ARTICLE

Conformationally-Locked *C*-Glycosides: Tuning Aglycone Interactions for Optimal Chaperone Behaviour in Gaucher Fibroblasts

C. D. Navo,[a] F. Corzana,[a] E. M. Sánchez-Fernández,[b] J. H. Busto,[a] A. Avenoza,[a] M. M. Zurbano,[a] E. Nanba,[c] K. Higaki,[c] C. Ortiz Mellet,*[b] J. M. García Fernández*[d] and J. M. Peregrina*[a]

A series of conformationally locked *C*-glycosides based on the 3-aminopyrano[3,2-*b*]pyrrol-2(1*H*)-one (APP) scaffold has been synthesized. The key step involved a totally stereocontrolled *C*-Michael addition of a serine-equivalent *C*-nucleophile to tri-*O*-benzyl-2-nitro-D-galactal. Stereoselective transformations of the Michael adduct allowed us the synthesis of compounds with mono- or diantennated aglycone moieties and different topologies. In vitro screening showed highly selective inhibition of bovine liver β-glucosidase/β-galactosidase and specific inhibition of human β-glucocerebrosidase among lysosomal glycosidases for compounds bearing palmitoyl chains in the aglycone, with a marked dependence of the inhibition potency upon their number and location. Molecular dynamics simulations highlighted the paramount importance of an optimal orientation of the hydrophobic substituent to warrant efficient non-glycone interactions, which are critical for the binding affinity. The results provide a rationale for the strong decrease of the inhibition potency of APP compounds on going from neutral to acidic pH. The best candidate was found to behave as pharmacological chaperone in Gaucher fibroblasts with homozygous N370S and F213I mutations, with enzyme activity enhancements similar to those encountered for the reference compound Ambroxol®.

Introduction

Glycosidases are enzymes that catalyze the hydrolysis of glycosidic bonds, thereby playing a key role in biochemical routes that are essential for life such as the processing of glycoproteins and the catabolism of polysaccharides and glycoconjugates. Deregulation of any of these enzymes generally translates into significant biological and pathological consequences, which has fuelled research in compounds interfering with their activity as potential drug candidates.[1] The carbohydrate mimics (glycomimetics) of the iminosugar type, such as the piperidine derivative 1-deoxynojirimicyn (DNJ) or the indolizidine analogue castanospermine (CS, Figure 1), represent by far the most studied family of molecules towards these channels.[1,2] Their polyhydroxylated structure emulates that of the natural monosaccharides, enabling them to compete with the substrate for the glycone pocket in the active site of glycosidases, behaving as competitive inhibitors. However, classical iminosugars cannot fully reproduce the structure of the putative glycoside substrates due to the instability of aminoacetal functional groups, which often translates into poor selectivity. Even though rigidification of the azaheterocyclic core in bicyclic derivatives can be exploited to improve the discrimination capacity among isoenzymes, the absence of a defined anomeric configuration generally results in simultaneous inhibition of glycosidases acting on anomeric substrates, i.e. α- and β-glucosidases in the case of DNJ and CS, which represents a serious drawback for clinical applications.[3]

The urgent need for more specific glycosidase inhibitors has fuelled research on synthetic glycomimetics targeting medically relevant enzymes.[4,5] In principle, molecular prototypes mirroring the full structure of glycosides, that is incorporating both glycone- and aglycone-like portions and benefiting from a defined pseudoanomeric configuration, are better suited for that purpose.[6] For example, sp²-iminosugar cores,[7] in which the endocyclic nitrogen is part of a pseudoamide functionality, are compatible with the incorporation of axially-oriented pseudoanomeric substituents through stable *O*-, *S*- or *N*-glycosidic linkages (Figure 1). This feature has been exploited in the design of specific α-glucosidase inhibitors exhibiting anticancer and antileishmanial activities.[8] *N*-alkylated bicyclic isoureas derived from aminocyclitol scaffolds (e.g., benzo[*d*]oxazolimine derivatives, Figure 1) behaved instead as very selective inhibitors of human lysosomal β-glucosidase, exhibiting strong pharmacological chaperone potential for the treatment of Gaucher disease.[9]

[a] Departamento de Química and Centro de Investigación en Síntesis Química, Universidad de La Rioja, c/ Madre de Dios, 53, 26006 Logroño, Spain.
[b] Departamento de Química Orgánica, Facultad de Química, Universidad de Sevilla, c/Profesor García González 1, 41012 Sevilla, Spain.
[c] Division of Functional Genomics, Research Center for Bioscience and Technology, Tottori University, 86 Nishi-cho, Yonago 683-8503, Japan.
[d] Instituto de Investigaciones Químicas (IIQ), CSIC – Universidad de Sevilla, Avda. Américo Vespucio 49, 41092 Sevilla, Spain.
† Corresponding authors: For C.O.M.: e-mail, mellet@us.es; for J.M.G.F.: jogarcia@iiq.csic.es; for J.M.P.: jesusmanuel.peregrina@unirioja.es. The authors declare no competing financial interest.
Electronic Supplementary Information (ESI) available: [1D and 2D NMR spectra for all new compounds, inhibition studies with enzymes, and data corresponding to molecular dynamics simulations]. See DOI: 10.1039/x0xx00000x
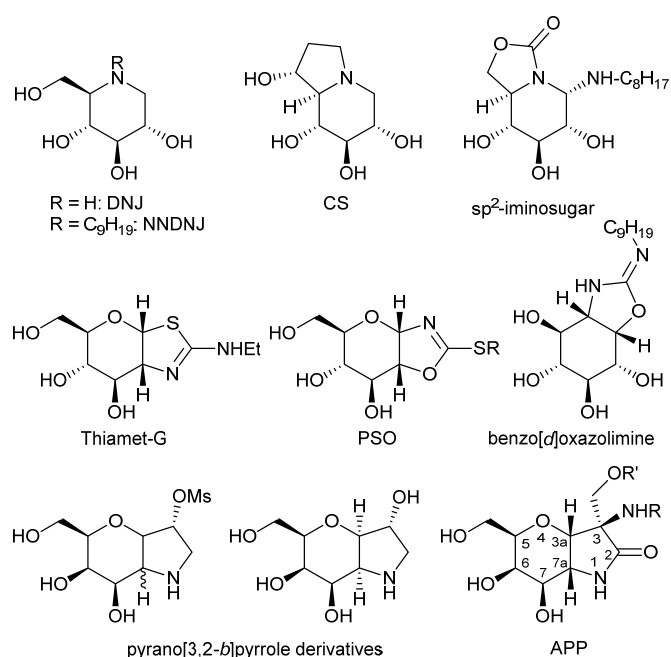
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Org. Biomol. Chem., 2015, **00**, 1-11 | 1

Figure 1. Some iminosugars and bicyclic derivatives glycosidase inhibitors.

Interestingly, some examples of glycosidase inhibitors keeping the pyranose ring as the glycone constituent have also been reported, which presents advantages in term of synthetic procedures. An example is Thiamet G (Figure 1), a fused pyranose-thiazoline derivative, that acts as a potent inhibitor of *O*-linked 2-acetamino-2-deoxy-β-D-glucopyranoside hydrolysis currently in preclinical evaluation as a potential therapeutic for the treatment of Alzheimer's disease.[10] The conformationally-locked bicyclic *N*-glycoside derivatives of the pyranose-sulfanyl-1,3-oxazoline family (PSO, Figure 1) exhibit β-glucosidase inhibitory activity that is strongly dependent on the nature of the pseudoaglycone *S*-substituent, some representatives behaving as pharmacological chaperones for Gaucher disease.[11]

The pyrano[3,2-*b*]pyrrole framework (Figure 1) has also been suggested as a suitable scaffold for the preparation of glycosidase inhibitors.[12] However, the lack of effective synthetic methods compatible with the incorporation of aglycone-type substituents has burdened optimization of the affinity and selectivity towards biomedically relevant enzyme targets.[13] The development of efficient approaches towards this framework through synthetic routes compatible with structurally diversity-oriented strategy is therefore highly wanted. The possibility of accessing conformationally-restricted multiantennated derivatives with *C*-glycoside structure is particularly appealing[14] given that many medically relevant glycosidases, such as those that are dysfunctional in glycosphingolipid-related metabolic disorders, act on substrates with branched aglycone segments.[15]

With this background, we have now envisioned the synthesis of 3-**a**mino-6,7-dihydroxy-3,5-bis(hydroxymethyl)hexahydro**p**yrano[3,2-*b*]**p**yrrol-2(1*H*)-one derivatives (APP, Figure 1) as a new family of non-iminosugar-type glycosidase inhibitors. The disubstitution pattern at the exocyclic quaternary anomeric carbon, which bears simultaneously a hydroxymethyl and an amino group

susceptible of chemical modification, offers broad opportunities for the incorporation of non-glycone moieties. The optimization of the synthesis of a key synthetic precursor for APP synthesis, the subsequent transformation into a broad battery of mono- and diantennated APP derivatives and the assessment of the affinity and selectivity of the final compounds against a panel of commercial and human glycosidase enzymes is presented.

Results and discussion

Recently, a totally stereocontrolled *C*-Michael addition of a serine-equivalent *C*-nucleophile to tri-*O*-benzyl-2-nitro-D-galactal (nitrogalactal) was used as the key step to synthesize several pyrano[3,2-*b*]pyrrole structures, e.g. compound **1**, following reduction of the nitro group in the corresponding Michael adduct.[16] Interestingly, spirocycle **1** can be regarded as a protected form of the target APP derivatives (Scheme 1). To explore this channel, an optimized preparation of **1** in gram scale was a prerequisite. This goal was conveniently achieved by using platinized Raney Ni under an atmosphere of hydrogen[17] in the reduction step. The transient amino group reacted spontaneously with the methyl ester group to close the corresponding five-membered lactam ring with retention of the configuration at both stereocenters.

Compound **1** was next used as a precursor for the synthesis of a battery of APP derivatives. In a first step, concomitant hydrolysis of the acetal and cyclic carbamate groups, by treatment with 4 M aqueous HCl at 40 °C for 12 h and liberation of the resulting ammonium hydrochloride salt by the action of propylene oxide in ethanol at reflux, provided aminoalcohol **2** in good yield (71%). Acylation of **2** with palmitoyl chloride in the presence of triethylamine at room temperature for 12 h afforded the diantennated derivative **3** (70% yield), which was subsequently de-*O*-benzylated by hydrogenolysis in acidified methanol, using palladium on carbon as a catalyst, to give the unprotected APP **4** in only three steps and 62% overall yield (Scheme 2).
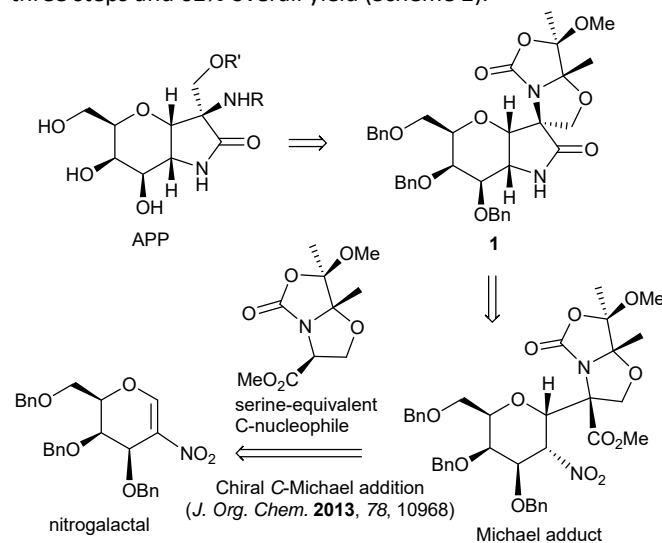

Scheme 1. Retrosynthetic analysis of APP derivatives.
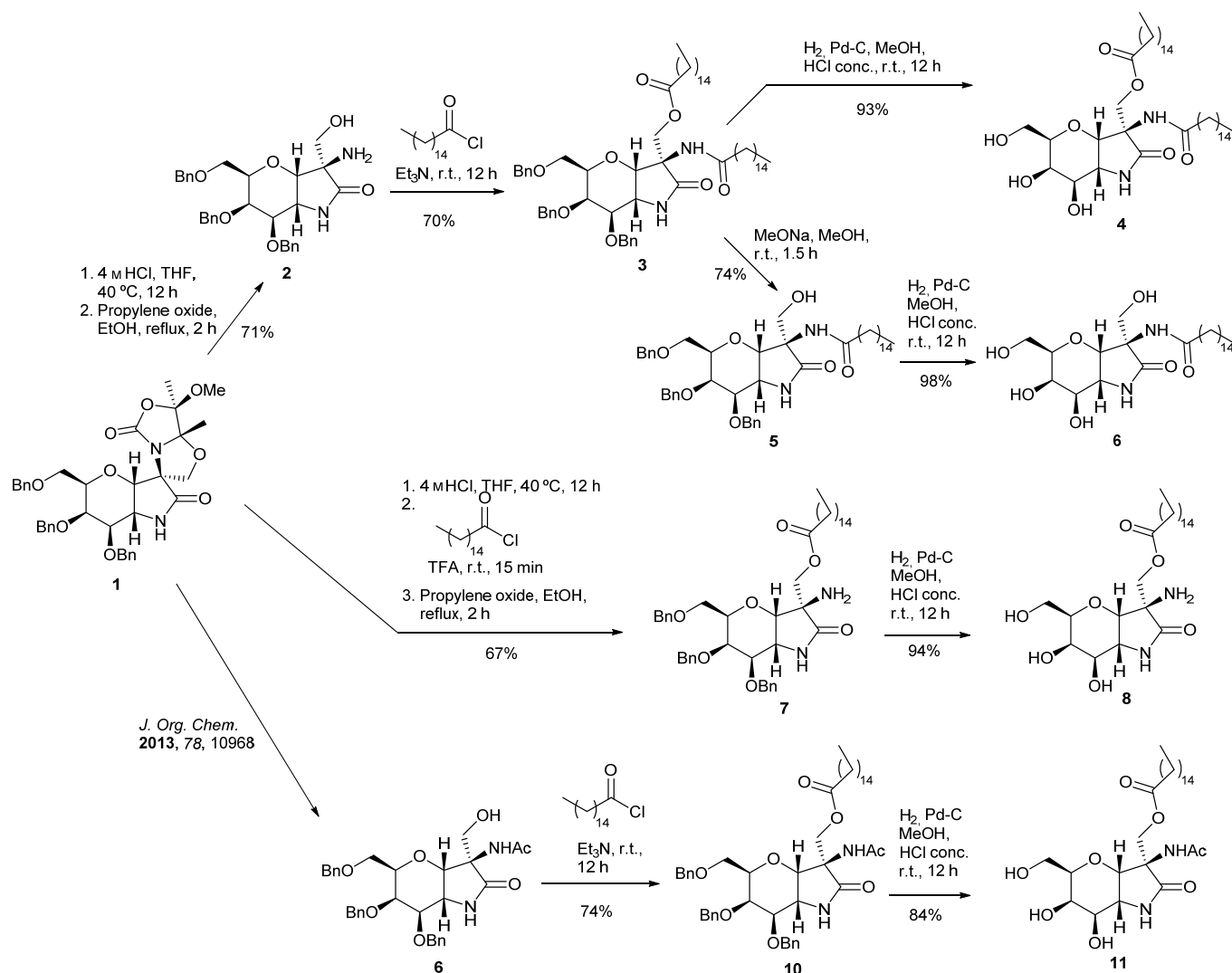
Scheme 2. Synthesis of target compounds **4**, **6**, **8** and **11** incorporating palmitoyl subtituents.

Compound **3** was also selectively de-*O*-acylated with sodium methoxide in methanol at pH 9 to give the corresponding alcohol **5**, which after debenzylation following the above procedure afforded the corresponding APP derivative **6**, bearing a single-chain *N*-palmitoyl aglycone moiety (Scheme 2). The homologous *O*-palmitoyl monoantennated adduct **8**, keeping a free amino group in the aglycone, was obtained from the pivotal precursor **1** after acid hydrolysis, in situ regioselective *O*-acylation with palmitoyl chloride, neutralization with propylene oxide in ethanol and hydrogenolysis of the tribenzyl ether **7** (62% overall yield; Scheme 2).

The possibility of conducting chemoselective acylation of the masked hydroxy and amino groups in **1** is further compatible with the incorporation of different substituent at each position. Thus, palmitoylation of the previously reported[16] *N*-acetylated compound **9** followed by hydrogenolysis of the resulting ester **10** led to the new APP derivative **11** (Scheme 2). Finally, compounds **13**-**15**, featuring *O*-acetyl and a free amino groups, free hydroxyl and amino groups, or free hydroxy and *N*-acetyl groups in the aglycone segment, respectively, were prepared as controls to assess the influence of the presence of the long hydrophobic segments in the glycosidase inhibitory properties of the APP derivatives (Scheme 3).
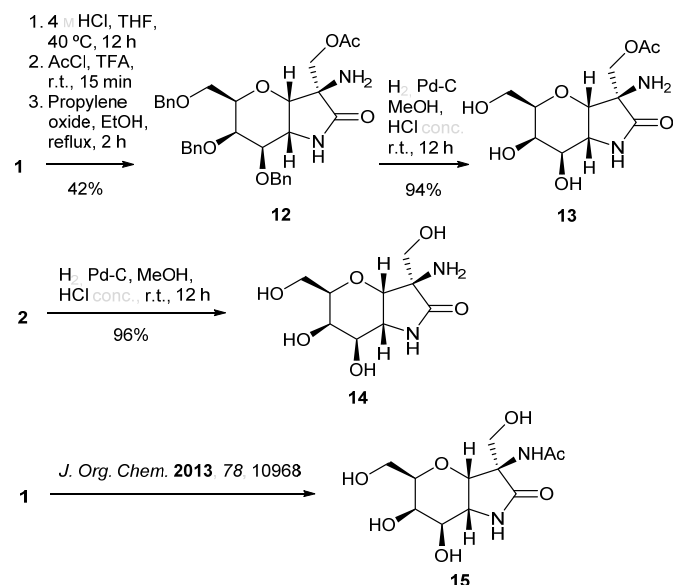
Scheme 3. Synthesis of target compounds **13**-**15** without large alkyl chains.

Compounds **4**, **6**, **8**, **11**, **13**, **14** and **15** were first evaluated against a panel of commercially available glycosidase enzymes including α-glucosidase (baker yeast), isomaltase (baker yeast), amyloglucosidase (*A. niger*), β-glucosidase/β-galactosidase (bovine liver), β-galactosidase (*E. coli*), α-galactosidase (green coffee), β-glucosidase (almonds), α-mannosidase (Jack bean), β-mannosidase (*H. pomatia*) and β-*N*-acetylglucosaminidase (human placenta, bovine kidney and Jack bean). Inhibition constants (K_i, μM) were determined from the slope of Lineweaver-Burk plots (see Figure 2 for a representative example) and are collected in Table 1. The data confirm that the presence of a hydrophobic segment in the aglycone (**4**, **6**, **8** and **11**) is necessary to elicit a significant activity, isomaltase (K_i, 13-80 μM) and β-glucosidase/β-galactosidase (K_i, 5.8-86 μM) being the more sensitive enzymes to APP inhibition. The presence of a bulky substituent at the nitrogen atom (**4** and **6**) is detrimental for α-glucosidase and α-galactosidase inhibition, whereas it has the opposite effect in the case of the mammalian β-glucosidase/β-galactosidase enzyme. Compound **8**, keeping a free amino group in the aglycone, additionally behaves as a μM inhibitor of α-glucosidase (K_i, 27 μM) and α-galactosidase (K_i, 67 μM).

Table 1. Glycosidase inhibitory activities (K_i, μM) for APP derivatives against commercial glycosidases.[a]
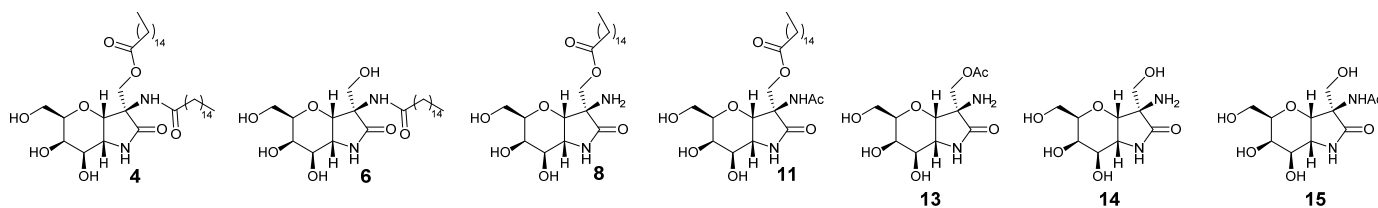
Enzymes	APP **4**	APP **6**	APP **8**	APP **11**	APP **13**	APP **14**	APP **15**
α-Glucosidase (Baker yeast) (pH 6.8)	225±25	124±13	27±3	244±28	n.i.	532±58	n.i.
Isomaltase (Baker yeast) (pH 6.8)	80±9	34±4	13±1	19±2	97±11	283±31	n.i.
β-Glucosidase/β-Galactosidase (Bovine liver) (pH 7.3)	10±1[b]	5.8±0.6[b]	86±9	9.2±8[b]	n.i.	n.i.	n.i.
α-Galactosidase (Green coffee) (pH 6.8)	657±72	515±56	67±7	n.i.	n.i.	n.i.	n.i.

[a] Inhibition, when detected, was competitive in all cases. No inhibition was detected for any of the compounds at concentrations up to 2 mM against amyloglucosidase (*A. niger*) (pH 5.5), β-glucosidase (almonds) (pH 7.3), β-galactosidase (*E. coli*) (pH 5.5), α-mannosidase (Jack bean) (pH 5.5), β-mannosidase (*H. pomatia*) (pH 5.5) and β-*N*-acetylglucosaminidase (human placenta, bovine kidney and Jack bean) (pH 5.5). [b] Determined from the slope of Lineweaver_Burk Plots and Double Reciprocal Analysis. n.i. not inhibition detected at concentrations up to 2 mM.

Inhibition of bovine liver β-glucosidase/β-galactosidase has been often used as a preliminary parameter to select candidates as pharmacological chaperones for mutant forms of human β-glucocerebrosidase (GCase) associated with Gaucher disease.[18] Although the predictive character of the data must be taken with care,[19] the significant inhibitory potential and high selectivity encountered for some of the new APP compounds in Table 1 against the commercial enzyme warranted further evaluation in this sense.

The concentrations of the di- (**4**) and monoantennated derivatives (**6**, **8** and **11**) given 50% inhibition of human GCase are collected in Table 2. The corresponding data for the non-glycomimetic type pharmacological chaperone Ambroxol® (ABX), currently in preclinical studies for the treatment of Gaucher disease,[20] are included for comparative purposes (Supporting Information).
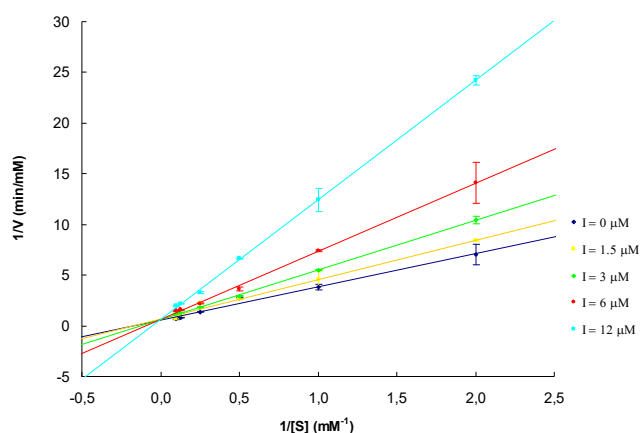
4 | *Org. Biomol. Chem.*, 2015, **00**, 1-11

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Figure 2. Lineweaver-Burk plot for K_i determination (5.8 μM) of APP **6** against β-galactosidase (bovine liver) (pH 7.3). We have performed two replicas of each experiment.

Table 2. Glycosidase inhibitory activities in vitro (K_i, μM) for APP derivatives (**4**, **6**, **8** and **11**) against human GCase.

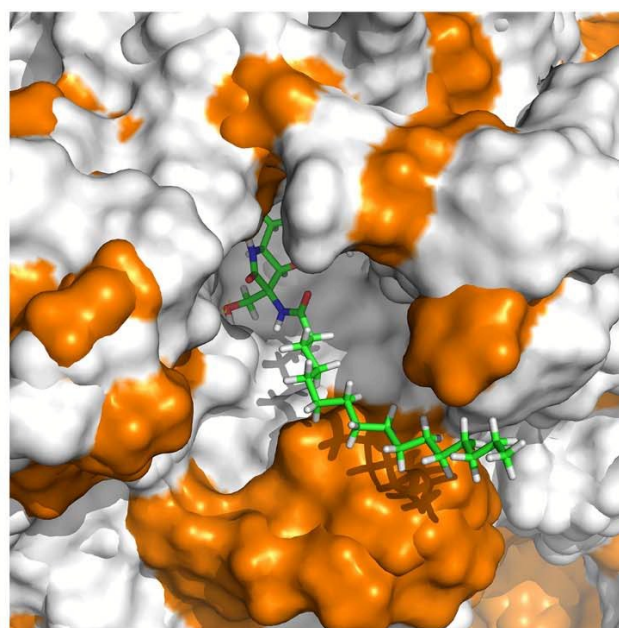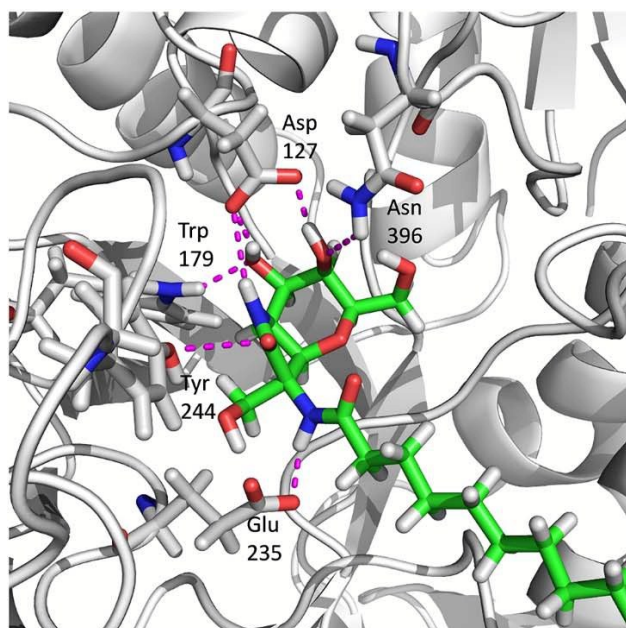| Compound	GCase pH 7	GCase pH 5
4	33±4	334±12
6	20±1	204±7
8	37±2	410±14
11	52±2	537±10

Figure 3. Representative frame obtained from the 50 ns MD simulation on showing the hydrogen bonds between the glycone unit of compound **6** and the residues in the binding site (left panel). The side chain of compound APP **6** is engaged in hydrophobic contacts with the surface of the enzyme (right panel). The hydrophobic residues of the enzyme are shown in orange.

The inhibition potency against GCase followed a slightly different trend as compared to that encountered for the commercial mammalian β-glucosidase/β-galactosidase. The presence of the *O*-acyl substituent in the aglycone seems to be detrimental for the human enzyme. Thus, compound **6** (K_i 20.3 μM at pH 7), missing this group, is an about 1.5- to 2.5-fold stronger inhibitor than the three other APP derivatives assayed K_i 33-52 μM at pH 7), though it is weaker than the reference compound ABX (Supporting Information). No inhibition of other lysosomal enzymes, such as α-glucosidase, α-galactosidase, β-galactosidase, and β-hexosaminidase, was observed. Most interestingly, an about one-order-of-

magnitude decrease in the GCase inhibition strength was observed at pH 5, a favourable feature for chaperone candidates.[21]

The ensemble of data in Tables 1 and 2 highlight the paramount importance of non-glycone interactions in the affinity and selectivity towards glycosidases. They also underline the need for implementing strategies allowing structural modifications with a relatively low synthetic cost to optimize such interactions. The superiority of compound **6** as GCase inhibitor within the APP series is remarkable. To get further structural information, we performed 50 ns MD simulations[22] on **6** in complex with the human enzyme (pdb code: 2WCG).[23] The results support that the pyranoid glycone

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Org. Biomol. Chem., 2015, **00**, 1-11 | **5**

moiety is engaged in hydrogen bond networking in a similar manner to that previously encountered for other glycomimetic-type competitive inhibitors (Figure 3). Indeed, although the putative substrate of GCase is D-glucosylceramide, it has been shown that D-*galacto* configured glycomimetics do also fit well in the catalytic site.[24] Noticeably, the calculations predict that the exocyclic amide nitrogen is involved in a hydrogen bond with the catalytic glutamic acid nucleophile Glu235. Protonation of this residue may be the responsible for the large difference observed in the inhibitory potency of APP compounds at neutral and acidic pH. Moreover, this hydrogen bond orients the aliphatic *N*-substituent towards the hydrophobic region at the entrance of the active site of the enzyme, reinforcing the stability of the complex.

All the four amphiphilic APP compounds were further assayed for their chaperoning capabilities using healthy and Gaucher fibroblasts from patients having the N370S/N370S, F213I/F213I or L444P/L444P GCase mutations. These variants are associated to the non-neuronopathic (type 1) and neuronopathic (types 2 and 3) phenotypes of Gaucher disease, respectively.[25] Only the first one, with the highest prevalence and located in the catalytic domain of GCase, is responsive to the enzyme replacement or substrate reduction therapies currently available. The F213I/F213I mutation is also located in the catalytic domain of the enzyme, while the L444P/L444P mutation is located in a noncatalytic domain, which makes it less prone to rescuing by pharmacological chaperones.[26] The cells were cultured for 5 days in the absence and in the presence of 2 or 20 μM concentrations of the APP compounds, then lysed and the GCase activity determined using 4-methylumbelliferyl β-D-glucopyranoside as a substrate. Enzyme activity variations relative to the control in the absence of any compound were thus monitored. Statistically significant activity enhancements were observed only for the N370S/N370S and F213I/F213I GCase mutants after treatment with compound **6** at 20 μM concentration, with relative increases of 1.3- and 1.5-folds, respectively, which approach those achieved with the reference compound ABX (1.5- and 1.8-fold, respectively, Figure 4). None of the APP derivatives did exhibit toxic effect on any of the normal or mutant cell lines assayed for 5 days incubation.
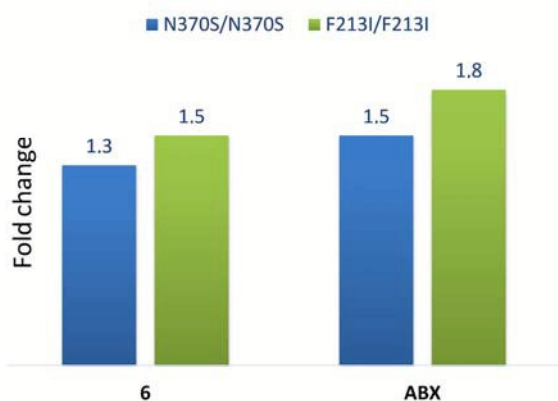

Figure 4. Comparative effect of compound **6** and ABX at 20 μM concentration on the activity enhancements for N370S/N370S (blue bars) and F213I/F213I (green bars) GCase mutants.

Conclusions

We have devised an efficient methodology for the synthesis of conformationally locked *C*-glycosides based on the 3-amino-3-hydroxymethylpyrano[3,2-*b*]pyrrol-2(1*H*)-one (APP) scaffold compatible with the incorporation of mono and multibranched aglycone moieties. Evaluation against a variety of glycosidase enzymes showed that amphiphilic derivatives bearing long hydrophobic substituents behaved as μM competitive inhibitors of bovine liver β-glucosidase/β-galactosidase. Most interestingly, they also behaved as selective inhibitors of human lysosomal β-glucosidase (β-glucocerebrosidase) at neutral pH, with a remarkable decrease in the inhibitory potency upon acidification (pH 5). The pH dependency of glucocerebrosidase binding makes this type of compounds good candidates as pharmacological chaperones for Gaucher disease. Indeed, compound **6**, having a palmitoylamido segment in the aglycone, was able to increase the activity of N370S/N370S and F213I/F213I glucocerebrosidase mutants in fibroblasts of Gaucher patients with an efficiency similar to that of the reference compound Ambroxol®. MD simulations support the existence of a hydrogen bond involving the amide proton of the chaperone and the carboxylate group of the catalytic glutamic acid residue Glu235 that favourably orients the palmitoyl chain towards a hydrophobic pocket in the enzyme. Since the protonation state of Glu235 changes in the pH 7-5 range, this structural feature offers further interesting opportunities for pH-dependent chaperone design. Research in that direction is currently sought in our laboratories.

Experimental

General Information

Solvents were purified according to standard procedures. All reactions were followed by thin layer chromatography (TLC) where practical, using silica gel 60 F254 fluorescence treated silica gel plates, which were visualized under UV light (250 nm). Column chromatography was performed using silica gel 60 (230–400 mesh). ^{1}H and ^{13}C NMR spectra were recorded on a 400 MHz spectrometer using CDCl$_3$, CD$_3$OD, or D$_2$O as the solvent; chemical shifts are reported in parts per million on the δ scale, and coupling constants are reported in Hertz. All of the resolved signals in the ^{1}H NMR spectra were assigned on the basis of coupling constants and ge-COSY and ge-HSQC experiments performed on the 400 MHz spectrometer. The results of these experiments were processed with MestReC and MestreNova software. Optical rotations were measured on a polarimeter from solutions in 1.0 dm cells of capacity 1.0 or 0.3 mL. Electrospray mass spectra were recorded on a micrOTOF spectrometer; accurate mass measurements were achieved by using sodium formate as an external reference. Copies of NMR spectra for all new compounds are provided in the Supporting Information.

(3*R*,3a'*S*,5'*R*,6'*R*,7*R*,7a*S*,7'*R*,7a'*S*)-6',7'-Bis(benzyloxy)-5'-((benzyloxy)methyl)-7-methoxy-7,7a-dimethylhexahydro-2*H*,5*H*,5'*H*-spiro[oxazolo[4,3-*b*]oxazole-3,3'-pyrano[3,2-*b*]pyrrole]-2',5(1'*H*)-dione 1

Compound **1** was obtained following the method previously published by us with slightly modification.[16] Raney Ni (2.00 g)

6 | *Org. Biomol. Chem.*, 2015, **00**, 1-11

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was suspended in H_2O (12 mL), and hexachloroplatinic acid (50 mg) and sodium hydroxide 20% (400 μL) were added under stirring. The mixture was heated at 50 °C. After the mixture was stirred for 2.5 h, sodium hydroxide 40% (6 mL) was added, keeping the stirring and the heating. After 1.5 h of stirring, a white cloud in the top of the flask appeared, which was removed by decantation; the resulting solution was then washed with warm water (3 × 15 mL) and ethanol (3 × 15 mL). The catalyst obtained was suspended in ethanol (10 mL) and prehydrogenated for 10 min. An ethanol/ethyl acetate solution (5:2, 7 mL) of Michael adduct (200 mg, 0.28 mmol) was added, and the mixture was stirred under molecular hydrogen at room temperature and atmospheric pressure for 5 h. The crude product was filtered, and the liquid phase was concentrated and purified by silica gel column chromatography (ethyl acetate/hexane, 7:3), to afford compound **1** (105 mg, 0.16 mmol, 57%) as a colorless oil. Physical data are agree with those reported in the literature.[16]

(3R,3aS,5R,6R,7R,7aS)-3-Amino-6,7-bis(benzyloxy)-5-(benzyloxymethyl)-3-(hydroxymethyl)hexahydropyrano[3,2-b]pyrrol-2(1H)-one 2

Compound **1** (105 mg, 0.163 mmol) was dissolved in THF (10 ml). A 4 M solution of HCl (3.6 mL) was then added and the mixture was heated up to 40 ºC for 12 h under stirring. The crude obtained after concentration was dissolved in absolute ethanol (5 mL). Propylene oxide (5 mL) was added and the mixture was heated to reflux for 2 h under stirring. Concentration and purification of the crude product by silica gel column chromatography (dichloromethane/methanol, 15:1) afforded compound **2** (60 mg, 0.116 mmol, 71%) as a colourless oil. $[\alpha]_D^{20}$ = +64.8 (c 1.0 in CHCl$_3$). HRMS ESI+ (m/z) = 519.2495 [M+H]$^+$; calculated for $C_{30}H_{35}N_2O_6^+$ = 519.2490. ^{1}H NMR (400 MHz, CDCl$_3$) δ = 3.52-3.61 (m, 2H, H^7, BnOCH^aH^b), 3.67 (d, 1H, J = 12.0 Hz, CH^{c}H^{d}OH), 3.71-3.83 (m, 2H, CH^{c}H^{d}OH, BnOCH^{a}H^b), 4.03-4.15 (m, 3H, H^5, H^6, H^{7a}), 4.24 (d, 1H, J = 4.8 Hz, H^{3a}), 4.48-4.59 (m, 4H, PhCH$_2$O, PhCH^{e}H^{f}O, PhCH^{g}H^{h}O), 4.68 (d, 1H, J = 11.6 Hz, PhCH^{e}H^{f}O, 4.84 (d, 1H, J = 11.6 Hz, PhCH^{g}H^{h}O), 6.84 (s, 1H, NH), 7.20-7.40 (m, 15H, Ph). ^{13}C NMR (100 MHz, CDCl$_3$) δ = 56.2 (C^{7a}), 62.3 (C^3), 65.1 (CH$_2$OH), 67.2 (BnOCH$_2$), 71.9 (PhCH^{e}H^{f}O), 72.5 (C^6), 73.6 (PhCH$_2$O), 74.5 (PhCH^{g}H^{h}O), 76.2 (C^5), 76.7 (C^{3a}), 81.3 (C^7), 127.8, 127.9, 128.0, 128.3, 128.5, 128.6, 128.8, 137.6, 137.9, 138.1 (Ph), 177.8 (CO).

General procedure for the synthesis of compounds 3 and 10

Compound **2** (0.086 mmol) or compound **9** (0.107 mmol) was dissolved in dichloromethane (2 mL) and palmitoyl chloride (80 μL, 0.26 mmol) was added. Et$_3$N (50 μL, 0.36 mmol) was then added and the mixture was stirred at room temperature for 12 h. A 0.5 M solution of HCl (2 mL) was added. The organic layer was separated and the aqueous one was washed with dichloromethane (3 × 2 mL). The organic phases were collected and dried with anhydrous Na$_2$SO$_4$. Concentration and purification of the crude product by silica gel column chromatography afforded compounds **3** or **10**, respectively.

[(3R,3aS,5R,6R,7R,7aS)-6,7-Bis(benzyloxy)-5-(benzyloxymethyl)-2-oxo-3-palmitamidooctahydropyrano[3,2-b]pyrrol-3-yl]methyl palmitate 3

Starting from compound **2** (45 mg, 0.086 mmol) and the corresponding purification by silica gel column chromatography (hexane/ethyl acetate, 65:35), compound **3** (60 mg, 0.06 mmol, 70%) was obtained as a colourless oil. $[\alpha]_D^{20}$ = +34.5 (c 1.0 in CHCl$_3$). HRMS ESI+ (m/z) = 995.7071 [M+H]$^+$; calculated for $C_{62}H_{95}N_2O_8^+$ = 995.7083. ^{1}H NMR (400 MHz, CDCl$_3$) δ = 0.88 (t, 6H, J = 6.5 Hz, 2CH$_3$), 1.19-1.35 (m, 48H, 2(CH$_2$)$_{12}$CH$_3$), 1.51-1.64 (m, 4H, 2CH$_2$(CH$_2$)$_{12}$CH$_3$), 2.18 (t, 2H, J = 7.6 Hz, NHCOCH$_2$), 2.24 (t, 2H, J = 7.6 Hz, OCOCH$_2$), 3.52-3.60 (m, 2H, H^7, BnOCH^aH^b), 3.63-3.70 (m, 1H, BnOCH^{a}H^b), 4.03-4.10 (m, 1H, H^5), 4.11-4.15 (m, 1H, H^6), 4.29-4.36 (m, 2H, H7a, CH^{c}H^{d}OCO), 4.42-4.54 (m, 4H, CH^{c}H^{d}OCO, PhCH$_2$O, PhCH^{e}H^{f}O), 4.58 (d, 1H, J = 11.6 Hz, PhCH^{g}H^{h}O), 4.66-4.72 (m, 2H, H^{3a}, PhCH^{e}H^{f}O), 4.88 (d, 1H, J = 11.6 Hz, PhCH^{g}H^{h}O), 6.06 (br s, 1H, NH), 6.32 (br s, 1H, NH), 7.22-7.40 (m, 15H, Ph). ^{13}C NMR (100 MHz, CDCl$_3$) δ = 14.2 (CH$_3$), 22.8, 24.9, 25.3, 29.3, 29.5, 29.6, 29.7, 29.8, 32.1 ((CH$_2$)$_{13}$CH$_3$), 34.2 (OCOCH$_2$), 36.2 (NHCOCH$_2$), 53.5 (C^3), 56.0 (C^{7a}), 63.1 (CH$_2$OCO), 67.8 (BnOCH$_2$), 71.5 (PhCH^{e}H^{f}O), 71.7 (C^6), 73.6 (PhCH$_2$O), 74.4 (PhCH^{g}H^{h}O), 75.9 (C^{3a}), 76.5 (C^5), 81.4 (C^7), 127.7, 127.8, 127.9, 128.2, 128.4, 128.6, 128.8, 137.6, 138.1, 138.4 (Ph), 172.4 (OCO), 173.2 (NHCO), 173.7 (NHCO).

[(3R,3aS,5R,6R,7R,7aS)-3-Acetamido-6,7-bis(benzyloxy)-5-(benzyloxymethyl)-2-oxooctahydropyrano[3,2-b]pyrrol-3-yl]methyl palmitate 10

Starting from compound **9** (60 mg, 0.107 mmol) and the corresponding purification by silica gel column chromatography (hexane/ethyl acetate, 35:65), compound **10** (63 mg, 0.079 mmol, 74%) was obtained as a colourless oil. $[\alpha]_D^{20}$ = +41.0 (c 1.0 in CHCl$_3$). HRMS ESI+ (m/z) = 799.4853 [M+H]$^+$; calculated for $C_{48}H_{67}N_2O_8^+$ = 799.4892. ^{1}H NMR (400 MHz, CDCl$_3$) δ = 0.88 (t, 3H, J = 6.7 Hz, CH$_3$), 1.21-1.30 (m, 24H, (CH$_2$)$_{12}$CH$_3$), 1.51-1.58 (m, 2H, CH$_2$(CH$_2$)$_{12}$CH$_3$), 1.98 (s, 3H, NHAc), 2.23 (t, 2H, J = 7.5 Hz, OCOCH$_2$), 3.53-3.59 (m, 2H, BnOCH^aH^b, H^7), 3.66 (dd, 1H, J = 9.5, 6.9 Hz, BnOCH^{a}H^b), 4.07 (dd, 1H, J = 11.1, 6.3 Hz, H^5), 4.13 (d, 1H, J = 4.2 Hz, H^6), 4.27-4.34 (m, 2H, H^{7a}, CH^{c}H^{d}OCO), 4.42-4.52 (m, 4H, CH^{c}H^{d}OCO, PhCH$_2$O, PhCH^{e}H^{f}O), 4.58 (d, 1H, J = 11.6, PhCH^{g}H^{h}O), 4.66-4.72 (m, 2H, PhCH^{e}H^{f}O, H^{3a}), 4.87 (d, 1H, J = 11.6 Hz, PhCH^{g}H^{h}O), 6.07-6.18 (m, 1H, NHAc), 6.38 (s, 1H, NHCO), 7.22-7.40 (m, 15H, Ph). ^{13}C NMR (100 MHz, CDCl$_3$) δ = 14.1 (CH$_3$), 23.1 (NHCOCH$_3$), 22.7, 24.8, 29.2, 29.3, 29.4, 29.5, 29.7, 31.9, 34.1 (CO(CH$_2$)$_{14}$CH$_3$), 55.9 (C^{7a}), 63.0 (CH$_2$OCO), 63.2 (C^3) 67.7 (BnOCH$_2$), 71.4 (PhCH^{e}H^{f}O), 71.6 (C^6), 73.5 (PhCH$_2$O), 74.3 (PhCH^{g}H^{h}O), 75.7 (C^{3a}), 76.4 (C^5), 81.2 (C^7), 127.7, 127.8, 127.9, 128.2, 128.3, 128.5, 128.7, 137.5, 138.0, 138.2 (Ph), 170.0 (OCO), 172.3 (NHCOCH$_3$), 173.6 (NHCO).

[(3R,3aS,5R,6R,7R,7aR)-6,7-Dihydroxy-5-(hydroxymethyl)-2-oxo-3-palmitamidooctahydropyrano[3,2-b]pyrrol-3-yl]methyl palmitate 4

Hydrogenolysis of a methanol solution (5 mL) of compound **3** (37 mg, 0.037 mmol) was held under atmospheric pressure and room temperature, using Pd-C (35 mg) and HCl conc. (3 drops) as a catalyst. The reaction was performed for 12 h and the catalyst was filtered over diatomaceous earth. The liquid phase was concentrated affording compound **4** (25 mg, 0.034 mmol, 93%) as a yellow oil. $[\alpha]_D^{20}$ = +20.6 (c 0.99 in methanol). HRMS ESI+ (m/z) = 725.5669 [M+H]$^+$; calculated for $C_{41}H_{77}N_2O_8^+$ = 725.5674. ^{1}H NMR (400 MHz, CD$_3$OD) δ = 0.89 (t, 6H, J = 6.5 Hz, 2CH$_3$), 1.24-1.35 (m, 48H, 2(CH$_2$)$_{12}$CH$_3$), 1.54-1.67 (m, 4H, 2CH$_2$(CH$_2$)$_{12}$CH$_3$), 2.23 (t, 2H, J = 6.7 Hz,

NHCOCH_2), 2.36 (t, 2H, J = 7.3 Hz, OCOCH_2), 3.70-3.78 (m, 3H, CH_2OH, H[7]), 3.79-3.85 (m,1H, H[5]), 3.91-4.02 (m, 2H, H[6], H[7a]), 4.31 (d, 1H, J = 10.7 Hz, CH^aH^{b}OCO), 4.45-4.52 (m, 1H, CH^{a}H^{b}OCO), 4.83 (m, 1H, H[3a]). ^{13}C NMR (100 MHz, CD$_3$OD) δ = 14.4 (CH$_3$), 23.7, 25.8, 26.8, 30.2, 30.4, 30.5, 30.6, 30.7, 30.8, 33.0 ((CH$_2$)$_{13}$CH$_3$), 34.9 (OCOCH_2), 36.7 (NHCOCH_2), 56.9 (C[7a]), 61.6 (CH$_2$OH), 62.6 (CH$_2$OCO), 63.1 (C[3]), 69.4 (C[6]), 73.3 (C[7]), 76.0 (C[3a]), 78.8 (C[5]), 174.7 (OCO), 175.7 (NHCO), 176.0 (NHCO).

N-[(3R,3aS,5R,6R,7R,7aS)-6,7-Bis(benzyloxy)-5-(benzyloxymethyl)-3-hydroxymethyl-2-oxooctahydropyrano[3,2-b]pyrrol-3-yl]palmitamide 5

A methanol solution of sodium methoxide 0.5 M (2 mL) was added to a methanol solution (4 mL) of the compound **3** (76 mg, 0.076 mmol). The mixture was stirred for 1.5 h and sulfonic acid resine Dowex® was then added. The liquid phase was filtered, concentrated and the residue was purified by silica gel column chromatography (hexane/ethyl acetate, 15:85) to afford compound **5** (40 mg, 0.053 mmol, 70%) as a colourless oil. $[\alpha]_D^{20}$ = +66.0 (c 1.0 in CHCl$_3$). HRMS ESI+ (m/z) = 757.4788 [M+H]$^+$; calculated for C$_{46}$H$_{65}$N$_2$O$_7^+$ = 757.4786. ^{1}H NMR (400 MHz, CDCl$_3$) δ = 0.88 (t, 3H, J = 6.7 Hz, CH$_3$), 1.22-1.33 (m, 24H, (CH$_2$)$_{12}$CH$_3$), 1.55-1.65 (m, 2H, CH_2(CH$_2$)$_{12}$CH$_3$), 2.20 (t, 2H, J = 7.6 Hz, NHCOCH_2), 3.48 (dd, 1H, J = 10.3, 4.7 Hz, BnOCH^aH^b), 3.56 (d, 1H, J = 8.1 Hz, H[7]) , 3.67-3.75 (m, 2H, BnOCH^{a}H^b, CH^{c}H^{d}OH), 3.98 (d, 1H, J = 12.0 Hz, CH^{c}H^{d}OH), 4.09 (d, 1H, J = 4.9 Hz, H[6]), 4.11-4.18 (m, 1H, H[5]), 4.38 (t, 1H, J = 7.2 Hz, H[7a]), 4.47 (d, 1H, J =11.9 Hz, PhCH^eH^{f}O), 4.49 (s, 2H, PhCH_2O), 4.55 (d, 1H, J = 11.8 Hz, PhCH^gH^{h}O), 4.61 (d, 1H, J = 6.3, H[3a]) 4.68 (d, 1H, J = 11.8 Hz, PhCH^{e}H^{f}O), 4.87 (d, 1H, J = 11.8 Hz, PhCH^{g}H^{h}O), 6.22 (br s, 1H, NH), 6.56 (br s, 1H, NH), 7.20-7.39 (m, 15H, Ph). ^{13}C NMR (100 MHz, CDCl$_3$) δ = 14.1 (CH$_3$), 22.7, 25.3, 29.2, 29.4, 29.5, 29.7, 32.0 ((CH$_2$)$_{13}$CH$_3$), 35.8 (NHCOCH_2), 56.3 (C[7a]), 63.5 (C[3]), 64.0 (CH$_2$OH), 67.4 (BnOCH_2), 71.5 (C[6]), 71.6 (PhCH^eH^{f}O), 73.5 (PhCH$_2$O), 74.2 (PhCH^gH^{h}O), 76.7 (C[5], C[3a]), 81.4 (C[7]), 127.6, 127.8, 127.9, 128.2, 128.4, 128.5, 128.7, 137.5, 137.8, 138.0 (Ph), 173.5 (NHCO), 173.9 (NHCO).

Deprotection of benzyloxy groups. General procedure for the synthesis of compounds 6, 8, 11, 13 and 14

Hydrogenolysis of a methanol solution (5 mL) of the corresponding benzyloxy-protected carbohydrate derivatives was held under atmospheric pressure and room temperature, using Pd-C (35 mg) and HCl conc. (3 drops) as a catalyst. The reaction was performed for 12 h and the catalyst was filtered over diatomaceous earth. The liquid phase was concentrated affording compound **6**, **8**, **11**, **13** or **14**.

N-[(3R,3aS,5R,6R,7R,7aR)-6,7-Dihydroxy-3,5-bis(hydroxymethyl)-2-oxooctahydropyrano[3,2-b]pyrrol-3-yl]palmitamide 6

Starting from compound **5** (35 mg, 0.046 mmol), compound **6** (22 mg, 0.045 mmol, 98%) was obtained as a colourless oil. $[\alpha]_D^{20}$ = +15.1 (c 1.0 in methanol). HRMS ESI+ (m/z) = 487.3386 [M+H]$^+$; calculated for C$_{25}$H$_{47}$N$_2$O$_7^+$ = 487.3378. ^{1}H NMR (400 MHz, CD$_3$OD) δ = 0.83 (t, 3H, J = 5.8 Hz, CH$_3$), 1.13-1.36 (m, 24H, (CH$_2$)$_{12}$CH$_3$), 1.49-1.62 (m, 2H, CH_2(CH$_2$)$_{12}$CH$_3$), 2.38 (t, 2H, J = 6.7 Hz, NHCOCH_2), 3.65-4.02 (m, 6H, CH_2OH, H[5], H[6], H[7], H[7a]), 4.39 (d, 1H, J = 11.3 Hz, C[3]CH^aH^{b}OH), 4.48 (d, 1H, J = 11.7 Hz, C[3]CH^{a}H^{b}OH), 4.74 (d, 1H, J = 5.3 Hz, H[3a]). ^{13}C NMR (100 MHz, CD$_3$OD) δ = 14.3 (CH$_3$), 23.5, 25.5, 30.0, 30.3, 30.4, 30.5, 30.6, 32.9 ((CH$_2$)$_{13}$CH$_3$), 34.4 (NHCOCH_2), 55.8 (C[7a]), 61.6 (C[3]CH$_2$OH), 61.9 (CH$_2$OH), 63.0 (C[3]), 69.2 (C[6]), 73.3 (C[7]), 74.6 (C[3a]), 79.0 (C[5]), 174.1 (2 NHCO).

[(3R,3aS,5R,6R,7R,7aR)-3-Amino-6,7-dihydroxy-5-(hydroxymethyl)-2-oxooctahydropyrano[3,2-b]pyrrol-3-yl]methyl palmitate 8

Starting from compound **7** (50 mg, 0.065 mmol), compound **8** (30 mg, 0.062 mmol, 94%) was obtained as a colourless oil. $[\alpha]_D^{20}$ = -3.9 (c 1.0 in methanol). HRMS ESI+ (m/z) = 487.3388 [M+H]$^+$; calculated for C$_{25}$H$_{47}$N$_2$O$_7^+$ = 487.3378. ^{1}H NMR (400 MHz, CD$_3$OD) δ = 0.89 (t, 3H, J = 6.4 Hz, CH$_3$), 1.22-1.39 (m, 24H, (CH$_2$)$_{12}$CH$_3$), 1.58-1.67 (m, 2H, CH_2(CH$_2$)$_{12}$CH$_3$), 2.43 (t, 2H, J = 7.4 Hz, OCOCH$_2$), 3.70-3.78 (m, 3H, CH_2OH, H[7]), 3.79-3.85 (m, 1H, H[5]), 3.93 (t, 1H, J = 7.6 Hz, H[7a]), 3.99 (s, 1H, H[6]), 4.44 (d, 1H, J = 12.1 Hz, CH^aH^{b}OCO), 4.53 (d, 1H, J = 11.9 Hz, CH^{a}H^{b}OCO), 4.77 (d, 1H, J = 7.2 Hz, H[3a]). ^{13}C NMR (100 MHz, CD$_3$OD) δ = 14.4 (CH$_3$), 23.7, 25.7, 30.1, 30.4, 30.6, 30.7, 30.8, 33.0 ((CH$_2$)$_{13}$CH$_3$), 34.5 (OCOCH_2), 55.9 (C[7a]), 61.7 (CH$_2$OCO), 62.0 (CH$_2$OH), 63.1 (C[3]), 69.3 (C[6]), 73.5 (C[7]), 74.8 (C[3a]), 79.2 (C[5]), 170.3 (OCO), 174.1 (NHCO).

[(3R,3aS,5R,6R,7R,7aR)-3-Acetamido-6,7-dihydroxy-5-(hydroxymethyl)-2-oxooctahydropyrano[3,2-b]pyrrol-3-yl]methyl palmitate 11

Starting from compound **10** (63 mg, 0.079 mmol), compound **11** (35 mg, 0.066 mmol, 84%) was obtained as a yellow oil. $[\alpha]_D^{20}$ = +22.7 (c 1.0 in H$_2$O). HRMS ESI+ (m/z) = 529.3493 [M+H]$^+$; calculated for C$_{27}$H$_{49}$N$_2$O$_8^+$ = 529.3483. ^{1}H NMR (400 MHz, CD$_3$OD) δ = 0.88 (t, 3H, J = 6.4 Hz, CH$_3$), 1.19-1.37 (m, 24H, (CH$_2$)$_{12}$CH$_3$), 1.53-1.66 (m, 2H, CH_2(CH$_2$)$_{12}$CH$_3$), 2.26-2.39 (m, 2H, OCOCH_2), 3.68-4.03 (m, 6H, CH_2OH, H[5], H[6], H[7], H[7a]), 4.25-4.35 (m, 1H, CH^aH^{b}OCO), 4.46-4.54 (m, 1H, CH^{a}H^{b}OCO), 4.75-4.85 (m, 1H, H[3a]). ^{13}C NMR (100 MHz, CD$_3$OD) δ = 14.3 (CH$_3$), 23.6, 25.8, 25.9, 30.1, 30.2, 30.3, 30.4, 30.5, 30.6, 30.7, 32.9 ((CH$_2$)$_{13}$CH$_3$), 34.7 (OCOCH_2), 56.9 (C[7a]), 61.6 (CH$_2$OH), 62.2 (CH$_2$OCO), 64.9 (C[3]), 69.3 (C[6]), 73.1 (C[7]), 75.9 (C[3a]), 78.7 (C[5]), 171.4 (OCO), 174.6 (NHCO), 176.0 (NHCO).

[(3R,3aS,5R,6R,7R,7aR)-3-Amino-6,7-dihydroxy-5-(hydroxymethyl)-2-oxooctahydropyrano[3,2-b]pyrrol-3-yl]methyl acetate 13

Starting from compound **12** (27 mg, 0.048 mmol), compound **13** (13 mg, 0.044 mmol, 94%) was obtained as a yellow oil. $[\alpha]_D^{20}$ = +42.3 (c 1.0 in H$_2$O). HRMS ESI+ (m/z) = 291.1188 [M+H]$^+$; calculated for C$_{11}$H$_{19}$N$_2$O$_7^+$ = 291.1187. ^{1}H NMR (400 MHz, D$_2$O) δ = 2.17 (s, 3H, CH$_3$), 3.69-3.84 (m, 2H, CH_2OH), 3.84-4.08 (m, 4H, H[5], H[6], H[7], H[7a]), 4.51 (d, 1H, J = 12.3 Hz, CH^aH^{b}OAc), 4.66 (d, 1H, J = 12.3 Hz, CH^{a}H^{b}OAc), 4.96 (d, 1H, J = 8.1 Hz, H[3a]). ^{13}C NMR (100 MHz, D$_2$O) δ = 20.1 (CH$_3$), 53.1 (C[7a]), 61.1 (CH$_2$OAc), 61.4 (CH$_2$OH), 61.8 (C[3]), 67.4 (C[6]), 71.8 (C[7]), 74.3 (C[3a]), 77.4 (C[5]), 169.7 (COAc), 172.6 (NHCO).

(3R,3aS,5R,6R,7R,7aR)-3-Amino-6,7-dihydroxy-3,5-bis(hydroxymethyl)hexahydropyrano[3,2-b]pyrrol-2(1H)-one 14

Starting from compound **2** (35 mg, 0.067 mmol), compound **14** (16 mg, 0.064 mmol, 96%) was obtained as a yellow oil. $[\alpha]_D^{20}$ = +32.6 (c 1.0 in H$_2$O). HRMS ESI+ (m/z) = 249.1087 [M+H]$^+$; calculated for C$_9$H$_{17}$N$_2$O$_6^+$ = 249.1081. ^{1}H NMR (400 MHz, D$_2$O) δ = 3.71-3.84 (m, 2H, CH_2OH), 3.90-4.16 (m, 6H, H[5], H[6], H[7], H[7a], C[3]CH_2OH), 4.96 (d, 1H, J = 5.6 Hz, H[3a]). ^{13}C NMR (100 MHz, D$_2$O) δ = 53.0 (C[7a]), 59.0 (C[3]CH$_2$OH), 61.4 (CH$_2$OH), 63.6 (C[3]), 67.4 (C[6]), 71.8 (C[7]), 73.8 (C[3a]), 77.3 (C[5]), 170.7 (NHCO).

General procedure for the synthesis of compounds 7 and 12

Compound **1** (0.12 mmol) was dissolved in THF (8 mL). A 4 M solution of HCl (2.5 mL) was then added and the mixture was heated up to 40 ºC for 12 h under stirring. The crude obtained after concentration was dissolved in TFA (4 mL) and palmitoyl chloride (for **7**) or acetyl chloride (for **12**) (1.2 mmol) was added. The mixture was stirred at room temperature for 15 min and absolute ethanol (10 mL) was added to quench the reaction. After concentration, the crude was dissolved in absolute ethanol (5 mL). Propylene oxide (5 mL) was added and the mixture was heated to reflux for 2 h under stirring. Concentration and purification of the crude product by silica gel column chromatography afforded compound **7** or **12**.

[(3R,3aS,5R,6R,7R,7aS)-3-Amino-6,7-bis(benzyloxy)-5-(benzyloxymethyl)-2-oxooctahydropyrano[3,2-b]pyrrol-3-yl]methyl palmitate 7

Starting from compound **1** (82 mg, 0.127 mmol) and the corresponding purification by silica gel column chromatography (hexane/ethyl acetate, 65:35), compound **7** (64 mg, 0.085 mmol, 67%) was obtained as a colourless oil. $[\alpha]_D^{20}$ = +52.9 (c 1.0 in CHCl$_3$). HRMS ESI+ (m/z) = 757.4788 [M+H]$^+$; calculated for C$_{46}$H$_{65}$N$_2$O$_7^+$ = 757.4786. ^{1}H NMR (400 MHz, CDCl$_3$) δ = 0.88 (t, 3H, J = 6.7 Hz, CH$_3$), 1.20-1.36 (m, 24H, (CH$_2$)$_{12}$CH$_3$), 1.54-1.64 (m, 2H, CH$_2$(CH$_2$)$_{12}$CH$_3$), 2.27 (t, 2H, J = 7.8 Hz, OCOCH$_2$), 3.55 (d, 1H, J = 5.3 Hz, H^7), 3.70 (d, 2H, J = 5.2 Hz, BnOCH$_2$) , 4.05-4.12 (m, 2H, H^{3a}, H^5), 4.14-4.20 (m, 2H, H^{7a}, H^6), 4.47 (d, 2H, J = 2.7 Hz, CH$_2$OCO), 4.49-4.61 (m, 4H, PhCH^{a}H^{b}O, PhCH^{c}H^{d}O, PhCH$_2$O), 4.69 (d, 1H, J = 11.8 Hz, PhCH^{a}H^{b}O), 4.81 (d, 1H, J = 11.5 Hz, PhCH^{c}H^{d}O), 6.57 (br s, 1H, NH), 7.24-7.38 (m, 15H, Ph). ^{13}C NMR (100 MHz, CDCl$_3$) δ = 14.2 (CH$_3$), 22.8, 26.1, 29.3, 29.5, 29.6, 29.7, 29.8, 32.0 ((CH$_2$)$_{13}$CH$_3$), 28.2 (NHCOCH$_2$), 57.3 (C^{7a}), 67.2 (BnOCH$_2$), 68.3 (CH$_2$OCO), 72.0 (PhCH^{a}H^{b}O), 73.0 (C^{3a}), 73.5 (PhCH$_2$O), 74.2 (PhCH^{c}H^{d}O), 74.6 (C^6), 76.2 (C^5), 78.7 (C^3), 80.1 (C^7), 127.8, 127.9, 128.2, 128.5, 128.6, 128.7, 137.7, 138.1, 138.2 (Ph), 170.9 (OCO), 175.5 (NHCO).

[(3R,3aS,5R,6R,7R,7aS)-3-Amino-6,7-bis(benzyloxy)-5-(benzyloxymethyl)-2-oxooctahydropyrano[3,2-b]pyrrol-3-yl]methyl acetate 12

Starting from compound **1** (73 mg, 0.113 mmol) and the corresponding purification by silica gel column chromatography (ethyl acetate), compound **12** (27 mg, 0.048 mmol, 42%) was obtained as a colourless oil. $[\alpha]_D^{20}$ = +76.7 (c 1.0 in CHCl$_3$). HRMS ESI+ (m/z) = 561.2590 [M+H]$^+$; calculated for C$_{32}$H$_{37}$N$_2$O$_7^+$ = 561.2595. ^{1}H NMR (400 MHz, CDCl$_3$) δ = 1.99 (s, 3H, CH$_3$), 3.54 (s, 1H, H^7), 3.67-3.73 (m, 2H, BnOCH$_2$), 4.06-4.12 (m, 2H, H^{3a}, H^5), 4.15-4.20 (m, 2H, H^6, H^{7a}), 4.41-4.49 (m, 2H, CH$_2$OAc), 4.49-4.61 (m, 4H, PhCH^{a}H^{b}O, PhCH^{c}H^{d}O, PhCH$_2$O), 4.70 (d, 1H, J = 11.8 Hz, PhCH^{a}H^{b}O), 4.82 (d, 1H, J = 11.4 Hz, PhCH^{c}H^{d}O), 6.51 (s, 1H, NH), 7.24-7.39 (m, 15H, Ph). ^{13}C NMR (100 MHz, CDCl$_3$) δ = 14.0 (CH$_3$), 57.4 (C^{7a}), 67.2 (BnOCH$_2$), 68.6 (CH$_2$OAc), 71.9 (PhCH^{a}H^{b}O), 72.9 (C^{3a}), 73.5 (PhCH$_2$O), 74.3 (PhCH^{c}H^{d}O), 74.6 (C^6), 76.2 (C^5), 78.8 (C^3), 80.2 (C^7), 127.8, 127.9, 128.3, 128.5, 128.6, 128.8, 137.7, 138.1, 138.2 (Ph), 167.8 (COCH$_3$), 175.5 (NHCO).

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, or o-nitrophenyl β-D-galactopyranoside (for β-galactosidases), in the presence of compounds **4**, **6**, **8**, **11**, **13**, **14** and **15**. Each essay was performed in phosphate buffer 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 °C, and the reaction was quenched by addition of 1 M Na$_2$CO$_3$. 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 nm. 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.[27] Briefly, cells were scraped in ice-cold 0.1% Triton X-100 in water. After centrifugation (6,000 rpm for 15 min at 4 ºC) to remove insoluble materials, protein concentrations were determined using Protein Assay Rapid Kit (Wako, Tokyo, Japan). The lysates were incubated at 37 ºC with the corresponding 4-methylumbelliferyl β-D-glycopyranoside solution in 0.1 M citrate buffer (pH 4.5). The liberated 4-methylumbelliferone was measured with a fluorescence plate reader (excitation 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 the tested derivatives.

Measurement of purified human GCase inhibition activities in vitro. Purified human GCase, obtained from Genzyme (Genzyme Japan, Tokyo, Japan) was mixed with the indicated concentrations of each chaperone. The incubation and the measurement of 4-methylumbelliferyl β-D-glycopyranoside substrates in the mixture was performed at pH 7 as described. In a parallel series of experiments, the inhibition activities were determined at pH 5 (citrate buffer).

Cell culture and GCase activity enhancement assay. Human skin fibroblasts from a healthy and three Gaucher disease patients (with N370S/N370S, F213I/F213I 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 the candidates or DMSO alone (as a control) for 5 days and harvested by scraping.[26] Cytotoxicity of the compounds was monitored by measuring the lactate dehydrogenase activities in the cultured supernatants (LDH assay kit, Wako, Tokyo, Japan).

Unrestrained Molecular Dynamics simulations. All molecular dynamics simulations were carried out on the Finis-Terrae cluster of the *Centro de Supercomputación de Galicia* (CESGA), Spain. Starting geometries for complexes were generated from the available data deposited in the Protein Data Bank (pdb code: 2WCG for β-galactosidase) and modified accordingly. Each model complex was immersed in a 10 Å-sided cube with pre-equilibrated TIP3P water molecules. To equilibrate the system, we followed a protocol consisting of 10 steps. Firstly, only the water molecules are minimized, and then heated to 300 K. The water box, together with Na$^+$ ions, was then

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Org. Biomol. Chem., 2015, **00**, 1-11 | 9

minimized, followed by a short MD simulation. At this point, the system was minimized in the four following steps with positional restraints imposed on the solute, decreasing the force constant step by step from 20 to 5 kcal·mol^{-1}. Finally, a non-restraint minimization was performed. The production dynamics simulations were accomplished at a constant temperature of 300 K (by applying the Berendsen coupling algorithm for the temperature scaling) and constant pressure (1 bar). Particle Mesh Ewald Method, to introduce long-range electrostatic effects, and periodic boundary conditions were also used. SHAKE algorithm for hydrogen atoms, which allows using a 2 fs time step, was also employed. Finally, a 9Å cutoff was applied for the Lennard-Jones interactions. MD simulations were performed with the sander module of AMBER 11.0 (parm99 force field), which was implemented with GAFF parameters[28] to accurately simulate the corresponding ligands. A simulation length of 100 ns and the trajectory coordinates were saved each 0.5 ps.

Acknowledgements

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