3088, 3063, 3030, 2926, 2854, 1549, 1097, 1070, 696 cm^{-1}; ^1H NMR (δ, 500 MHz, $CDCl_3$): 0.88 (t, 3H, *J* = 7.2 Hz), 1.10−1.35 (m, 14H), 2.86−3.18 (m, 2H), 3.30−3.45 (m, 1H), 3.47−3.55 (m, 1H), 3.58 (t, 1H, *J* = 8.7 Hz), 3.89 (t, 1H, *J* = 9.8 Hz), 3.94 (t, 1H, *J* = 9.4 Hz), 4.18−4.28 (m, 1H), 4.62−5.00 (m, 8H), 6.40−6.90 (br s, 1H), 7.26−7.43 (m, 20H); ^{13}C NMR (δ, 100 MHz, $CDCl_3$): 14.2, 22.8, 27.0, 28.7, 29.3, 29.5, 31.9, 45.6, 56.9, 68.7, 73.0, 75.9, 76.0, 80.4, 81.1, 83.7, 127.7−128.7, 137.6, 138.4, 138.6, 182.8. HRMS calcd for $C_{44}H_{57}N_2O_5S$: 725.3988 [M + H]$^+$. Found: 725.4008.

General Procedure for the Preparation of Bicyclic Isoureas and Guanidines: Synthesis of (−)-22c as a Representative Example. Yellow mercury oxide (II) (117 mg, 0.54 mmol) was added in one portion to a solution of the thiourea (+)-**18c** (130 mg, 0.18 mmol) in toluene (15 mL). The reaction mixture was stirred at 115 °C for 24 h under argon atmosphere. After cooling, the mixture was filtered through Celite, and the plug of Celite was washed several times with CH_2Cl_2. The filtrate and washings were combined, evaporated, and purified by flash chromatography (2:1 to 1:2 hexane/EtOAc gradient) to afford 112 mg (0.16 mmol, 89%) of (−)-**22c**. $[\alpha]_D^{25}$ − 2.4 (*c* 0.8, $CHCl_3$); IR (film): υ = 3330, 3086, 3063, 3030, 2926, 2854, 1718, 1671, 1454, 1092, 1069, 1028, 696 cm^{-1}; ^1H NMR (δ, 500 MHz, $CDCl_3$): 0.89 (t, 3H, *J* = 7.0 Hz, CH_3), 1.20−1.34 (m, 12H, 6xCH_2), 1.41−1.51 (m, 2H, CH_2), 3.14 (t, 2H, *J* = 7.2 Hz, NCH_2), 3.73 (t, 1H, *J* = 5.2 Hz, H-5), 3.87 (t, 1H, *J* = 5.0 Hz, H-4), 3.89 (dd, 1H, *J* = 4.9, 8.2 Hz, H-6), 4.01 (dd, 1H, *J* = 3.4, 8.2 Hz, H-7), 4.22 (dd, 1H, *J* = 4.6, 8.7 Hz, H-3a), 4.52−4.82 (m, 9H, H-7a, 4x$PhCH_2O$), 7.25−7.41 (m, 20H, Ph); ^{13}C NMR (δ, 100 MHz, $CDCl_3$): 14.2 (CH_3), 22.8 (CH_2), 26.9 (CH_2), 29.37 (CH_2), 29.40 (CH_2), 29.6 (CH_2), 29.8 (CH_2), 32.0 (CH_2), 43.1 (NCH_2), 67.3 (C3a), 72.7 ($PhCH_2O$), 72.8 ($PhCH_2O$), 73.2 ($PhCH_2O$), 73.7 ($PhCH_2O$), 77.1 (C7), 78.7 (C7a), 79.7 (C4), 81.0 (C6), 82.5 (C5), 127.6−128.5 (CHar), 138.3 (Car), 138.57 (Car), 138.59 (Car), 160.8 (C2). HRMS calcd for $C_{44}H_{55}N_2O_5$: 691.4111 [M + H]$^+$. Found: 691.4140.

General Procedure for *O*-Debenzylation with Boron Trichloride: Synthesis of (+)-28c and (+)-4c as a Representative Example. A solution of the starting benzylated bicyclic isourea (−)-**22c** (112 mg, 0.16 mmol) in anhydrous CH_2Cl_2 (10 mL) at −78 °C was treated with 1 M BCl_3 solution in heptane (1.6 mL, 1.6 mmol, 2.5 equiv for *O*-benzyl group). The reaction was stirred 2 h at −78 °C and 5 h at −40 °C. The mixture was then cooled to −78 °C and quenched with methanol (2 mL), and the solvents were removed under reduced pressure. Purification by flash chromatography (20:1 to 10:1 CH_2Cl_2/MeOH gradient) provided (+)-**28c** (10 mg, 0.02 mmol, 14%), followed by (+)-**4c** (36 mg, 0.11 mmol, 68%).

Data for (+)-**28c**: white solid; $[\alpha]_D^{25}$ + 10.7 (*c* 0.9, MeOH); ^1H NMR (δ, 500 MHz, CD_3OD): 0.90 (t, 3H, *J* = 7.0 Hz, CH_3), 1.20−1.39 (m, 12H, 6xCH_2), 1.54−1.63 (m, 2H, CH_2), 3.22 (t, 2H, *J* = 7.0 Hz, NCH_2), 3.61−3.67 (m, 3H, H-4, H-5, H-6), 3.98 (dd, 1H, *J* = 3.6, 7.1 Hz, H-7), 4.14−4.20 (m, 1H, H-3a), 4.74 (d, 1H, *J* = 11.4 Hz, $PhCH_2O$), 4.85 (d, 1H, *J* = 11.4 Hz, $PhCH_2O$), 4.95−5.05 (m, 1H, H-7a), 7.26−7.43 (m, 5H, Ph); ^{13}C NMR (δ, 100 MHz, CD_3OD): 14.4 (CH_3), 23.7 (CH_2), 27.7 (CH_2), 30.2 (CH_2), 30.39 (CH_2), 30.42 (CH_2), 30.6 (CH_2), 33.0 (CH_2), 43.8 (NCH_2), 62.0 (C3a), 71.0 (C7), 74.3 (C6), 75.4 (C5), 76.5 ($PhCH_2O$), 82.5 (C4), 84.3 (C7a), 128.7 (CHar), 129.1 (2xCHar), 129.3 (2xCHar), 139.7 (Car), 163.4 (C2). HRMS calcd for $C_{23}H_{37}N_2O_5$: 421.2702 [M + H]$^+$. Found: 421.2704.

Data for (+)-**4c**: white solid; $[\alpha]_D^{25}$ + 16.2 (*c* 0.9, MeOH); ^1H NMR (δ, 500 MHz, CD_3OD): 0.90 (t, 3H, *J* = 6.9 Hz, CH_3), 1.25−1.42 (m, 12H, 6xCH_2), 1.57−1.65 (m, 2H, CH_2), 3.25−3.30 (m, 2H, NCH_2), 3.34−3.38 (m, 1H, H-5), 3.66 (t, 1H, *J* = 6.0 Hz, H-6), 3.81 (t, 1H, *J* = 8.3 Hz, H-4), 3.97−4.04 (m, 1H, H-7), 4.09−4.14 (m, 1H, H-3a), 5.13−5.24 (m, 1H, H-7a); ^{13}C NMR (δ, 100 MHz, CD_3OD): 14.4 (CH_3), 23.7 (CH_2), 27.6 (CH_2), 30.3 (CH_2), 30.4 (CH_2), 30.6 (CH_2), 33.0 (CH_2), 44.0 (NCH_2), 61.4 (C3a), 71.4 (C7), 74.5 (C6), 75.8 (C5), 76.5 (C4), 84.5 (C7a), 163.2 (C2). HRMS calcd for $C_{16}H_{31}N_2O_5$: 331.2233 [M + H]$^+$. Found: 331.2235.

ASSOCIATED CONTENT

Supporting Information. Complete refs 6, 16a, and 19 general experimental protocols, structural features, and compound characterization data for all new compounds as well as experimental procedure for glycosidase inhibition assays, GCS inhibition in cell homogenates, inhibition of GCase (imiglucerase and wt human fibroblasts), cytotoxicity, stabilization ratios after thermal denaturation of recombinant GCase (imiglucerase), and the effects of compounds on L444P or N370S GCase activity in GD lymphoblasts. This material is available free of charge via the Internet at http://pubs.acs.org.

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