Journal of Medicinal Chemistry

Article

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Conformationally-Locked *N*-Glycosides with Selective β-Glucosidase Inhibitory Activity: Identification of a New Non-Iminosugar-Type Pharmacological Chaperone for Gaucher Disease

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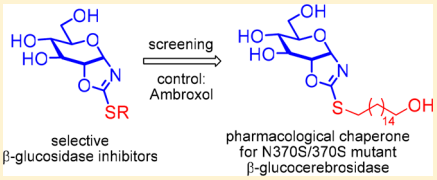
Supporting Information

ABSTRACT: A series of conformationally locked *N*-glycosides having a cis-1,2-fused pyranose−1,3-oxazoline-2-thione structure and bearing different substituents at the exocyclic sulfur has been prepared. The polyhydroxylated bicyclic system was built in only three steps by treatment of the corresponding readily available 1,2-anhydrosugar with KSCN using $TiO(TFA)_2$ as catalyst, followed by S-alkylation and acetyl deprotection. In vitro screening against several glycosidase enzymes showed highly specific inhibition of mammalian β-glucosidase with a marked dependence of the potency upon the nature of the exocyclic substituent. The most potent representative, bearing an *S*-(ω-hydroxyhexadecyl) substituent, was further assayed as inhibitor of the human lysosomal β-glucocerebrosidase and as pharmacological chaperone in Gaucher disease fibroblasts. Activity enhancements in N370S/N370S mutants analogous to those achieved with the reference compound ambroxol were attained with a more favorable chaperone/inhibitor balance.



INTRODUCTION

Given the broad range of biological and pathological processes in which glycosidases are involved, from the catabolism of sugars to the biosynthesis of the complex oligosaccharide chains in glycoproteins and glycolipids, specific inhibitors of these enzymes bear strong potential for the development of new pharmaceuticals. Examples include the treatment of viral infections,[1,2] such as human immunodeficiency virus (HIV), human hepatitis C (HCV) or dengue virus, cancer,[3−5] diabetes,[6−8] tuberculosis,[9,10] and lysosomal storage diseases (LSDs),[11−16] which has strongly stimulated research in this area of glycobiology.[17,18] With few exceptions,[19,20] the glycosidase inhibitors under study as drug candidates mimic the glycone moiety of the putative substrate, which is shared within a series of isoenzymes and enzymes acting on anomeric substrates. For instance, the piperidine-type iminosugar 1-deoxynojirimycin (DNJ) can be regarded as a stereochemical mimic of D-glucose, which is consistent with its behavior as a potent inhibitor of mammalian and plant α- and β-glucosidases.[21] The indolizidine-type iminosugar (+)-castanospermine (CS), with an identical hydroxylation profile at the six-membered ring, exhibits higher enzyme specificity compared with DNJ, which is ascribed to the conformational restriction imposed by the rigid bicyclic structure.[22] In any case both DNJ and CS can simultaneously inhibit several α- as well as β-glucosidases in humans, which is a serious drawback for clinical application (Figure 1).[23]

In connection with the design of more fine-tuned inhibitors, numerous syntheses of DNJ and CS analogues have been reported.[24−34] Incorporation of alkyl substituents at the nitrogen atom or at its vicinity in monocyclic iminosugar frameworks, e.g., as in *N*-(*n*-nonyl)-1-deoxynojirimycin (NNDNJ)[35,36] or α-1-C-nonyl-1,5-dideoxy-1,5-iminoxylitol (α-1-C-nonyl-DIX),[37] has been shown to improve the affinity toward certain glycosidases (Figure 1). Further chemical and

Received: May 3, 2012
Published: July 4, 2012

   dx.doi.org/10.1021/jm3006178 | *J. Med. Chem.* 2012, 55, 6857−6865

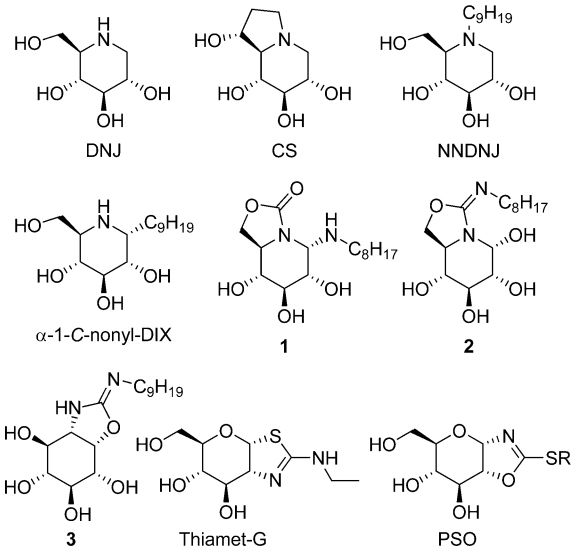


Figure 1. Structures of representative iminosugar (DNJ, CS, NNDNJ, α-1-C-nonyl-DIX), sp^2-iminosugar (**1** and **2**), and bicyclic non-iminosugar (**3** and thiamet G) glycosidase inhibitors and general structures of the pyranose−sulfanyloxazoline (PSO) glycomimetics reported in this work.

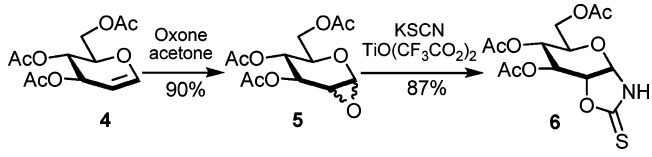
structural evidence indicated that once the azaheterocycle ring occupies the glycone site of the enzyme, the substituent can favorably interact with other regions of the protein.[38−40] This result strongly suggests that exploiting non-glycone interactions has the potential to become a general strategy to elaborate selective glycosidase inhibitors. Implementing molecular diversity-oriented strategies in conformationally locked bicyclic glycomimetics is particularly attractive toward these channels. Several polyhydroxylated bicyclic cores armed with anchoring functionalities compatible with library generation schemes have been proposed, among which the so-called sp^2 iminosugars have proven particularly useful (Figure 1).[41−47] This family of sugar mimics incorporates a pseudoamide group in the structure that facilitates the installation of substituents either at the pseudoanomeric position (e.g., in the carbamate-type bicyclic nojirimycin derivative **1**)[48,49] or at an exocyclic nitrogen (e.g., in the isourea-type bicyclic nojirimycin derivative **2**).[50,51] Total discrimination between α- and β-glucosidase enzymes and even between closely related α-glucosidase isoenzymes could be achieved in this manner, which translated into interesting lead compounds in view of developing drug candidates for the treatment of breast cancer[49] and Gaucher disease, the LSD with the highest prevalence.[52,53] In the first case, the biological activity was ascribed to selective inhibition of the neutral α-glucosidases at the endoplasmic reticulum (ER). In the second case, binding of the glycomimetic to the active site of mutant β-glucocerebrosidase (GCase) in the ER restored trafficking to the lysosome, acting as pharmacological chaperone. Similarly, N′-alkylated bicyclic isoureas derived from aminocyclitol scaffolds (e.g., **3**) were also shown to behave as very selective GCase inhibitors with strong pharmacological chaperone potential (Figure 1).[54]

The above examples illustrate the suitability of six-membered−five-membered bicyclic glycomimetics to achieve strong and selective glycosidase inhibition after incorporation of substituents susceptible to participating in non-glycone interactions. They also demonstrate that the presence of a basic amine-type nitrogen in the six-membered ring is not a prerequisite for strong enzyme affinity. Actually, protonation under physiological conditions is probably responsible for the broad range rather than selective activity of iminosugars.[55,56] In principle, the pyranose ring present in the natural glycoside substrate could be directly incorporated in the molecular design of bicyclic competitive inhibitors toward a target glycosidase to account for the glycone specificity, which would greatly simplify the synthetic scheme. Thus, thiamet G (Figure 1), a fused pyranose−thiazoline derivative recently developed by Vocadlo and co-workers,[57] behaves as a potent inhibitor of O-linked 2-acetamino-2-deoxy-β-D-glucopyranoside hydrolysis, being investigated as a potential therapeutic target that could hinder progression of Alzheimer's disease.[58,59] To further test this hypothesis, we have now prepared a new family of glycomimetics having a cis-1,2-fused pyranose−2-alkylsulfanyl-1,3-oxazoline structure (PSO). PSO derivatives share with compound **1** the *N*-glycoside-type character (Figure 1). Actually, they can be formally considered as conformationally locked *N*-glycoside derivatives, which should warrant chemical and enzymatic stability and at the same time impart selectivity.[60] On the other hand, similar to compounds **2** and **3** bearing an imine type nitrogen, the PSO structure is also very well suited for the incorporation of a broad battery of substituents on the exocyclic heteroatom. Here we report the synthesis of the key synthetic precursor, the scope of the approach, the assessment of the affinity and selectivity of the final compounds against a panel of commercial glycosidases, and the evaluation of a selected candidate as pharmacological chaperone in human Gaucher disease fibroblasts.

RESULTS

Synthesis. The initial synthetic objective of this research was the preparation of *S*-alkyl cis-1,2-fused pyranose−(2-alkylsulfany-1,3-oxazoline) carbohydrate derivatives bearing different substituents at the exocyclic sulfur atom. A new methodology has been developed for the construction of the heteroatomic bicycle system that exploits the reactivity of sugar epoxides. The reaction sequence started with the conventional epoxidation of commercial tri-*O*-acetyl-D-glucal (**4**) to afford a mixture of the corresponding tri-*O*-acetyl-1,2-anhydrosugars (**5**, D-gluco/D-manno ratio of 7:1) in 90% yield.[61] Treatment of **5** with potassium thiocyanate and catalytic amounts of $TiO(CF_3CO_2)_2$ led to the key thionocarbamate **6** in 87% yield (Scheme 1).[62] The ^1H NMR coupling constants of the

Scheme 1. Synthesis of 3,4,6-Tri-*O*-acetyl-1,2-dideoxy-α-D-glucopyranoside[1,2-*d*]-1,3-oxazolidine-2-thione (6)

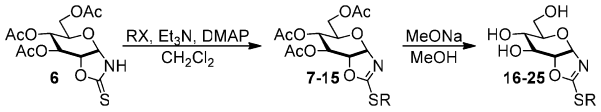


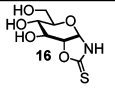
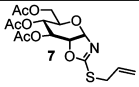
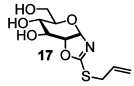
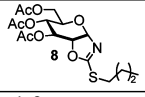
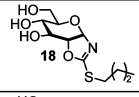
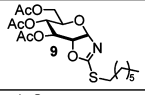
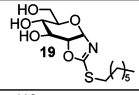
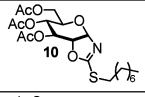
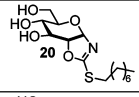
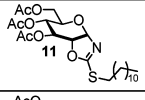
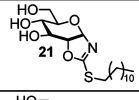
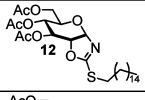
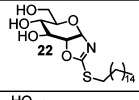
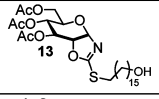
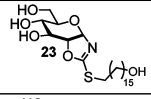
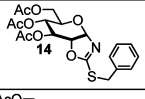
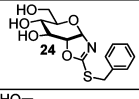
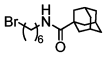
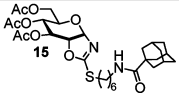
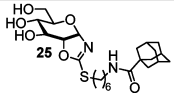
pyranose ring suggested a skew-boat conformation close to $^0S_2$, instead of the $^4C_1$ chair conformation typical of monocyclic D-glucosyl derivatives, which is in agreement with previously reported data for structurally related bicyclic cis-1,2-fused glucopyranose structures in solution.[63,64]

Thionocarbamates display ambident functionality, offering diverse reactivity.[65,66] The different properties of both the N and S electron-rich centers are explained by Pearson's hard−soft acid−base (HSAB) theory,[67] where the nitrogen atom acts

dx.doi.org/10.1021/jm3006178 | J. Med. Chem. 2012, 55, 6857−6865

Table 1. Synthesis of 2-*S*-Alkylsulfanyl-1,3-oxazoline D-glucopyranose Derivatives



		S-alkylation[a]			O-deprotection[b]		
E	Alkyl Bromide	Product	t (h)	yield (%)	Product	t (h)	yield (%)
1	-	-	-	-		0.5	97
2			3	91		0.5	98
3			3	69		0.5	90
4			3	82		0.5	89
5			4	77		3	96
6			6	90		3	97
7			24	90		20	92
8			24	86		20	89
9			3	75		0.5	98
10			24	78		5	99

[a]Carried out at rt with 1.0 equiv of substrate, 3.0 equiv of alkyl bromide, 3.0 equiv of Et_3N, and 2 mol % DMAP in CH_2Cl_2. [b]Carried out at rt with 1.0 equiv of of S-alkylated substrate and 5 mol % MeONa in MeOH.

Table 2. K_i Values (μM) against Bovine Liver (Cytosolic) β-Glucosidase[a] and Inhibition of Human GCase Relative to Control (%) for Compounds 16–25

enzyme	16	17	18	19	20	21	22	23	24	25
β-glucosidase (bovine liver)	NI[b]	220	580	310	280	73	52	12	210	230
GCase (*Homo sapiens*)	99	97	92	91	91	87	86	36	88	91

[a]Inhibition was competitive in all cases. No inhibition was observed for any of the compounds at 2 mM on almonds β-glucosidase, yeast α-glucosidase, Jack bean α-mannosidase, *Helix* pomatia β-mannosidase, pig kidney trehalase, *Aspergillus* niger amyloglucosidase, *Penicillium decumbens* naringinase, green coffee α-galactosidase, *E. coli* β-galactosidase, or yeast isomaltase. [b]NI: no inhibition observed at 2 mM.

as a hard basic center while the sulfur atom shows a soft base character. This reactivity has been extensively investigated by Rollin et al.[68−72] Reagents of R-X type are mostly considered as soft electrophilic species, providing high-yielding S-alkylation.[73−75]

Reaction of **6** with a series of different alkyl bromides in basic medium led to the expected 2-alkylsulfanyl-1,3-oxazoline derivatives **7**−**15** in 69−91% yield (Table 1). Structures of the S-alkylated compounds were ascertained by ^{13}C NMR spectroscopy by comparison with previously synthesized thionocarbamates.[68−72] The chemical shifts for the quaternary sp^2 carbon atom at position 2 of the five-membered heterocycle varied from roughly 190 ppm (−N−C═S in 1,3-oxazolidine-2-thione **6**) to approximately 170 ppm (−N═C−SR in 2-alkylsulfanyl-1,3-oxazoline- derivatives **7**−**15**). Final removal of the acetyl protecting groups using methanol under standard NaOMe-catalyzed conditions provided the requested PSO glucomimetics **16**−**25** in 89−99% yields (Table 1). Both the alkylation and the deprotection steps requested longer reaction times for the bulkier alkyl groups of the series (entries 6−8 and

dx.doi.org/10.1021/jm3006178 | J. Med. Chem. 2012, 55, 6857−6865

10, Table 1). The unprotected S-alkylated derivatives **17**−**25** maintain the skewed boat conformation already observed for their corresponding acetylated precursors **7**−**15** and the parent compound **6**.

Evaluation of the Glycosidase Inhibitory Activity and Chaperone Effect. All the new cis-1,2-fused D-glucopyranose−(2-alkylsulfanyl-1,3-oxazoline) derivatives were first screened as inhibitors against a panel of commercial glycosidases including α-glucosidase (yeast), β-glucosidase (almonds and bovine liver, cytosolic), α-mannosidase (Jack bean), β-mannosidase (*Helix pomatia*), trehalase (pig kidney), amyloglucosidase (*Aspergillus niger*), naringinase (β-glucosidase/α-L-rhamnosidase, *Penicillium decumbens*), α-galactosidase (green coffee beans), β-galactosidase (*E. coli*), and isomaltase (yeast). The corresponding inhibition constants (K_i) are collected in Table 2.

The S-unsubstituted PSO derivative **16** did not inhibit any of the assayed glycosidases at concentrations up to 2 mM. Interestingly, the S-substituted derivatives **17**−**25** behaved as specific, though modest, inhibitors of the bovine liver β-glucosidase among the 11 glycosidases tested. The capacity to discriminate between the mammalian and the plant β-glucosidases is particularly remarkable. Both the enzyme from almonds and the enzyme from bovine liver belong to the same glycosyl hydrolase family GH1 in the CAZy classification,[76] meaning that they bear considerable similarities within their active sites. The results support that subtle differences must exist in areas close to the active site and further substantiate that non-glycone interactions are better suited than glycone interactions to attain high levels of selectivity among isoenzymes.

A structure−activity relationship analysis within the PSO series **17**−**25** indicated a notable influence of the nature of the exocyclic substituent at the sulfur functionality on the inhibitory potency. For linear alkyl substituents, a progressive decrease of the corresponding K_i values, indicative of increased binding affinity, with the chain length was observed, going from 584 μM for the *n*-butyl derivative **18** to 52 μM for the *n*-hexadecyl derivative **22**, which is compatible with accommodation of the aliphatic chain into a hydrophobic pocket of the protein. No significant improvement was observed when aromatic (**24**) or adamantyl residues (**25**) were present. Noteworthy, installation of a terminal hydroxyl group at the hexadecyl chain (**23**) further improved binding by a factor of 4.3-fold (K_i = 12.1 μM), probably due to adventitious hydrogen-bonding interactions.

The molecular basis for the unprecedented specificity of the PSO family toward the mammalian β-glucosidase is still unknown. The presence of the glycosidic nitrogen atom anchored in the α-configuration might seem to mismatch the β-anomeric selectivity of this enzyme. However, recent X-ray evidence on human GCase-inhibitor complexes has shown that α-configured glycomimetics can be accommodated at the active site in a skew-boat conformation in which the pseudoanomeric substituent adopts a pseudoequatorial disposition.[77] PSO derivatives are preorganized in such a binding conformation, which could be the origin of the observed specificity. In any case, the results reported here represent a proof of concept of the utmost importance for implementing non-glycone interactions in the design of potent and specific glycosidase inhibitors.

Inhibition of bovine liver β-glucosidase is often used as a preliminary parameter to select candidates as pharmacological chaperones for mutant human β-glucocerebrosidase (GCase) associated with Gaucher disease. The significant inhibitory potential and total selectivity encountered for compound **23** against the commercial enzyme warranted further evaluation in this sense. Determination of the inhibition activity on human GCase, relative to control, for the whole set of compounds at 100 μM confirmed this point; only compound **23** within the PSO derivatives surpassed 50% inhibition at this concentration (Table 2). In further studies, ambroxol (ABX), a non-glycomimetic-type GCase inhibitor under investigation as a pharmacological chaperone for Gaucher disease,[78] was assayed in parallel. Inhibition studies in cell lysates from healthy fibroblasts (Figure 2) indicated that **23** inhibited lysosomal GCase slightly less potently compared to ABX (IC_{50} of 11.4 versus 4.1 μM). No inhibition of other lysosomal enzymes, such as α-glucosidase, α-galactosidase, β-galactosidase, and β-hexosaminidase, was observed, reproducing the selectivity pattern already found in commercial enzymes.

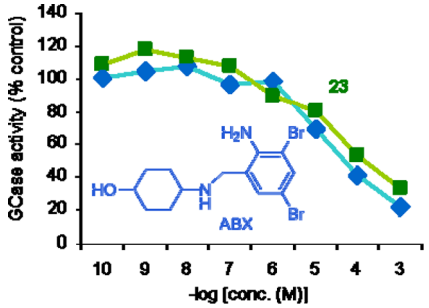


Figure 2. Effects of ambroxol (ABX) and PSO **23** on lysosomal enzyme activities in lysate from human normal fibroblasts. Enzyme activity in normal cell lysates was determined in the absence or presence of increasing concentrations of chaperones. Each point represents the mean of triplicate determinations obtained in a single experiment. Values were expressed relative to the activity in the absence of compounds (100%). 4-Methylumbelliferyl β-D-glucopyranoside was used as substrate.

Further comparative enzyme activity enhancement assays were conducted in healthy and Gaucher fibroblasts from patients having the N370S/N370S or the L444P/L444P mutations. The first one, the most common mutation among Gaucher patients, is located in the catalytic domain of the enzyme, while the second one is located in a noncatalytic domain. The cells were cultured for 5 days in the absence and in the presence of various concentrations of **23** or ABX, then lysed and the β-glucocerebrosidase activity determined using 4-methylumbelliferyl β-D-glucopyranoside as substrate. In normal cells, **23** had no effect on GCase activity, whereas ABX induced a statistically significant activity enhancement compared to the control at 10 and 30 μM. In N370S/N370S Gaucher fibroblasts both **23** and ABX significantly up-regulated the activity of the mutant enzyme. In the case of ABX a decrease in the relative activity increase occurs from 30 μM, however, meaning that the inhibitory activity overcomes the chaperone effect (Figure 3). In contrast, the mutant enzyme activity enhancement induced by PSO **23** steadily increased in a dose dependent manner in the range 30−90 μM, indicating a more favorable chaperone/inhibitor balance. Neither **23** nor ABX was effective at increasing the activity in the case of the L444P/L444P mutant GCase, nor did they exhibit toxic effect on any of the normal or mutant cell lines assayed for 5 days of incubation.

dx.doi.org/10.1021/jm3006178 | J. Med. Chem. 2012, 55, 6857−6865

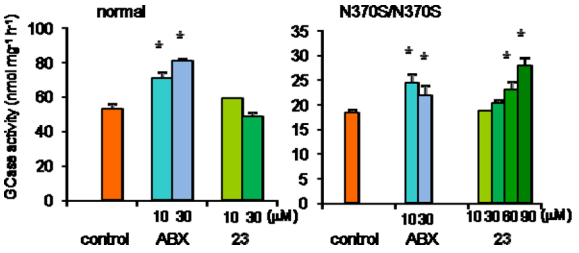


Figure 3. Effect of ABX and PSO **23** in GCase activity in normal and N370S/N370S fibroblasts. Fibroblasts from patients were cultured in the absence or presence of the indicated concentrations of the chaperone for 96 h, and the GCase activities in lysates were measured using 4-methylumbelliferyl β-D-glucopyranoside as substrate. Each bar represents the mean ± SEM of three determinations each done in triplicate. The asterisks indicate highly significant statistical difference ($p < 0.01$) from the values in the absence of the compound (t test).

Altogether, the above data support that compound **23**, similar to the reference compound ABX, behaves as an active site-directed pharmacological chaperone, although the observed activity increase (1.8-fold) does not surpass the values reported for the most effective iminosugar-type chaperones. For instance, NNDNJ elicits up to 2.3-fold activity enhancement in the N370S mutant and α-1-C-nonyl-DIX, developed by Compain and co-workers, already reaches 1.8-fold increase at 10 nM.[37] They also provide a proof of concept that non-glycone interactions can be advantageously exploited to endow a rigid pyranoid glycone moiety with high binding affinity and selectivity toward a given glycosidase. Most importantly, the synthetic methodology developed is very well adapted to molecular-diversity-oriented strategies and compatible with lead identification and optimization of pharmacological chaperones for LSDs.

EXPERIMENTAL SECTION

General Methods. All chemicals were reagent grade and used as supplied unless otherwise specified. $TiO(CF_3COO)_2$ catalyst was prepared following a previously reported procedure.[79] ^1H and ^{13}C NMR spectra were recorded on a Varian Mercury VX 400 (400 and 100.6 MHz, respectively) and Varian 400-MR spectrometer in $CDCl_3$ or CD_3OD as solvent, with the solvent resonance (δ) as the internal standard ($CDCl_3$ δ 7.26 ppm for ^1H, δ 77.23 ppm for ^{13}C; CD_3OD δ 3.31 ppm for ^1H, δ 49.14 ppm for ^{13}C) or using Me_4Si as an internal reference (δ 0.00 ppm for ^1H and ^{13}C). The 2D correlation spectra (gCOSY, NOESY, gHSQC, gHMBC) were visualized using the VNMR program (Varian). ESI-MS was run on an Agilent 1100 series LC/MSD instrument. Melting points (Mp) were measured on a Griffin melting point apparatus and are uncorrected. Optical rotations were measured at 598 nm at room temperature in a Perkin-Elmer 241 MC apparatus with 10 cm cells. IR spectra were recorded on a JASCO FT/IR-600 plus Fourier transform infrared spectrometer ATR Specac Golden Gate in the Servei de Recursos Científics (SRCiT-URV). Reactions were monitored by TLC carried out on 0.25 mm E. Merck silica gel 60 F254 glass or aluminum plates. The plates were visualized under a short-wave UV lamp (250 nm) or after dipping in a suitable developing solution. Flash column chromatography was carried out using forced flow of the indicated solvent on Fluka or Merck silica gel 60 (230−400 mesh). Radial chromatography was performed on 1 or 2 mm plates of Kieselgel 60 PF254 silica gel, depending on the amount of product. Compounds **5**[61] (as a mixture of the D-gluco and D-manno epimers), **6**,[62] and **16**[62] were synthesized as previously reported. Purity of all compounds was confirmed to be >95% by LC/MS and ^1H NMR.

General Procedure for the S-Alkylation of Cis-1,2-Fused D-Glucopyranose−1,3-Oxazolidine-2-thione Derivatives. To a solution of **6** (1.00 mmol) in CH_2Cl_2 (3.5 mL) were added the appropriate alkyl bromide (3.00 mmol), Et_3N (3.00 mmol), and DMAP (0.2 mmol) followed by stirring at room temperature. After completion of the reaction, the mixture was washed with saturated $NaHCO_3$ and brine, dried, and concentrated. Chromatographic purification afforded the S-alkylated compounds in the yields shown in Table 1 (see Supporting Information for details).

General Procedure for Acetyl Deprotection of Cis-1,2-Fused D-Glucopyranose−2-Alkylsulfanyl-1,3-oxazoline Derivatives. Sodium methoxide (0.05 mmol) was added to a solution of protected 1,3-oxazoline carbohydrate (1.00 mmol) in methanol (20 mL), followed by stirring at room temperature. Upon completion of the reaction, the solvent was removed in vacuo, and the crude product was purified by flash chromatography on silica gel to afford the deprotected compounds in the yields shown in Table 1.

1,2-Dideoxy-α-D-glucopyranoside[1,2-*d*]allylsulfanyl-1,3-oxazoline (17). The title compound was prepared following the general procedure for acetyl deprotection of cis-1,2-fused 1,3-oxazoline carbohydrate derivatives starting from **7** (100 mg, 0.26 mmol), MeONa (8 mg, 14 μmol), and MeOH (5.2 mL). The reaction mixture was stirred at room temperature for 30 min. After standard workup, the crude was purified by flash chromatography (1:9 $MeOH/CH_2Cl_2$) to afford the desired compound (66.0 mg, 98% yield) as colorless syrup. R_f (1:9 $MeOH/CH_2Cl_2$): 0.45. $[\alpha]_D$ +18 (c 1.0, MeOH). FT-IR (neat, cm^{-1}): 3361, 1703, 1638, 1582, 1477, 1362, 1296, 1229, 1114, 1046, 992, 949. ^1H NMR (400 MHz, CD_3OD) δ in ppm: 5.90 (ddt, 1H, J = 17.2, 10.4 Hz, J = 6.8 Hz), 5.78 (d, 1H, J = 7.2 Hz), 5.28 (dd, 1H, J = 17.2, 0.8 Hz), 5.19 (dd, 1H, J = 10.4, 0.8 Hz), 4.48 (dd, 1H, J = 7.2, 5.2 Hz), 3.74 (dd, 1H, J = 12.0, 2.8 Hz), 3.69 (dd, 1H, J = 12.0, 5.2 Hz), 5.20 (t, 1H, J = 6.8 Hz), 3.46 (dd, 1H, J = 8.8, 6.8 Hz), 3.51−3.30 (m, 3H). ^{13}C NMR (100.6 MHz, CD_3OD) δ in ppm: 171.8, 134.5, 119.6, 94.4, 84.4, 75.9, 75.6, 69.9, 63.3, 35.5. +TOF MS calcd for $C_{10}H_{15}NO_5S$ m/z $[M - H]^+$: 262.0749. Found: 262.0737.

1,2-Dideoxy-α-D-glucopyranoside[1,2-*d*]butylsulfanyl-1,3-oxazoline (18). The title compound was prepared following the general procedure for acetyl deprotection of cis-1,2-fused 1,3-oxazoline carbohydrate derivatives starting from **8** (50.1 mg, 0.12 mmol), MeONa (3.8 mg, 6 μmol), and MeOH (2.5 mL). The reaction mixture was stirred at room temperature for 30 min. After standard workup, the crude was purified by flash chromatography (1:9 $MeOH/CH_2Cl_2$) to afford the desired compound (31.0 mg, 90% yield) as colorless syrup. R_f (1:9 $MeOH/CH_2Cl_2$): 0.16. $[\alpha]_D$ + 92 (c 2.5, MeOH). FT-IR (neat, cm^{-1}): 3342, 2930, 2872, 1579, 1455, 1294, 1144, 1114, 952. ^1H NMR (400 MHz, CD_3OD) δ in ppm: 5.77 (d, 1H, J = 7.2 Hz), 4.47 (dd, 1H, J = 7.2, 5.2 Hz), 3.78 (dd, 1H, J = 12.0, 2.8 Hz), 3.72 (dd, 1H, J = 12.0, 5.2 Hz), 3.68 (dd, 1H, J = 6.8, 5.2 Hz), 3.47 (dd, 1H, J = 9.2, 6.8 Hz), 3.34−3.29 (m, 1H), 3.03 (t, 2H, J = 7.2 Hz), 1.71−1.65 (m, 2H), 1.47−1.41 (m, 2H), 0.92 (t, 3H, J = 7.2 Hz). ^{13}C NMR (100.6 MHz, CD_3OD) δ in ppm: 170.8, 92.4, 82.3, 74.0, 73.7, 68.0, 61.4, 31.4, 30.7, 21.3; 12.5. +TOF MS calcd for $C_{11}H_{19}NO_5S$ m/z $[M - H]^+$: 278.1062. Found: 278.1054.

1,2-Dideoxy-α-D-glucopyranoside[1,2-*d*]heptylsulfanyl-1,3-oxazoline (19). The title compound was prepared following the general procedure for acetyl deprotection of cis-1,2-fused 1,3-oxazoline carbohydrate derivatives starting from **9** (51.6 mg, 0.12 mmol), MeONa (3.8 mg, 6 μmol), and MeOH (2.5 mL). The reaction mixture was stirred at room temperature for 30 min. After standard workup, the crude was purified by flash chromatography (1:9 $MeOH/CH_2Cl_2$) to afford the desired compound (33.0 mg, 89% yield) as a white solid. R_f (0.5:9.5 $MeOH/CH_2Cl_2$): 0.09. Mp: 48−50 °C. $[\alpha]_D$ +80.2 (c 5.5, MeOH). FT-IR (neat, cm^{-1}): 3372, 2925, 2856, 1582, 1456, 1295, 1149, 1117, 954. ^1H NMR (400 MHz, CD_3OD) δ in ppm: 5.75 (d, 1H, J = 7.2 Hz), 4.44 (dd, 1H, J = 7.2, 5.2 Hz), 3.75 (dd, 1H, J = 12.0, 2.8 Hz), 3.66 (dd, 1H, J = 12.0, 5.2 Hz), 3.66 (dd, 1H, J = 6.8, 5.2 Hz), 3.44 (dd, 1H, J = 9.2, 6.8 Hz), 3.32−3.27 (m, 1H), 3.0 (t, 2H, J = 7.2 Hz), 1.72−1.65 (m, 2H), 1.40−1.25 (m, 8H), 0.87 (t, 3H, J = 7.2 Hz). ^{13}C NMR (100.6 MHz, CD_3OD) δ in ppm: 172.7, 94.2, 84.2; 75.9, 75.6, 69.9, 63.4, 33.4, 32.9, 31.2, 30.4, 30.1, 24.1, 14.9. +TOF MS calcd for $C_{14}H_{25}NO_5S$ m/z $[M - H]^+$: 320.1532. Found: 320.1519.

1,2-Dideoxy-α-D-glucopyranoside[1,2-*d*]octylsulfanyl-1,3-oxazoline (20). The title compound was prepared following the

dx.doi.org/10.1021/jm3006178 | J. Med. Chem. 2012, 55, 6857−6865

general procedure for acetyl deprotection of cis-1,2-fused 1,3-oxazoline carbohydrate derivatives starting from **10** (73.2 mg, 0.16 mmol), MeONa (5.1 mg, 8 μmol), and MeOH (3.3 mL). The reaction mixture was stirred at room temperature for 30 min. After standard workup, the crude was purified by flash chromatography (1:9 MeOH/CH_2Cl_2) to afford the desired compound (51.0 mg, 96% yield) as a white solid. R_f (5:95 MeOH/CH_2Cl_2): 0.16. Mp: 56−58 °C. $[\alpha]_D$ +44 (*c* 5.6, MeOH). FT-IR (neat, cm^{-1}): 3259, 2923, 2854, 1578, 1467, 1352, 1289, 1163, 1099, 1046, 957. ^1H NMR (400 MHz, CD_3OD) δ in ppm: 5.75 (d, 1H, J = 7.2 Hz), 4.44 (dd, 1H, J = 7.2, 5.2 Hz), 3.75 (dd, 1H, J = 12.0, 2.8 Hz), 3.70 (dd, 1H, J = 12.0, 5.2 Hz), 3.66 (dd, 1H, J = 6.8, 5.2 Hz), 3.44 (dd, 1H, J = 9.2, 6.8 Hz), 3.31−3.26 (m, 1H, H-5), 2.99 (t, 2H, J = 7.6 Hz), 1.71−1.64 (m, 2H), 1.40−1.27 (m, 10 H), 0.87 (t, 3H, J = 7.2 Hz). ^{13}C NMR (100.6 MHz, CD_3OD) δ in ppm: 171.7, 93.3; 83.2, 74.7, 73.6; 68.9, 62.4, 32.5, 32.0, 30.2, 29.8, 29.7, 29.2, 23.2, 13.9. +TOF MS calcd for $C_{15}H_{27}NO_5S$ m/z $[M - H]^+$: 334.1688. Found: 334.1672.

1,2-Dideoxy-α-D-glucopyranoside[1,2-*d*]dodecylsulfanyl-1,3-oxazoline (21). The title compound was prepared following the general procedure for acetyl deprotection of cis-1,2-fused 1,3-oxazoline carbohydrate derivatives starting from **11** (49.17 mg, 0.10 mmol), MeONa (2.7 mg, 4 μmol), and MeOH (1.7 mL). The reaction mixture was stirred at room temperature for 30 min. After standard workup, the crude was purified by flash chromatography (1:9 MeOH/CH_2Cl_2) to afford the desired compound (36.0 mg, 97% yield) as colorless syrup. R_f (5:95 MeOH/CH_2Cl_2): 0.26. $[\alpha]_D$ +50 (*c* 1.0, MeOH). FT-IR (neat, cm^{-1}): 3300, 2919, 2850, 1573, 1468, 1349, 1292, 1163, 959. ^1H NMR (400 MHz, CD_3OD) δ in ppm: 5.8 (d, 1H, J = 7.2 Hz), 4.47 (dd, 1H, J = 7.2, 5.2 Hz), 3.74 (dd, 1H, J = 12.0, 2.8 Hz), 3.69 (dd, 1H, J = 12.0 Hz, 5.2 Hz), 3.65 (dd, 1H, J = 6.8, 5.2 Hz), 3.43 (dd, 1H, J = 8.8, 6.8 Hz), 3.3−3.26 (m, 1H), 3.03 (t, 2H, J = 7.6 Hz), 1.71−1.63 (m, 2H), 1.39−1.25 (m, 18H), 0.92 (t, 3H, J = 7.6 Hz). ^{13}C NMR (100.6 MHz, CD_3OD) δ in ppm: 172.7, 94.3, 84.3, 75.9, 75.6, 69.9, 63.4, 33.6, 33.0, 31.3, 31.2, 31.1, 31.0, 30.7, 30.2, 24.2, 15.0. +TOF MS calcd for $C_{19}H_{35}NO_5S$ m/z $[M - H]^+$: 390.2314. Found: 390.2298.

1,2-Dideoxy-α-D-glucopyranoside[1,2-*d*]hexadecylsulfanyl-1,3-oxazoline (22). The title compound was prepared following the general procedure for acetyl deprotection of cis-1,2-fused 1,3-oxazoline carbohydrate derivatives starting from **12** (83.7 mg, 0.15 mmol), MeONa (0.5 mg, 9 μmol), and MeOH (3.0 mL). The reaction mixture was stirred at room temperature for 20 h. After standard workup, the crude was purified by flash chromatography (2:98 MeOH/CH_2Cl_2) to afford the desired compound (60.1 mg, 92% yield) as a white solid. R_f (1:1 AcOEt/hexane): 0. Mp: 81−82 °C. $[\alpha]_D$ +0.58 (*c* 1.2, CD_3OD). FT-IR (neat, cm^{-1}): 3352, 2916, 2848, 1745, 1592, 1369, 1241, 1219, 1166, 1041. ^1H NMR (400 MHz, CD_3OD) δ in ppm: 5.79 (d, 1H, J = 7.6 Hz), 4.47 (dd, 1H, J = 7.2, 5.2 Hz), 3.78 (dd, 1H, J = 12.0, 2.8 Hz), 3.73 (dd, 1H, J = 12.0, 5.2 Hz), 3.69 (dd, 1H, J = 6.8, 5.2 Hz), 3.48 (dd, 1H, J = 9.0, 6.8 Hz), 3.32 (m, 1H); 3.03 (t, 2H, J = 7.2 Hz), 1.72 (q, 2H, J = 7.6 Hz), 1.44−1.22 (m, 26H), 0.90 (t, 3H, J = 7.0 Hz). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ in ppm: 172.7, 94.3; 84.3, 75.9, 75.6, 69.9, 63.4, 33.6, 33.0, 31.3, 31.2, 31.1, 31.0, 30.7, 30.2, 24.3, 15.0. +TOF MS calcd for $C_{23}H_{43}NO_5S$ m/z $[M - H]^+$: 446.2940. Found: 446.2920.

1,2-Dideoxy-α-D-glucopyranoside[1,2-*d*]-(16-hydroxyhexadecyl)sulfanyl-1,3-oxazoline (23). The title compound was prepared following the general procedure for acetyl deprotection of cis-1,2-fused 1,3-oxazoline carbohydrate derivatives starting from **13** (95.5 mg, 0.16 mmol), MeONa (0.6 mg, 10 μmol), and MeOH (3.2 mL). The reaction mixture was stirred at room temperature for 20 h. After standard workup, the crude was purified by flash chromatography (2:98 MeOH/CH_2Cl_2) to afford the desired compound (66.8 mg, 89% yield) as colorless syrup. R_f (1:1 AcOEt/hexane): 0. $[\alpha]_D$ +0.67 (*c* 1.2, CD_3OD). FT-IR (neat, cm^{-1}): 3397, 2918, 2849, 1745, 1587, 1464, 1158, 1219, 1039, 986. ^1H NMR (400 MHz, CD_3OD) δ in ppm: 5.78 (d, 1H, J = 7.0 Hz), 4.47 (dd, 1H, J = 7.0, 5.0 Hz), 3.77 (dd, 1H, J = 11.6, 2.8 Hz), 3.72 (dd, 1H, J = 11.6, 5.4 Hz), 3.69 (dd, 1H, J = 6.4, 5.2 Hz), 3.53 (t, 2H, J = 6.6 Hz), 3.48 (dd, 1H, J = 8.8, 6.4 Hz), 3.31 (m, 1H, H-5), 3.03 (t, 2H, J = 7.4 Hz), 1.71 (q, 2H, J = 7.6 Hz), 1.53 (q, 2H, J = 6.8 Hz), 1.42−1.23 (m, 26H). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ in ppm: 172.4, 94.0, 83.9, 75.6, 75.3, 69.6, 63.2, 63.1, 33.8, 32.6, 30.9, 30.9, 30.8, 30.8, 30.7, 30.4, 29.8, 27.1. +TOF MS calcd for $C_{23}H_{43}NO_6S$ m/z $[M - H]^+$: 462.2884. Found: 463.2888. Calcd $[M - Na]^+$: 484.2703. Found: 484.2684.

1,2-Dideoxy-α-D-glucopyranoside[1,2-*d*]benzylsulfanyl-1,3-oxazoline (24). The title compound was prepared following the general procedure for acetyl deprotection of cis-1,2-fused 1,3-oxazoline carbohydrate derivatives starting from **14** (100 mg, 0.23 mmol), MeONa (6.8 mg, 12 μmol), and MeOH (4.6 mL). The reaction mixture was stirred at room temperature for 30 min. After standard workup, the crude was purified by flash chromatography (1:9 MeOH/CH_2Cl_2) to afford the desired compound (70.0 mg, 98% yield) as a colorless syrup. R_f (1:9 MeOH/CH_2Cl_2): 0.16. $[\alpha]_D$ +68 (*c* 1.0, MeOH). FT-IR (neat, cm^{-1}): 3345, 2923, 1579, 1495, 1453, 1296, 1144, 1113, 1045, 993, 949. ^1H NMR (400 MHz, CD_3OD) δ in ppm: 7.39−7.22 (stack, 5H), 5.78 (d, 1H, J = 7.2 Hz), 4.47 (dd, 1H, J = 7.2, 5.2 Hz), 4.29 (d, 1H, J = 13.4 Hz), 4.26 (d, 1H, J = 13.4 Hz), 3.74 (dd, 1H, J = 12.0, 2.8 Hz), 3.69 (dd, 1H, J = 12.0, 5.2 Hz), 3.65 (dd, 1H, J = 6.8, 5.2 Hz), 3.45 (dd, 1H, J = 8.8, 6.8 Hz), 3.30−3.26 (m, 1H). ^{13}C NMR (100.6 MHz, CD_3OD) δ in ppm: 171.6, 138.1, 130.2, 129.9, 128.9, 94.1, 84.2, 75.6, 75.3, 69.6, 63.0, 36.8. +TOF MS calcd for $C_{14}H_{17}NO_5S$ m/z $[M - H]^+$: 312.0906. Found: 312.0895.

1,2-Dideoxy-α-D-glucopyranoside[1,2-*d*]-(6-(1-adamantanecarboxamido)hexyl)sulfanyl-1,3-oxazoline (25). The title compound was prepared following the general procedure for acetyl deprotection of cis-1,2-fused 1,3-oxazoline carbohydrate derivatives starting from **15** (36.9 mg, 0.06 mmol), MeONa (0.2 mg, 3 μmol), and MeOH (1.2 mL). The reaction mixture was stirred at room temperature for 5 h. After standard workup, the crude was purified by flash chromatography (2:98 MeOH/CH_2Cl_2) to afford the desired compound (29.0 mg, 99% yield) as colorless syrup. R_f (1:1 AcOEt/hexane): 0. $[\alpha]_D$ +0.54 (*c* 1.3, CD_3OD). FT-IR (neat, cm^{-1}): 3334, 2904, 2850, 1630, 1587, 1530, 1451, 1367, 1289, 1097. ^1H NMR (400 MHz, CD_3OD) δ in ppm: 5.78 (d, 1H, J = 7.2 Hz), 4.47 (dd, 1H, J = 7.2, 5.2 Hz), 3.78 (dd, 1H, J = 12.0, 2.8 Hz), 3.73 (dd, 1H, J = 12.0, 5.2 Hz), 3.69 (dd, 1H, J = 6.8, 5.2 Hz), 3.47 (dd, 1H, J = 9.2, 6.8 Hz), 3.32 (m, 1H, H-5), 3.15 (t, 2H, J = 7.0 Hz), 3.03 (t, 2H, J = 7.4 Hz), 1.98 (m, 3H), 1.81 (m, 6H), 1.76−1.65 (m, 8H), 1.50−1.24 (m, 6H). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ in ppm: 181.0, 172.3, 94.0, 83.9, 75.6, 75.2, 69.6, 63.1, 41.9, 40.4, 40.3, 37.8, 32.5, 30.8, 30.5, 29.8, 29.4, 27.5. +TOF MS calcd for $C_{24}H_{38}NO_6S$ m/z $[M - H]^+$: 483.2523. Found: 483.2515. Calcd $[M - Na]^+$: 505.2343. Found: 505.2335.

Inhibition Studies with Commercial Enzymes. Inhibition constant (K_i) values were determined by spectrophotometrically measuring the residual hydrolytic activities of the glycosidases against the respective *p*-nitrophenyl α- or β-D-glycopyranoside, *o*-nitrophenyl β-D-galactopyranoside (for β-galactosidases), or α,α′-trehalose (for trehalase) in the presence of compounds **16**−**25**. Each essay was performed in phosphate buffer or phosphate−citrate buffer (for α- or β-mannosidase and amyloglucosidase) at the optimal pH of each enzyme. The reactions were initiated by addition of enzyme to a solution of the substrate in the absence or presence of various concentrations of inhibitor. The mixture was incubated for 10−30 min at 37 or 55 °C (for amyloglucosidase), and the reaction was quenched by addition of 1 M Na_2CO_3 or by heating and subsequent addition of a solution of GLC-Trinder (Sigma, for trehalase). Reaction times were appropiate to obtain 10−20% conversion of the substrate in order to achieve linear rates. The absorbance of the resulting mixture was determined at 405 or 492 nm (for trehalase). Approximate values of K_i were determined using a fixed concentration of substrate (around the K_m value for the different glycosidases) and various concentrations of inhibitor. Full K_i determinations and enzyme inhibition mode were determined from the slope of Lineweaver−Burk plots and double reciprocal analysis.

Lysosomal Enzyme Activity Assay. Lysosomal enzyme activities in cell lysates were determined as described previously.[79,80] Briefly, cells were scraped in ice-cold 0.1% Triton X-100 in water. After centrifugation (6000 rpm for 15 min at 4 °C) to remove insoluble materials, protein concentrations were determined using protein assay

rapid kit (Wako, Tokyo, Japan). The lysates were incubated at 37 °C with the corresponding 4-methylumbelliferyl β-D-glycopyranoside solution in 0.1 M citrate buffer (pH 4). The librated 4-methylumbelliferone was measured with a fluorescence plate reader (exitation 340 nm; emission 460 nm; Infinite F500, TECAN Japan, Kawasaki, Japan). For enzyme inhibition assay, cell lysates from normal skin fibroblasts were mixed with the 4-methylumbelliferyl β-D-glycopyranoside substrates in the absence or presence of increasing concentrations of ABX or PSO **23**.

Cell Culture and GCase Activity Enhancement Assay. Human skin fibroblasts from a healthy and two Gaucher disease patients (with N370S/N370S and L444P/L444P mutations) were maintained in our laboratory with DMEM supplemented with 10% FBS as the culture medium. For enzyme activity enhancement assay, cells were cultured in the presence of different concentrations of ABX or PSO **23** or DMSO alone (as a control) for 5 days and harvested by scraping.[80,81] Cytotoxicity of the compounds was monitored by measuring the lactate dehydrogenase activities in the cultured supernatants (LDH assay kit, Wako, Tokyo, Japan).

■ ASSOCIATED CONTENT

Supporting Information

General experimental methods, experimental procedures, compound characterization data, and NMR spectra for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The Spanish Ministerio de Economía y Competitividad (Contracts CTQ-2008-01569, CTQ2010-15848, and SAF2010-15670), the Ministry of Health, Labour and Welfare of Japan (H19-Nanji-Ippan-002, H22-Nanji-Ippan-002), and the Japan Science and Technology Agency (AS232Z00009G) are acknowledged. Cofinancing from the Fondo Europeo de Desarrollo Regional (FEDER), the Fundación Ramón Areces, and the Junta de Andalucía (P08-FQM-03711) are also thanked. J.C. and R.R. thank the Ministerio de Educación for predoctoral fellowships. D.C. thanks CONACYT (México) for a grant.

■ ABBREVIATIONS USED

LSD, lysosomal storage disorder; DNJ, 1-deoxynojirimycin; CS, (+)-castanospermine; NNDNJ, *N*-(*n*-nonyl)-1-deoxynojirimycin; DIX, 1,5-dideoxy-1,5-iminoxylitol; ER, endoplasmic reticulum; GCase, β-glucocerebrosidase; PSO, cis-1,2-fused pyranose−2-alkylsulfanyl-1,3-oxazoline; HSAB theory, hard−soft acid−base theory; ABX, ambroxol

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