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# Multivalent pyrrolidines acting as pharmacological chaperones against Gaucher disease

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**ABSTRACT.** A concise asymmetric synthesis of clickable enantiomeric pyrrolidines was achieved using Crabbé-Ma allenation. The synthesized iminosugars were grafted by copper-free strain-promoted alkyne-azide cycloaddition onto phosphorus dendrimers. The hexavalent and dodecavalent pyrrolidines were evaluated as  $\beta$ -glucocerebrosidase inhibitors. The level of inhibition suggests that monofluorocyclooctatriazole group may contribute to the affinity for the protein leading to potent multivalent inhibitors. Docking studies were carried out to rationalize these results. Then, the iminosugars clusters were evaluated as pharmacological chaperones in Gaucher patients' fibroblasts. An increase in  $\beta$ -glucocerebrosidase activity was observed with hexavalent and dodecavalent pyrrolidines at concentrations as low as 1  $\mu$ M and 0.1  $\mu$ M, respectively. These iminosugar clusters constitute the first example of multivalent pyrrolidines acting as pharmacological chaperones against Gaucher disease.

**Keywords:** multivalency, pyrrolidines, glucocerebrosidase inhibition, chaperones

## 1. Introduction

Gaucher disease (GD) is a rare lysosomal storage disorder affecting about 20 000 people worldwide [1]. Many mutations of the *GBA1* gene cause  $\beta$ -glucocerebrosidase (GCCase, E.C.3.2.1.45) misfolding and its premature degradation in the endoplasmic reticulum (ER). The resulting enzymatic deficiency leads to lysosomal accumulation of glucosylceramide and subsequent enlargement of the spleen and the liver, bones abnormalities, hematological dysfunctions and in the most severe cases, neurological impairments [2]. Three types of GD are distinguished depending on the symptoms severity and the central nervous system involvement: the most common and least severe non-neuronopathic type 1 (GD1), the acute neuronopathic type 2 (GD2), and the chronic neuronopathic type 3 (GD3).[2] To date, more than 300 *GBA1* variants have been identified in patients with GD. Among them, N370S (c.1226A>G) and L444P (c.1448T>C) mutations are the two most prevalent missense mutant forms of the protein (>60% of mutations).[3] Moreover, heterozygous *GBA1* mutations are also associated with an increased risk of Parkinsonism [4,5] or with the possibility to develop different forms of cancer [2,6].

The two following therapeutic approaches are currently employed for the GD treatment [7,8]. The enzymatic replacement therapy consists in the periodic infusion of the

recombinant protein (Cerezyme®, Vpriv®, Elelyso®). The substrate reduction therapy aims at decreasing the lysosomal storage of glucosylceramide by the use of an inhibitor of glucosylceramide biosynthesis. Due to its many side effects, the glucosylceramide synthase inhibitor, *N*-butyl-D-deoxyojirimycin (NB-DNJ, Zavesca®), has gradually been replaced by the more selective eliglustat (Cerdelga®). All these treatments are quite effective and well tolerated but none of them address neurological damage. Consequently, new therapies are still needed and much hope has been placed in pharmacological chaperone (PC) therapy.

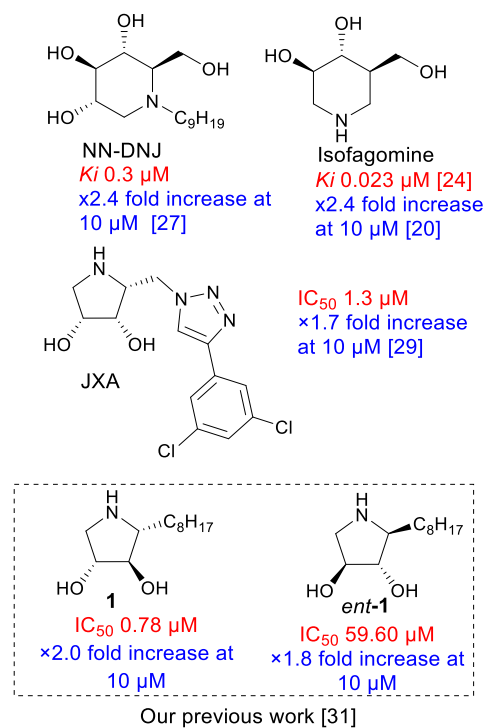
PC are specific protein ligands able to promote the stabilization or to induce the proper folding of a misfolded but yet catalytically active GCCase protein. Thus, PC avoid the premature degradation of the protein by the ER-associated degradation systems and improve its trafficking to the lysosome to catabolize the accumulated glucosylceramide [9,10]. Paradoxically, in most cases, PC are competitive inhibitors of the protein used at sub-inhibitory concentrations and listed as active-site specific chaperones. Since the pioneering work of Fan and co-workers [11], a large number of PC have been described for various protein misfolded diseases [9][12–17] including GD [18]. Whereas 1-deoxygalactonojirimycin (Galafold®) has been marketed since 2016 for Fabry disease patients displaying a PC-amenable *GLA* mutation [19], no PC drug is available against GD to date. Most of the reported

GCCase PC are piperidinic iminosugars, representative examples of which are isofagomine D-tartrate [20–24] (Plicera®) and *N*-nonyl-deoxynojirimycin [25–27] (NN-DNJ) (Fig. 1). Interestingly, only few pyrrolidines have been shown to also act as GCCase PC [28–30]. Recently, we reported an expedient asymmetric synthesis of the enantiomeric *C*-alkyl hydroxypyrrolidines **1** and *ent*-**1** via chiral aminoallenes (Fig. 1) [31]. We also demonstrated that both pyrrolidines were able to enhance residual lysosomal GCCase activity in N370S homozygous Gaucher fibroblasts.

Multivalency offers a promising path towards new PC design [32]. Since the seminal work of Kovensky and coworkers in 2009 [33], the search for a multivalent effect in glycosidase inhibition has grown exponentially [34–37]. A positive multivalent effect is characterized by an increased relative potency (rp) of the ligand in the multivalent system as compared to the monovalent one, taking into account the valency (*n*) [36]. Several potential interactions have been proposed to explain the multivalent effect, such as the “bind-and-recapture” process, the chelate effect, the substrate-binding effect or the clustering effect [37]. Since studies have suggested that the protein exists in dimeric or tetrameric structures, GCCase is a promising candidate for multivalent interactions [38]. In 2012, a step forward has been taken with Compain and coworkers with the first report of multivalent iminosugars targeting GCCase [39]. A heptavalent deoxynojirimycin (DNJ) built on a cyclodextrin scaffold showed a high multivalent inhibitory effect (rp/*n* = 28) towards GCCase and allowed an enhancement of the enzyme activity in N370S fibroblasts (1.6 fold increase at 10 μM). However, since this promising work, little progress has been made and only a few multivalent GCCase PC have been described [40–44].

Recently, we reported the two cyclotriphosphazene-based dendrimers **2** and **3** presenting six or twelve monofluorocyclooctyne units on their surface, respectively (Fig. 2) [43]. These monofluorocyclooctyne units were chosen because of the easy access to the 1-fluorocyclooct-2-yne-1-carboxylic acid [45] and because of the increase of the cyclooctyne reactivity due to the fluorine [46]. A strain-promoted alkyne-azide cycloaddition (SPAAC) reaction allowed the straightforward assembly of dendrimers **4** and **5** bearing DNJ inhiotopes (Fig. 2). These dendrimers, showing a modest multivalent effect on GCCase inhibition, induced a significant increase of the GCCase activity at concentrations down to the nanomolar range. Such results prompted us to investigate the chaperone activity of multivalent platforms decorated with our original pyrrolidinic pharmacophores **1** and *ent*-**1**. Indeed, despite of being a relevant alternative, multivalent pyrrolidines have been much less studied than their piperidine counterparts [47]. Notably, not only multivalent pyrrolidines have been scarcely described as GCCase inhibitor [42], but none has shown GCCase chaperone activity to date. The present study describes the

asymmetric synthesis of the enantiomeric ω-azido *C*-alkyl hydroxypyrrolidines and their strained-promoted ligation on the cyclooctyne-decorated cyclotriphosphazene-based dendrimers **2** and **3**. The inhibition of GCCase as well as their ability to increase enzymatic activity on GD patient's fibroblasts were also assessed.

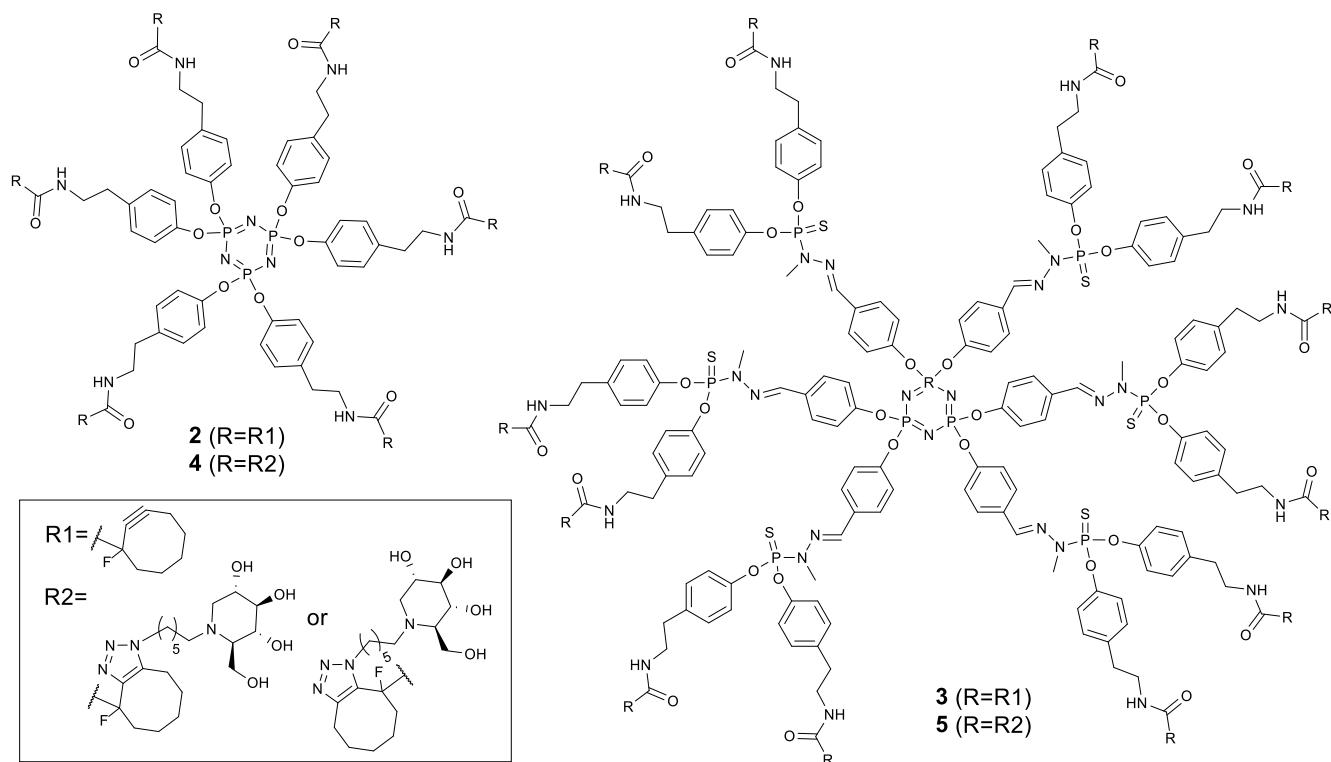


**Fig. 1.** Examples of GCCase PC. In red, GCCase inhibition. In blue, GCCase activity enhancement in GD patients' fibroblasts with N370S mutation.

## 2. Results and discussion

### 2.1 Synthesis of the ω-azido pyrrolidines **14** and *ent*-**14**

Based on our previous results [31], we developed the enantioselective synthesis of the “clickable” ω-azido *C*-alkyl hydroxypyrrolidines **14** and *ent*-**14** with high yield and optical purity. Our strategy was based on the asymmetric synthesis of the two aminoallenes **8** and *ent*-**8** using a CuBr<sub>2</sub>-catalysed Crabbé-Ma enantioselective reaction [48]. Thus, the reaction between *O*-silylated aldehyde **7** and *N*-Boc-propargylamine performed in the presence of the suitable enantiomer of diphenylprolinol as chiral inductor provided the expected aminoallenes **8** and *ent*-**8** with 60% and 53% yield, respectively (Scheme 1). High enantiomeric excesses were obtained (98% for **8**, 93 % for *ent*-**8**), as determined by chiral HPLC analysis (see Supporting Information). Intramolecular cyclisation of the aminoallene in the presence of AgNO<sub>3</sub> as π-electrophilic Lewis acid led to the pyrroline **9** (or *ent*-**9**) quantitatively with a complete transfer of the chirality [31]. The *m*-CPBA epoxidation of pyrroline **9** (or *ent*-**9**) proceeded with total diastereoselectivity to give epoxy pyrrolidines **10** (or *ent*-**10** respectively). Silyl deprotection with TBAF, primary alcohol



**Fig. 2.** Cyclotriphosphazene-based dendrimers before SPAAC (dendrimers **2** and **3**) and after SPAAC reaction with  $\omega$ -azidoheptyl DNJ (dendrimers **4** and **5**)

tosylation and nucleophilic substitution with sodium azide allowed introduction of the terminal azido group. Finally, regioselective acidic hydrolysis of the oxirane led to the desired clickable C-alkyl hydroxypyrrolidines **14** (and *ent*-**14**) with all-trans relative configuration.

## 2.2. Synthesis of multivalent pyrrolidines by SPAAC

The strain-promoted click reaction between  $\omega$ -azido C-alkyl pyrrolidine **14** (or *ent*-**14**) and cyclooctyne **15** [43] was first performed by a simple stirring at 40 °C for 3 hours in a THF/MeOH mixture (Scheme 2). It delivered the monovalent reference compound **16** (or **17**, respectively) presenting a chain similar to that of a single branch of cyclotriphosphazene-based dendrimers. After purification on silica gel column chromatography, the monovalent compounds **16** and **17** were isolated with 74 and 87% yield, respectively, as a non-separable mixture of 1,3- and 1,5-regioisomers (55:45 ratio).

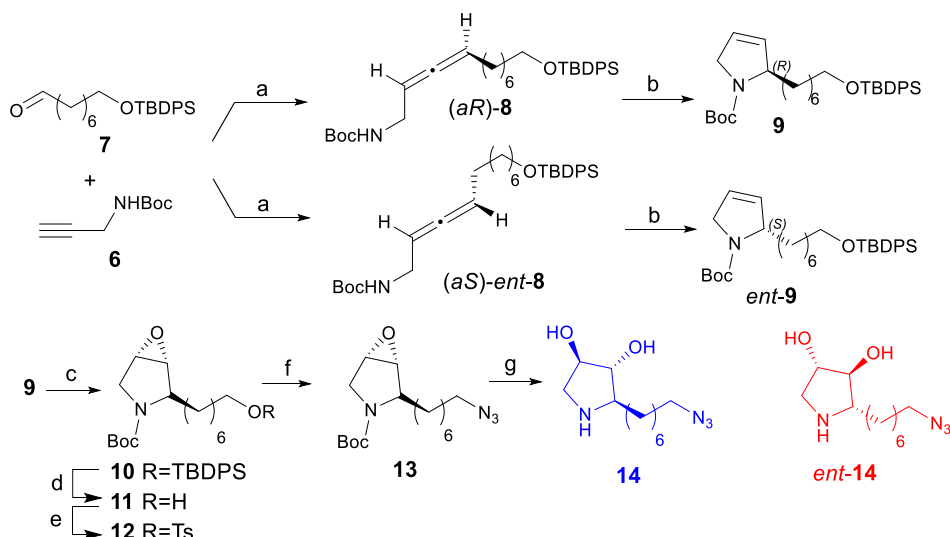
Pyrrolidine **14** (or *ent*-**14**) and the cyclooctyne-decorated dendrimer **2** were then stirred in a THF/methanol/water mixture at 40 °C for 3 hours to give hexavalent compounds **18** (or **19**, respectively) (Scheme 2). Purification on Sephadex column gave pure hexavalent clusters **18** and **19** with 56 and 50% yield, respectively, as a non-separable mixture of regioisomers. The same protocol was applied for the preparation of dodecavalent compounds **20** and **21** starting from dendrimer **3**. After Sephadex purification, compounds **20** and **21** were

obtained with 58 and 76% yield, respectively. The purity of the multivalent constructs **18-21** was assessed by  $^{19}\text{F}$  NMR and by HR-ESI mass spectrometry (see Supporting Information). In the case of the dodecavalent iminosugars **20** and **21**,  $^{31}\text{P}$  NMR spectra were also recorded (see Supporting Information).

## 2.3. Synthesis of multivalent pyrrolidines by CuAAC

For the sake of comparison, tetravalent and hexavalent scaffolds **22** and **23** were prepared following previously described procedures [49,50]. These well-known platforms deriving from a pentaerythritol core were propargylated allowing the subsequent copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) reaction with azido pyrrolidines **14** or *ent*-**14**. The hexavalent compound **23**, possessing the same valency as the multivalent iminosugars derived from **2**, was of particular interest. Moreover, the respective influence of the triazole and the monofluorocyclooctatriazole groups on the inhibition of GCase could thus be compared.

CuAAC reactions between the tetravalent scaffold **22** and **14** or *ent*-**14** were performed at 60 °C in the presence of tris(benzyltriazolylmethyl)amine (TBTA) in MeOH/H<sub>2</sub>O with a complete conversion (Scheme 3). Of note, the use of microwave activation led to degradation products and a significant decrease in yield (down to 30% yield). Tetravalent pyrrolidines **24** and **25** were then treated with



**Scheme 1.** Synthesis of clickable pyrrolidines **14** and *ent*-**14**. a) CuBr<sub>2</sub>, a) (*S*)-diphenylprolinol for (a*R*)-**8** and (*R*)-diphenylprolinol for (a*S*)-*ent*-**8**, dioxane, 130 °C, overnight, ((a*R*)-**8**: 98% yield, 98% ee; (a*S*)-*ent*-**8**: 53% yield, 93% ee); b) AgNO<sub>3</sub>, acetone, r.t., 48h (**9**: 98% yield; *ent*-**9**: 95% yield); c) *m*-CPBA, CH<sub>2</sub>Cl<sub>2</sub>, r.t., 48h (**10**: 87% yield; *ent*-**10**: 76% yield); d) TBAF, THF, overnight; e) *p*-TsCl, pyridine, CH<sub>2</sub>Cl<sub>2</sub>, r.t. (**12**: 92% yield over two steps; *ent*-**12**: 77% yield over two steps); f) NaN<sub>3</sub>, DMF, 40 °C, overnight (**13**: 84 % yield; *ent*-**13**: 85% yield); g) H<sub>2</sub>SO<sub>4</sub>, dioxane/H<sub>2</sub>O, 1:1 (**14**: 64% yield; *ent*-**14**: 52% yield)

Quadrasil® resin to remove copper residues and then subjected to purification on a Sephadex® column. The same procedure allowed complete conversion of the starting materials to form the hexavalent compounds **26** and **27**. Monovalent reference compounds **28** and **29** were also synthesized in order to quantify the multivalent effect. The latter were obtained by CuAAC reaction between 1-pentyne and ω-azido *C*-alkyl hydroxypyrrolidines **14** or *ent*-**14**, respectively.

#### 2.4. Inhibition of human GCCase

The inhibitory properties of all multivalent pyrrolidines and their corresponding monomeric references were evaluated against recombinant human GCCase (Table 1). In agreement with our previous studies on **1** and its enantiomer *ent*-**1** [31], derivatives of **14** were more potent GCCase inhibitors than derivatives of the enantiomer *ent*-**14**. These results confirm the importance of the iminosugar stereochemistry in GCCase inhibition as observed by others [51]. The data also indicate that GCCase is able to discriminate between the two enantiomeric inhitopes grafted onto the surface of a multivalent object. These observations contrast with the previous results obtained by Carmona, Moreno-Vargas and coll. who observed that that GCCase was unable to discriminate between two epimeric iminosugar inhitopes [42]. The monovalent compounds **28** and **29** prepared by CuAAC were less potent inhibitors than the starting pyrrolidines **1** and *ent*-**1**. This trend suggests that the triazole group could have a detrimental effect on ligand binding to GCCase. On the other hand, the monovalent references **16** and **17** obtained by strain-

promoted click chemistry were stronger inhibitors than **1** and *ent*-**1**. As observed previously [43], the monofluorocyclooctatriazole group seems to play a favorable role on the affinity of the ligand for GCCase. More generally, the iminosugar derivatives obtained by SPAAC with a monofluorocyclooctatriazole group displayed a greater GCCase inhibitory potency than their respective triazole analogues obtained by CuAAC. The most efficient inhibitor was the dodecavalent derivative **20** with an IC<sub>50</sub> of 4.8 nM and is, to our knowledge, among the best GCCase inhibitors described to date.

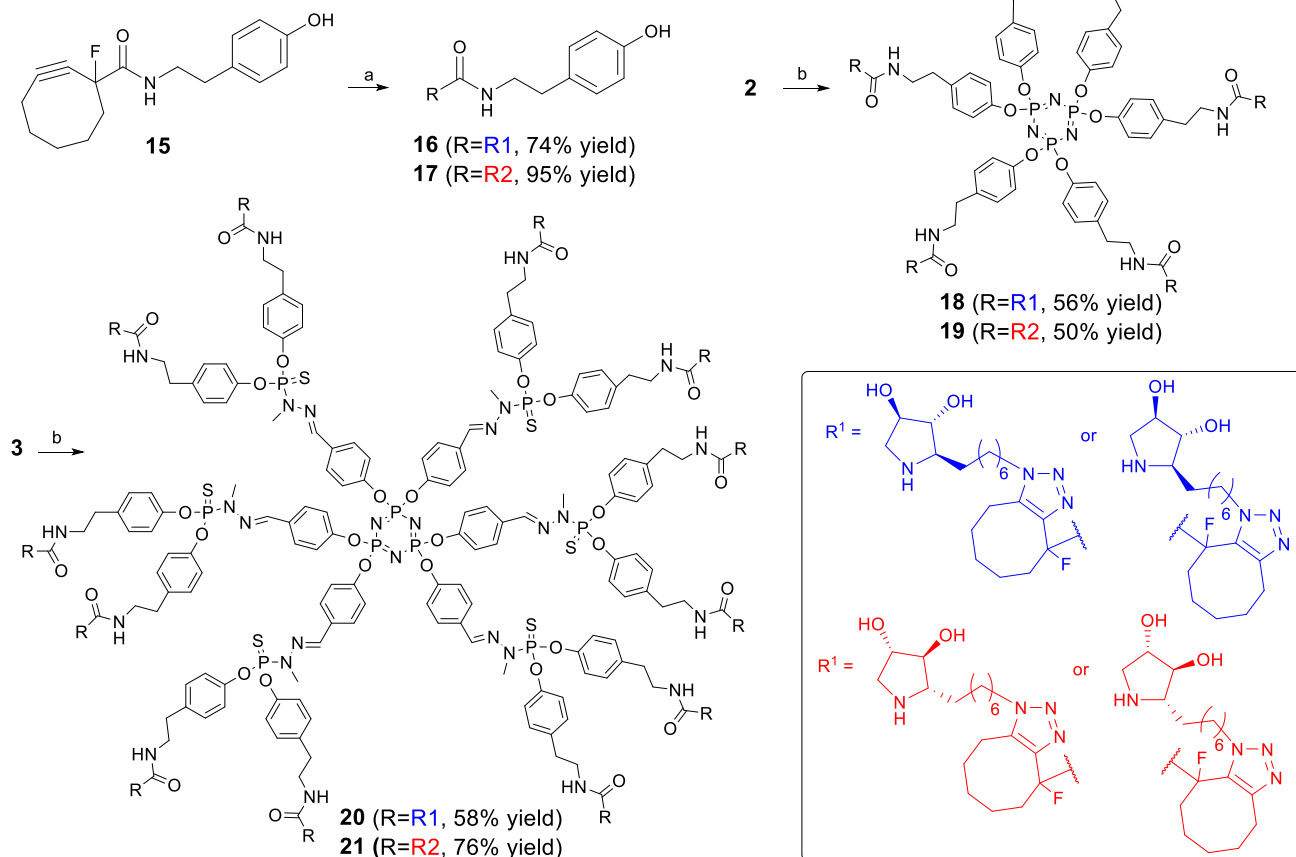
To quantify the multivalent effect towards GCCase, the relative inhibitory potencies (rp) (given by the ratio of the IC<sub>50</sub> values of the monovalent pyrrolidine to that of the multivalent compounds) per pyrrolidinic motif (rp/n) were calculated (Table 1). A multivalent effect was observed for all multivalent compounds assessed in this study (1.6 < rp/n < 8). GCCase is reported to be sensitive to multivalency although it has a single and buried active site as many other glycosidases [39,40]. Thus, the weak multivalent effects might be ascribed to statistical rebinding mode as previously proposed by others [41]. Moreover, independently of the enantiomeric ligand series or of the dendrimeric scaffold, we observed that the multivalent effect is increasing with the object valency, reaching rp/n values of 7.7 and 8.0 for compound **20** and **27** respectively.

#### 2.5. Docking studies

In order to gain insights into the influence of lipophilic appendage on the binding mode to GCCase, docking studies were performed with compounds **28** and the two regioisomers

of **16** (**16a** and **16b**, see Supporting Information). Compounds **28** and **16** exhibited a mixed-type inhibition with respective  $K_i$

of 14.83  $\mu\text{M}$  and 0.34  $\mu\text{M}$  (see Supporting Information). Molecular modeling studies were carried with



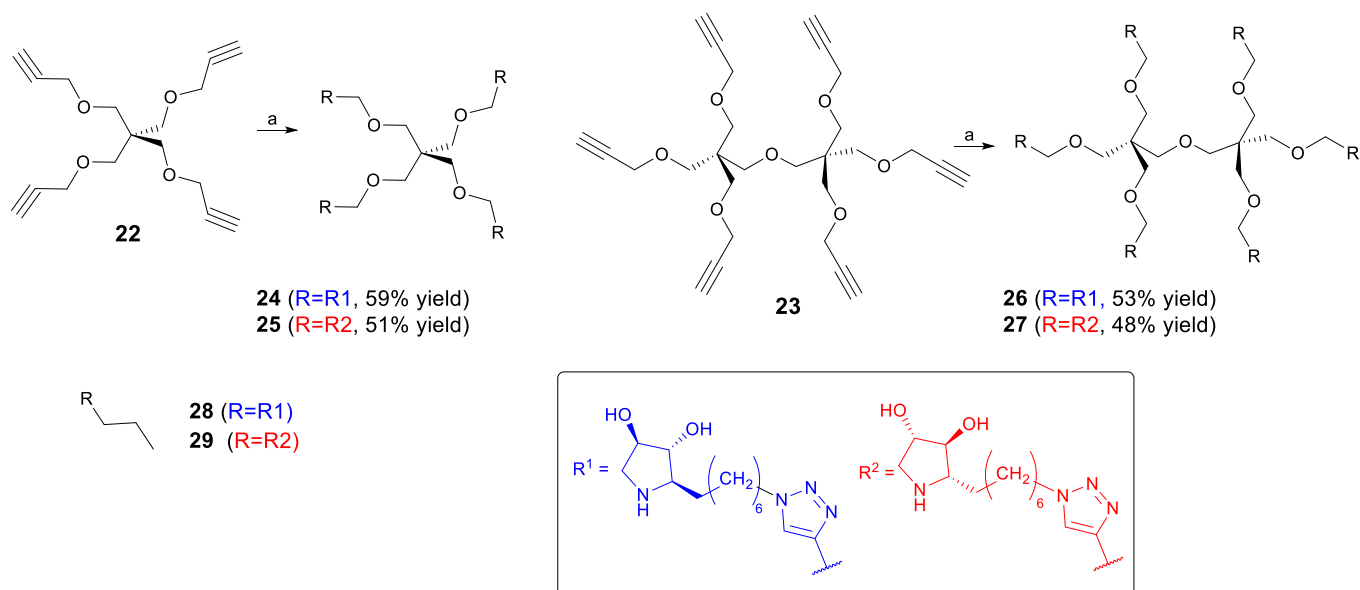
**Scheme 2.** SPAAC synthesis of monovalent references **16** and **17** and dendrimers **18-21**. a) 0.7 eq of pyrrolidines **14** or *ent*-**14**, THF/MeOH/H<sub>2</sub>O, 40 °C, 3 h. b) 1.4 eq of pyrrolidines **14** or *ent*-**14**, THF/MeOH/H<sub>2</sub>O, 40 °C, overnight

Molegro Virtual Docker 6 software (www.molexus.com) using crystal structures in complex with isofagomine (PDB code: 3GXFb and 2NSXb, ligand code: IFM) [52,53]. The docked poses were compared to the co-crystal structure with JXA pyrrolidine (PDB code: 6MOZ, ligand code: JXA, Fig. 1) [29]. In the three structures, the active site topology is organized with several channels around the catalytic pocket, in particular a minor channel (open state in 2NSXb, close state in 3GXFb, see Supporting Information) controlled by LYS346 and a main entrance with a side channel near SER237 (Fig. 4). Since **16** and **28** exhibited a mixed-type inhibition, they should be docked in both the active site and allosteric sites of the protein. To date, knowledge of the allosteric sites of GCCase is still limited [54–56]. Therefore, the docking study was restrained to the active site.

The calculated docked poses of the ligand **28** were equally spread into two populations in 3GXFb (Fig. 3). The first group has a binding mode close to that of JXA with a similar interaction network for the pyrrolidine scaffold. The

positions and interactions of the nitrogen atom with GLU340 and the oxygens atoms of the secondary alcohols with ASN396, ASP127, TRP179 were similar (Fig. 3). However, in the second group, the ligand **28** adopts an “inverted” pose with interactions between the nitrogen atom and ASP127 and between the secondary alcohols and GLU340, GLU235, ASN234. In both groups, the chain was found to fluctuate in the side channel and the triazole ring of the compound **28** may interact with SER237 or GLN284. In 2NSXb, the compound **28** was able to use the open minor channel favouring an “inverted” pose (See Supporting Information). In this case, an additional interaction between triazole group and SER345 in the minor channel can be noticed.

The two regioisomers of ligand **16** were also able to dock in the same manner as JXA ligand or with “inverted” poses in equal proportions in 3GXFb (Fig. 4) and with similar interaction network as compound **28**. For both regioisomers, the chain fluctuations were strong with different positions of monofluorocyclooctatriazole group,



**Scheme 3.** CuAAC synthesis of monovalent references **28** and **29** and pentaerythritol-based dendrimers **24-27**. a) alkyne (1 eq) and pyrrolidines **14** or *ent-14* (1.1 eq per alkyne), TBTA (0.1 eq per alkyne) CuSO<sub>4</sub>·5H<sub>2</sub>O (0.3 eq per alkyne) and *L*-ascorbic acid sodium salt (3 eq per Cu<sup>II</sup>), CH<sub>3</sub>OH/water, 60 °C

the latter being alternatively located in the side channel or in the minor channel. The triazole may interact with SER237 for both populations (See Supporting Information). Furthermore, an additional stabilizing interaction is observed between the hydroxyl function of the phenol and SER345 (Fig. 5). Favorable hydrophobic interactions can also be observed between the monofluorocyclooctyl group and LEU314, LEU286 and LEU241 (See Supporting Information). In 2NSXb, some poses were found roughly similar, with a JXA-type conformation. An additional population of poses is also encountered that follow the minor channel and tends to deviate significantly from JXA-type or "inverted" pyrrolidine conformations and interactions. Indeed, in order to follow the minor channel, the interaction of the triazole group with SER345 seems to be important. The phenolic hydroxyl group shows also interactions with CYS342 (backbone), GLY344 and GLU349. Additional favorable interactions at the active site were thus observed in the case of compound **16**, albeit not discriminating enough to explain the differences in inhibitory activity of compounds **16** and **28**. This suggests that the allosteric component of the activity of **16** and **28** may significantly contribute to their inhibitory potency.

#### 2.6. Evaluation of pharmacological chaperone activity in treated fibroblasts from type 1 GD patients

The potential cytotoxicity of multivalent C-alkyl hydroxypyrrolidines with a cyclotriphosphazene core was assessed on healthy fibroblasts as well as on N370S homozygous GD fibroblasts. No cytotoxicity was observed

up to a concentration of 10 μM (see Supporting Information). We then investigated the effect on GCCase activity in N370S GD fibroblasts. The cells were grown in the presence or absence of up the selected compounds (at 5 μM, 1 μM or 0.1 μM, Table 2) and the GCCase activity was assayed after 3 days and compared to the untreated control assay. NN-DNJ (at 10 μM) was used as a positive control, the latter leading to a typical 2.3-fold increase at this concentration. A similar fold increase was observed with the monovalent compound **17** used at 10 μM (Table 2). At 5 μM, only monovalent references **16** and **17** allowed an increase of GCCase activity, reaching 2.9-fold increase for compound **16**. At such a concentration, hexavalent compounds **18**, **19** and dodecavalent compounds **20**, **21** led to a decrease of the enzymatic activity, consistently with their high inhibitory potency.

To observe a chaperone effect, we anticipated that lower concentrations would be required. Indeed, at 1 μM a 2.4 and 2.5-fold GCCase activity enhancement was observed with hexavalent dendrimers **18** and **19**. Due to their high inhibitory potencies, we chose to evaluate the dodecavalent compounds **20** and **21** at 0.1 μM. At this concentration, the dodecavalent structures retained a GCCase activity increase of 1.3-1.8-fold. This need to decrease the concentration of a multivalent compound to observe an increase in GCCase activity has already been observed in our previous studies [43]. Thus, all the multivalent pyrrolidines showed a GCCase activity increase of 1.3 to 2.5-fold at the suitable concentration.

Remarkably, while compounds **17**, **19** and **21** bearing the pharmacophore of *ent-1* were less potent inhibitors than those presenting **1** (compounds **16**, **18**, **20**,

respectively), their chaperone effects were quite comparable. These results confirm that the inhibitory potency does not necessarily correlate with the chaperone

**Table 1.** Inhibition of recombinant human GCCase

Core	Derivatives of pyrrolidine <b>14</b>				Derivatives of pyrrolidine <i>ent-14</i>			
	Compound	Valency	IC <sub>50</sub> [μM] <sup>a</sup>	Rp/n	Compound	Valency	IC <sub>50</sub> [μM] <sup>a</sup>	Rp/n
None	<b>1</b>	1	0.78 ± 0.14 <sup>b</sup>	-	<i>ent-1</i>	1	59.60 ± 5.17 <sup>b</sup>	-
Cyclotriphosphazene	<b>16</b>	1	0.463±0.022	-	<b>17</b>	1	1.799±0.296	-
	<b>18</b>	6	0.018±0.001	3.8	<b>19</b>	6	0.196±0.011	1.6
	<b>20</b>	12	0.0048±0.0004	7.7	<b>21</b>	12	0.052±0.006	2.9
Pentaerythritol	<b>28</b>	1	6.6±0.4	-	<b>29</b>	1	105.1±14.0	-
	<b>24</b>	4	0.642±0.040	2.5	<b>25</b>	4	4.1±0.5	6.4
	<b>26</b>	6	0.327±0.010	3.3	<b>27</b>	6	2.2±0.7	8.0

<sup>a</sup> IC<sub>50</sub> values were determined by measuring GCCase activity at different concentrations of each compound and assays were performed in triplicate. <sup>b</sup> Data taken from ref [31] (IC<sub>50</sub> value obtained using the same experimental protocol and substrate concentration of this manuscript).

activity, as mentioned previously [31]. In addition, this feature is also advantageous since the undesired inhibitory activity of PCs may hamper their use as pharmacological agents. Decoupling their pharmacological chaperone capacity from their inhibitory activity in selecting the most appropriate enantiomeric series thus offers promising prospects.

### 3. Conclusion

This study reports the enantioselective synthesis of ω-azido C-alkyl hydroxypyrrolidines **14** and *ent-14* via chiral aminoallenes. These enantiomeric inhitopes were grafted onto hexa- and dodecavalent dendrimers with cyclotriphosphazene core by strain-promoted copper-free click reaction. The hexavalent (compounds **18** and **19**) and dodecavalent (compounds **20** and **21**) dendrimers were obtained with yields ranging from 50% to 76%. Tetra- and hexavalent derivatives were also synthesized through CuAAC using pentaerythritol-core dendrimers to compare the influence of the triazole and monofluorocyclooctatriazole groups.

The inhibitory potency of the multivalent pyrrolidines was evaluated towards recombinant human GCCase. Compounds obtained by SPAAC proved to be more potent inhibitors than those synthesized by CuAAC, indicating that the monofluorocyclooctatriazole group could improve the affinity of clicked compounds for the protein. Docking studies showed that both regioisomers of **16**, despite their large size, could bind to the active site. The most potent inhibitor was the dodecavalent compound **20** decorated with inhitope **1**, displaying an IC<sub>50</sub> of 4.8 nM and a rp/n ratio of 8. Of note, this compound is among the best inhibitors described to date. These results also show that GCCase is able to distinguish between the two enantiomeric inhitopes grafted onto the surface of a multivalent scaffold. Thus, derivatives of **14** are more potent GCCase inhibitors than derivatives of the *ent-14* enantiomer. Moreover, all multivalent objects with a cyclotriphosphazene core behaved as PC inducing a GCCase enhancement on Gaucher fibroblasts. However, due to the strong inhibition

of GCCase by multivalent pyrrolidines, cellular studies on their chaperone effect required an optimization of the concentration to be used in the culture medium. The hexavalent derivatives **18** and **19** showed chaperone effects at a concentration of 1 μM similar to those observed for NN-DNJ at a ten-fold higher concentration of 10 μM. An increased GCCase activity was also observed using dodecavalent compounds **20** and **21** at concentrations down to 0.1 μM. Thus, the compounds reported herein represent the first examples of enantiomeric multivalent pyrrolidines acting as pharmacological chaperones of GCCase in Gaucher disease patient cells.

### 4. Methods

#### 4.1. Synthetic chemistry

##### General procedure for CuAAC.

Solvents were first degassed under nitrogen. Then, a solution of CuSO<sub>4</sub>·5H<sub>2</sub>O (0.3 eq per alkyne) and L-ascorbic acid sodium salt (3 eq per Cu<sup>II</sup>) in water ([Cu<sup>II</sup>]= 0.01 M) were added to a solution of alkyne (1 eq) and azide derivate (1.1 eq per alkyne) and TBTA (0.1 eq per alkyne) in THF or DMF ([Alkyne]= 0.01 M).

##### General procedure for SPAAC reaction.

THF, CH<sub>3</sub>OH and water were first degassed under argon. Then the monovalent cyclooctyne (1.3 eq) or dendrimer derivative (0.7 eq) and azide (1 eq) were dissolved in a THF/CH<sub>3</sub>OH (1:1, [Alkyne] = 0.01 M) before adding water (1/3 of the final volume). The mixture was stirred at 40 °C at 1200 rpm.

#### 4.2. Inhibition assays on recombinant GCCase

Recombinant Human GCCase/GBA (7410-GH), purchased from R&D was used in the inhibition studies. GCCase activity was determined using 4-methylumbelliferyl-β-D-glucopyranoside (purchased from Sigma-Aldrich) as previously reported [31,43] Briefly, enzyme solutions (25 μL from a stock solution containing 0.6 μg mL<sup>-1</sup>) in the



presence of 0.25% (w/v) sodium taurocholate and 0.1% (v/v) Triton X-100 in McIlvaine buffer (100 mM sodium citrate and 200 mM sodium phosphate buffer, pH 5.2) were incubated at 37 °C without (control) or with inhibitor at a final volume of 50  $\mu$ L for 30 min. After addition of 25  $\mu$ L 4-methylumbelliferyl- $\beta$ -D-glucopyranoside (7.2 mM, McIlvaine buffer pH 5.2), the samples were incubated at 37 °C for 10 min. Enzymatic reactions were stopped by the addition of aliquots (100  $\mu$ L) of glycine/NaOH buffer (100 mM, pH 10.6). The amount of 4-methylumbelliferone formed was determined with a FLUOstar microplate reader (BMG Labtech) at 355 nm (excitation) and 460 nm (emission) wavelengths. Type of inhibition and  $K_i$  values for compounds **16** and **28** were determined by Lineweaver-Burk plots of assays performed with 4 concentrations of inhibitor and substrate.

#### 4.3. Cell viability evaluation

Cultured primary skin fibroblasts from control individuals were obtained from the Laboratoire de Biochimie Métabolique, CRB, IFB, CHU Toulouse, France and CBC Biotec biobank BB-0033-00046 (3809 F01 and 1541 F02 cell lines), Hospices Civils de Lyon, France. Cells were immortalized after transfection with a plasmid encoding the SV40 large T antigen. Cells were routinely cultured in DMEM medium supplemented with 10% inactivated fetal calf serum. After incubation for 72 h in the presence of the chemical compounds or vehicle (ethanol) only, cell viability was evaluated using the MTT test as reported earlier.[57] Briefly, cells were plated in 24-well plates (30,000 cells/well) and at 50% confluency cells were incubated with fresh medium supplemented with 2, 5 and 10  $\mu$ M of compounds. After 72 h, 500  $\mu$ g of MTT was added to each well. After 2 h of incubation at 37 °C, supernatants were discarded, and the remaining material was dissolved by 1 mL of DMSO. After 30 min, absorbance was measured at 560 nm by using a microplate reader. Background values were subtracted from all other values, and viability was expressed as percentage compared with untreated controls.

#### 4.4. Docking studies

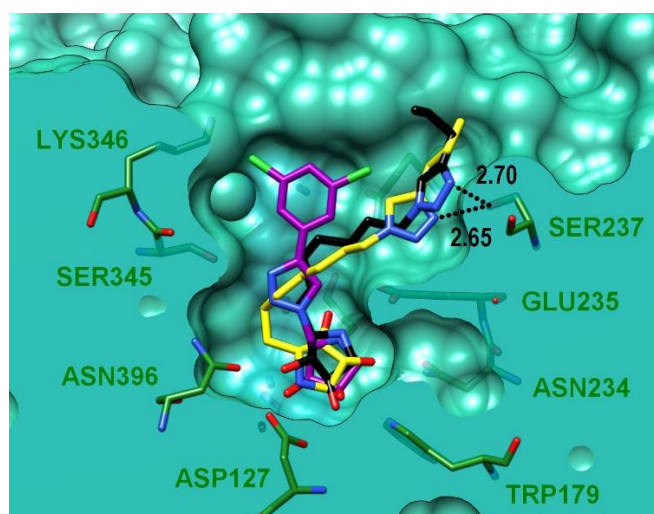
##### *Molecular graphics and molecules.*

Molecular graphics were performed with the UCSF Chimera package [58]. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by the NIGMS P41-GM103311). The protein structures used in this paper were downloaded from the RCSB Protein Database [59] and structurally aligned [60] on a reference structure 1OGS chain A [61]. The protein structures, were prepared (structure checks, rotamers, hydrogenation, splitting of chains) using Biovia ([www.3dsbiovia.com](http://www.3dsbiovia.com)) Discovery Studio Visualizer 2016 (DSV) and UCSF Chimera. The new compounds were sketched using ChemAxon Marvin 21 ([www.chemaxon.com](http://www.chemaxon.com)). All ligands were

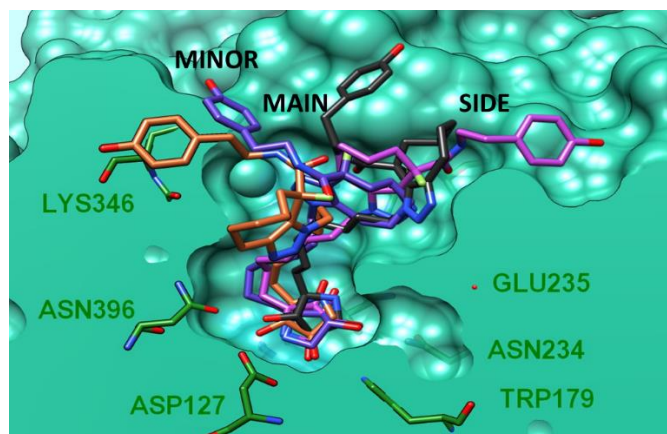
checked (hybridization, hydrogenation, some geometry optimizations, 3D sketching) and merged in SDF libraries using DSV.

##### *Molecular modelling studies*

Molecular modelling studies were carried with Molegro Virtual Docker 6 software ([www.molexus.com](http://www.molexus.com)) using 2NSXb and 3GXFb structures. A rough and non-flexible CPU-type docking protocol was used and, no displaceable water molecule was considered at the interface, no pharmacophoric profiles were added to calculations. Docking process used 3000 iteration steps, for 30 independent runs, a grid resolution of 0.3 Å. MSE optimizer was used, with a population size of 50, a creation EnergyTheshold of 100, maxsimplex and simplexsteps were set to 1500 and simplex distance factor to 2. The convergence was reached for all ligands, other parameters were let as default. A fast minimization and hydrogen bonds optimization were carried post-docking. Clustering of poses (tabu clustering) was set with an RMS threshold of 1 Å in order to enrich results with best poses. Positive charges were eventually added for nitrogen ligands, according to pKa calculations at neutral pH (ChemAxon). Final MolDock and Rerank [62] scores were calculated post-docking and post-minimization. This unique protocol was found to work well in the case of the two structures (2NSXb and 3GXFb), the same calculation conditions were used and checked successfully on piperidine-based (IFM and NND) and pyrrolidine-based ligands used as test (JXA,).



**Fig.3:** Best docked poses of **28**: JXA-type conformation (black) and inverted conformation (yellow) in 3GXFb (green) with triazole group near SER237 and GLN284 (masked) directed to the side channel of main entrance. Interatomic distances (heavy atoms) are expressed in Angstroms. \*The JXA (purple) from aligned 6MOZa is also shown.



**Fig. 4:** Typical fluctuation of docked poses for regioisomers of **16a** (pink and dark purple) and **16b** (grey and dark orange) in 3GFXb (clipped molecular surface in green) with JXA-type or "inverted" poses

**Table 2.** GCCase activity in N370S fibroblasts

	Compound	Valency	Fold increase <sup>a</sup>
	NN-DNJ		2.3 at 10 $\mu$ M
Derivatives of pyrrolidine <b>14</b>	<b>16</b>	1	2.9 at 5 $\mu$ M
	<b>18</b>	6	0.7 at 5 $\mu$ M
			2.5 at 1 $\mu$ M
	<b>20</b>	12	0.1 at 5 $\mu$ M
			1.3 at 0.1 $\mu$ M
Derivatives of pyrrolidine <i>ent-14</i>	<b>17</b>	1	2.3 at 10 $\mu$ M
			1.9 at 5 $\mu$ M
	<b>19</b>	6	0.9 at 5 $\mu$ M
			2.4 at 1 $\mu$ M
	<b>21</b>	12	0.5 at 5 $\mu$ M
			1.8 at 0.1 $\mu$ M

<sup>a</sup>GCCase activity in fibroblasts from GD patients bearing the N370S mutation. The fibroblasts were incubated in the presence of compounds at the chosen concentration for three days and compared to the untreated control assay. Mean values are shown for triplicate experiments.

#### 4.5. Assay of $\beta$ -GCCase activity in human fibroblasts

Cultured primary skin fibroblasts from patients affected with Gaucher disease (carrying the N370S mutation) were obtained from CBC Biotech biobank BB-0033-00046 (3809 F01 and 1541 F02 cell lines), Hospices Civils de Lyon, France. Cultured primary skin fibroblasts from control individuals were from the Laboratoire de Biochimie Métabolique, CRB, IFB, CHU Toulouse, France. Informed, signed consent was obtained from all patients for molecular genetic studies and cell cultures. All experiments were performed in compliance with the French rules and institute policies on ethics and human material usage. The activity of preparation and conservation of biological material for research purposes is the subject of a declaration to the "Ministère de l'Enseignement Supérieur, de la Recherche et de l'innovation (MESRI)", to the "Comité de protection des Personnes Sud-Est IV" and at "the Agence Régionale d'Hospitalisation" registered under number DC-2008-72 and amendments DC-2011-1437, DC-2015-2566, DC-

2019-3464 and DC-2020-3919. The activity of biological material transfer is subject to MESRI authorizations registered under numbers AC-2008-73, AC-2013-1867, AC-2015-2576, AC-2019-3465 and AC-2020-3918. The biobank has also been certified according to the NF S-96 900 standard since 2011. Cells were harvested and pelleted. Cell lysates were prepared in 0.2% Triton X100 by brief sonication. GCCase enzyme activity was determined on cell lysates in sodium acetate buffer pH 5.6 using 4-methylumbelliferyl- $\beta$ -D-glucopyranoside (Sigma-Aldrich, St Louis, MO, USA) as substrate.

#### Conflicts of interest

Cédric-Olivier Turrin is cofounder and CEO of IMD-Pharma SAS and Abdelouahd Oukhrib is employed by this company.

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