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N-Benzylgalactonoamidines as potent β-galactosidase inhibitors

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ABSTRACT

A series of *N*-benzylgalactonoamidines was synthesized to probe their inhibitory ability during the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside by β-galactosidase (*Aspergillus oryzae*). All compounds are characterized as potent competitive inhibitors with inhibition constants (K_i) in the low nanomolar range (12–48 nM). The structure of the inhibitors mimics the bond-lengthening during the hydrolysis and the aromatic aglycon of the substrate. The electronic nature of the substituent in *p*-position of the aglycon influences the overall inhibitory ability most when compared to the unsubstituted parent compound.

1. Introduction

Emerging evidence suggests that the mechanistic pathways for glycoside hydrolyses are distinctively different within the glycosidase family.[1] Given this observation, the prediction of whether or not a given compound is a true transition state analog (TSA) for the cleavage of a glycosidic bond by the glycosidase of interest remains difficult and tied to experimental evaluation.[1–3] While many natural and rationally designed compounds are described as TSAs for the hydrolysis of glycosides in excellent reviews,[3–11] only a few of these have binding affinities in the submicromolar concentration range accounting for such TSA-like character.[1]

About 20 years ago, glyconoamidines were identified as competitive inhibitors and putative TSAs due to their similarity in structure, shape, and charge to the half-chair oxocarbenium ion anticipated during the transition state of most enzymatically promoted hydrolyses of glycosides.[12–15] However, only a few notable examples of the inhibitors in this compound class display inhibition of glycosidases in the nanomolar range.[1] Efforts to improve the inhibitory ability of glyconoamidines of unsubstituted *gluco*- and benzylmannoamidines[16–18] focused on the incorporation of supplementary electrostatic interaction to the glyconoamidine core by using aliphatic aglycons with and without additional *N*- or *O*-binding motifs,[1] and the introduction of hydrophobic groups.[19]

Considerably less attention has been given to account for the lengthening of the glycosidic bond during the glycoside hydrolysis, which might play an important role for the interaction of an inhibitor with the active site in the enzyme. We and others[16,20] hypothesized that the aglycon moiety of a substrate might have additional or different interactions with the active site in a glycosidase during the transition state that can only be identified by inhibitors that account for the bond-lengthening during the reaction. The spacer length was shown to influence the inhibitory ability of glucono- and mannonoamidines to some extent.[1,20] The flexibility of the chosen aliphatic aglycons, however, renders a correlation between their structure in an active site and the observed inhibitory activity ambiguous.

In order to verify or reject our hypothesis, we designed a series of *N*-substituted benzylgalactonoamidines as a first step toward the development of potent TSAs of glycosidases and glycosyltransferases. The targeted compounds account for both the bond-lengthening during the hydrolysis and the hydrophobic aromatic character of the aglycon in a model substrate, such as *o*-nitrophenyl-β-D-galactopyranoside (**1**). Substrate **1** is routinely employed to assess the activity of glycosidases and glycosyltransferases. The methylene group in the benzyl moiety of the substituted *N*-benzylgalactonoamidines accounts for the anticipated bond-lengthening in the transition state during the hydrolysis of **1**.[16] The influences of the electronic properties of the substituents of the aromatic aglycon in the designed inhibitors were additionally characterized. Both an electron-withdrawing fluorine and an electron-donating methyl group were incorporated at various positions of the aglycon of *N*-benzylgalactonoamidines. A structure–activity relationship between the

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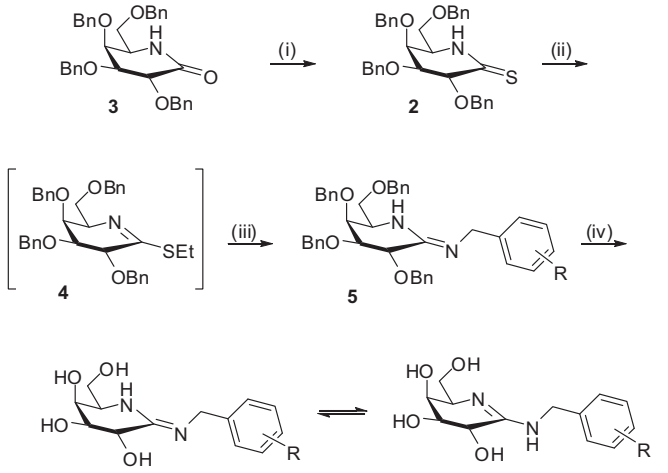
electronic properties of the aromatic aglycon and the inhibitor performance was elaborated.

2. Results and discussion

2.1. Synthesis of *N*-benzylgalactonoamidines

The synthesis of the *N*-benzylgalactonoamidines followed a strategy we reported recently.[21] In brief, 2,3,4,6-tetra-*O*-benzyl-D-galactothionolactam (**2**) was obtained as the main product in a 10g-scale synthesis from 2,3,4,6-tetra-*O*-benzyl-D-galactonolactam (**3**) and Lawesson's reagent (Scheme 1).[22] In situ activation of **2** with Meerwein's salt and coupling of the resulting galactoiminothioether (**4**) with dried *N*-benzylamine provided *N*-benzyl-2,3,4,6-tetra-*O*-benzyl-D-galactono-amidine (**5**) after chromatographic purification.[21] Quantitative O-debenzylation of **5** yielded *N*-benzyl-D-galactonoamidine (**6**) by hydrogenation in the presence of Pd/C and TFA at ambient temperature in 100–150 mg scale reactions.[21]



Scheme 1. Synthesis of *N*-benzylgalactonoamidines. Reaction conditions: (i) Lawesson's reagent, benzene, reflux, 2 h, 93%; (ii) Meerwein salt, CH_2Cl_2, 0 °C, 2 h; (iii) R–$C_6H_5CH_2NH_2$, 0 °C to rt, 16 h, 80% over two steps; (iv) H_2, Pd/C, TFA, EtOH, rt 1 atm, 16 h, quantitatively.

The subsequent syntheses of *N*-(2-methylbenzyl)-D-galactonoamidine (**6a**), *N*-(3-methylbenzyl)-D-galactono-amidine (**6b**), *N*-(4-methylbenzyl)-D-galactonoamidine (**6c**), *N*-(2-fluorobenzyl)-D-galactonoamidine (**6d**), *N*-(3-fluorobenzyl)-D-galactonoamidine (**6e**), *N*-(4-fluorobenzyl)-D-galactonoamidine (**6f**), were conducted likewise using the corresponding methyl- and fluorobenzylamines (Scheme 1). In contrast to previous reports on other glycoamidines,[20] all compounds isolated herein are stable in aqueous solution at −18 °C for at least six months. Despite all attempts, compound **6e** was not obtained in sufficient purity, and was therefore neither fully characterized nor evaluated as a potential inhibitor for β-galactosidases (see Supplementary data).

2.2. Evaluation of *N*-benzylgalactonoamidines as inhibitors for β-galactosidase (*Aspergillus oryzae*)

To assess the inhibitory ability of the synthesized *N*-benzylgalactonoamidines **6a**–**d** and **f**, β-galactosidase from *Aspergillus oryzae* (*A. oryzae*) was employed. The commercially available protein is supplied as a purified, lyophilized powder, stabilized on dextrin with a molar mass in a range between 45 and 113 kDa. Prior to the determination of the kinetic parameters in the presence of absence of *N*-benzylgalactonoamidines, we thus determined the exact molar mass of the enzyme, re-assessed its purity, and the determined concentration of the supplied protein in the freeze-dried powder batch.

2.3. Determination of the molar mass of the enzyme

The molar mass of the enzyme was determined by microchip gel electrophoresis under reducing and non-reducing conditions in the presence of a protein ladder for the 10–260 kDa range. The peak migration times of the samples were normalized using internal size markers and compared to the peak migration in the protein ladder. The molar mass of the tetrameric β-galactosidase (*A. oryzae*) used in this study was determined as 86.8 kDa; no other protein that would interfere with the planned analysis was detected (see Supplementary data).[23] Due to the known limited stability of the enzyme in its pure form and its observed higher activity when immobilized,[24] the dextrin-stabilized enzyme was used as received. The exact protein concentration in the supplied freeze-dried powder was determined by the BCA assay using a known concentration of bovine serum albumin (BSA) as standard.

2.4. Determination of the enzyme activity

The hydrolytic activity of β-galactosidase (*A. oryzae*) was then determined employing **1** as substrate in 50 mM acetate buffer at pH 5 and 30 °C under steady state conditions (Fig. 1).

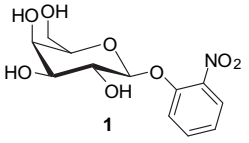


Fig. 1. Structure of the substrate (**1**).

The absorbance recorded for the product formation was transformed into concentration by using the extinction coefficient of *o*-nitrophenolate obtained separately from a calibration curve (ε_{400}=816±56 cm^{-1} M^{-1}) under identical conditions. Initial rates of the hydrolysis were obtained from plots of product formation over time. The apparent Michaelis–Menten constant (K_m=2.8±0.1 mM) and the rate constant (k_{cat}=4100±20 min^{-1}) were determined by plotting the initial rates corrected for the enzyme concentration (3×10^{-8} M) versus the substrate concentration (2–22 mM), and fitting the hyperbolic data by non-linear regression applying the Michaelis–Menten model (Fig. 2a). All experiments were conducted in triplicate, and the resulting data averaged. The obtained kinetic parameters are in good agreement with to those reported for the same enzyme and substrate under similar conditions by others (pH 4.5, K_m=1 mM).[16]

2.5. Inhibition of the enzyme activity

The kinetic experiments were then repeated in the presence of three different concentrations (0.2–0.5 μM) of compounds **6a**–**d** and **6f** to characterize their inhibitory activity during the enzymatic hydrolysis of **1**. Typically, the value of the apparent Michaelis–Menten constant (K'_m) increases, while the apparent rate constant remains uneffected in the presence of **6a**–**d** and **6f**. The hyperbolic data, Lineweaver–Burk, Eadie–Hofstee, and Hanes–Woolf plots obtained from the kinetic data in the presence of **6c** are depicted as representative examples for all others visualizing the observed competitive inhibition during the hydrolysis of **1** (Fig. 2a–d).

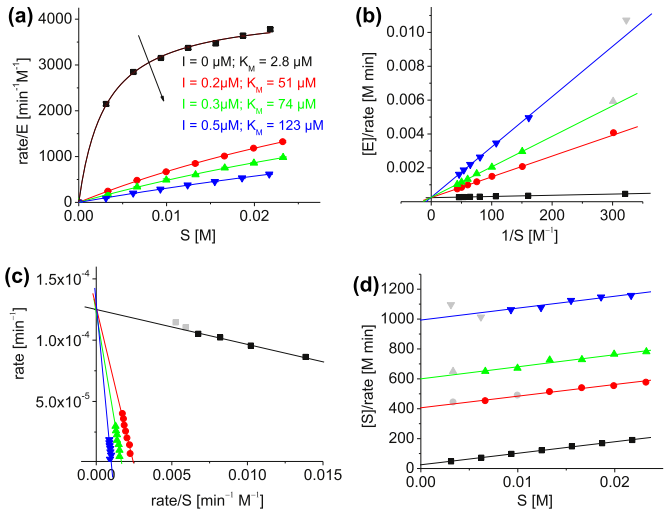


Fig. 2. Inhibitory effect of **6c** on the enzymatic hydrolysis of **1** as (a) hyperbolic data, (b) Lineweaver–Burk, (c) Hanes–Woolf, and (d) Eadie–Hofstee plot; data for I=0 μM are shown in black; I=0.2 μM (red), I=0.3 μM (green), I=0.5 μM (blue); data points not considered during data fitting (gray).

Competitive inhibition constants (K_i) were estimated from the apparent Michaelis–Menten constants in the presence and absence of **6**, **6a**–**d**, and **6f**, and are given as an average of three independent experiments for each concentration (Table 1). All evaluated *N*-benzylgalactonoamidines showed remarkably high potency as competitive inhibitors of the enzymatic hydrolysis of **1** with inhibition constants (K_i) in the low nanomolar range (12–48 nM) rendering compounds **6**, **6a**–**d**, and **6f** among the most potent competitive inhibitors for glycosidases known to date.[1] The result is even more significant when compared to the inhibition ability previously reported for *N*-benzylmannonoamidine;[16] the inhibitor was found to be inactive toward β-galactosidase from *A. oryzae* and showed inhibition ability toward mannosidases in the low micromolar concentration range (6–25 μM).[16] Distinctly different mechanistic pathways for the hydrolysis of glycosides by the enzymes explored might account for this observation.[1]

Table 1
Competitive inhibition constants (K_i) for *N*-arylgalactonoamidines **6**, **6a**–**d**, **6f**

Entry	Inhibitor	$K_i \pm \Delta K_i$ [nM]
1	**6**	21±0.1
2	**6a**	19±0.4
3	**6b**	21±0.1
4	**6c**	12±0.1
5	**6d**	16±0.1
6	**6f**	48±1.7

Notably, the substitution of the *N*-benzyl aglycon in the *p*-position leads to a noteworthy alteration of the inhibitory ability of the corresponding glycoamidine, which depends on the electronic nature of the substituent. Incorporation of a methyl group increases the electron density in the aromatic ring system of the aglycon and decreases the inhibition constant by one half rendering the inhibitor more potent (**6c**<**6**, Table 1, entries 1 and 4); the substitution in the same position with an electron-withdrawing fluoro atom increases the inhibition constant more than twofold rendering the inhibitor less efficient than the unsubstituted parent compound (**6**<**6f**, Table 1, entries 1 and 6). Alterations of **6** with methyl- and fluoro-substituents in the *o*- or *m*-position have a less pronounced effect on the inhibitory ability of the resulting compounds, and will be consequently not further pursued (Table 1, entries 2,3, and 5).

3. Conclusion

In summary, six new *N*-benzylgalactonoamidines (**6a**–**f**) were synthesized. Their inhibitory ability on the hydrolysis of 2-nitrophenyl-β-D-galactopyranoside (**1**) by β-galactosidase (*A. oryzae*) in 50 mM acetate buffer at pH 5 was explored. All compounds are characterized as very potent competitive inhibitors with inhibition constants in the low nanomolar range (12–48 nM), which renders them among the most potent glycosidase inhibitors known up to date. The electronic nature of the substituent in the *p*-position of the aromatic aglycon influences the overall inhibitory ability in opposite directions when compared to the unsubstituted parent compound. Current studies focus on the evaluation of compound **6c** as transition state analog, the determination of the inhibitory selectivity of **6**, **6a**–**d**, and **6f** among glycosidases, and screens of all inhibitors as antiviral drugs.

4. Experimental section

4.1. Instrumentation

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV400 (400.2 MHz for ^{1}H, and 100.6 MHz for ^{13}C). Chemical shifts (δ) in ^{1}H NMR are expressed in parts per million and coupling constants (J) in hertz. Signal multiplicities are denoted as s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Deuterated chloroform and deuterium oxide were used as solvents, and chemical shift values are reported relative to the residual signals of these solvents ($CDCl_3$: δ=7.29 for ^{1}H and δ=77.0 for ^{13}C; D_2O: δ=4.80 for ^{1}H and δ=49.0 for ^{13}C after addition of a few drops of CH_3OD). The MS analysis of the samples was performed using an Ultra Performance LC Systems (ACQUITY, Waters Corp., Milford, MA, USA) coupled with a quadrupole time-of-flight mass spectrometer (Q-TOF Premier, Waters) with electrospray ionization (ESI) in both ESI-MS and ESI-MS/MS modes operated by the Masslynx software (V4.1). Each sample in 50% aqueous acetonitrile was directly injected into the ESI source at a flow rate of 50 mL/min. The ion source voltages were set at 3 kV for positive and negative ion mode acquisitions, respectively. The sampling cone was set at 37 V and the extraction cone was at 3 V. In both modes the source and desolvation temperature was maintained at 120 °C and 225 °C, respectively, with the desolvation gas flow at 200 L/h. IR spectra of oils were recorded as thin films on KBr discs; solids were measured as KBr pellets with a resolution of 0.5 cm^{-1} using a Shimadzu IR Prestige-21 FT-Infrared Spectrometer; ν in cm^{-1}. Thin layer chromatography (TLC) was performed using silica gel TLC plates from SORBENT Technologies, 200 μm, 4×8 cm, aluminum backed, with fluorescence indicator F_{254} and detection by UV light or by charring with an aqueous vanillin–sulfuric acid reagent and subsequent heating of the TLC plate. Column chromatography was carried out using silica gel 60 from Silicycle® (40–63 μm, 230–240 mesh) as stationary phase or basic aluminum oxide (pH range 9.7±0.4, activity I), 32–63 μm, surface area 150 m^2 g^{-1} from Sorbent Technologies. The UV/vis spectra were recorded on a Cary WinUV, Vers. 3.0, Analysis Suite with Suprasil standard cells (200–2000 nm) of 1 cm thickness and 4.5 ml volume at 30 °C over a range of 200–800 nm pH values were measured using a Beckman Φ 250 pH meter equipped with refillable long Futura pH electrode of 0.7 mm thickness. The pH meter was calibrated before each set of readings with standard buffer solutions for pH 4, 7, and 10. Nanopure water was obtained from an EASYpure® II water system from Barnstead (18.2 MΩ/cm).

4.2. Chemicals

Benzylamine, *o*-methylbenzylamine, *m*-methylbenzylamine, *p*-methylbenzylamine, *o*-fluorobenzylamine, *m*-fluorobenzylamine,

and *p*-fluorobenzylamine were obtained from Acros, distilled in vacuum and stored over molecular sieves 3 Å prior to use; dichloromethane (Pharmco-Aaper) and benzene (Sigma–Aldrich) were dried dynamically over neutral aluminum oxide from Acros or basic aluminum oxide from EMD. *o*-Nitrophenol was obtained from Acros and recrystallized from Ethanol prior to use. β-Galactosidase [3.2.1.23] from *A. oryzae* was obtained as lyophilized powder stabilized on dextrin from Sigma–Aldrich and stored at −18 °C prior to use. All other chemicals were reagent grade or better, and were used as received.

4.3. General procedure for the synthesis of *N*-aryl-2,3,4,6-tetra-*O*-benzyl-D-galactonoamidines 5a–f

Meerwein's salt (1.5 equiv) is added under argon to a solution of 2,3,4,6-tetra-*O*-benzyl-D-galactothionolactam (**2**) (200 mg, 0.362 mmol, 1 equiv) in dry CH_2Cl_2 at 0 °C. The resulting suspension is stirred at 0 °C for 2 h, and is used directly for the next step without further purification. A solution of the corresponding benzylamine (2 equiv) in dry CH_2Cl_2 (2 mL) is then added under argon at 0 °C. The reaction mixture is allowed to warm to ambient temperature and is stirred overnight.

*4.3.1. 2-Methylbenzyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidine (**5a**).* Yellow oil (90 mg, 43%); δ_H ($CDCl_3$) 7.57–7.27 (m, 24H, Ph*H*), 5.12 (d, 11.4, 1H, PhCH_2), 5.04 (d, 11.5, 1H, PhCH_2), 4.95 (d, 11.8, 1H, PhCH_2), 4.86 (d, 11.4, 1H, PhCH_2), 4.80–4.69 (m, 5H, −C*H*, OCH_2), 4.50–4.32 (m, 3H, −C*H*, NCH_2), 4.12 (dd, 1.4, 9.7, 1H, −C*H*), 3.99–3.82 (m, 3H, −C*H*, −CH_2), 2.37 (s, 3H, −CH_3), 1.43 (br s, 1H, N*H*); δ_C ($CDCl_3$) 155.7, 139.1, 138.4, 138.1, 137.7, 137.0, 136.4, 130.1–125.8, 81.9, 75.3, 74.4, 74.0, 73.7, 73,3, 71.2, 71.0, 59.6, 42.9, 18.9; ν_{max} (KBr, thin film) 3432, 3018, 2914, 2858, 1642, 1455, 1091, 739, 694 cm^{-1}; ESI-TOF MS *m*/*z* calcd for $C_{42}H_{45}N_2O_4$ $(M+H)^+$: 641.3375; found: 641.3375.

*4.3.2. 3-Methylbenzyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidine (**5b**).* Yellow oil (140 mg, 55%); δ_H ($CDCl_3$) 7.52–7.08 (m, 24H, Ph*H*), 5.06 (d, 11.3, 1H, PhCH_2), 4.99 (d, 11.4, 1H, PhCH_2), 4.90 (d, 12.1, 1H, PhCH_2), 4.80 (d, 11.4, 1H, PhCH_2), 4.75–4.66 (m, 5H, −C*H*, OCH_2), 4.43 (t, 2.9, 1H, −C*H*), 4.35 (q, 14.3, 2H, NCH_2), 4.08 (dd, 1.8, 9.7, 1H, −C*H*), 3.93–3.77 (m, 3H, −C*H*, −CH_2), 2.43 (s, 3H, −CH_3), 1.39 (br s, 1H, N*H*); δ_C ($CDCl_3$) 156.1, 139.0, 138.4, 138.0, 137.7, 128.5–127.4, 124.8, 81.7, 75.2, 74.5, 73.9, 73.6, 73.3, 71.0, 45.2, 21.3; ν_{max} (KBr, thin film) 3440, 2919, 2863, 1650, 1494, 1092, 743, 694 cm^{-1}; ESI-TOF MS *m*/*z* calcd for $C_{42}H_{45}N_2O_4$ $(M+H)^+$: 641.3375; found: 641.3389.

*4.3.3. 4-Methylbenzyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidine (**5c**).* Yellow oil (180 mg, 51%); δ_H ($CDCl_3$) 7.43–7.24 (m, 20H, Ph*H*), 7.35 (s, 4H, Ph*H*), 4.98 (d, 11.4, 1H, PhCH_2), 4.91 (d, 11.5, 1H, PhCH_2), 4.82 (d, 12.0, 1H, PhCH_2), 4.73 (d, 11.4, 1H, PhCH_2), 4.68–4.58 (m, 5H, −C*H*, OCH_2), 4.35 (t, 2.0, 1H, −C*H*), 4.25 (q, 14.1, 2H, NCH_2), 4.0 (dd, 1.8, 9.7, 1H, −C*H*), 3.84–3.69 (m, 3H, −C*H*, −CH_2), 2.38 (s, 3H, −CH_3), 1.32 (br s, 1H, N*H*); δ_C ($CDCl_3$) 155.9, 139.0, 138.4, 138.1, 137.7, 136.5, 136.2, 129.0–127.4, 81.8, 75.3, 74.5, 73.9, 73.7, 73,3, 71.2, 71.0, 59.6, 44.9, 21.1; ν_{max} (KBr, thin film) 3431, 3023, 2930, 2878, 1653, 1498, 1091, 730, 696 cm^{-1}; ESI-TOF MS *m*/*z* calcd for $C_{42}H_{45}N_2O_4$ $(M+H)^+$: 641.3375; found: 641.3370.

*4.3.4. 2-Fluorobenzyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidine (**5d**).* Colorless oil (100 mg, 43%); δ_H ($CDCl_3$) 7.44–7.2 (m, 22H, Ph*H*), 7.10–7.0 (m, 2H, Ph*H*), 4.98 (d, 11.3, 1H, PhCH_2), 4.92 (d, 11.4, 1H, PhCH_2), 4.82 (d, 11.5, 1H, PhCH_2), 4.74–4.58 (m, 6H, −C*H*, OCH_2), 4.40–4.32 (m, 3H, −C*H*, NCH_2), 3.99 (dd, 1.7, 9.7, 1H, −C*H*), 3.83–3.67 (m, 3H, −C*H*, −CH_2), 1.31 (s, 1H, N*H*); δ_C ($CDCl_3$) 162.3, 155.6, 139.0, 138.4, 138.1, 137.8, 130.1 (d, 4.4, −C*H*), 128.5–127.3, 123.9 (d, 2.9, −C*H*), 115.1 (d, 21.6, −C*H*), 81.8, 75.4, 74.4, 73.7, 73.3, 71.2, 71.0, 59.6, 38.5; ν_{max} (KBr, thin film) 3440, 3058, 3028, 2915, 2863, 1653, 1492, 1087, 733, 699 cm^{-1}; ESI-TOF MS *m*/*z* calcd for $C_{41}H_{41}FN_2O_4$ $(M+H)^+$: 645.3129; found: 645.3129.

*4.3.5. 3-Fluorobenzyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidine (**5e**).* Colorless oil (140 mg, 60%); δ_H ($CDCl_3$) 7.49–7.30 (m, 21H, Ph*H*), 7.04–6.94 (m, 3H, Ph*H*), 5.30 (d, 11.3, 1H, PhCH_2), 4.98 (d, 11.4, 1H, PhCH_2), 4.88 (d, 11.7, 1H, PhCH_2), 4.80–4.60 (m, 6H, −C*H*, OCH_2), 4.41–4.27 (m, 3H, −C*H*, NCH_2), 4.03 (dd, 1.7, 9.7, 1H, −C*H*), 3.85–3.70 (m, 3H, −C*H*, −CH_2), 1.35 (s, 1H, N*H*); δ_C ($CDCl_3$) 164.0, 155.7, 142.0, 140.0, 138.3, 138.0, 137.7, 129.7 (d, 8.3, −C*H*), 128.6–127.4, 123.2, 114.5 (d, 20.4, −C*H*), 113.7 (d, 21, −C*H*), 82.0, 75.2, 74.4, 74.2, 73.7, 73.3, 71.1, 71.0, 59.5, 44.4; ν_{max} (KBr, thin film) 3426, 3031, 2853, 1640, 1487, 1092, 744, 691 cm^{-1}; ESI-TOF MS *m*/*z* calcd for $C_{41}H_{41}FN_2O_4$ $(M+H)^+$: 645.3129; found: 645.3126.

*4.3.6. 4-Fluorobenzyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidine (**5f**).* Colorless oil (90 mg, 39%); δ_H ($CDCl_3$) 7.48–7.30 (m, 20H, Ph*H*), 7.22–7.15 (m, 2H, Ph*H*), 7.04–6.97 (m, 2H, Ph*H*), 5.01 (d, 11.3, 1H, PhCH_2), 4.95 (d, 11.4, 1H, PhCH_2), 4.85 (d, 11.8, 1H, PhCH_2), 4.77–4.60 (m, 6H, −C*H*, OCH_2), 4.38 (t, 1.9, 1H, −C*H*), 4.28 (q, 14.4, 2H, NCH_2), 4.01 (dd, 1.7, 9.7, 1H, −C*H*), 3.85–3.68 (m, 3H, −C*H*, −CH_2), 1.32 (s, 1H, N*H*); δ_C ($CDCl_3$) 163.1, 155.8, 139.0, 138.4, 138.0, 137.7, 129.4 (d, 7.6, −C*H*), 128.6–127.4, 115.1 (d, 21.4, −C*H*), 81.9, 75.3, 74.5, 74.1, 73.7, 71.1, 71.0, 59.5, 44.3; ν_{max} (KBr, thin film) 3430, 3027, 2859, 1646, 1503, 1085, 744, 694 cm^{-1}; ESI-TOF MS *m*/*z* calcd for $C_{41}H_{41}FN_2O_4$ $(M+H)^+$: 645.3129; found: 645.3128.

4.4. General procedure for the O-debenzylation of *N*-aryl-2,3,4,6-tetra-*O*-benzyl-D-galactonoamidines; synthesis of *N*-aryll-D-galactonoamidines 6a–f

The perbenzylated amidine (40–80 mg) and Pd/C (80–160 mg, 200% w/w) were stirred in 5 mL of ethanol in the presence of TFA (1 mL, 13.5 mmol) under hydrogen at room temperature. After 16 h, a TLC analysis on silica gel with cyclohexane/EtOAc (1/1; v/v) as eluent showed complete transformation of the starting material toward a more polar spot at the baseline. The reaction mixture was diluted with EtOH (10 mL) and filtered on Celite. The filtrate was concentrated under reduced pressure at 37 °C and dried in vacuum for 16 h at room temperature.

*4.4.1. 2-Methylbenzyl-D-galactonoamidine (**6a**).* The hydrogenation of **5a** (80 mg, 0.125 mmol) yielded **6a** as colorless oil (30 mg, 86%); δ_H (D_2O) 7.4–7.3 (m, 4H, Ph*H*), 4.70–4.60 (m, 3H, −C*H*, −NCH_2), 4.30 (t, 1H, 2.1, −C*H*), 3.99 (dd, 1H, 10.7, 2.4, −C*H*), 3.83–3.70 (m, 3H, −C*H*, −CH_2), 2.30 (s, 3H, −CH_3); δ_C (D_2O/MeOD) 165.0, 137.8, 132.3, 131.6, 129.5, 128.4, 127.3, 71.4, 67.8, 67.2, 60.8, 58.2, 44.8, 18.8; ν_{max} (KBr, thin film): 3425, 2921, 1670, 1199, 1134 cm^{-1}; HRMS (+TOF-MS) *m*/*z* calcd for $C_{14}H_{21}N_2O_4$ $(M+H)^+$: 281.1501; found: 281.1501.

*4.4.2. 3-Methylbenzyl-D-galactonoamidine (**6b**).* The hydrogenation of **5b** (70 mg, 0.11 mmol) yielded **6b** as a yellowish oil (30 mg, quantitative) and analyzed by ^1H NMR spectroscopy in D_2O; δ_H (D_2O) 7.33–7.10 (m, 4H, Ph*H*), 4.63–4.55 (m, 3H, −C*H*, −NCH_2), 4.25 (t, 1H, 2.2, −C*H*), 3.95 (dd, 1H, 10.3, 2.4, −C*H*), 3.77–3.64 (m, 3H, −C*H*, −CH_2), 2.30 (s, 3H, −CH_3); δ_C (D_2O/CD_3OD) 165.3, 140.4, 134.7, 130.1, 128.9, 125.3, 71.7, 68.0, 67.5, 61.0, 58.4, 46.2, 21.3; ν_{max} (KBr, thin film): 3420, 2924, 1676, 1201, 1139 cm^{-1}; HRMS (+TOF-MS) *m*/*z* calcd for $C_{14}H_{21}N_2O_4$ $(M+H)^+$: 281.1501; found: 281.1490.

*4.4.3. 4-Methylbenzyl-D-galactonoamidine (**6c**).* The hydrogenation of **5c** (40 mg, 0.063 mmol) yielded **6c** as a colorless oil (17 mg, quantitatively); δ_H (D_2O) 7.26 (dd, 4H, 12.5, 8.2, Ph*H*), 4.63–4.55 (m, 3H, −C*H*, −NCH_2), 4.28 (t, 3H, 1.8, −C*H*), 3.97 (dd, 1H, 10.1, 2.4, −C*H*), 3.68–3.58 (m, 3H, −C*H*, −CH_2), 2.32 (s, 3H, −CH_3); δ_C (D_2O/CD_3OD) 165.2, 134.0, 131.7, 130.7, 128.4, 71.6, 68.0, 67.5, 61.0, 58.4, 46.0, 21.1;

ν_{max} (KBr, thin film) 3427, 2975, 1667, 1430, 1202, 1137 cm^{-1}; HRMS (+TOF-MS) *m*/*z* calcd for $C_{14}H_{21}N_2O_4$ $(M+H)^+$: 281.1501; found: 281.1496.

*4.4.4. 2-Fluorobenzyl-D-galactonoamidine (**6d**).* The hydrogenation of **5d** (70 mg, 0.11 mmol) yielded **6d** as a colorless oil (30 mg, 97%); δ_H (D_2O) 7.46–7.13 (m, 4H, Ph*H*), 4.66 (s, 2H, PhCH_2), 4.61 (d, 1H, 10.1, C*H*), 4.29 (d, 1H, 1.6, C*H*), 3.97 (dd, 1H, 10.2, 1.7, C*H*), 3.86–3.68 (3H, m, C*H*, CH_2); δ_C (D_2O/CD_3OD) 165.2, 131.8, 130.6, 125.8, 116.8, 116.6, 71, 6, 68.0, 67.5, 61.1, 40.8; ν_{max} (KBr, thin film) 3278, 2931, 1676, 1497, 1459, 1200, 1137 cm^{-1}; ESI-TOF MS *m*/*z* calcd for $C_{13}H_{18}N_2O_4F$ $(M+H)^+$: 285.1251; found: 285.1251.

*4.4.5. 3-Fluorobenzyl-D-galactonoamidine (**6e**).* The hydrogenation of **5e** (70 mg, 0.11 mmol) yielded **6e** as a colorless oil in insufficient purity as judged by ^1H NMR spectroscopy (31 mg, quantitative); ESI-TOF MS *m*/*z* calcd for $C_{13}H_{18}N_2O_4F$ $(M+H)^+$: 285.1251; found: 285.1248.

*4.4.6. 4-Fluorobenzyl-D-galactonoamidine (**6f**).* The hydrogenation of **5f** (40 mg, 0.062 mmol) yielded **6f** as a colorless oil (17 mg, quantitative); δ_H (D_2O) 7.34 (dd, 2H, 8.5, 5.4, Ph*H*), 7.14 (t, 2H, 8.9, Ph*H*), 4.65–4.58 (m, 3H, −C*H*, −NCH_2), 4.28 (t, 1H, 2.3, −C*H*), 3.97 (dd, 1H, 10.1, 2.4, −C*H*), 3.83–3.64 (m, 3H, −C*H*, −CH_2); δ_C (D_2O/MeOD) 165.3, 130.4, 130.3, 117.0, 116.7, 71.7, 68.0, 67.5, 61, 0., 58.5, 45.6; ν_{max} (KBr, thin film): 3378, 2922, 1673, 1202, 1138 cm^{-1}; HRMS (+TOF-MS) *m*/*z* calcd for $C_{13}H_{18}N_2O_4F$ $(M+H)^+$: 285.1251; found: 285.1245.

4.5. Assay for the activity of β-galactosidase (*A. oryzae*)

The activity of tetrameric β-galactosidase (*A. oryzae*, MW 347.2 kDa, unit: 86.6 kDa) was determined in 50 mM buffered solutions at pH 5 using *o*-nitrophenyl-β-D-galactopyranoside as substrate.

4.5.1. Enzyme stock solution and concentration. Typically, 10 mg of the lyophilized protein were dissolved in 10 mL of a 50 mM acetate solution yielding a 3×10^{-8} M stock solution; the concentration was determined by comparison of the absorbance at 280 nm to a calibration curve prepared by the BCA assay using BSE as standard.

4.5.2. Substrate stock solution and kinetic assay. Typically, 10 mL of a 33 mM substrate stock solution (100.0 mg, 0.323 mmol) were prepared and 100–700 μL aliquots were diluted into 990 μL with buffer solution, and thermostated for 30 min at 30 °C. After addition of 10 μL aliquots of the enzyme stock solution and thorough mixing, the formation of *o*-nitrophenolate was followed spectrophotometrically at 400 nm for 15 min.

4.5.3. Data analysis. The absorbance recorded for the product formation was transformed into concentration by using the extinction coefficient of *o*-nitrophenolate at pH 5 (ε_{400}=816±56 cm^{-1} M^{-1}), which was obtained from a calibration curve. Initial rates of the reaction were obtained from plots of product concentration over time. Apparent Michaelis–Menten constants (K_m) and rate constants (k_{cat}) were determined by plotting the initial rates corrected for the enzyme concentration versus the substrate concentration, and fitting the hyperbolic data by non-linear regression. All experiments were conducted in triplicate, and the resulting data averaged.

4.6. Assay for the inhibition of the enzymatic 2-nitrophenyl-β-D-galactopyranoside hydrolysis with substituted *N*-benzylgalactonoamidines

4.6.1. Stock solutions. The enzyme and substrate stock solutions were prepared as described for the non-inhibited hydrolysis (vide infra). Typically, 2–4 mg of the inhibitors were dissolved in nanopure water yielding 6–15 mM aqueous solutions that were stored at −18 °C prior to use. Appropriate volumes of the aqueous inhibitor solutions were diluted into 1000 μL of buffer solutions to obtain 20–50 μM inhibitor stock solution, which were then used for the kinetic analysis.

4.6.2. Kinetic assay. Initially, 10 μL of the inhibitor stock solutions were added to 100–700 μL aliquots of the substrate solution; the resulting solution was diluted with buffer solution to a total volume of 990 μL and equilibrated at 30 °C. After 30 min, 10 μL of the enzyme stock solution were added, the solutions mixed, and the product formation followed spectrophotometrically at 400 nm for 15 min.

4.6.3. Data analysis. The absorbance recorded for the product formation was transformed into concentration by the extinction coefficient of *o*-nitrophenolate at pH 5; initial rates of the reaction were obtained from plots of the product concentration over time. Apparent Michaelis–Menten constants (K'_m) and rate constants (k'_{cat}) were determined by plotting the initial rates corrected for the enzyme concentration versus the substrate concentration, and fitting the hyperbolic data by non-linear regression. All experiments were conducted in triplicate and the data were averaged. Kinetic parameters for three different inhibitor concentrations were determined, and Lineweaver–Burk ([*E*]/rate vs 1/[*S*]), Eadie–Hofstee (rate vs rate/[*S*]), and Hanes plots ([*S*]/rate vs [*S*]) constructed to visualize and examine the inhibition type. The inhibition constant (K_i) for competitive inhibition was calculated from Eq. 1, where K'_m and K_m are Michaelis–Menten constants in the presence and absence of the inhibitor:

$$K'_m = K_m \times (1 + ([I]/K_i)) \quad (1)$$

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Supplementary data

^1H, ^{13}C NMR, IR, and MS spectra of all new compounds **5a**–**f**, **6a**–**f**; gel electrophoresis experiments on the microchip. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tet.2011.10.048.

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