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Conformational analysis of the mannosidase inhibitor kifunensine: a quantum mechanical and structural approach

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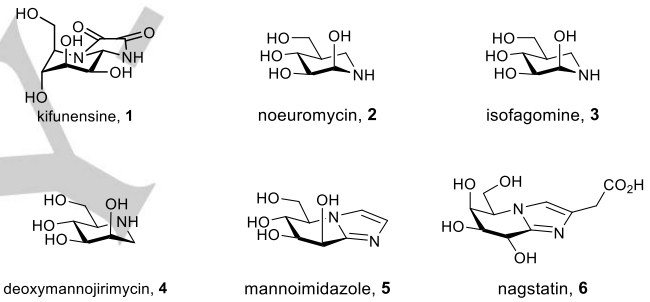
Abstract: The varied yet family-specific conformational pathways utilized by individual glycoside hydrolases (GHs) offer a tantalising prospect for the design of tight binding and specific enzyme inhibitors. A cardinal example of a GH family specific inhibitor, and one that finds widespread practical use, is the natural product kifunensine, which is a low nanomolar inhibitor selective for GH family 47 inverting α-mannosidases. Here we show, through quantum mechanical approaches, that kifunensine is restrained to a 'ring-flipped' $^{1}C_{4}$ conformation with another accessible, but higher-energy, region around the $^{1,4}B$ conformation. The conformations of kifunensine in complex with a range of GH47 enzymes including an atomic level (1 Å) resolution structure of kifunensine with *Caulobacter* sp. *Ck*GH47 reported herein, and on GH family 38 and 92 α-mannosidases, were mapped onto the kifunensine free energy landscape. These studies revealed that kifunensine has the ability to mimic the product state of GH47 enzymes but cannot mimic any conformational states relevant to the reaction coordinate of mannosidases from other families.

There is compelling evidence that the enzymatic hydrolysis of glycosides, catalyzed by glycoside hydrolases (GHs) or glycosidases, occurs via a transition-state(s) with significant oxocarbenium ion character. For pyranoside-active enzymes, Sinnott was the first to argue that the allowed canonical conformations of the transition state sugar ring were two half-chairs ($^{4}H_{3}$ and $^{3}H_{4}$; and their closely related envelope conformations) and two boats ($^{2,5}B$ and $B_{2,5}$).[1] Sustained efforts to map the conformational reaction pathways of glycosidases leading from substrate to product via the transition state(s) have revealed that individual glycosidases are optimized to act on substrates and follow a defined conformational itinerary through a specific transition-state conformation.[2] Glycosidases that are sequence related (for family classification see www.cazy.org; www.cazypedia.org)[3] and act on sugars with the same configuration are believed to act with identical conformational reaction itineraries. It is also apparent that GHs from different families that act on substrates with the same stereochemical configuration can utilize different conformational itineraries during catalysis. Given that mimicry of the enzymatic transition-state is a powerful approach to inhibitor design and enzyme inhibition,[4] the potential exists for molecules to be designed or discovered with intrinsically-biased conformations that could act as GH family-specific enzyme inhibitors. However, to achieve specificity inhibitor design might not be limited to transition state mimicry but could target any distinctive conformational state along the reaction coordinate. However, while highly appealing, efforts to design molecular scaffolds that predispose inhibitors to conformations matching that of the transition state of a specific GH have been disappointing. For example, molecular constraints that restrict conformational mobility typically result in steric clashes that prevent efficient binding.[5] As well, a recent study that identified a unique stereoelectronic bias of mannopyranosiduronic iminosugars failed to deliver effective inhibition of a conformationally-matched α-mannosidase, presumably because of the inability to accommodate the requisite carboxylate group.[6] In the context of the failure thus far of rational design methods to achieve conformationally-specific inhibition, one compound from nature, kifunensine **1**, stands prominent as a powerful and family-specific enzyme inhibitor. Kifunensine **1** (Scheme 1) is an unusual oxalamide-fused iminosugar with high specificity for α-mannosidases of GH family 47.



Scheme 1. Assorted mannosidase inhibitors.

Kifunensine is produced by the actinobacterium *Kitasatosporia kifunense* strain No. 9482.[7] Originally isolated on the basis of immunomodulatory properties, it was soon identified as a potent inhibitor of selected α-mannosidases.[8] The major contemporary applications of kifunensine stem from its activity as a specific inhibitor of class I GH47 α-mannosidases involved in glycoprotein biosynthesis within the secretory pathway that that are specific for the hydrolysis of α-1,2-glycosidic bonds.[9] During glycoprotein biosynthesis, glucosylated high mannose N-glycans are appended to nascent unfolded peptide chains in the endoplasmic reticulum (ER), whereupon they undergo a range of trimming reactions in the ER that assist in the unfolded peptide achieving the folded state, and subsequent trimming reactions in the Golgi apparatus that remove additional mannose residues prior to late stage glycosylation reactions.[10] As part of this process, a quality control mechanism termed ER associated degradation (ERAD) extracts terminally-misfolded proteins from the secretory pathway for proteosomal degradation.[11] Kifunensine-sensitive α-mannosidases are found within both the normal trimming pathway (ER mannosidase I, Golgi mannosidase I), and within the ERAD pathway (ER degradation-enhancing mannosidase-like proteins 1, 2 and 3).[12]

The powerful and specific inhibition of GH47 α-mannosidases by kifunensine has led to its widespread use for manipulation of N-glycan structure. In the structural biology context it is used to improve the homogeneity and crystallization

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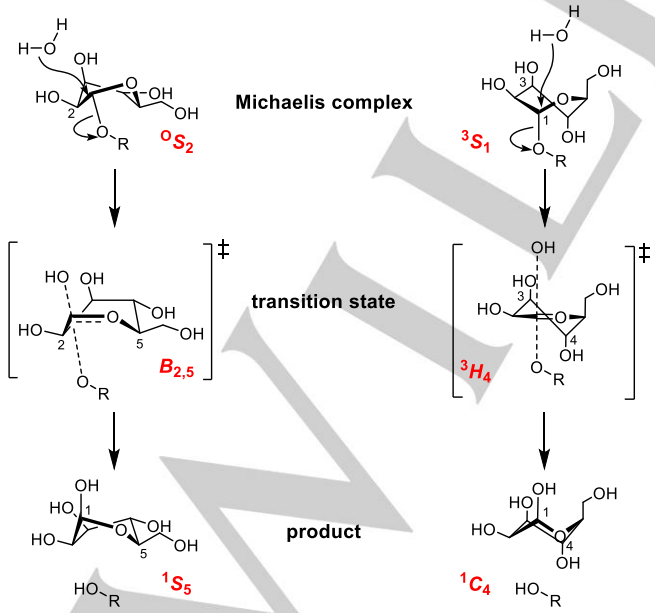
of proteins by arresting glycan remodeling to yield high mannose glycans that are more easily cleaved by endoH.[13] Kifunensine is also used in the production of therapeutic proteins. The effectiveness of acid β-glucocerebrosidase as a treatment for the lysosomal storage disorder Gaucher's disease depends upon the presence of high mannose N-glycans of this protein, which enable ligation to mannose receptors and delivery to lysosomes.[14] The lysosomal replacement therapy protein Velaglucerase alfa (acid β-glucocerebrosidase) is produced by Shire Plc by culturing HT1080 fibrosarcoma cells expressing acid β-glucocerebrosidase in the presence of kifunensine—this promotes the biosynthesis of enzyme decorated by immature high mannose-type N-linked glycan chains.[15]

Structural studies on GH47 enzymes, firstly the seminal work by the Howell group on the human ER ManB1 α-1,2-mannosidase,[16] and subsequent work on a *Penicillium citrinum* homolog[12c] revealed kifunensine to bind in a 'ring-flipped' $^1C_4$ conformation. This conformation is unusual for an iminosugar; relative to the proposed conformational pathway, $^3S_1 \rightarrow [^3H_4]^{\ddagger} \rightarrow {}^1C_4$ (Scheme 2),[2b, 16-17] for this family of enzymes it provides conformational mimicry of the product. Of relevance to this observation, a complex of noeuromycin **2** bound to a bacterial α-mannosidase of family GH47, *Caulobacter* sp. K31 (*Ck*GH47) also adopted a $^1C_4$ conformation, which on the basis of computational work was assigned as a product-mimicking species.[17a] However, in the case of noeuromycin, the computational analysis of the inhibitor reveals that it favours a $^4C_1$ conformation,[18] and the observed conformation on-enzyme would therefore appear to be as a consequence of the enzyme restricting its shape to a higher energy conformation. Because of the widespread application of kifunensine, and its unique specificity as a GH47 selective α-mannosidase inhibitor, we sought to understand its specificity by developing a quantitative view of the conformational preferences of this compound when isolated, relative to bound states on α-mannosidases from GH family 47 and other families.



Scheme 2. Conformational itineraries for inverting α-mannosidases proceeding through (left) $^OS_2 \rightarrow [B_{2,5}]^{\ddagger} \rightarrow {}^1S_5$ (e.g. GH family 125) and (right) $^3S_1 \rightarrow [^3H_4]^{\ddagger} \rightarrow {}^1C_4$ (GH family 47) pathways.

Structures are available for kifunensine bound to *Homo sapiens*[16] and *P. citrinum*[12c] family 47 α-mannosidases at medium resolutions of 1.75 Å and 2.20 Å, respectively. In order to allow comparison to conformations that the α-mannoside substrate follows during catalysis, we sought to obtain a higher resolution structure on *Ck*GH47 α-mannosidase, which we have previously shown to provide atomic resolution diffraction data (Table 1). Kifunensine, shown here using isothermal titration calorimetry, is a tight binding (K_D = 39 nM) inhibitor of *Ck*GH47 (Figure 1a). Crystals of a complex of *Ck*GH47 bound to kifunensine diffracted to 1.05 Å resolution (Figure 1b). Kifunensine binds to *Ck*GH47 in a $^1C_4$ conformation, in line with the conformation observed in the structures with the *H. sapiens* and *P. citrinum* GH47 α-mannosidases.

Table 1. Data and structure quality for **1** in complex with *Ck*GH47 (PDB code 5NE5).

Resolution (Å)	72-1.05
R_{merge}	0.090 (0.88)
CC(1/2)	1.00 (0.53)
Completeness (%)	99.2 (99.6)
R_{work} / R_{free}	0.12/0.14
$rmsd_{bonds}$ (Å)	0.020

Conformational free energy landscapes (FELs) are quantitative maps of the energy of the full suite of conformations of a molecule, and provide insight into locally and globally stable conformations and the barriers that must be crossed in order to achieve them. In order to understand the conformational landscape of kifunensine, and how this contributes to its GH47 specific inhibition, its conformational FEL was calculated by *ab initio* metadynamics[19] using methods we have developed and applied to a range of glycosidase inhibitors.[18, 20] The kifunensine FEL (Figure 2, top) exhibits a strong preference for the 'southern hemisphere' $^1C_4$ conformation, which is consistent with the conformation observed in a small molecule, single crystal X-ray structure.[8, 21] A low energy zone is also seen around the $^1S_3/^{1,4}B/^1S_5$ region of the FEL, which at approximately 7-8 kcal/mol higher in energy, and with a minimal barrier, can be considered energetically accessible. Overall, the isolated kifunensine FEL can be considered strongly constrained to a ring-flipped $^1C_4$ conformation, with limited ability to adopt a small set of 'equatorial' conformations. Plotting the conformations of kifunensine observed in representative GH47 crystal structures (for enzymes from *H. sapiens* and *B. thetaitaomicron*), onto the isolated kifunensine FEL reveals that they lie close together in the low energy zone, with similar conformations to that of the small molecule, single crystal X-ray structure, suggesting that little distortion occurs upon binding to GH47 enzymes (the apparent differences reflect distortion near the poles of a Mercator projection (for a Polar projection and direct comparison, see SI Figure S1). Confluent with the FEL for **1**, an FEL calculated for methyl α-mannoside within the active-centre environment of the *Ck*GH47 (Figure 2, bottom) revealed that this molecule is heavily distorted away from its preferred $^4C_1$ conformation when bound to the enzyme.[17a]

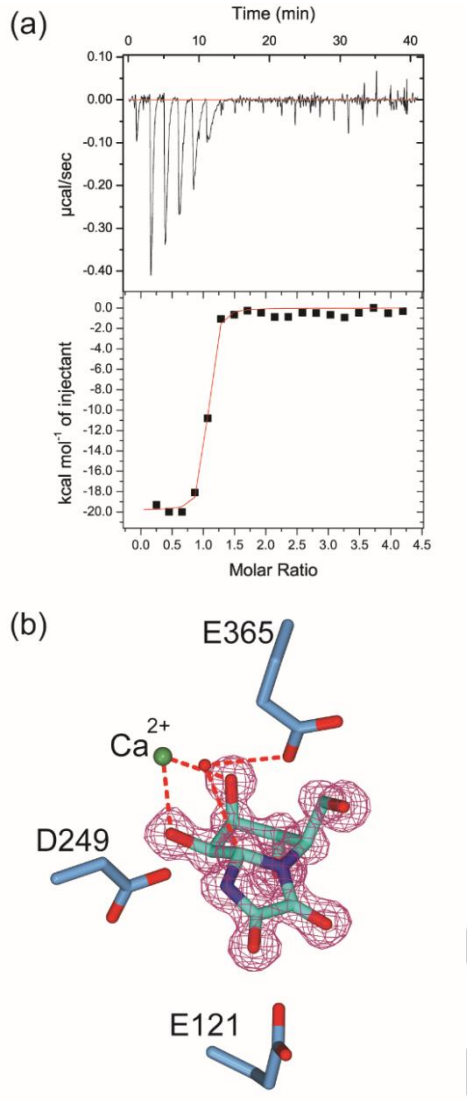


Figure 1. Kifunensine is a potent inhibitor of *Caulobacter* sp. K31 (*Ck*GH47) α-mannosidase and binds in a $^1C_4$ conformation. a) Thermodynamics of binding between CkGH47 and kifunensine obtained by ITC. The raw data are shown in the injection profile (upper panel) and the titration curve (bottom panel). The stoichiometry, *n*, is 0.98 ± 0.01 sites. The association constant, K_A, is (2.6 ± 1.1) x 10^7 M^{-1}. The enthalpy change, ΔH, is 19.8 ± 0.3 kcal mol^{-1}. b) Complex with kifunensine showing catalytic residues, the Ca^{2+} ion, and the nucleophilic water molecule. Electron density map is REFMAC maximum-likelihood/σA-weighted 2*Fo* – *Fc* synthesis contoured at 0.80 electrons per $Å^3$, respectively.

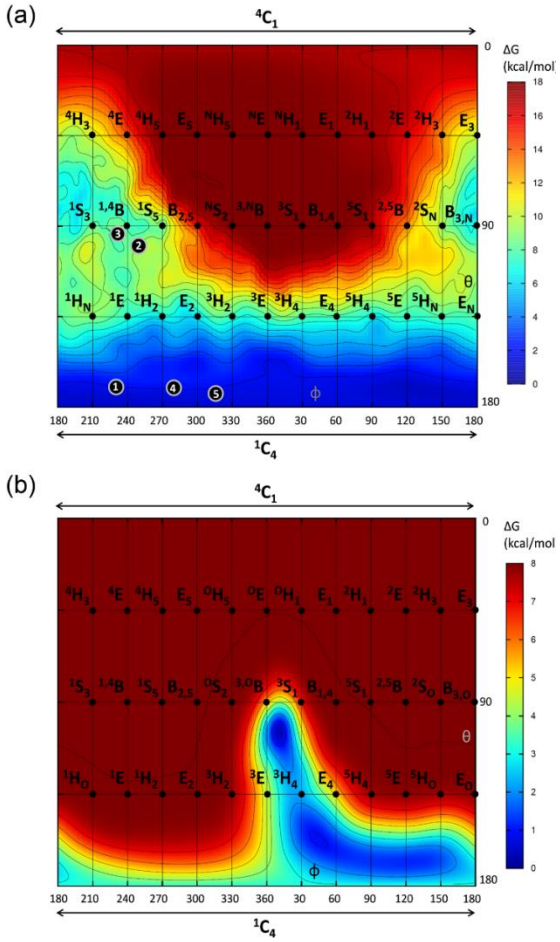


Figure 2. Mercator projections of conformational free energy landscapes (FELs) of kifunensine and methyl α-mannopyranoside obtained by *ab initio* metadynamics simulations. (Top) FEL for kifunensine *in vacuo*. The indicated observed kifunensine conformations, shown in numbered circles, are for the following α-mannosidases: ❶ *H. sapiens* GH47 (PDB 1FO3), ❷ *B. thetaiotaomicron* GH92 (PDB 2WVZ), ❸ *D. melanogaster* GH38 (PDB 1PS3), ❹ *Caulobacter* sp. *Ck*GH47 (this work) and ❺ the small molecule crystal structures of kifunensine (KASNOH/KASNOH10). ❸, ❹, and ❺ adopt almost identical conformations, the apparent differences reflect distortions nearing the poles of a Mercator projection (see SI Figure S1 for an alternative Polar projection). (Bottom) FEL for methyl α-mannopyranoside bound to *Ck*GH47 from Ref.[17a] Contour lines are separated by 1 kcal/mol.

The conformational bias of kifunensine towards the southern hemisphere is unusual for a glycosidase inhibitor. For example, FELs of the azasugar isofagomine reveal this molecule favours a 'normal' $^4C_1$ conformation,[20b] which is also expected for the iminosugar deoxymannojirimycin **4**. A range of iminosugars containing sp^2-hybridized atoms, best illustrated by mannoimidazole **5**, favor half-chair ($^4H_3$) and boat ($B_{2,5}$) conformations that lie along the FEL equator.[20] One unusual example worth mentioning that is similar to that observed here is a mannopyranosiduronic iminosugar, which under acidic conditions favors a $^1C_4$ chair, as shown by ^{1}H NMR analysis of vicinal coupling constants; however, the presence of the carboxylate group prevented binding to α-mannosidases.[6]

While kifunensine typically binds with nanomolar dissociation constants to GH47 α-mannosidases, it is usually a far poorer binder/inhibitor for GH families other than family 47. The available evidence suggests that all other *exo*-mannosidases operate through $^OS_2 \leftrightarrow [B_{2,5}]^{\ddagger} \leftrightarrow {}^1S_5$ conformational pathways [GH families 2 (retaining),[5b] 38 (retaining),[22] 92 (inverting)[23] and 125 (inverting)[24]]. Data are available for kifunensine binding to representatives of families 38 and 92. For the *Drosophila melanogaster* Golgi class II GH38 retaining α-mannosidase dGMII, **1** has a K_I value of 5 mM,[25] and it is reported to be a very poor inhibitor of jack bean GH38 α-mannosidase.[8, 12a] Similarly, for a range of *Bacteroides thetaiotaomicron* inverting GH92 α-mannosidases, **1** is reported to have K_I values in the range of 100-200 µM.[23] In both cases structures of **1** bound to GH38 (PDB 1PS3) and GH92 (PDB

2WVZ)[23] α-mannosidases revealed a $^{1,4}B$ conformation. Consideration of these results in the context of the kifunensine FEL reveals that while this conformation lies in the energetically accessible equatorial region (Figure 2, top), this conformation lies some distance away from the $^{O}S_2 \leftrightarrow [B_{2,5}]^{\ddagger} \leftrightarrow {}^{1}S_5$ conformational pathway proposed for both GH38 and 92 α-mannosidases, suggesting that the poor inhibition results from an inability of the inhibitor to adopt a conformation that matches species formed on the enzyme during catalysis.

Our conformational analysis of kifunensine **1** highlights the unique nature of this compound to target a region of the FEL that is poorly populated by other GH inhibitors. We suggest that the specificity and potency of **1** for family GH47 enzymes is a direct consequence of its strong preference for the southern hemisphere $^{1}C_4$ conformation, which matches that of the product state of the $^{3}S_1 \rightarrow [^{3}H_4]^{\ddagger} \rightarrow {}^{1}C_4$ conformational itinerary that this family of enzymes is believed to utilize.[2b, 16-17] We highlight that this analysis suggests that potency is achieved in the absence of transition state mimicry. It is surprising that this conformational restraint is achieved by introduction of an oxalamide bridge without interfering with binding in the active site, as a previous attempt to synthetically introduce a bridge into an iminosugar with a similar goal failed owing to steric clashes in the active site.[5] The conformational preference of kifunensine for a $^{1}C_4$ conformation likely arises not merely from the fusion of the bridge to the ring, but also from the sp^2-hybridization of the endocyclic nitrogen as part of an amide. Further, it is noteworthy that kifunensine is a neutral species, showing that unlike most aza/iminosugar glycosidase inhibitors, kifunensine achieves its potency through shape, rather than charge (for a discussion of shape versus charge mimicry for the inhibition of a GH99 α-mannosidase see Ref.[18]; for an example of a potent class of neutral GH inhibitors see Ref.[26]).

In conclusion, this work highlights the capacity of natural products to provide inspiration for achieving the goal of selective inhibition of glycosidases based on mechanistic principles. Aligned with this goal, we highlight the inspiration provided by the natural product nagstatin **6**,[27] which informed the development of the concept of lateral protonation by GHs with a catalytic acid or acid/base located anti to the C1-O5 bond, and the design of the glycoimidazole class inhibitors that are selective for anti-protonating GHs.[28] The existence of kifunensine as a GH47 selective inhibitor should inspire continuing efforts to develop selective and potent GH inhibitors based on targeting unique conformational features of their catalysis reaction coordinates. We highlight that one other mannosidase family operates through a reversed $^{1}C_4 \rightarrow [^{3}H_4]^{\ddagger} \rightarrow {}^{3}S_1$ conformational itinerary, namely GH family 134 β-mannanases.[29] Based on the analysis presented herein, this family is likely to be specifically targeted by substrate-mimicking kifunensine-derived oligosaccharides relative to the β-mannanases of GH26 and 113, which operate through $^{O}S_2 \leftrightarrow [B_{2,5}]^{\ddagger} \leftrightarrow {}^{1}S_5$ conformational pathways.[20b]

Experimental Section

C*k*GH47 protein was cloned, expressed and purified as described previously.[17a] The crystallisation conditions were the same as discussed in Ref.[6] Mature crystals were soaked in 1 mM kifunensine for 15 hours. Data were collected on the Diamond I04-1 MX beamline. After data collection, the diffraction images were integrated using xia2[30] and reintegrated using AIMLESS from the CCP4 software suite.[31] The Free R flag data set was copied from 4AYO. Refinement of the model was conducted using multiple rounds of REFMAC and manual model building in COOT.[32] Waters were added in using FIND WATERS in COOT and validated. The conformation of the ligand was validated using PRIVATEER. Coordinates have been deposited on PDB with accession code 5NE5; details of refinement quality are shown in Table1 and the PDB header. Figures of the structure were produced using CCP4mg.[33]

ITC was performed using a MicroCal ITC_{200} calorimeter at 25 °C with 20 injections. *Ck*GH47 and DMJ were transferred into matching buffer by dialysis into 25 mM HEPES pH 7.0, 50 mM NaCl and 2 mM $CaCl_2$. The *Ck*GH47 concentration in the cell was 50 μM and the ligand concentration was 500 μM. The binding affinity was calculated using the Origin7 software.

The free energy landscape of kifunensine was obtained by density functional theory-based metadynamics,[19] using the Car-Parrinello (CP) method.[34] The molecule was enclosed in an isolated cubic box of 14.0 Å × 14.0 Å × 14.0 Å. A fictitious electron mass of 700 au and a time step of 0.12 fs ensured a proper conservation of the total energy during the simulation. The Kohn-Sham orbitals were expanded in a plane wave (PW) basis set with a kinetic energy cutoff of 70 Ry. *Ab initio* pseudopotentials, generated within the Troullier-Martins scheme,[35] were employed. The Perdew, Burke and Ernzerhoff generalized gradient-corrected approximation (PBE)[36] was selected in view of its good performance in our previous works.[37] Two collective variables of the puckering coordinates of Cremer and Pople (θ and φ) were used to explore the conformational space.[38] Initially, the height/width of these Gaussian terms was set at 0.6 kcal·mol^{-1} / 0.10 Å and a new Gaussian-like potential was added every 250 MD steps. Once the whole free energy space was explored, the height of the Gaussian terms was reduced to half of its initial value (0.3 kcal·mol^{-1}) and a new Gaussian-like potential was added every 500 MD steps. The simulation was stopped when energy differences among wells remained constant, which was further confirmed by a time-independent free energy estimator.[39] The phase space was fully explored in less than 50 ps and the simulation was further extended up to 95 ps. The error in the energy difference of the principal minima, taken as a standard deviation (SD) from the last 30 ps, is below 0.6 kcal mol^{-1}.

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Keywords: ab initio calculations • enzymes • iminosugar • carbohydrates • hydrolases

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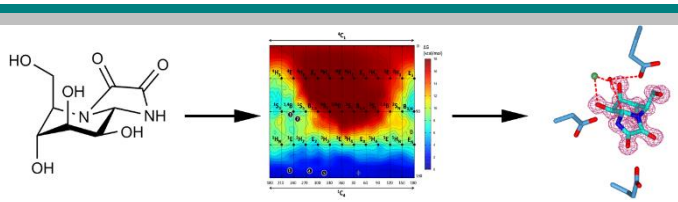

Perfect fit: Kifunensine is an iminosugar inhibitor with high selectivity for α-mannosidases of glycoside hydrolase family 47. The free energy landscape for kifunensine reveals a strong preference for an inverted $^{1}C_{4}$ chair that matches its conformation when bound to GH47 enzymes and predicted for the enzyme-product state. The selectivity of kifunensine for GH47 α-mannosidases derives from its ability to mimic a conformation of the reaction coordinate for GH47 that is distinct from that for α-mannosidases of other families.

*Alexandra Males, Lluís Raich, Spencer J Williams, Carme Rovira and Gideon J Davies**

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Conformational analysis of the mannosidase inhibitor kifunensine: a quantum mechanical and structural approach