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Evaluating hydrophobic galactonoamidines as transition state analogs for enzymatic β -galactoside hydrolysis

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Abstract

A spectroscopic examination of six galactonoamidines with inhibition constants and efficacy in the low nanomolar concentration range ($K_i = 6\text{--}11\text{ nM}$, $IC_{50} = 12\text{--}36\text{ nM}$) suggested only two of them as putative transition state analogs for the hydrolysis of β -galactosides by β -galactosidase (*A. oryzae*). A rationale for the experimental results was elaborated using docking and molecular dynamics studies. An analysis of the combined observations reveals several common factors of the compounds suggested as transition state analogs (TSAs): the putative TSAs have a similar orientation in the active site; show conserved positioning of the glycon; display a large number of H-bond interactions toward the catalytically active amino acid residues via their glycon; and exhibit hydrophobic interactions at the outer rim of the active site with small changes of the position and orientation of their respective aglycons.

Graphical Abstract

Keywords

Inhibitor; transition state analogs; galactonoamidines; β -galactosidase; molecular dynamics; hydrophobic loops

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Declaration of interests: none

Supplementary Information:

Supplementary data for all molecular docking and molecular dynamic simulations between **1a–f** and β -galactosidase (*A. oryzae*, 4IUG) can be found online at doi:10.1016/j.carres.2017.xx.xx. including output files from AutoDock Vina, input files for molecular dynamic studies, and movies visualizing the results of all simulations over 30 ns.

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1. Introduction

The ubiquity of glycosidases in biological systems and the complexity of manipulating the glycosidic bonds in oligosaccharides indicate a need for evaluating mechanistic details of specific glycosidases.[1] These enzymes cleave glycosidic bonds either by retention or inversion of the configuration at the anomeric carbon atom of a glycoside substrate in a S_N1 or S_N2 -like manner.[2–4] Both mechanisms go through oxocarbenium ion-like transition states during the substrate hydrolyses.[2] As β -galactosidases are prominent in many diseases, [5–7] a detailed knowledge of their mechanistic function is advantageous.

A common strategy toward this goal relies on the design of inhibitors possessing currently known features of the transition states of glycoside hydrolyses. The derived compounds often display an oxocarbenium ion-like feature with a flattened half-chair conformation and a sp^2 -like character at the anomeric center.[8] Additionally, a positive charge at the location of the ring oxygen atom was found to be important.[9] Lastly, spacers between glycon and aglycon of the inhibitors were designed to mimic the lengthening of the glycosidic bond during cleavage.[9, 10] The transition states of enzymatic reactions have been examined by various techniques including studies based on spectroscopic evaluations, [11] kinetic isotope effects, [12] X-ray diffraction, [13, 14] and mutations of residues within the active sites[15] in combination with molecular dynamics simulations.[16] In the absence of crystallographic data, an in-depth evaluation of the corresponding enzymes by a combination of spectroscopic and molecular modeling studies is often used to provide mechanistic insights for the development of new drugs in future therapeutic treatments.[17–21]

In this context, we previously synthesized a small library of 7 galactonoamidines and evaluated their ability to inhibit β -galactosidase (*A. oryzae*).[22] The compounds inhibit the selected β -galactosidase competitively and show low nanomolar inhibition constants (K_i = 8–60 nM).[22] However, only *p*-methylbenzyl galactonoamidine (**1a**) was characterized as a putative transition state analog using experimental methods described by Bartlett et al.[23, 24] It is hypothesized that the nature of the aglycon of the respective galactonoamidines is responsible for the decisive differences in the stabilization of the transition state during enzymatic glycoside hydrolysis. This hypothesis prompted a detailed structure-activity relationship study after extending the library to 25 galactonoamidines while installing aglycons that support hydrophobic, hydrophilic, π - π stacking and H-bond donor or acceptor interactions.[18, 25] Although all members in the library were classified as competitive inhibitors with inhibition constants in the nanomolar concentration range, [26] only six of those amidines (**1b–g**) displayed inhibition constants below 15 nM and IC_{50} values below 36 nM similar to **1a** (Chart 1).

The respective aglycons of the six inhibitors encompass a large structural variety that includes aromatic residues with extended spacer (**1b**), cyclic aliphatic moieties with cycloheptyl (**1c**) or cyclohexyl (**1d**) rings, a branched aliphatic 2-ethylhexyl residue (**1e**), a very small cyclopropyl group (**1f**) and a linear aliphatic heptyl chain (**1g**). The noted similarities in inhibition efficacy and inhibition constants, despite the structural diversity of the aglycons, prompted our interest in an in-depth evaluation of the selected galactonoamidines as putative transition state analogs of the enzymatic glycoside hydrolysis

by β -galactosidase (*A. oryzae*). Our results from the spectroscopic investigation of the inhibitor interactions in the active site of the selected enzyme, related docking studies, and subsequent molecular dynamic analyses are summarized below.

2. Results and discussion

2.1. Molecular docking study

Prior to extensive experimental evaluation of inhibitors as putative transition state analogs (TSAs), Autodock Vina was used for an initial estimate of docked inhibitor-enzyme assemblies. The structure of β -galactosidase (*A. oryzae*, 4IUG) is known from X-ray diffraction studies.[27] Its active site encompasses residues of 8 amino acids, including catalytically active Glu²⁰⁰ (proton donor) and Glu²⁹⁸ (nucleophile) (Figure 1).[27]

The docked inhibitor-enzyme assemblies were examined for their H-bonding interactions, and for the orientation of the galactonoamidines within the active site. Particular scrutiny was paid to the interactions of **1a–g** with the residues of the two catalytically active amino acids. The predicted free energies of all docked conformations of the enzyme-amidine assemblies vary by 1.7 kcal/mol or less confirming the noted similarity of the experimentally determined inhibition ability of **1a–g**.

The docking study suggest H-bonding interactions of the inhibitors with both catalytically active amino acid residues and Ala¹⁴¹ (Table 1). The inhibitors may act as a H-bond acceptor toward Asn¹⁹⁹, Tyr⁹⁶, and Tyr³⁴², and as a H-bond donor toward the catalytically active amino acid residues and Glu¹⁴². The position of the glycon moiety in the docked inhibitor-protein assemblies seems conserved.

By contrast, remarkable differences in the orientation of the aglycon moieties in the docked inhibitor-enzyme assemblies were observed upon examination of interactions between **1a–g** and amino acid residues at the outer rim of the active site (Ala²³⁷, Leu²⁶², Phe²⁶⁴, Phe³⁰⁴, and Trp⁸⁰⁶). The important role of such hydrophobic interactions for the stabilization of substrates during catalytic turnover was recently disclosed in a crystallographic study using β -galactosidase (*A. niger*) from the same family of glycoside hydrolases.[15]

Here, the hydrophobic residues create a tunnel-like binding pocket around the aglycons of **1a–g**. As galactonoamidine **1a** was previously classified as a TSA, the planar orientation of its methylbenzyl aglycon was used as a standard. The aglycons of amidines **1b** and **1d** are slightly tilted out of a plane formed by the aglycon of **1a**. [28] The cycloheptyl and cyclopropyl aglycons of amidines **1c** and **1e** show large deviations in their orientation relative to **1a**. The aliphatic branched and linear aliphatic residues in amidines **1e** and **1g** appear in a perpendicular orientation relative to the aglycon in **1a** (Figure 2a–f).

The docking study indicates for all inhibitors a large number of conserved H-bonding interactions with amino acids in the active site. The orientation of the glycon is conserved, while the orientation of the aglycons is influenced by interactions with hydrophobic amino acid residues at the outer rim of the active site. High similarity of the galactonoamidine orientation in protein-inhibitor assemblies are noted for **1a**, **1b** and **1d**. However, further

insights for classification of a compound as a putative TSA were obtained by spectroscopic evaluation of the inhibitors with binding energies within 1.2 kcal/mol relative to **1a** (i.e. **1e–f**), and followed by molecular dynamics simulations.

3. Spectroscopic evaluation of galactonoamidines as putative transition state analogs

Following a spectroscopic method introduced by Bartlett et al. for the characterization of competitive inhibitors as putative transition state analogs based on Eyrings' transition state theory, [23, 29] we evaluated the selected galactonoamidines **1b–f**. The enzyme-catalyzed hydrolysis of nine structurally related nitrophenyl- β -D-galactopyranoside substrates **2a–i** was monitored by UV/Vis spectroscopy in presence and absence of **1b–f** (Scheme 1).

The galactonoamidines were used in a 0.25–1 mM concentration range. The determined inhibition constants (K_i) are given as an average of independent experiments using three or more different inhibitor concentrations (Table 2). Nanomolar inhibition constants ($K_i = 6–170$ nM) were observed for the enzymatic hydrolysis of all substrates in the presence of **1b–f**.

Subsequently, the determined inhibition constants (K_i) were correlated to the catalytic efficiency (k_{cat}/K_M) of the enzymatically catalyzed substrate hydrolysis in absence of inhibitors (Figure 3). The evaluation of correlation coefficients and standard deviations for a linear fit of the data in double logarithmic plots results in an estimate of the ability of the inhibitors to function as a TSA of the catalyzed glycoside hydrolysis.[23] Ideally, a linear fit of the data with a slope of 1, and a standard deviation factor R^2 of 1 is expected.[23] Deviations from this ideal exclude the compound under investigation from further consideration as a putative TSA.[23] Given the criteria introduced above, galactonoamidines **1b** and **1d** qualify as putative TSAs for the hydrolysis of β -galactosides by β -galactosidase (*A. oryzae*) (Figure 3a), while all other amidines **1c** and **1e–f** are very potent competitive inhibitors that do not fully resemble the features of the transition state of the reaction (Figure 3b).

Interestingly, the experimental selection of amidines (**1a**, **1b** and **1d**) with putative transition state-like character is identical to those suggested by molecular docking studies (see above). Their docked inhibitor-protein conformations show the aglycon in a hydrophobic tunnel-like loop that is formed by several amino acids at the outer rim of the active side. In order to further illuminate the underlying stabilizing forces for the inhibitor-protein assemblies, molecular dynamics simulations were employed.[30]

4. Molecular dynamic studies of galactonoamidine-protein interactions

The interactions between the galactonoamidines **1a–f** and amino acids within and outside of the active site of β -galactosidase (*A. oryzae*, 4IUG) were further examined by molecular dynamics simulations employing the GROMOS 96-43A1 model.[30] Similar investigations to describe assemblies between enzymes and carbohydrate analogs were reported by others recently.[31, 32] Interactions of an inhibitor with both catalytically active amino acid

residues within the active site were previously suggested as a prerequisite for the designation of the compound as a TSA.[33] Along these lines, the probability of H-bond formation between the hydroxyl groups of the glycons of galactonoamidines **1a–f** and all amino acid residues within the active site was examined with an focus on H-bond interactions toward the catalytically active amino acid residues Glu²⁹⁸ (nucleophile) and Glu²⁰⁰ (donor) (Figures 4a and b).

The amidines **1a–b** and **1d–e** show higher probability to form an overall larger number of H-bond interactions within the active site than inhibitors **1c** and **1f** (Figure 4a). Over most of the simulation time, all galactonoamidines display high probability for H-bond formation with catalytically active residue Glu²⁹⁸ (Figure 4b). Similar interactions with Glu²⁰⁰ were noted for amidines **1a–d**, but not for **1e–f**. The molecular dynamics simulations further indicate almost identical orientations of the glycon moieties of the inhibitors of **1a**, **1b** and **1d** in the active site (RMSD = 0.65 Å).

The combined observations of the modeling study support considerations of amidines **1a–d** as transition state analogs, while rendering the selection of **1e–f** unlikely. Further insights were then obtained by comparing simulations of the protein in the absence and presence of galactonoamidines **1a–f**, focusing on the orientation and location of their respective aglycon in the active site (Figure 5).

The simulations disclose the presence of a triangular hydrophobic tunnel and its induced fit movement upon binding of **1a**, **1b** and **1d** in the active site. The hydrophobic tunnel encompasses Ala²³⁷, Leu²⁶², Phe²⁶⁴, and Trp⁸⁰⁶ at the outer rim of the active site as revealed by the rigid docking study, and extends into a hydrophobic loop comprised of Pro²⁶¹, Phe²⁷⁸, Phe²⁸¹, Ala³⁰¹, Ala³⁰³, Phe³⁰⁴, Ala³¹⁷, Val³¹⁹, and Met³⁴³ upon relaxation with molecular dynamics.

During the simulation, the aglycon of **1a** orients itself via the *p*-methyl substituent toward several residues of the hydrophobic tunnel (Ala²³⁷, Pro²⁶¹, Leu²⁶², Phe²⁶⁴, Pro²⁷⁸ and Phe²⁸¹) (Figure 6a). Likewise, the flexibility of the extended spacer of the aglycon in **1b** allows the inhibitor to adapt a conformation that provides proximity of the propylene spacer to hydrophobic residues (Ala²³⁷, Pro²⁶¹, Leu²⁶², Phe²⁶⁴, Ala³⁰¹, Ala³⁰³, Phe³⁰⁴, Ala³¹⁷, Val³¹⁹, Met³⁴³, and Trp⁸⁰⁶) at the outer rim of the active site (Figure 6b). Similar hydrophobic interactions for the cyclohexyl aglycon of **1d** are noted and accompanied by an induced fit movement of the respective hydrophobic residues as well (Figure 6d). Lastly, π - π stacking interactions to support inhibitor binding are not apparent, and were therefore excluded from any further consideration as means of inhibitor stabilization in the active site of β -galactosidase (*A. oryzae*).

By contrast, galactonoamidines **1c**, **1e** and **1f** (Figure 5c–e, f) interact differently in the active site of β -galactosidase (*A. oryzae*). Over the 30 ns simulation time, larger conformational fluctuations of glycon and aglycon of these inhibitors in location and orientation were noted that indicate overall weaker binding interactions. While the aglycons from **1c**, **1e** and **1f** could support hydrophobic binding interactions, the simulation indicates their perpendicular orientation away from the hydrophobic tunnel. An induced fit upon

binding of **1c**, **1e** and **1f** in the active site and accompanying movement of the hydrophobic tunnel is not apparent. The combined observations are in line with the experimental evaluation described above, that disclosed a very poor correlation between inhibition constant and catalytic efficiency for **1c**, **e–f** (→Figure 2b), and suggest to exclude those amidines from further consideration as TSAs.

5. Conclusion

A previously synthesized library of 25 galactonoamidines contains 7 potent galactonoamidines **1a–g** with inhibition constants below 15 nM and IC₅₀ values between 12–36 nM.[25] Amidine **1a** was then experimentally characterized as a TSA by spectroscopic analysis, and hydrophobic interactions within the active site were suggested as source for its proficiency.[26] All other amidines **1b–g** under investigation here contain aglycons in a large structural variety encompassing aliphatic linear, branched or cyclic moieties as well as aromatic groups. Despite their apparent structural diversity, the noted similarities in inhibition constants and efficacy of galactonoamidines **1a–g** prompted our interest in an in-depth evaluation of the underlying forces to discriminate potent competitive inhibitors from putative TSAs of the enzymatic glycoside hydrolysis by β -galactosidase (*A. oryzae*).

The spectroscopic evaluation identified amidines **1a**, **1b** and **1d** as putative TSAs, which is consistent with computational investigations based on a combination of interactions. Most importantly, the study disclosed an induced fit movement of the enzyme upon interaction with amidines **1a**, **1b** and **1d** that is not apparent for all others. Thereby, a loop encompassing an array of hydrophobic amino acids at the outer rim of the active site is suggested to sustain hydrophobic interaction with the identified amidines over their aglycons. Additionally, amidines **1a**, **1b** and **1d** show a high probability for interactions with both catalytically active amino acid residues, an almost conserved orientation of their glycon in the active site, and similar orientation of their aglycons over the simulation time. All other inhibitors lack support upon interaction in the active site by a similar combination of interactions. Evidence for π - π stacking interactions through aromatic residues was not obtained experimentally or by molecular dynamics simulations for any amidine. The study overall identified hydrophobic interactions and induced fit movements of β -galactosidase (*A. oryzae*) as a decisive driving force for interactions with TSA-like inhibitors and will further support ongoing efforts for the design of potent glycosidase inhibitors.

6. Experimental Section

6.1. Instrumentation

The FilterMax F5 Multi-Mode Microplate reader (Molecular Devices) was employed for all kinetic assays based on UV/Vis spectroscopy using flat bottom transparent 96-well plates (Bio- Greiner One). All pH values were obtained using a Φ 250 pH meter (Beckman) equipped with a refillable ROSS combination pH electrode (Orion) with an 8 mm semi-micro tip and epoxy body. The pH meter was calibrated using standard buffers prior to use. Nanopure water at a resistance of 18.2 M Ω .cm was obtained from a water purification system (ThermoScientific Barnstead E-pureTM).

6.2. Materials

Acetic acid at 99.7% purity was obtained from VWR. β -galactosidase (*A. oryzae*) and sodium hydroxide at 99.999% purity were obtained from Sigma-Aldrich. The enzyme was received as a lyophilized powder stabilized with dextrin and stored at -18°C prior to use. Previous BCA assays confirmed a protein content of 10 %. [34] The molar weight was determined as $86,800\text{ g mol}^{-1}$. [34] The galactonoamidine inhibitors **1a–g** and the nitrophenyl- β -D-galactopyranoside substrates **2a–i** were synthesized as described. [25, 26]

6.3. Spectroscopic evaluation of galactonoamidines as TSAs

6.3.1. Acetate buffer—Typically, a 500 mL aliquot of a 50 mM acetate buffer at pH 5.00 was prepared using standard methods at ambient temperature accounting for use at 30°C . The buffer was stored at ambient temperature and used within 10 days or discarded.

6.3.2. Stock solution of β -galactosidase (*A. oryzae*)—Typically, 4.5 mg of lyophilized β -galactosidase (*A. oryzae*) were dissolved in 5 mL of buffer solution. A 500 μL aliquot was further diluted into 10 mL with the same buffer yielding a 518 nM enzyme stock solution that was stored on ice until use.

6.3.3. Stock solutions of nitrophenyl- β -D-galactopyranoside substrates **2a–i**—Typically, 7–11 mg of the respective nitrophenyl- β -D-galactopyranosides **2a–i** were dissolved in 5 mL of acetate buffer. The resulting solution was kept at ambient temperature and used within 4 hr of preparation.

6.3.4. Stock solutions for galactonoamidine inhibitors **1b–f**—5 mL of 0.25, 0.50, 0.75 and 1.0 μM solutions of inhibitors **1b–f** were prepared by serial dilution of appropriate volumes of 1 mM solutions in nanopure water. The resulting four stock solutions were kept at ambient temperature until use.

6.3.5. Kinetic assay—Initially, 0–70 μL of the substrate stock solution was added in 10 μL increments in 96-well plates. A constant 10 μL aliquot of the inhibitor stock solution and appropriate amounts of buffer solution were added to keep the volume in each well at 80 μL . After equilibration at 30°C , a 20 μL aliquot of the enzyme stock solution was added to initiate the glycoside hydrolysis. The change in absorbance due to product formation was followed at 405 nm over 15 min in 27s intervals for a total of 34 readings.

6.3.6. Data analysis—The absorbance data were initially transferred into product concentrations using previously determined apparent extinction coefficients. [24] The change in product concentration was then plotted over time, and the initial rates were determined by linear regression of the resulting data. A plot of respective rates corrected for enzyme concentration versus substrate concentration yielded hyperbolic data. The Michaelis-Menten model was applied to determine the catalytic rate constant and the apparent binding affinity for each substrate in absence (k_{cat} , K_{M}) and presence (k'_{cat} , K'_{M}) of inhibitors **1b–f** using non-linear regression. [35] The inhibition constants (K_{i}) were then determined from the kinetic parameters using equation (1)

$$K_i = \frac{K_M [I]}{K_M - K_M} \quad (1)$$

The kinetic parameters were determined in triplicate, and the given inhibition constants are reported as an average of data obtained from 3–4 different inhibitor concentrations.

6.4. Docking studies

The structure of β -galactosidase (*A. oryzae*, 4IUG) was obtained from the Protein Data Bank (<http://www.rcsb.org/pdb/>).[36] The structures of galactonoamidines **1a–g** were minimized using an MM2 force field and converted from a mol file to a pdb file using PYMOL. The pdb files were then imported into the PMV-1.5.6 software[37] and converted into pdbqt files while allowing all non-ring bonds to rotate. All water molecules, solvents, and substrates were removed from the enzyme structure file prior to the docking study. The coordinates of the enzyme were transferred from pdb into pdbqt files with the addition of Kollman partial charges on all atoms, polar H-atoms were added to all residues using AutoDock Vina Tools.[38] A 30×30×30 Å³ grid box was then placed at the center of the active site of the β -galactosidase. The exhaustiveness was set to 100 to account for the size of the grid box, while all other parameters were set to defaults of the AutoDock Vina 1.1.2 Software. The calculations yielded nine conformations that were scored based on their binding affinity in kcal/mol. The docked inhibitor-enzyme assembly with the lowest free energy estimate was selected for further investigation.

6.5. Molecular dynamic studies

Molecular dynamic simulations for the galactonoamidines **1a–f** in complex with β -galactosidase (*A. oryzae*) were performed with GROMACS 4.6.7 software using the GROMOS96 43A1 force field. [39] The coordinate and topology files of the enzyme were generated with the pdb2gmx program.[30] The lowest free energy conformation of each docked inhibitor was imported into PRODRG 2.5 software also using the GROMOS96 43A1 model.[40, 41] Point charges assigned by the PRODRG program may not conform with those of the 43A1 force field, and were consequently corrected.[41] The inhibitor-protein assemblies were prepared by adding coordinates and topology files of **1a–f** to those of β -galactosidase (*A. oryzae*). The resulting protein-inhibitor complexes were then solvated in a 2389 nm³ cubic box with 75,211–75,214 water molecules described by the simple point charge (SPC) water model.[42] Last, 28 randomly selected water molecules were replaced by sodium ions to neutralize the system.

The neutralized systems were minimized using the steepest-decent protocol to avoid any steric clashes.[30] Using a stochastic scaling thermostat, a NVT simulation over 100 ps was done at experimental conditions (303K), and followed by a NPT simulation at 1 bar over 500 ps.[43]. Finally, 31 ns NPT simulations were carried out using the Nosé-Hoover thermostat[44] and the Parrinello-Rahman barostat[45] with a thermostat relaxation time constant of 2.0 ps and a barostat relaxation time constant of 5.0 ps.. The last 30 ns of the NPT simulation were treated as production. All molecular dynamics simulations were performed using an integration time step of a 2 fs with a particle mesh Ewald treatment for

coulombic interactions. Additional details of the molecular dynamics protocol have been described previously.[46]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Spectroscopic identification of inhibitors as transition state analogs (TSAs)
- Molecular dynamics studies reveal key features of TSA characteristics
- Hydrophobic loop stabilizes aglycon during enzymatic glycoside hydrolysis

Figure 1.
Amino acids of β -galactosidase (*A. oryzae*) comprising the active site and part of the hydrophobic loops at its outer rim (margenta).[27]

Figure 2.

Galactonoamidines **1b-g** (blue) docked in the active site of β -galactosidase (*A. oryzae*, 4IUG) and depicted relative to **1a** (yellow) are shown in two different perspectives (a) **1b**, (b) **1c**, (c) **1d**, (d) **1e**, (e) **1f**, and (f) **1g**; residues of catalytically active amino acids (Glu²⁹⁸ and Glu²⁰⁰) in green, all others in grey; H-bonding interactions between the proton donor (Glu²⁰⁰) and **1b-g** at the endocyclic NH group (green) and the exocyclic NH group (dark cyan).

Figure 3. Double-logarithmic plot of the inhibition constant (K_i) and the catalytic efficiency (k_{cat}/K_M) for the hydrolysis of nitrophenyl- β -D-galactopyranosides **2a–i** with linear fit in the presence of (a); **1b** ($\blacktriangle$) $a = 1.02 \pm 0.10$, $R^2 = 0.93$; and **1d** ($\blacksquare$) $a = 1.08 \pm 0.10$, $R^2 = 0.95$; and (b) **1c** ($\blacksquare$) $a = 1.17 \pm 0.32$, $R^2 = 0.69$; **1e** ($\blacklozenge$) $a = 1.16 \pm 0.21$, $R^2 = 0.83$; **1f** ($\blacktriangle$) $a = 0.44 \pm 0.19$, $R^2 = 0.46$;

Figure 4.

(a) Probability of the overall number of H-bond interactions between **1a–f** and β -galactosidase (*A. oryzae*); (b) occupancy of the catalytic amino acid residues Glu²⁹⁸ (nucleophile) and Glu²⁰⁰ (donor) with **1a–f**

Figure 5. Overlay of simulated structures of β -galactosidase (*A. oryzae*) in presence (grey) and absence (purple) of galactonoamidine **1a** (orange).

Figure 6. Orientation of galactonoamidines within the active sites of β -galactosidase (*A. oryzae*) as predicted with molecular dynamic simulations; (a) **1a** (b) **1b** (c) **1c**, (d) **1d** (e) **1e**; and (f) **1f**.

Scheme 1.

Enzymatic hydrolysis of model substrates **2a–i** in the presence of galactonoamidines **1b–f** in 50 mM acetate buffer at pH 5.00, 30.0 ± 0.1 °C.

Chart 1.
Structures of galactonoamidines **1a–g**

Binding free energies G (kcal/mol) and H-bonding interactions via the glycon of galactonoamidines **1a–f** and amino acid residues in the active site of β -galactosidase (*A. oryzae*).

Table 1

Entry	Compound	G (kcal/mol)	H-bond interactions involving					
			OH (C-2)	OH (C-3)	OH (C-4)	OH (C-6)	NH (endocyclic)	NH (exocyclic)
1	1a	-7.5	E ⁴⁴²	E ²⁹⁸	A ¹⁴¹ , Y ³⁴²	E ²⁰⁰	-	-
2	1b	-7.4	E ⁴⁴²	E ²⁹⁸	A ¹⁴¹ , E ⁴⁴²	N ¹⁹⁹	E ²⁰⁰	E ²⁰⁰
3	1c	-7.4	-	-	A ¹⁴¹ , Y ³⁴²	E ²⁹⁸ , N ¹⁹⁹	E ²⁰⁰	E ²⁰⁰
4	1d	-7.2	-	E ²⁹⁸	A ¹⁴¹	N ¹⁹⁹ , Y ⁹⁶	E ²⁰⁰	E ²⁰⁰
5	1e	-6.6	-	E ²⁹⁸	A ¹⁴¹ , Y ³⁴²	E ²⁹⁸ , N ¹⁹⁹	E ²⁰⁰	E ²⁰⁰
6	1f	-6.3	-	E ²⁹⁸	A ¹⁴¹	E ²⁹⁸ , N ¹⁹⁹	E ²⁰⁰	-
7	1g	-5.8	-	E ²⁹⁸	A ¹⁴¹ , Y ³⁴²	E ²⁹⁸ , N ¹⁹⁹	E ²⁰⁰	E ²⁰⁰

Inhibition constants (K_i) for the enzymatic hydrolysis of nitrophenyl- β -D-galactopyranosides **2a-i** in the presence of galactonoamidines **1b-f** in 50 mM acetate buffer at pH 5.00 and 30.0 ± 0.1 °C.

Table 2

Entry	S	$k_{cat}/K_M \times 10^6$ [min ⁻¹ M ⁻¹][25]	K_i [nM] [1b]	K_i [nM] [1c]	K_i [nM] [1d]	K_i [nM] [1e]	K_i [nM] [1f]
1	2a	3.61	17.4	30.4	24.2	28.6	35.8
2	2b	3.23	11.1	42.8	20.3	20.8	37.9
3	2c	3.76	13.5	13.3	20.5	31.9	29.7
4	2d	4.31	11.8	39.2	20.1	26.5	14.6
5	2e	2.18	21.2	135	35.5	81.5	30.6
6	2f	9.86	60.7	168	87.5	145	64.9
7	2g	3.85	15.7	61.8	16.2	40.2	35.2
8	2h	3.99	14.2	40.6	18.7	36.7	51.5
9	2i	3.59	8.6 [25]	6.3 [25]	11.3 [25]	7.8 [25]	9.5 [25]