Stereoselective Synthesis of C-2 Alkylated Trihydroxypiperidines:
Novel Pharmacological Chaperones for Gaucher DiseaseFrancesca Clemente,[†] Camilla Matassini,^{*,†,‡} Andrea Goti,^{†,‡,§} Amelia Morrone,[#] Paolo Paoli,[&] and Francesca Cardona^{*,†,‡,§}[†]Department of Chemistry “Ugo Schiff”, University of Firenze, via della Lastruccia n. 3-13, 50019 Sesto Fiorentino (FI), Italy[‡]CNR-INO and LENS, Via N. Carrara 1, 50019 Sesto Fiorentino (FI), Italy[§]Consorzio Interuniversitario Nazionale di ricerca in Metodologie e Processi Innovativi di Sintesi (CINMPIS), 70125 Bari, Italy[#]Paediatric Neurology Unit and Laboratories, Neuroscience Department, Meyer Children’s Hospital, and Department of Neurosciences, Pharmacology and Child Health, University of Florence, Viale Pieraccini n. 24, 50139 Firenze, Italy[&]Department of Experimental and Clinical Biomedical Sciences, University of Florence, Viale Morgagni n. 50, 50134 Florence, Italy

Supporting Information

ABSTRACT: Pharmacological chaperones (PCs) are small molecules that bind and stabilize enzymes. They can rescue the enzymatic activity of misfolded or deficient enzymes when they are used at subinhibitory concentration, thus with minimal side effects. Pharmacological Chaperone Therapy (PCT) is an emerging treatment for many lysosomal storage disorders (LSDs) including Gaucher disease, the most common, which is characterized by a deficiency in the GCase enzyme. We report herein a straightforward synthetic strategy to afford C-2 substituted trihydroxypiperidines with different alkyl chains starting from low cost D-mannose. Stereoselective Grignard reagent addition onto a key nitron intermediate in the presence or absence of a suitable Lewis acid afforded both epimers of the target compounds, after a final reductive amination-ring closure step. We show that the shift of the alkyl chain from the endocyclic nitrogen to the C-2 position leads to a considerable increase in chaperoning efficacy, affording a new compound (**4a**) able to induce a remarkable 1.9-fold maximal increase in GCase activity.

KEYWORDS: Lysosomal storage disorders, Gaucher disease, pharmacological chaperones, iminosugars, trihydroxypiperidines

Gaucher disease (GD) is the most common lysosomal storage disorder (LSD) of glycosphingolipids and is caused by mutations in *GBA* gene (chromosome: 1q21–22), that encodes for the lysosomal enzyme acid- β -glucosidase (glucocerebrosidase, also known as GCase, EC 3.2.1.45, MIM*606463).¹ The *GBA* mutations lead to total or partial deficiency of GCase, the enzyme responsible for the hydrolysis of the fatty acid glucosylceramide (GlcCer (**1**), Figure 1) to ceramide and glucose, with consequent accumulation of GlcCer in the lysosomes, mainly in the liver, spleen, and bone marrow, causing organ enlargement and inflammation.¹

Gaucher disease is classified into three phenotypes on the basis of the presence or absence of neurological involvement: Type 1, the most common form (OMIM#230800), which was considered non-neuronopathic until recent discoveries; Type 2, acute neuronopathic (OMIM#230900), the rarest and most severe form; Type 3, subacute chronic neuronopathic (OMIM#231000), with later onset and a slower progressive course.² At present, more than 490 *GBA* gene mutations have been reported in Gaucher patients (data from HGMD professional 2018.3; <http://www.hgmd.cf.ac.uk/ac>). The presence of the N370S allele mutation correlates with the most

Figure 1. Natural substrate of the GCase enzyme, glucosyl ceramide (GlcCer, **1**), a pharmacological chaperone previously synthesized in our group (**2**), and the new chaperones **3** and **4** described in this work.

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common Type 1 phenotype, causing the enzyme malfunction due to incorrect folding.

The non-neuronopathic forms of Gaucher disease are currently treated with Enzyme Replacement Therapy (ERT), which involves infusion of the recombinant enzyme (with consequent frequent hospitalization and high costs), or with Substrate Reduction Therapy (SRT), which inhibits the biosynthesis of GlcCer.³

Pharmacological Chaperone Therapy (PCT) is an emerging approach to LSDs and has recently provided the first oral drug on the market for the treatment of Fabry disease (another LSD) in Europe (Migalstat, Galafold, Amicus Therapeutics). Pharmacological chaperones (PCs) are small molecules that bind proteins, inducing a template-based rescue of correct folding, with effective recovery of enzyme activity when they are used at subinhibitory concentration, thus minimizing side effects. They commonly behave as reversible inhibitors of the enzyme at higher concentrations. PCs bind to the active site of the enzyme and promote its correct folding, helping its translocation to the lysosomes where they are displaced by the natural substrate present in high concentrations. One of the main advantages of PCs over ERT and SRT is that they may address also the forms of the disease with CNS involvement, by correcting the endogenous mutated protein.^{4,5} Moreover, the pharmacological chaperone approach could be applied to a whole range of diseases related to protein misfolding, such as Alzheimer's, Parkinson's, Huntington's, or amyotrophic lateral sclerosis.⁶

In terms of chemical structure, the most investigated class of PCs for LSDs are glycomimetics and, in particular, iminosugar derivatives, nitrogenated glycomimetics with a nitrogen atom in the ring.^{7,8} However, unmodified iminosugars behaving as competitive inhibitors of GCase (such as isofagomine, IFG) failed to reach the market due to their high hydrophilicity, which hampered an efficient transport to the lysosomes. Iminosugars with *N*-linked alkyl chains showed better metabolic properties as potential PCs for GD.⁸

We recently reported the synthesis of the *N*-alkylated iminosugar **2** (Figure 1), which showed measurable chaperone activity with human fibroblasts derived from Gaucher patients bearing the N370/RecNcil mutation (1.25-fold increase of GCase activity at 100 μ M concentration).⁹

C-Alkylated iminosugars with different configuration at the centers bearing the hydroxyl groups were also reported as PCs for GD.^{10,11} However, they were obtained by lengthy syntheses with low overall yields.

In this context, we designed a new straightforward synthetic strategy to furnish structural analogues of compound **2** where the alkyl chain is moved to a vicinal position, and we investigated their activity. We report herein the synthesis of trihydroxypiperidines **3** and **4** (Figure 1), bearing alkyl chains at C-2 with both configurations. 3,4,5-Trihydroxypiperidines, belonging to the family of 1,5-dideoxyiminosugar natural products, demonstrated several biological activities including glycosidase inhibition, immunosuppressant, and antibacterial activities.¹²

The adopted synthetic strategy to access C-2 substituted trihydroxypiperidines relies on a stereodivergent approach that employs a carbohydrate-derived nitron **6**, readily derived from aldehyde **5**, as the key intermediate (Scheme 1). We recently observed that the stereoselectivity of the addition of MeMgBr to nitron **6** can be controlled by the presence or absence of Lewis acids, affording two diastereoisomeric hydroxylamines in

Scheme 1. Stereodivergent Strategy for the Synthesis of Trihydroxypiperidines **3 and **4****

diastereomeric ratio up to 7:1 when a suitable Lewis acid was added.¹³ This approach opens the possibility of investigating the effect on pharmacological activity of varying the configuration of the newly created C-2 stereogenic center, as well as the effect of the chain length introduced in the addition step.

In this work, we exploit the above strategy employing Grignard reagents generated from primary alkyl halides with long chains to access hydroxylamines **7** and **8** with opposite configuration at C-2. Since the nitrogen atom is already installed on the molecules and the hydroxylamines can be directly employed for the final cyclizations reactions, the stereochemistry at C-2 of the target trihydroxypiperidines depends on the stereoselectivity of this reaction. Final reductive amination (RA) afforded compounds **3** and **4** (Scheme 1), which were assayed as pharmacological chaperones for Gaucher disease.

Aldehyde **5** was synthesized in four steps from D-mannose on gram scale.^{14,15} Nitron **6**¹⁶ was readily accessed from **5** in 85% yield by reaction with *N*-benzyl hydroxylamine in dry CH_2Cl_2 .¹⁷ The addition of organometallic reagents both to α -alkoxy^{18,19} and *N*-benzyl glycosyl nitrones^{20,21} has been extensively investigated in the presence or absence of a Lewis acid.

The best results obtained in the Grignard addition onto **6** are shown in Table 1 (see Supporting Information for more comprehensive results). On the basis of our previous findings with MeMgBr, $\text{BF}_3 \cdot \text{Et}_2\text{O}$ was chosen as the Lewis acid for inverting the stereoselectivity of the addition.²²

The addition of different Grignard reagents RMgBr (R = octyl, undecyl, dodecyl, tridecyl) to nitron **6** was initially investigated in THF as a solvent at -78°C for 2 h without BF_3 .²³ The corresponding hydroxylamines were obtained as a mixture of two diastereoisomers **7** and **8** with good isolated yields (from 57% to 70%). In all cases, hydroxylamines **7** with the (*S*) absolute configuration at the newly formed stereocenter were obtained as the major diastereoisomers with *dr* ranging from 4.2:1 (entry 4, Table 1) to 5.6:1 (entry 1, Table 1). By increasing the reaction temperature from -78 to -30°C , the selectivity slightly decreased (entry 2 vs entry 1). The addition of $\text{BF}_3 \cdot \text{Et}_2\text{O}$ (1.0 equiv) resulted in the formation of hydroxylamines **7** and **8** with excellent yields (from 70% to 87%, entries 3, 5, 7, 9). More importantly, the Lewis acid addition completely reversed the selectivity^{24,25} in favor of the (*R*) hydroxylamines **8**, with *dr* up to 9:1 in the case of hydroxylamine **8c** (entry 7, Table 1). Other Lewis acids were

Table 1. Addition Reactions of Grignard Reagents to Nitrone 6 in THF, in the Presence or Absence of Lewis Acid

reagent RMgBr	entry	Grignard reagent equiv	Lewis acid (1 equiv)	temp(°C)	time (h)	7/8 ratio ^a	yield (%) (7+ 8) ^b
R = octyl (a)	1	1.8	none	−78	2	7a/8a	5.6:1
	2	1.8	none	−30	2		4.7:1
	3	1.8	BF ₃ ·Et ₂ O	−30	2		1:5.6
R = undecyl (b)	4	4	none	−78	4	7b/8b	4.2:1
	5	4	BF ₃ ·Et ₂ O	−30	4		1:3.0
R = dodecyl (c)	6	4.5	none	−78	4	7c/8c	4.5:1
	7	2.3	BF ₃ ·Et ₂ O	−30	2		1:9.0
R = tridecyl (d)	8	3	none	−78	3.5	7d/8d	4.2:1
	9	2	BF ₃ ·Et ₂ O	−30	2		1:6.0

^aDetermined by integration of signals in the ¹H NMR spectra of the crude reaction mixture. ^bDetermined on the basis of the total amount of R and S adducts recovered after purification by column chromatography.

tested (e.g., MgCl₂, InCl₃, Et₂AlCl) in this reaction (see [Supporting Information](#)), but no better selectivity was obtained. Thus, the presence or absence of BF₃·Et₂O in this reaction allowed the stereodivergent synthesis of both diastereoisomers 7 and 8, which were readily separable by flash column chromatography. However, they are not stable and undergo slow oxidation by air to the corresponding nitrones 9 and 10 ([Scheme 2](#)).

Scheme 2. Ring-Closure Reductive Amination (RA) Step: Synthesis of Trihydroxypiperidines 3a–d, 4a–d, and 14a^a

^aReagents and conditions: (a) Pd/C, H₂, CH₃COOH, MeOH, r.t., 2 d; (b) Ambersep 900-OH, r.t., 40 min; (c) HCl, MeOH, r.t., 16 h; (d) Pd/C, H₂, HCl, MeOH, r.t., 2 d.

This fact did not affect our aim since the conditions employed for the ring-closure reductive amination step (H₂, Pd/C, cat. CH₃COOH in MeOH) were also effective on the hydroxylamine/nitrone mixtures, affording the piperidines 11 and 12 in excellent yields ([Scheme 2A](#)). The yields of this reaction are remarkable, considering that they result from several distinguished synthetic steps (*N*- and *O*-debenzylation, hydroxylamine/nitrone reduction, condensation with sugar

aldehyde, and C=N reduction) in a one-pot reaction. When the reaction was performed in the presence of HCl (instead of CH₃COOH), the *N*-methylpiperidine 13a was obtained with a low yield ([Scheme 2B](#)). Final deprotection of the acetonide protecting groups performed under acidic conditions (aqueous HCl in MeOH) led to the final trihydroxypiperidines 3 and 4 with high yields and to compound 14a ([Scheme 2](#)).

With the aim of comparing the activity of the *N*-alkylated iminosugar 2 with the C-2 alkylated trihydroxypiperidines 3 and 4, the whole set of newly synthesized compounds was first tested at 1 mM for GCCase inhibition in human leukocyte homogenates.²⁶ The percentages of inhibition, together with the corresponding IC₅₀ values, are shown in [Table 2](#).

Table 2. Inhibitory Activity of Compounds 2, 3a–d, 4a–d, and 14a toward GCCase

entry	compd	GCCase inhibition [%] ^a	IC ₅₀ [μM] ^b
1	2	98 ^c	30.0 ± 1.0 ^c
2	3a	80	93.5 ± 5.3
3	3b	96	100.0 ± 40.0
4	3c	91	23.3 ± 4.0
5	3d	88	160.0 ± 30.0
6	4a	100	29.3 ± 1.8
7	4b	100	7.0 ± 1.0
8	4c	95	1.5 ± 0.1
9	4d	98	9.0 ± 2.0
10	14a	50	>1000

^aPercentage inhibition of GCCase in human leukocytes extracts incubated with iminosugars (1 mM). ^bIC₅₀ values were determined by measuring GCCase activity at different concentrations of each inhibitor. ^cData taken from ref 9 (IC₅₀ value obtained using the same experimental protocol and substrate concentration of this Letter).

All the newly synthesized trihydroxypiperidines showed inhibitory activity higher than 80%, with the only exception of *N*-methylated compound 14a, which exhibited only 50% inhibitory activity ([Table 2](#), entry 10). This result may suggest that the simultaneous presence of two alkyl chains at the nitrogen atom and at C-2 is detrimental for inhibition.

[Table 2](#) clearly shows that, among the newly synthesized compounds, trihydroxypiperidines 4 with an (*R*) configuration at C-2 ([Table 2](#), entries 6–9) are more active than epimeric trihydroxypiperidines 3 ([Table 2](#), entries 2–5). If we compare, for instance, compounds 4d and 3d, bearing a tridecyl alkyl chain at C-2, their IC₅₀ values differ by more than 1 order of magnitude (IC₅₀ = 9 μM vs IC₅₀ = 160 μM, entry 9 vs entry 5, [Table 2](#) and Figure S28 of the [Supporting Information](#)). The

shift of the octyl chain from nitrogen to C-2 provoked a slight decrease in activity for the (*S*) configuration at C-2 (Table 2, entry 2 vs entry 1; compound 3a vs compound 2), while the inhibitory activity was maintained for compound 4a, with the (*R*) configuration (Table 2, entry 6 vs entry 1; compound 4a vs compound 2).

When comparing the effect of the chain length of the trihydroxypiperidines with the same configuration at C-2, it appears that the dodecyl alkyl chain imparts the best inhibitory activity to the final compounds for both series (compound 3c, entry 4 and compound 4c, entry 8 and Figure S28 of the Supporting Information). Overall, compound 4c is the best inhibitor among the whole series, with an $IC_{50} = 1.5 \mu M$.

To gain insight into the mechanism of action of the best inhibitor 4c and the best chaperone (4a, see below), kinetic analyses were performed and revealed that both compounds act as competitive inhibitors of GCase, with K_i values of 3.6 and $18.5 \mu M$, respectively (see Supporting Information).

The ability of the C-2 alkylated trihydroxypiperidines 3 and 4 to enhance the activity of GCase was next assayed in human fibroblasts derived from Gaucher patients bearing the N370/RecNcil mutation. Interestingly, except for derivative 3d, all new compounds showed an increase in GCase activity from 1.2-fold to 1.9-fold, after incubation (4 days) with mutated fibroblasts (see Supporting Information). Very similar results, in terms of enhancement and concentration were observed for couples of compounds with the same alkyl chain regardless of the configuration of the substituent. Indeed, a 1.4-fold maximal increase was obtained for both dodecyl trihydroxypiperidines 3c and 4c at $1 \mu M$ concentration. Analogously, treatment with 3b and 4b (undecyl trihydroxypiperidines) led to 1.3-fold and 1.2-fold maximal increase, respectively, at 50 nM concentration. It should be noted that the best inhibitory activity, shown by 4c (entry 8, Table 2), did not correspond to the best chaperoning efficacy (Figure 2A), which was obtained with octyl trihydroxypiperidines 3a and 4a (Figure 2B,C). In fact, compound 3a (entry 2, Table 2), despite being a poorer inhibitor than 4c ($IC_{50} = 93.5$ vs $1.5 \mu M$) showed similar enhancement (1.3 vs 1.4), but at a lower 10 nM concentration. These data confirm that chaperoning activity does not necessarily parallel *in vitro* GCase inhibition, as already observed with both amphiphilic DNJ derivatives²⁷ and multivalent iminosugars.²⁸

In terms of GCase activity enhancement, the best result was obtained with compound 4a, showing a 1.6-fold increase at $10 \mu M$ and a remarkable 1.9-fold maximal increase at $50 \mu M$ (Figure 2C). The latter result demonstrates that a simple shift of the octyl chain from the endocyclic nitrogen (compound 2, Figure 1) to C-2, leads to a remarkable increase in chaperoning efficacy (1.9-fold at $50 \mu M$ vs 1.26-fold at $100 \mu M$). Moreover, the chaperoning activity of 4a compares well with that of *N*-octyl-deoxynojirimycin (*N*-octyl-DNJ, 1.7-fold at $20 \mu M$) reported by Asano et al.²⁹ Compound 4a was afforded by a concise and highly stereoselective synthetic strategy that, starting from cheap D-mannose, produced the final compound with a remarkable overall yield of 42% over eight steps. Considering that similar enhancement values were previously observed with C-6 nonyl IFG¹⁰ and α -1-C-nonyl DIX¹¹ (although at lower concentrations), obtained from much more expensive L-xylose with lower overall yields (5–6%), 4a reasonably represents an appealing compound for further optimization. In order to endorse the chaperoning effect of compound 4a, an additional GD patient cell line carrying at

Figure 2. GCase activity in human fibroblasts derived from GD patients bearing N370/RecNcil mutations, measured after 4 days of incubation without (ctrl) or with compound 4c (A), 3a (B), or 4a (C).

homozygous level the L444P GBA gene mutation was tested. A remarkable rescue of 1.8-fold in GCase activity was observed at $100 \mu M$ (see Supporting Information).

In summary, a series of remarkable GCase inhibitors of human GCase, the deficient enzyme in Gaucher disease, was identified among trihydroxypiperidines bearing an alkyl chain (8, 11, 12, or 13 carbon atoms) at C-2. These compounds were prepared by applying a straightforward, stereodivergent synthetic strategy that exploits the Grignard addition to the carbohydrate-derived nitron 6 as the key step, followed by reductive amination/cyclization. Trihydroxypiperidines (*R*)-configured at C-2 showed higher inhibitory activities toward GCase than those with the opposite configuration, with the best result obtained for the *N*-dodecyl derivative 4c ($IC_{50} = 1.5 \mu M$). Most of the newly synthesized compounds, when incubated in fibroblasts from GD patients, showed GCase enhancements of 1.2–1.4-fold at low micromolar or high nanomolar concentrations. Remarkably, the *N*-octyl derivative with (*R*) configuration at C-2 (compound 4a) was able to enhance the GCase activity up to 1.9-fold at $50 \mu M$. Given that this compound was obtained from D-mannose with the high overall yield of 42%, it represents a promising target for future development and clinical application. Further optimization of its physicochemical and pharmacokinetic properties will be the topic of future investigations.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.8b00602.

General synthetic procedures and characterization data of key compounds, general procedure for biochemical characterization toward human GCase and other lysosomal enzymes, IC₅₀ graphs and chaperoning activity graphs for 3a–d and 4a–d, and kinetic analysis for compounds 4a and 4c (PDF)

AUTHOR INFORMATION

Corresponding Authors

*Phone (+39)0554573504. E-mail: francesca.cardona@unifi.it.

*Phone (+39)0554573536. E-mail: camilla.matassini@unifi.it.

ORCID

Francesca Clemente: 0000-0002-9899-0836

Camilla Matassini: 0000-0002-8336-383X

Andrea Goti: 0000-0002-1081-533X

Paolo Paoli: 0000-0001-6448-7266

Francesca Cardona: 0000-0002-6766-4624

Author Contributions

F. Clemente carried out the syntheses and the biological experiments. F. Cardona and C.M. designed the syntheses. C.M. designed and carried out the biological experiments. P.P. carried out IC₅₀ and K_i calculation. A.M. supervised of the biological experiments. F. Cardona, C.M., and A.G. wrote the manuscript. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

GD, Gaucher disease; LSD, lysosomal storage disorder; GlcCer, glucosylceramide; ERT, Enzyme Replacement Therapy; SRT, Substrate Reduction Therapy; PCT, Pharmacological Chaperone Therapy; PC, pharmacological chaperone; IFG, isofagomine; DNJ, deoxynojirimycin; DIX, 1,5-dideoxy-1,5-imino-D-xylitol

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