

## Cyclodextrin-mediated crystallization of acid $\beta$ -glucosidase in complex with amphiphilic bicyclic nojirimycin analogues†

Boris Brumshtein,<sup>a</sup> Matilde Aguilar-Moncayo,<sup>b</sup> Juan M. Benito,<sup>c</sup> José M. García Fernández,<sup>c</sup> Israel Silman,<sup>d</sup> Yoseph Shaaltiel,<sup>e</sup> David Aviezer,<sup>e</sup> Joel L. Sussman,<sup>a</sup> Anthony H. Futerman<sup>f</sup> and Carmen Ortiz Mellet<sup>\*b</sup>

Received 5th February 2011, Accepted 7th March 2011

DOI: 10.1039/c1ob05200d

Cyclodextrin-based host–guest chemistry has been exploited to facilitate co-crystallization of recombinant human acid  $\beta$ -glucosidase ( $\beta$ -glucocerebrosidase, GlcCerase) with amphiphilic bicyclic nojirimycin analogues of the  $sp^2$ -iminosugar type. Attempts to co-crystallize GlcCerase with 5-*N*,6-*O*-[*N'*-(*n*-octyl)iminomethylidene]nojirimycin (NOI-NJ) or with 5-*N*,6-*S*-[*N'*-(*n*-octyl)iminomethylidene]-6-thionojirimycin (6S-NOI-NJ), two potent inhibitors of the enzyme with promising pharmacological chaperone activity for several Gaucher disease-associated mutations, were unsuccessful probably due to the formation of aggregates that increase the heterogeneity of the sample and affect nucleation and growth of crystals. Cyclomaltoheptaose ( $\beta$ -cyclodextrin,  $\beta$ CD) efficiently captures NOI-NJ and 6S-NOI-NJ in aqueous media to form inclusion complexes in which the lipophilic tail is accommodated in the hydrophobic cavity of the cyclooligosaccharide. The dissociation constant of the complex of the amphiphilic  $sp^2$ -iminosugars with  $\beta$ CD is two orders of magnitude higher than that of the corresponding complex with GlcCerase, allowing the efficient transfer of the inhibitor from the  $\beta$ CD cavity to the GlcCerase active site. Enzyme–inhibitor complexes suitable for X-ray analysis were thus grown in the presence of  $\beta$ CD. In contrast to what was previously observed for the complex of GlcCerase with the more basic derivative, 6-amino-6-deoxy-5-*N*,6-*N*-[*N'*-(*n*-octyl)iminomethylidene]nojirimycin (6N-NOI-NJ), the  $\beta$ -anomers of both NOI-NJ and 6S-NOI-NJ were seen in the active site, even though the  $\alpha$ -anomer was exclusively detected both in aqueous solution and in the corresponding  $\beta$ CD: $sp^2$ -iminosugar complexes. Our results further suggest that cyclodextrin derivatives might serve as suitable delivery systems of amphiphilic glycosidase inhibitors in a biomedical context.

### Introduction

Acid  $\beta$ -glucosidase ( $\beta$ -glucocerebrosidase, GlcCerase; EC 3.2.1.45) is responsible for the lysosomal hydrolysis of glucosylceramide (GlcCer), an intermediate in the catabolism of

glycosphingolipids.<sup>1</sup> Mutations in the gene encoding for this enzyme result in Gaucher disease (GD), the lysosomal storage disorder with the highest prevalence.<sup>2–4</sup> Some mutations in GlcCerase cause misfolding of the protein in the endoplasmic reticulum, and as a result, less GlcCerase is transported to the lysosome leading to progressive accumulation of GlcCer, particularly in macrophages. The most common clinical manifestations are hepatosplenomegaly, anemia, bone lesions, respiratory failure and, in the most severe cases, central nervous system (CNS) dysfunction.<sup>5,6</sup>

The main treatment for Gaucher disease is enzyme replacement therapy (ERT), in which defective GlcCerase is supplemented by exogenous enzyme, administered to patients intravenously, usually every two weeks.<sup>7–10</sup> Three sources of recombinant GlcCerase are available, namely imiglucerase (Cerezyme®), a recombinant GlcCerase expressed in Chinese hamster ovary (CHO) cells,<sup>11</sup> velaglucerase  $\alpha$  (VPRIV®), produced in a human cell line using gene-activation technologies,<sup>12</sup> and taliglucerase  $\alpha$ , a recombinant GlcCerase expressed in transgenic carrot cells (prGCD).<sup>13</sup>

The iminosugar glycosidase inhibitor *N*-(*n*-butyl)-1-deoxynojirimycin (NB-DNJ; miglustat, Zavesca™) is used

<sup>a</sup>Department of Structural Biology, Weizmann Institute of Science, Rehovot, 76100, Israel

<sup>b</sup>Departamento de Química Orgánica, Facultad de Química, Universidad de Sevilla, C/Profesor García González 1, 41012, Sevilla, Spain. E-mail: mellet@us.es; Fax: +34 954624960; Tel: +34 954559806

<sup>c</sup>Instituto de Investigaciones Químicas, CSIC – Universidad de Sevilla, Américo Vespucio 49, Isla de la Cartuja, 41092, Sevilla, Spain

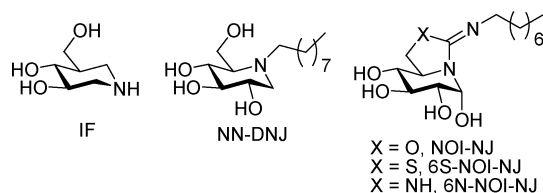
<sup>d</sup>Department of Neurobiology, Weizmann Institute of Science, Rehovot, 76100, Israel

<sup>e</sup>Protalix Biotherapeutics, 2 Snunit Street, Science Park, Carmiel, 20100, Israel

<sup>f</sup>Department of Biological Chemistry, Weizmann Institute of Science, Rehovot, 76100, Israel

† Electronic supplementary information (ESI) available: Experimental procedures for  $K_i$  and tensiometry determinations, titration plots showing the least-square fitted curves, Lineweaver–Burk plots and NMR spectra of NOI-NJ and 6S-NOI-NJ in the absence and in the presence of  $\beta$ -cyclodextrin. See DOI: 10.1039/c1ob05200d

in a second treatment modality known as substrate reduction therapy (SRT), in which partial inhibition of glycosphingolipid biosynthesis results in decreased accumulation of glycosphingolipids, including GlcCer.<sup>14–17</sup> A third therapeutic paradigm has been recently introduced, which advocates use of active site-directed competitive inhibitors to restore enzyme activity in the lysosome, namely pharmacological chaperone therapy.<sup>18,19</sup> This counterintuitive strategy relies on the ability of such inhibitors to promote the correct folding of mutant forms of lysosomal enzymes and to stabilize them, as they pass through the secretory pathway.<sup>20–22</sup> Based on this rationale, the 1-azasugar glycomimetic, isofagomine (IF; Fig. 1) was considered a candidate for the treatment of type 1 (non-neuronopathic) GD,<sup>23–25</sup> although it was discarded after phase II trials due to the absence of appreciable clinical effects.<sup>26</sup>



**Fig. 1** Chemical structures of isofagomine (IF), *N*-(*n*-nonyl)-1-deoxynojirimycin (NN-DNJ) and the bicyclic nojirimycin derivatives NOI-NJ, 6S-NOI-NJ and 6N-NOI-NJ.

Isofagomine is not specific for GlcCer; furthermore, its high hydrophilicity may hinder its movement across biological membranes. To overcome these problems, a battery of amphiphilic glycomimetics,<sup>27–31</sup> as well as non-carbohydrate chaperones,<sup>32–34</sup> are currently under preclinical investigation. However, simultaneous inhibition of acid  $\alpha$ -glucosidase remains a major problem for many of these compounds. This is the case, for instance, for the iminosugar-type chaperone *N*-(*n*-nonyl)-1-deoxynojirimycin (NN-DNJ), which binds to lysosomal  $\alpha$ - and  $\beta$ -glucosidases with a very similar potency.<sup>18</sup> Recently, we developed a new family of glycosidase inhibitors, namely  $sp^2$ -iminosugars, in which the anomeric selectivity can be finely tuned for  $\alpha$ - or  $\beta$ -glucosidases by the incorporation of pseudoaglyconic substituents onto a bicyclic nojirimycin skeleton.<sup>35–38</sup> Thus, the isourea, isothioureia and guanidine derivatives, 5-*N*,6-*O*-[*N'*-(*n*-octyl)iminomethylidene]nojirimycin (NOI-NJ), 5-*N*,6-*S*-[*N'*-(*n*-octyl)iminomethylidene]-6-thionojirimycin (6S-NOI-NJ) and 6-amino-6-deoxy-5-*N*,6-*N*-[*N'*-(*n*-octyl)iminomethylidene]nojirimycin (6N-NOI-NJ), behaved as selective inhibitors of GlcCer among lysosomal glycosidases, and exhibited remarkable chaperone effects for several GD mutations (Fig. 1).<sup>39</sup> Their capacity to cross biological membranes, as well as the rescuing mechanism, were confirmed by use of fluorescently labeled derivatives.<sup>40,41</sup> A comparative study of NOI-NJ, 6S-NOI-NJ, 6N-NOI-NJ and NN-DNJ revealed further significant differences in both their inhibitory and chaperone activities, with the  $sp^2$ -iminosugars being more potent chemical chaperones than NN-DNJ for mutations associated with neuronopathic forms of GD.<sup>39</sup> Correlation of the observed differences with structural information at the atomic level is a prerequisite for improving molecular design so as to produce pharmacological chaperones for GD that cope with individual mutations. In this context, a systematic study of the X-ray structures of complexes

of  $sp^2$ -iminosugar with  $\beta$ -glucosidases, including human acid  $\beta$ -glucosidase, is currently underway.<sup>42–44</sup>

The crystal structures of imiglucerase,<sup>45</sup> prGCD<sup>13,46</sup> and velaglucerase  $\alpha$ <sup>47</sup> have been determined, demonstrating that GlcCer consists of a ( $\beta/\alpha$ )<sub>8</sub> (TIM) barrel containing the catalytic residues, and two additional non-catalytic domains. A number of crystal structures of complexes or conjugates of small molecules with GlcCer have also been solved,<sup>48–51</sup> including that of the NN-DNJ/prGCD complex.<sup>50</sup> Initial attempts to obtain crystal complexes of GlcCer with amphiphilic  $sp^2$ -iminosugars were hampered by difficulties in co-crystallization due to aggregation of the amphiphiles. This difficulty was only overcome using the positively charged guanidine derivative 6N-NOI-NJ.<sup>44</sup> Crystallization of proteins in the presence of amphiphiles is, in general, a serious methodological problem, since micelle formation increases the heterogeneity of the sample, thus affecting both nucleation and crystal growth.<sup>52–54</sup>

Sequestration of amphiphilic molecules by exploiting host-guest chemistry with cyclodextrins (CDs), cyclic oligosaccharides composed of  $\alpha(1\rightarrow4)$ -linked D-glucopyranosyl units, has already been demonstrated to be a powerful tool for the crystallization of membrane proteins.<sup>55</sup> We speculated that using CD inclusion complexes of amphiphilic  $sp^2$ -iminosugars would provide a homogeneous crystallization environment in which transfer of the glycomimetic from the CD cavity to the active site of GlcCer might increase the accessible surface area of the protein for crystal contacts. Here we demonstrate that the sequestration of NOI-NJ and 6S-NOI-NJ by cyclomaltoheptaose ( $\beta$ CD) provides a means of obtaining crystalline complexes of both these sugars with prGCD, permitting subsequent resolution of their X-ray structures. The new crystal structures are analyzed, and compared to those of the corresponding complexes with NN-DNJ and 6N-NOI-NJ.

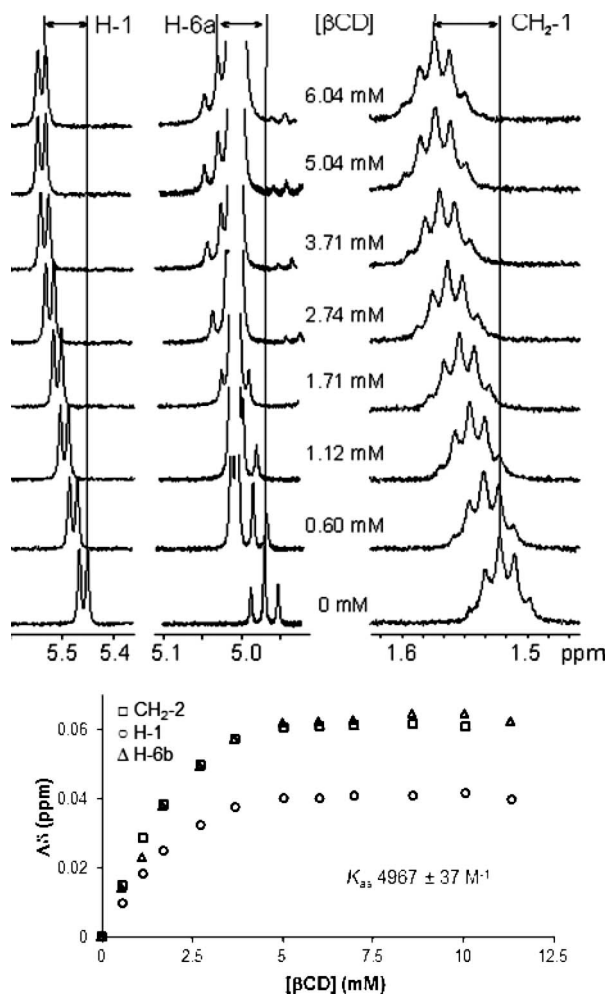
## Results and discussion

### Complexation of amphiphilic $sp^2$ -iminosugars by $\beta$ CD

The rigid truncated cone shape of  $\beta$ CD creates a cavity inside the ring that binds hydrophobic or amphiphilic guest molecules, but is unlikely to interact with hydrophobic patches of folded proteins. To test whether crystallization of amphiphilic inhibitor:GlcCer complexes could be controlled by transfer of the inhibitor from a preformed complex with  $\beta$ CD, we first characterized the effect of inclusion of the  $sp^2$ -iminosugar in  $\beta$ CD on the homogeneity of the samples as well as on the thermodynamics of the process. 0.1 M aqueous solutions of NOI-NJ and 6S-NOI-NJ were prepared as stock solutions. 10 mM solutions prepared in 0.02% (w/v) sodium azide/7% (v/v) ethanol/10 mM citric acid/sodium citrate buffer (pH 5.5), served as standard solutions for screening of crystallization conditions. In both these solutions aggregation was visually observed, but it disappeared upon addition of  $\beta$ CD, dramatically improving the homogeneity of the samples.

<sup>1</sup>H NMR titration experiments performed in pure D<sub>2</sub>O at 298 K revealed significant changes in the spectrum as the  $\beta$ CD concentration increased. The changes in chemical shift were particularly pronounced for the pseudoanomeric H-1 proton of the bicyclic  $sp^2$ -iminosugar skeleton and for the methylene protons

of the octyl chain (Fig. 2 and 3). These observations are consistent with the formation of inclusion complexes in which the aliphatic chain is deeply inserted into the  $\beta$ CD cavity, with the hydroxyl groups remaining in contact with bulk water. The corresponding binding isotherms fitted well to a 1:1 host:guest model (Fig. 2 and 3), with association constants ( $K_{as}$ ) of  $4967 \pm 37$  and  $2113 \pm 24 \text{ M}^{-1}$  for the NOI-NJ: $\beta$ CD and 6S-NOI-NJ: $\beta$ CD complexes, respectively.

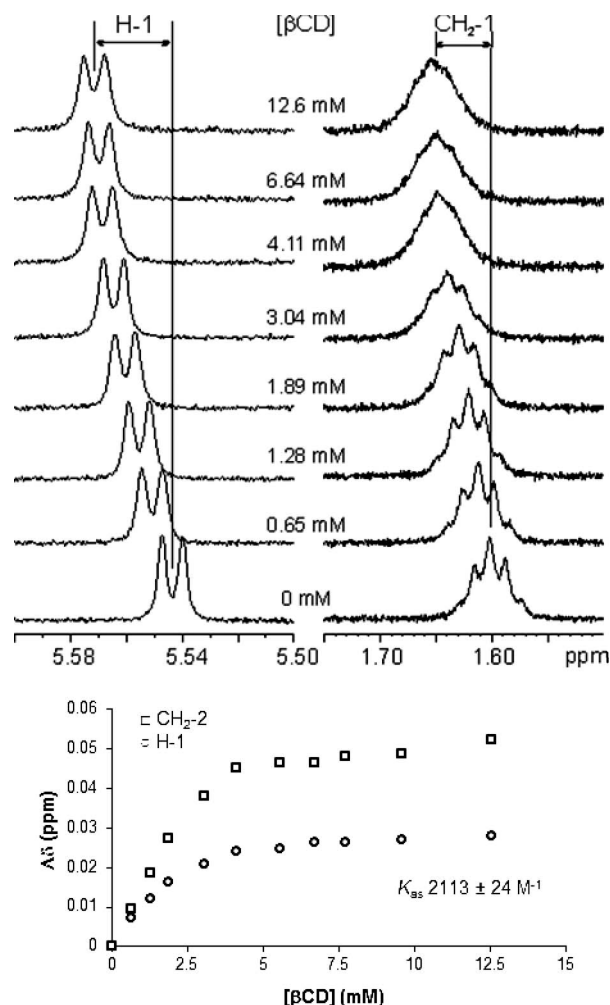


**Fig. 2** Stacked  $^1\text{H}$  NMR spectra (500 MHz, 298 K,  $\text{D}_2\text{O}$ ; selected regions) of NOI-NJ (2.64 mM) obtained in the presence of increasing concentrations of  $\beta$ CD (upper panel). Titration plots obtained from the corresponding changes in chemical shift (lower panel).

The measured  $K_{as}$  values imply that in equimolar mixtures of the iminosugar and  $\beta$ CD at 10 mM, the concentration of free inhibitor is in the range 1–2  $\mu\text{M}$ , well below the critical aggregation concentration of  $\sim 50 \mu\text{M}$ . To ensure full complexation, a 50% molar excess of cyclodextrin was used in the crystallization studies, meaning that the concentration of free inhibitor in the solution would be  $\sim 1 \text{ nM}$ .

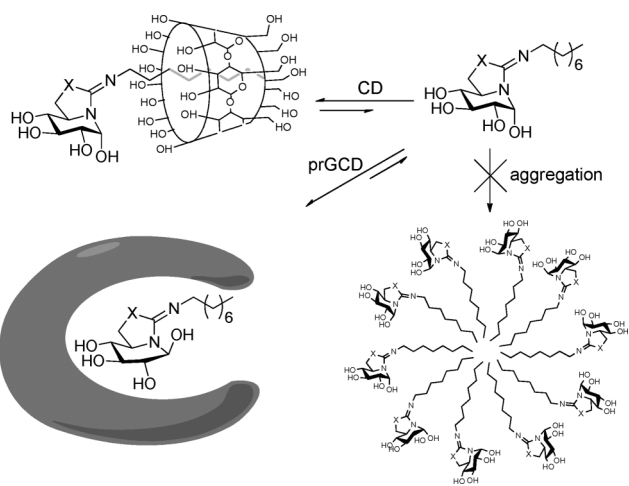
### prGCD inhibition profiling

The inhibition constants for inhibition of prGCD by NOI-NJ and 6S-NOI-NJ were found to be  $0.11 \pm 0.02$  and  $0.95 \pm 0.05 \mu\text{M}$  at pH 7.3, and  $8.7 \pm 0.3$  and  $3.5 \pm 0.2 \mu\text{M}$  at pH 5.5,



**Fig. 3** Stacked  $^1\text{H}$  NMR spectra (500 MHz, 298 K,  $\text{D}_2\text{O}$ ; selected regions) of 6S-NOI-NJ (2.41 mM) recorded in the presence of increasing concentrations of  $\beta$ CD (upper panel), and titration plots obtained from the corresponding changes in chemical shift (lower panel).

respectively. Inhibition by both inhibitors was competitive. Less potent inhibition by  $\text{sp}^2$ -iminosugars at acidic rather than neutral pH was previously reported for other  $\beta$ -glucosidases, including wild-type and mutant human GlcCerase. It has been argued that this lower potency is partially responsible for their effectiveness in rescuing misfolded GlcCerase at the endoplasmic reticulum, in facilitating trafficking of the enzyme to the Golgi apparatus, and in liberating the enzyme within the lysosome in the presence of excess substrate.<sup>39,41</sup> In any event, these dissociation constants are approximately two orders of magnitude lower than the dissociation constants for the respective complexes of the  $\text{sp}^2$ -iminosugars with  $\beta$ CD. Since the kinetics of interaction of the inhibitors with both  $\beta$ CD and with the enzyme are fast, as shown by the NMR titration data and the competitive inhibition experiments, transfer of the amphiphilic inhibitor from the  $\beta$ CD cavity to the active site of the enzyme should occur (Scheme 1) without accumulation of a high concentration of free amphiphile, thus precluding aggregation that might impede crystallization (Scheme 1). This assumption was further supported by prGCD inhibition experiments using 1:1.5 mixtures of the  $\text{sp}^2$ -iminosugars and  $\beta$ CD. The  $K_i$  values thus



**Scheme 1** Schematic representation of the dynamic processes in a solution containing the amphiphilic  $sp^2$ -iminosugars NOI-NJ or 6S-NOI-NJ,  $\beta$ CD and prGCD. The enzyme-bound inhibitor is depicted in the  $\beta$ -configuration whereas the  $\alpha$ -anomer is the only species detected in the free iminosugar as well as in the corresponding  $\beta$ CD complex.

obtained were not significantly different from those obtained in the absence of  $\beta$ CD (not shown).

### Structure determination and analysis of the crystal structures of the crystalline $sp^2$ -iminosugar/prGCD complexes obtained in the presence of $\beta$ CD

To validate the approach outlined above, crystallization of prGCD was attempted in the presence of a 1 : 1.5 mixture of either NOI-NJ or 6S-NOI-NJ and  $\beta$ CD under otherwise identical conditions to those previously used for crystallization of the 6N-NOI-NJ/prGCD complex.<sup>44</sup> Whereas no crystals could be obtained in the absence of  $\beta$ CD, use of the molecular host allowed us to obtain diffracting crystals in both cases. The enzyme:inhibitor complexes crystallized in the  $P2_1$  space group (Table 1), with two protein molecules in the asymmetric unit. The packing and unit cell dimensions were similar to those reported for the crystal structure of 6N-NOI-NJ/prGCD (PDB code 2WCG).<sup>44</sup>

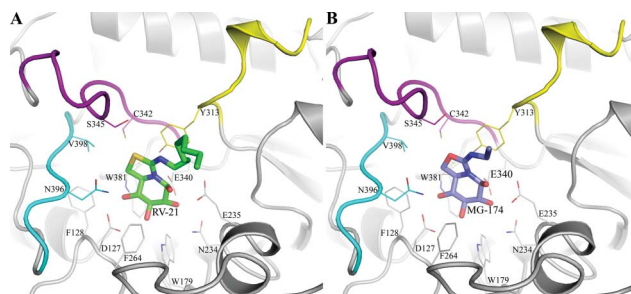
Superimposition of the structures of the new complexes (Fig. 4) on that of 6N-NOI-NJ/prGCD revealed very small rmsds (Fig. 5), indicating that complexation did not generate any global conformational changes in crystal structures. Difference maps revealed positive electron density in the prGCD active site that could be fitted by the bound inhibitors. Although we cannot rigorously exclude the possibility, it seems unlikely that the bicyclic polyhydroxylated core of the  $sp^2$ -iminosugars could bind to the active site while the hydrophobic chain, essential for strong binding, remained enveloped by the hydrophilic and relatively bulky cyclooligosaccharide. Indeed, no electron density could be assigned to  $\beta$ CD, confirming that it was unlikely to have remained associated with the bound inhibitor in the inhibitor/enzyme complex. We thus attribute the fact that diffracting crystals were obtained to the role of  $\beta$ CD in preventing aggregate formation rather than to binding of an iminosugar/ $\beta$ CD complex to the enzyme.

In the crystal structures of the two new complexes, the inhibitors were bound in the same orientation, although slight differences in the conformations of their aliphatic moieties were detected. In the

**Table 1** Data processing and refinement statistics for the complexes of 6S-NOI-NJ and NOI-NJ with prGCD

	6S-NOI-NJ	NOI-NJ
ESRF beamline	ID23eh1	ID23eh2
Resolution, Å	19.8–2.31 (2.37–2.31) <sup>a</sup>	29.9–2.66 (2.72–2.66) <sup>a</sup>
Space group	$P2_1$	$P2_1$
Unit cell parameters	$a = 68.1$ Å $b = 96.7$ Å $c = 83.0$ Å $\alpha = \gamma = 90^\circ$ $\beta = 102.9^\circ$	$a = 68.0$ Å $b = 96.6$ Å $c = 83.2$ Å $\alpha = \gamma = 90^\circ$ $\beta = 103.7^\circ$
$R_{\text{sym}}$ (%)	15.0 (44.7) <sup>a</sup>	17.5 (54.9) <sup>a</sup>
Mean $I/\sigma(I)$	33.6 (9.6) <sup>a</sup>	12.2 (3.0) <sup>a</sup>
Completeness (%)	96.5 (86.4) <sup>a</sup>	99.7 (99.4) <sup>a</sup>
Redundancy	7.2 (6.2) <sup>a</sup>	7.7 (6.3) <sup>a</sup>
No. unique reflections	42 172	28 378
$R_{\text{work}}$	15.0	15.0
$R_{\text{free}}$	21.0	21.9
RMSD bonds (Å)	0.022	0.016
RMSD angles (°)	1.861	1.581
No. of refined atoms:		
Protein	7803	7721
Carbohydrates	98	98
Solvent & ions	483	316
Ligands	39	48
Ramachandran outliers (%)	0.3	0.5
PDB code	2XWE	2XWD

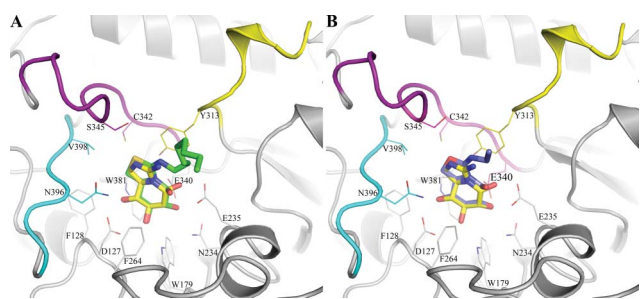
<sup>a</sup> Highest resolution shell.



**Fig. 4** Binding of 6S-NOI-NJ (A) and NOI-NJ (B) in the prGCD active site. The aliphatic tail of NOI-NJ in the crystal structure displayed several possible alternative conformations; therefore, only the two first carbon atoms were modeled. The isothiourae derivative, 6S-NOI-NJ, is shown in green, and the isourea analogue NOI-NJ, is in blue. Residues 341–349 (loop 1) are shown in green, residues 393–399 (loop 2) in cyan, and residues 312–319 (loop 3) in yellow. Active-site residues within 4 Å of the inhibitor are displayed as sticks.

case of the 6S-NOI-NJ complex, the aliphatic tail of the inhibitor was seen in only one of the two molecules in the asymmetric unit, and was therefore not modeled. In the NOI-NJ/prGCD complex, the inhibitor could be modeled in a unique conformation in one molecule, while in the other molecule of the asymmetric unit an electron density for the aliphatic tail of the inhibitor was ambiguous. It showed possible alternative conformations; therefore, only the first two carbon atoms of the octyl chain were modeled. As in previous cases, we did not observe significant alterations in the structure of the protein due to binding of the inhibitors.

The orientations of competitive inhibitors, such as NN-DNJ, are strongly dependent on the hydrogen bond network within the GlcCerase active site.<sup>50</sup> Like NN-DNJ, the non-anomeric



**Fig. 5** Overlays of the active site regions of the crystal structures of the 6S-NOI-NJ/prGCD complex and the NOI-NJ/prGCD complex on that of the previously reported 6N-NOI-NJ/prGCD complex. RMSD of 6S-NOI-NJ/prGCD and 6N-NOI-NJ/prGCD = 0.23 Å (A). RMSD of NOI-NJ/prGCD (B) and 6N-NOI-NJ/prGCD = 0.16 Å (B). 6S-NOI-NJ is displayed in green, NOI-NJ in blue, and 6N-NOI-NJ in yellow. Residues 341–349 (loop 1) are shown in purple, residues 393–399 (loop 2) in cyan, and residues 312–319 (loop 3) in yellow. Active-site residues within 4 Å of the inhibitors are displayed as sticks.

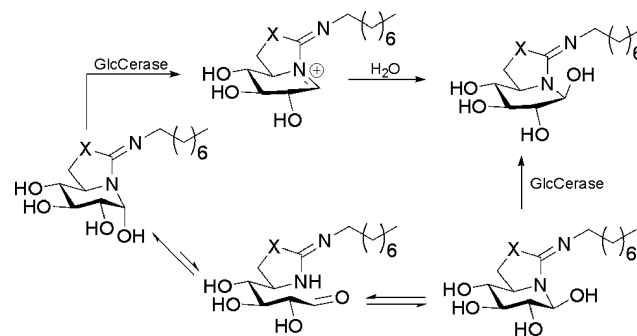
hydroxyl groups on the six-membered piperidine ring of NOI-NJ and 6S-NOI-NJ show stereochemical similarity, in terms of their orientations, to the glucose moiety of GlcCer. Superimposition of the corresponding structures reveals an almost total match in this region (Fig. 5). As already mentioned, in both the NOI-NJ and the 6S-NOI-NJ complexes, the electron density of the aliphatic tails is not well defined. However, based on the orientation imposed by the rigid bicyclic core, and on comparison with the structures of complexes of the enzyme with other amphiphilic inhibitors,<sup>50</sup> we infer that the aliphatic tail should be oriented towards the entrance to the active site. This entrance is confined by three loops in GlcCer, <sup>13,45–47</sup> and only one set of conformations of these loops has been observed so far in complexes with competitive inhibitors.<sup>50,51</sup>

Superimposition of the X-ray structures of NOI-NJ:prGCD or 6S-NOI-NJ:prGCD on that of 6N-NOI-NJ:prGCD revealed analogous conformational features, with C-1 slightly above the plane formed by C-2, C-3, C-5 and N-5 in a distorted <sup>4</sup>E arrangement (Fig. 5). The scenario is similar to that encountered for other reducing sp<sup>2</sup>-iminosugars having relatively flexible bicyclic frameworks in solution,<sup>56</sup> and also to that found<sup>43</sup> for NOI-NJ, 6S-NOI-NJ and their analogues with a D-galacto configuration in the active site of the β-glucosidase from *Thermotoga maritima* (TmGH1),<sup>57</sup> an enzyme belonging to family 1 and clan A, the same clan as human β-glucocerebrosidase, according to the CaZy classification.<sup>58</sup>

An important structural difference between the 1-deoxyiminosugar, NN-DNJ, and the sp<sup>2</sup>-iminosugars employed in this study is the presence in the latter of a pseudoanomeric hydroxyl group. Almost exclusively the α-anomer is detected in aqueous solutions of the free inhibitors (α : β ratio > 95 : 5) as well as in the corresponding inclusion complexes with βCD, with the pseudoanomeric OH group in axial orientation, as can be seen from the low coupling constant values between protons H-1 and H-2 ( $J_{1,2} = 3.8–4.0$  Hz) in the <sup>1</sup>H NMR spectra. This situation is in agreement with the strong contribution of orbital interactions involving the sp<sup>2</sup>-hybridized bridgehead nitrogen atom to the generalized anomeric effect.<sup>35–38</sup> However, in the crystal structures of both the NOI-NJ complex and the 6S-NOI-NJ complex, the

β-anomer, with the opposite configuration at C-1, was seen. This is in striking contrast to that previously observed for the guanidine analogue 6N-NOI-NJ in the 6N-NOI-NJ:prGCD complex.<sup>44</sup> In this case the anomeric OH retains its original α-like configuration, but changes from the axial to the equatorial disposition after distortion of the <sup>4</sup>C<sub>1</sub> ground state conformation of the six-membered ring.

The inversion of the pseudoanomeric configuration of NOI-NJ and 6S-NOI-NJ on going from the solution to the active site of GlcCer is intriguing. It was previously assumed that β-glucosidases could capture the “matching” β-anomer from an aqueous solution of the reducing sp<sup>2</sup>-iminosugar, even if it was almost undetectable by NMR techniques in the unbound state.<sup>43</sup> Anomerization would then proceed through the open chain *aldehyde* form of the inhibitor. However, it is very unlikely that this would occur in the βCD cavity. Taking into account the detection limit of proton NMR, the ratio of α to β-anomer in the unbound state must be at least 100 : 1, meaning that the concentration of the latter would, at best, be in the pM range in the presence of a 50% excess of βCD. Moreover, while sp<sup>2</sup>-iminosugar:βCD inclusion complex dissociation and binding of the inhibitor to the enzyme have fast dynamics, anomerization of reducing sugars is typically a relatively slow process. It is thus conceivable that epimerization may occur at the active site of the enzyme, presumably through an azacarbenium-type intermediate (Scheme 2). After distortion of the chair conformation of the piperidine ring, addition of a quasi-axially oriented incoming water molecule is stereoelectronically favored. Formally, the process can be seen as the glycosylation of a water molecule catalyzed by a β-glucosidase.

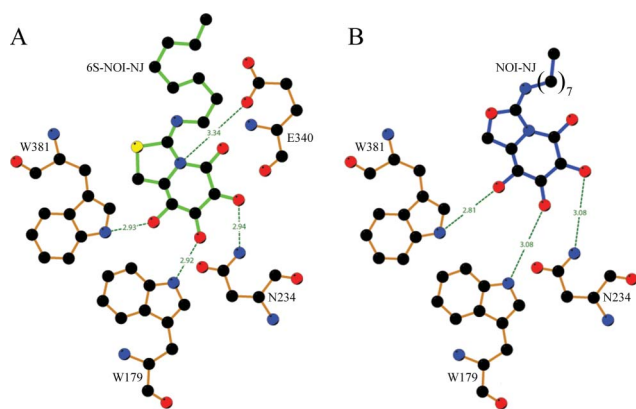


**Scheme 2** Possible mechanisms for the α-to-β anomerization reaction of the sp<sup>2</sup>-iminosugars NOI-NJ and 6S-NOI-NJ in the bulk aqueous medium (bottom) or in the active site of GlcCer (top).

Irrespective of the mechanism, the data presented here emphasize that reducing sp<sup>2</sup>-iminosugar glucomimetics can strongly bind to the active site of GlcCer in either the α- or β-anomeric configuration. The bicyclic structure is critical for the β-anomeric selectivity, but the nature of the endocyclic non-bridgehead heteroatom at the five-membered ring is irrelevant for binding (Fig. 6), suggesting that modification at this position might be useful in inhibitor/chaperone optimization strategies.

## Conclusions

Our data demonstrate that amphiphilic glycosidase inhibitors form inclusion complexes with βCD from which the inhibitor can be transferred to the active site of the enzyme while avoiding the



**Fig. 6** Hydrogen bonding of 6S-NOI-NJ (A) and NOI-NJ (B) in the active site of prGCD. Color coding for atoms: black-carbon, red-oxygen, blue-nitrogen, yellow-sulfur.

formation of aggregates. The approach should not be limited to study of complexes of  $sp^2$ -iminosugars with GlcCerase. Various types of amphiphilic ligands bearing alkyl chains or other hydrophobic moieties may be anticipated to form complexes with  $\beta$ CD with association constants in the appropriate range for host-guest mediated crystallization of the corresponding complexes with putative protein receptors and enzymes. Since CDs are inexpensive and widely available, this strategy could be readily incorporated into crystallization screenings involving amphiphilic compounds as ligands or inhibitors.

In this study, concentration-controlled delivery of the amphiphilic  $sp^2$ -iminosugars, using  $\beta$ CD as the host, led to nucleation and growth of crystals of the corresponding inhibitor/enzyme complex. The anomeric configuration of the two glycomimetics was  $\beta$  in their complexes with prGCD whereas it was almost exclusively  $\alpha$  in the unbound state as well as in the inclusion complex with  $\beta$ CD. Whether epimerization is driven by selection of the “matching”  $\beta$ -anomer from the solution or occurs after initial binding of the “wrong”  $\alpha$ -anomer, and involves a water molecule in a glycosylation-like step, remains to be clarified. Nevertheless, the data presented show that it is possible to design selective GlcCerase inhibitors with both matching and mismatching chirality at the pseudoanomeric position. Our results further suggest that cyclodextrin derivatives might serve as suitable delivery systems of amphiphilic glycosidase inhibitors in a biomedical context.

## Experimental section

### Materials and methods

The amphiphilic  $sp^2$ -iminosugars, NOI-NJ and 6S-NOI-NJ, were synthesized as previously reported<sup>41,43</sup> and their purity was established by spectroscopy and by combustion analysis. Commercial  $\beta$ -cyclodextrin (Roquette), containing about 11 mol of water per mol of  $\beta$ CD, was dried before use by heating at 80 °C under vacuum in the presence of  $P_2O_5$ , until constant weight. For the crystallization experiments, homogeneous mixtures of the corresponding iminosugar and  $\beta$ CD at a ratio of 1 : 1.5 were obtained by freeze-drying water solutions of both components at this same molar ratio. Purified prGCD was produced as described.<sup>46</sup>

### NMR titration experiments

Association constants ( $K_{as}$ ) were determined in  $D_2O$  at 298 K by measuring the proton chemical shift variations in the  $^1H$  NMR spectra (500 MHz) of solutions of the corresponding iminosugar, in the presence of increasing amounts of  $\beta$ CD. In a typical titration experiment, a solution of NOI-NJ (2.64 mM) or 6S-NOI-NJ (2.41 mM) in  $D_2O$  was prepared, a 500  $\mu$ L aliquot was transferred to a 5-mm NMR tube, and the initial NMR spectrum was recorded. A solution (30.22–33.48 mM) of  $\beta$ CD in the previous iminosugar solution (NOI-NJ at 2.64 mM or 6S-NOI-NJ at 2.41 mM in  $D_2O$ ) was prepared in order to maintain the guest concentration constant throughout the titration experiment. 10  $\mu$ L aliquots of this solution were sequentially added to the iminosugar solution and the corresponding NMR spectra recorded until 90–100% complexation of the guest had been achieved. The chemical shifts of the diagnostic signals obtained at 10–11 different host-guest concentration ratios were used in an iterative least-squares fitting procedure.<sup>59</sup>

### Crystallization and X-ray data collection

prGCD was diluted in crystallization buffer (10 mM citric acid/sodium citrate buffer, pH 5.5, 7% (v/v) ethanol and 0.02% (w/v)  $NaN_3$ ), washed three times, and concentrated to 4–5 mg  $ml^{-1}$  employing a Centricon<sup>®</sup> device, using a filter with a cut-off size of 30 kDa. The corresponding NOI-NJ/ $\beta$ CD or 6S-NOI-NJ/ $\beta$ CD complex, containing a 50% excess of  $\beta$ CD, prepared as described above, was dissolved in water to yield a stock solution with a 0.1 M concentration of the iminosugar, and subsequently added to prGCD to a final concentration of 10 mM. prGCD was co-crystallized with the inhibitors using the micro-batch technique under Al’s oil (1 : 1 v/v silicone and paraffin oils). The protein, together with the inhibitor and crystallization solutions, was dispensed into hydrophobic Vapor Batch crystallization plates under oil, such that the final solution of each crystallization drop contained a 1 : 1 ratio of the protein solution and of the crystallization solution. The crystallization solution contained 0.2 M  $(NH_4)_2SO_4$ , 0.1 M Tris pH 6.5, and 25% (w/v) PEG3350. Crystals were cryo-protected with a mixture of 80% crystallization solution and 20% (v/v) ethylene glycol solutions prior to cryo-cooling in liquid  $N_2$ . Diffraction was measured on the ID23eh1 and ID23eh2 beamlines at the ESRF synchrotron (Grenoble, France). Images were indexed with the HKL2000 software package and scaled with SCALEPACK.<sup>60</sup> The structure was solved *via* the molecular replacement method, using the crystal structure of prGCD<sup>13,46</sup> (PDB code 2V3F) as a starting model, and refined with Refmac<sup>561</sup> (Table 1). Model manipulation and water editing were performed using Coot graphics software.<sup>62</sup> Images were created with PyMol (www.pymol.org) and LIGPLOT.<sup>63</sup> Structures and structure factors were deposited in the PDB (PDB codes 2XWD and 2XWE).

### Acknowledgements

This study was supported by the Spanish Ministerio de Ciencia e Innovación (contract numbers CTQ2007-61180/PPQ, SAF2010-15670 and CTQ2010-15848), the Fundación Ramón Areces, the Junta de Andalucía (Project P08-FQM-03711), the European

Regional Development Fund, the EC 6th Framework Programme ('SPINE2-COMPLEXES' Project, Contract 03122, and 'TeachSG' Project, Contract ISSG-CT-2007-037198), the Bruce Rosen, William Singer, Divadol, and Neuman Foundations, and the Benozio Center for Neuroscience. J. L. S. is the Morton and Gladys Pickman Professor of Structural Biology, and A. H. F. is the Joseph Meyerhoff Professor of Biochemistry at the Weizmann Institute of Science. M. A.-M. was a FPU fellow.

## Notes and references

- 1 S. Kornfeld and I. Mellman, *Annu. Rev. Cell Biol.*, 1989, **5**, 483.
- 2 E. Beutler and G. A. Grabowski, in *The Metabolic and Molecular Bases of Inherited Disease*, ed. C. R. Scriver, W. S. Sly, B. Childs and A. L. Beaudet, McGraw-Hill, New York, 2001, pp. 2641–2670.
- 3 *Gaucher Disease*, ed. A. H. Futerman and A. Zimran, CRC Press, Boca Raton, 2006.
- 4 T. D. Butters, *Curr. Opin. Chem. Biol.*, 2007, **11**, 412.
- 5 A. Ballabio and V. Gieselmann, *Biochim. Biophys. Acta*, 2009, **1793**, 684.
- 6 A. H. Futerman and G. van Meer, *Nat. Rev. Mol. Cell Biol.*, 2004, **5**, 554.
- 7 N. W. Barton, R. O. Brady, J. M. Dambrosia, A. M. Di Bisceglie, S. H. Doppelt, S. C. Hill, H. J. Mankin, G. J. Murray, R. I. Parker and C. E. Argoff, *N. Engl. J. Med.*, 1991, **324**, 1464.
- 8 G. A. Grabowski, N. Leslie and R. Wenstrup, *Blood Rev.*, 1998, **12**, 115.
- 9 A. R. Sawkar, W. D'Haese and J. W. Kelly, *Cell. Mol. Life Sci.*, 2006, **63**, 1179.
- 10 R. O. Brady, *Annu. Rev. Med.*, 2006, **57**, 283.
- 11 N. J. Weinreb, *Expert Opin. Pharmacother.*, 2008, **9**, 1987.
- 12 A. Zimran, G. Altarescu, M. Phillips, D. Attias, M. Jmoudiak, M. Deeb, N. Wang, K. Bhirangi, G. M. Cohn and D. Elstein, *Blood*, 2010, **115**, 4651.
- 13 D. Aviezer, E. Brill-Almon, Y. Shaaltiel, S. Hashmueli, D. Bartfeld, S. Mizrahi, Y. Liberman, A. Freeman, A. Zimran and E. A. Galun, *PLoS One*, 2009, **4**, 1.
- 14 F. M. Platt, G. R. Neises, R. A. Dwek and T. D. Butters, *J. Biol. Chem.*, 1994, **269**, 8362.
- 15 T. Cox, R. Lachmann, C. Hollak, J. Aerts, S. van Weely, M. Hrebicek, F. Platt, T. Butters, R. Dwek, C. Moyses, I. Gow, D. Elstein and A. Zimran, *Lancet*, 2000, **355**, 1481.
- 16 G. M. Pastores, D. Elstein, M. Hrebicek and A. Zimran, *Clin. Ther.*, 2007, **29**, 1645.
- 17 F. M. Platt and M. Jeyakumar, *Acta Paediatr.*, 2008, **97**(Suppl. issue S497), 88.
- 18 A. R. Sawkar, W. C. Cheng, E. Beutler, C.-H. Wong, W. E. Balch and J. W. Kelly, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 15428.
- 19 H. Lin, Y. Sugimoto, Y. Ohsaki, H. Ninomiya, A. Oka, M. Taniguchi, H. Ida, Y. Eto, S. Ogawa, Y. Matsuzaki, M. Sawa, T. Inoue, K. Higaki, E. Nanba, K. Ohno and Y. Suzuki, *Biochim. Biophys. Acta*, 2004, **1689**, 219.
- 20 J.-Q. Fan, *Trends Pharmacol. Sci.*, 2003, **24**, 355.
- 21 Z. Q. Yu, A. R. Sawkar and J. W. Kelly, *FEBS J.*, 2007, **274**, 4944.
- 22 G. Parenti, *EMBO Mol. Med.*, 2009, **1**, 268.
- 23 R. A. Steet, S. Chung, B. Wustman, A. Powe, H. Do and S. A. Kornfeld, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 13813.
- 24 H. H. Chang, N. Asano, S. Ishii, Y. Ichikawa and J.-Q. Fan, *FEBS J.*, 2006, **273**, 4082.
- 25 R. Steet, S. Chung, W. S. Lee, C. W. Pine, H. Do and S. Kornfeld, *Biochem. Pharmacol.*, 2007, **73**, 1376.
- 26 (a) R. Khanna, E. R. Benjamin, L. Pellegrino, A. Schilling, B. A. Rigat, R. Soska, H. Nafar, B. E. Ranes, J.-F. Feng, Y. Lun, A.-C. Powe, D. J. Palling, B. A. Wustman, R. Schiffmann, D. J. Mahuran, D. J. Lockhart and K. J. Valenzano, *FEBS J.*, 2010, **277**, 1618; (b) J. M. Benito, J. M. García Fernández and C. Ortiz Mellet, *Expert Opin. Ther. Patents*, 2011, **21**, DOI: 10.1517/13543776.2011.569162, in press; see also Amicus Therapeutics webpage, available from: [www.amicustherapeutics.com/clinicaltrials](http://www.amicustherapeutics.com/clinicaltrials) (last accessed 1st March 2011).
- 27 L. Diaz, J. Bujons, J. Casas, A. Llebaria and A. Delgado, *J. Med. Chem.*, 2010, **53**, 5248.
- 28 M. Egido-Gabás, D. Canals, J. Casas, A. Llebaria and A. Delgado, *ChemMedChem*, 2007, **2**, 992.
- 29 P. Compain, O. R. Martin, C. Boucheron, G. Godin, L. Yu, K. Ikeda and N. Asano, *ChemBioChem*, 2006, **7**, 1356.
- 30 L. Yu, K. Ikeda, A. Kato, I. Adachi, G. Godin, P. Compain, O. R. Martin and N. Asano, *Bioorg. Med. Chem.*, 2006, **14**, 7736.
- 31 X. Zhu, K. A. Sheth, S. Li, H.-H. Chang and J.-Q. Fan, *Angew. Chem.*, 2005, **117**, 7616, (*Angew. Chem., Int. Ed.*, 2005, **44**, 7450).
- 32 W. Zheng, J. Padia, D. J. Urban, A. Jadhav, O. Goker-Alpan, A. Simeonov, E. Goldin, D. Auld, M. E. LaMarca, J. Inglese, C. P. Austin and E. Sidransky, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 13192.
- 33 M. B. Tropak, G. J. Kornhaber, B. A. Rigat, G. H. Maegawa, J. D. Buttner, J. E. Blanchard, C. Murphy, S. J. Tuske, S. J. Coales, Y. Hamuro, E. D. Brown and D. J. Mahuran, *ChemBioChem*, 2008, **16**, 2650.
- 34 G. H. Maegawa, M. B. Tropak, J. D. Buttner, B. A. Rigat, M. Fuller, D. Pandit, L. I. Tang, G. J. Kornhaber, I. Hamuro, J. T. R. Clarke and D. J. Mahuran, *J. Biol. Chem.*, 2009, **284**, 23502.
- 35 M. I. García Moreno, P. Diaz-Pérez, C. Ortiz Mellet and J. M. García Fernández, *Chem. Commun.*, 2002, 848.
- 36 M. I. García Moreno, P. Diaz-Pérez, C. Ortiz Mellet and J. M. García Fernández, *J. Org. Chem.*, 2003, **68**, 3578.
- 37 E. M. Sánchez-Fernández, R. Rísquez-Cuadro, M. Aguilar-Moncayo, M. I. García-Moreno, C. Ortiz Mellet and J. M. García Fernández, *Org. Lett.*, 2009, **11**, 3306.
- 38 (a) E. M. Sánchez-Fernández, R. Rísquez-Cuadro, M. Chasseraud, A. Ahidouch, C. Ortiz Mellet, H. Ouadid-Ahidouch and J. M. García Fernández, *Chem. Commun.*, 2010, **46**, 5328; (b) M. Aguilar-Moncayo, M. I. García-Moreno, A. Trapero, M. Egido-Gabás, A. Llebaria, José M. García Fernández and Carmen Ortiz Mellet, *Org. Biomol. Chem.*, 2011, DOI: 10.1039/C1OB05234A, in press.
- 39 Z. Luan, K. Higaki, M. Aguilar-Moncayo, L. Li, H. Ninomiya, E. Namba, K. Ohno, M. I. García-Moreno, C. Ortiz Mellet, J. M. García Fernández and Y. Suzuki, *ChemBioChem*, 2010, **11**, 2453.
- 40 M. Aguilar-Moncayo, M. I. García-Moreno, A. E. Stütz, J. M. García Fernández, T. M. Wrodnigg and C. Ortiz Mellet, *Bioorg. Med. Chem.*, 2010, **18**, 7439.
- 41 Z. Luan, K. Higaki, M. Aguilar-Moncayo, H. Ninomiya, K. Ohno, M. I. García-Moreno, C. Ortiz Mellet, J. M. García Fernández and Y. Suzuki, *ChemBioChem*, 2009, **10**, 2780.
- 42 M. Aguilar-Moncayo, T. M. Gloster, M. I. García-Moreno, C. Ortiz Mellet, G. J. Davies, A. Llebaria, J. Casas, M. Egido-Gabás and J. M. García Fernández, *ChemBioChem*, 2008, **9**, 2612.
- 43 M. Aguilar-Moncayo, T. M. Gloster, J. P. Turkenburg, M. I. García-Moreno, C. Ortiz Mellet, G. J. Davies and J. M. García Fernández, *Org. Biomol. Chem.*, 2009, **7**, 2738.
- 44 B. Brumshtein, M. Aguilar-Moncayo, M. I. García-Moreno, C. Ortiz Mellet, J. M. García Fernández, I. Silman, Y. Shaaltiel, D. Aviezer, J. L. Sussman and A. H. Futerman, *ChemBioChem*, 2009, **10**, 1480.
- 45 H. Dvir, M. Harel, A. A. McCarthy, L. Toker, I. Silman, A. H. Futerman and J. L. Sussman, *EMBO Rep.*, 2003, **4**, 704.
- 46 Y. Shaaltiel, D. Bartfeld, S. Hashmueli, G. Baum, E. Brill-Almon, G. Galili, O. Dym, S. A. Boldin-Adamsky, I. Silman, J. L. Sussman, A. H. Futerman and D. Aviezer, *Plant Biotechnol. J.*, 2007, **5**, 579.
- 47 B. Brumshtein, P. Salinas, B. Peterson, V. Chan, I. Silman, J. L. Sussman, P. J. Savickas, G. S. Robinson and A. H. Futerman, *Glycobiology*, 2010, **20**, 24.
- 48 L. Premkumar, A. R. Sawkar, S. Boldin-Adamsky, L. Toker, I. Silman, J. W. Kelly, A. H. Futerman and J. L. Sussman, *J. Biol. Chem.*, 2005, **280**, 23815.
- 49 Y. Kacher, B. Brumshtein, S. Boldin-Adamsky, L. Toker, A. Shainskaya, I. Silman, J. L. Sussman and A. H. Futerman, *Biol. Chem.*, 2008, **389**, 1361.
- 50 B. Brumshtein, H. M. Greenblatt, T. D. Butters, Y. Shaaltiel, D. Aviezer, I. Silman, A. H. Futerman and J. L. Sussman, *J. Biol. Chem.*, 2007, **282**, 29052.
- 51 R. L. Lieberman, B. A. Wustman, P. Huertas, A. C. Powe, Jr., C. W. Pine, R. Khanna, M. G. Schlossmacher, D. Ringe and G. A. Petsko, *Nat. Chem. Biol.*, 2007, **3**, 101.
- 52 M. C. Wiener, *Methods*, 2004, **34**, 364.
- 53 M. C. Wiener and C. F. Snook, *J. Cryst. Growth*, 2001, **232**, 426.
- 54 B. W. Berger, C. M. Gendron, C. R. Robinson, E. W. Kaler and A. M. Lenhoff, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, 2005, **61**, 724.
- 55 L. Li, S. Nachtergaele, A. M. Seddon, V. Tereshko, N. Ponomarenko and R. F. Ismagilov, *J. Am. Chem. Soc.*, 2008, **130**, 14324.

- 
- 56 M. Aguilar-Moncayo, C. Ortiz Mellet, J. M. García Fernández and M. I. García-Moreno, *J. Org. Chem.*, 2009, **74**, 3595.
- 57 T. M. Gloster, R. Madsen and G. J. Davies, *ChemBioChem*, 2006, **7**, 738.
- 58 B. L. Cantarel, P. M. Coutinho, C. Rancurel, T. Bernard, V. Lombard and B. Henrissat, *Nucleic Acids Res.*, 2008, **37**, D233.
- 59 The authors kindly thank Dr C. A. Hunter, for providing the titration isotherm curve-fitting programs. These programs use a Simplex procedure to fit the data to the appropriate binding model to yield the association constant, the bound chemical shift in the host:guest (HG) complex and, if required, the free chemical shifts of the unbound species. For a detailed description of the fitting methods and equations, see: (a) A. P. Bisson, C. A. Hunter, J. C. Morales and K. Young, *Chem.–Eur. J.*, 1998, **4**, 845; (b) A. P. Bisson, F. J. Carver, D. S. Eggleston, R. C. Haltiwanger, C. A. Hunter, D. L. Livingston, J. F. McCabe, C. Rotger and A. E. Rowan, *J. Am. Chem. Soc.*, 2000, **122**, 8856.
- 60 Z. Otwinowski and W. Minor, *Methods Enzymol.*, 1997, **276**, 307.
- 61 G. N. Murshudov, A. A. Vagin and E. J. Dodson, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, 1997, **53**, 240.
- 62 P. Emsley, B. Lohkamp, W. G. Scott and K. Cowtan, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, 2010, **66**, 486.
- 63 A. C. Wallace, R. A. Laskowski and J. M. Thornton, *Protein Eng.*, 1995, **8**, 127.