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Structure−Activity Relationship of Highly Potent Galactonoamidine Inhibitors toward β-Galactosidase (*Aspergillus oryzae*)

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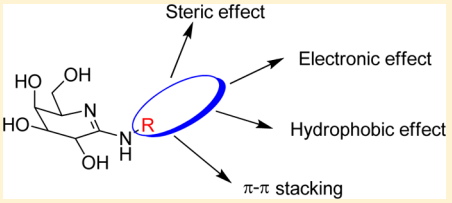
Supporting Information

ABSTRACT: A small library of 22 *N*-substituted galactonoamidines was synthesized, and their structure−activity relationship for inhibition of the hydrolytic activity of β-galactosidase (*Aspergillus oryzae*) was evaluated. A fast screening assay in 96-well plate format was used to follow the enzymatic hydrolysis of 2-chloro-4-nitrophenyl-β-D-galactopyranoside using UV−vis spectroscopy. The aglycon moiety of all compounds was found to have a profound effect on their inhibitory ability. In general, galactonoamidines derived from cyclic aliphatic and linear amines show higher inhibition activity than those derived from benzylamines. Hydrophobic interactions of the methyl group rather than π−π stacking interactions of the aromatic ring in *p*-methylbenzyl-D-galactonoamidine were identified to cause its transition-state-like character and the remarkably high inhibitory ability (K_i = 8 nM). A flexible 3-carbon methylene spacer between the exo N atom of the sugar moiety and a phenyl group furthermore increased the observed apparent inhibition drastically.



INTRODUCTION

Glycosidases are ubiquitous enzymes cleaving and transferring glycosidic bonds.[1] β-Galactosidases (EC 3.2.1.23), a subgroup of glycosidases, can be classified based on the reaction catalyzed, namely, hydrolysis of β-(1−3)- and β-(1−4)-galactosyl bonds, or based on sequence homology among the 90 glycosyl hydrolase families (GHFs). The currently known β-galactosidases belong to GHF-1, GHF-2, GHF-35, and GHF-42.[1]

Frequently, β-galactosidases are used in industrial processes requiring lactose hydrolysis of diary products.[2−4] Additional use of β-galactosidases is found as a reporter gene tool in molecular biology. Therapeutic benefits for treatment of lysosomal disorders have been associated with in vitro competitive inhibitors of lysosomal hydrolases that might also act as intracellular enhancers by stabilization of mutant proteins.[5,6] Due to the limited number of structural information for galactosidases across the corresponding families,[2,7−13] design of inhibitors to diminish or completely hamper glycosidase activity serves a dual purpose, namely, to elucidate the mechanism details and to interfere with the enzyme activity. Competitive inhibitors of human lysosomal β-galactosidase, the most prominent member of GHF-35, are envisioned as new agents for pharmacological chaperone therapy to treat metabolic disorders, such as GM1-gangliosidoses or Morquio B syndrome.[11,14−16]

Following the outlined strategy to elucidate mechanistic details of β-galactosidase activity, we recently focused on the design and evaluation of *N*-benzylgalactonoamidines as glycosidase inhibitors.[17,18] Glyconoamidines are known as potent inhibitors of glycosidases.[19−27] However, the early synthetic strategies developed by others focused on mannono- and gluconoamidines that did not give access to corresponding *N*-substituted galactonoamidines. We thus developed a synthetic strategy suitable for large-scale operation to fill this gap and evaluated the inhibitory ability of *N*-benzylgalactonoamidines and their derivatives toward β-galactosidase (*Aspergillus oryzae*) as a representative, conveniently accessible member of GHF-35.[17,18]

Designed inhibitors were found to be very potent competitive inhibitors with inhibition constants in the very low nanomolar concentration range (12−48 nM).[18] Encouraged by these results, we evaluated selected methyl- and fluoro-*N*-arylgalactonoamidines as putative transition-state analogs (TSA) following a kinetic procedure outlined by Barlett et al.[28,29] We thereby obtained experimental evidence suggesting *p*-methylbenzyl-D-galactonoamidine as a suitable candidate fulfilling the requirements as a TSA. The results furthermore pointed at a strong influence of the aglycon moiety on the overall inhibitory ability of the *N*-substituted galactonoamidines.[29]

These preliminary results prompted an in-depth investigation on the contributions of the aglycon moiety toward the interaction with the active site of the enzyme that may include steric, electronic, hydrophobic, and spacer effects, π−π stacking of the benzyl ring, and van der Waals interactions. We hypothesized that the transition-state-like character of *p*-methylbenzyl-D-galactonoamidine and its remarkably high inhibitory ability are caused by π−π stacking interactions of the aromatic ring of the aglycon moiety with aromatic side chains of amino acids in the active site of the β-galactosidase, by

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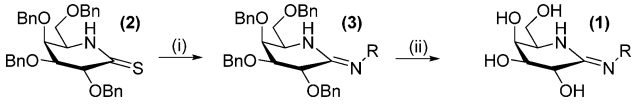
hydrophobic interaction of the methyl group in the para position of the *N*-benzyl ring with corresponding amino acids, or by a combination of both.

To verify or reject this hypothesis, we synthesized a small library of *N*-substituted galactonoamidines derived from benzylic and aliphatic amines and determined the correlation between galactonoamidine structure and resulting inhibitory activity toward *β*-galactosidase (*A. oryzae*). The synthetic efforts and determined bioactivity are summarized and discussed below.

RESULTS AND DISCUSSION

Chemistry. In order to verify or reject our hypothesis, we extended our previously synthesized small library of galactonoamidines composed of *N*-benzyl-D-galactonoamidine (**1a**),[17] 2-methyl-*N*-benzyl-D-galactonoamidine (**1b**),[17] 3-methyl-*N*-benzyl-D-galactonoamidine (**1c**),[17] 4-methyl-*N*-benzyl-D-galactonoamidine (**1d**),[17] 2-fluoro-*N*-benzyl-D-galactonoamidine (**1e**),[17] 3-fluoro-*N*-benzyl-D-galactonoamidine (**1f**),[29] and 4-fluoro-*N*-benzyl-D-galactonoamidine (**1g**)[17] by additional galactonoamidines derived from selected benzylic and aliphatic amines following the same synthetic strategy (Scheme 1).[17,20,30]

Scheme 1. Synthesis of Galactonoamidines 1[a]



R = $-CH_2-C_6H_4$	(**3a**)	(**1a**)
$-CH_2-C_6H_4$-*o*Me	(**3b**)	(**1b**)
$-CH_2-C_6H_4$-*m*Me	(**3c**)	(**1c**)
$-CH_2-C_6H_4$-*p*Me	(**3d**)	(**1d**)
$-CH_2-C_6H_4$-*o*F	(**3e**)	(**1e**)
$-CH_2-C_6H_4$-*m*F	(**3f**)	(**1f**)
$-CH_2-C_6H_4$-*p*F	(**3g**)	(**1g**)
$-CH_2-C_6H_4$-*p*^{t}Bu	(**3h**)	(**1h**)
$-CH_2-C_6H_4$-*p*OMe	(**3i**)	(**1i**)
$-CH_2-C_6H_4$-*p*CF_3	(**3j**)	(**1j**)
$-CH_2-C_6H_4$-*p*Cl	(**3k**)	(**1a**)
$-CH_2-C_6H_4$-*p*Br	(**3l**)	(**1a**)
$-CH_2-CH_2-CH_3$	(**3m**)	(**1m**)
$-CH_2-(CH_2)_5-CH_3$	(**3n**)	(**1n**)
$-CH_2-(CH_2)_{10}-CH_3$	(**3o**)	(**1o**)
$-CH_2-CH(C_2H_5)-(CH_2)_3-CH_3$	(**3p**)	(**1p**)
$-CH_2-(CH_2)_2-C_6H_5$	(**3q**)	(**1q**)
$-C_3H_5$	(**3r**)	(**1r**)
$-C_6H_{11}$	(**3s**)	(**1s**)
$-CH_2-C_3H_5$	(**3t**)	(**1t**)
$-CH_2-C_6H_{11}$	(**3u**)	(**1u**)
$-CH_2-C_7H_{13}$	(**3v**)	(**1v**)

[a]Reaction conditions: (i) $Et_3O^+BF_4^-$, CH_2Cl_2, 0 °C, 2 h; R–NH_2, 0 °C → ambient temperature, 17 h to 8 days; (ii) H_2, Pd/C, EtOH, ambient temperature, 24 h.

In short, we reacted 2,3,4,6-tetra-*O*-benzyl-D-galactothionolactam (**2**)[17,20] with five *p*-substituted benzylamines (namely, 4-*tert*-butylbenzylamine, 4-methoxybenzylamine, 4-trifluoromethylbenzylamine, 4-chlorobenzylamine, and 4-bromobenzylamine), five aliphatic linear amines (namely, propylamine, *n*-heptylamine, *n*-dodecylamine, 2-ethylhexylamine, and 3-phenylpropylamine), and five aliphatic amines with cyclic substituents (namely, cyclopropylamine, cyclohexylamine, cyclopropylmethylamine, cyclohexylmethylamine, and cycloheptylmethylamine). The selection of amines results in different electronic, steric, hydrophobic, and spacer effects, *π*–*π* stacking, van der Waals interaction, and a variety of ring sizes of the aglycon of the galactonoamidines used in the structure–activity study described below.

The coupling reactions between the selected amines and **2** yielded *N*-4-*tert*-butylbenzyl-2,3,4,6-tetra-*O*-benzyl-D-galactonoamidine (**3h**), *N*-4-methoxybenzyl-2,3,4,6-tetra-*O*-benzyl-D-galactonoamidine (**3i**), *N*-4-trifluoromethylbenzyl-2,3,4,6-tetra-*O*-benzyl-D-galactonoamidine (**3j**), *N*-4-chlorobenzyl-2,3,4,6-tetra-*O*-benzyl-D-galactonoamidine (**3k**), *N*-4-bromobenzyl-2,3,4,6-tetra-*O*-benzyl-D-galactonoamidine (**3l**), *N*-propyl-2,3,4,6-tetra-*O*-benzyl-D-galactonoamidine (**3m**), *N*-*n*-heptyl-2,3,4,6-tetra-*O*-benzyl-D-galactonoamidine (**3n**), *N*-*n*-dodecyl-2,3,4,6-tetra-*O*-benzyl-D-galactonoamidine (**3o**), *N*-2-ethylhexyl-2,3,4,6-tetra-*O*-benzyl-D-galactonoamidine (**3p**), *N*-3-phenylpropyl-2,3,4,6-tetra-*O*-benzyl-D-galactonoamidine (**3q**), *N*-cyclopropyl-2,3,4,6-tetra-*O*-benzyl-D-galactonoamidine (**3r**), *N*-4-cyclohexyl-2,3,4,6-tetra-*O*-benzyl-D-galactonoamidine (**3s**), *N*-cyclopropylmethyl-2,3,4,6-tetra-*O*-benzyl-D-galactonoamidine (**3t**), *N*-cyclohexylmethyl-2,3,4,6-tetra-*O*-benzyl-D-galactonoamidine (**3u**), and *N*-cycloheptylmethyl-2,3,4,6-tetra-*O*-benzyl-D-galactonoamidine (**3v**) in moderate to good yields after chromatographic purification over silica gel.

Removal of the protecting benzyl ethers in **3** using Pd on charcoal as catalyst in hydrogen atmosphere in the presence of trifluoroacetic acid (TFA) afforded galactonoamidines **1**, namely, *N*-4-*tert*-butylbenzyl-D-galactonoamidine (**1h**), *N*-4-methoxybenzyl-D-galactonoamidine (**1i**), *N*-4-trifluoromethylbenzyl-D-galactonoamidine (**1j**), *N*-propyl-D-galactonoamidine (**1m**), *N*-*n*-heptyl-D-galactonoamidine (**1n**), *N*-*n*-dodecyl-D-galactonoamidine (**1o**), *N*-2-ethylhexyl-D-galactonoamidine (**1p**), *N*-3-phenylpropyl-D-galactonoamidine (**1q**), *N*-cyclopropyl-D-galactonoamidine (**1r**), *N*-cyclohexyl-D-galactonoamidine (**1s**), *N*-cyclopropylmethyl-D-galactonoamidine (**1t**), *N*-cyclohexylmethyl-D-galactonoamidine (**1u**), and *N*-cycloheptylmethyl-D-galactonoamidine (**1v**) in very good to excellent yields after filtration of the reaction mixture over a pad of Celite, concentration of the filtrate to dryness, and freeze drying of the remaining residue.

Removal of the protecting groups under the elaborated conditions is effortless when the aglycon moiety of **3** is derived from substituted benzylamines.[17] However, applying these conditions to **3k** and **3l** leads to complete or partial dehalogenation of the *N*-benzyl group, yielding *N*-benzylgalactonoamidine **1a** as reaction product depending on the catalyst amount. Dehalogenation reactions of aryl halides under similar hydrogenation conditions are known.[31] As galactonoamidines derived from cyclic aliphatic and linear amines were found to show higher inhibition activity than those derived from benzylamines, a synthetic strategy to afford *p*-chloro- and *p*-bromo-substituted benzylgalactonoamidines **1k** and **1l** was not further explored. Deprotection of 2,3,4,6-tetra-*O*-benzylgalactonoamidines **3n** and **3p**–**3u** derived from aliphatic amines under the conditions resulted in formation of TFA salts that can be avoided by reducing the amount of TFA used during the deprotection reaction. Remaining traces of TFA in the raw products can then be removed by repetitive freeze-drying cycles to afford **1n** and **1p**–**1u** as free bases. For control experiments, D-galactonoamidine (**1w**)[27] and D-galactono-*δ*-lactam[30] (**1x**) were synthesized as described by others earlier. All compounds in the small library (**1a**–**x**) were subsequently employed to probe the stabilizing and destabilizing effects of the aglycon

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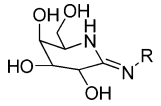
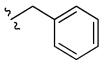
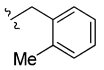
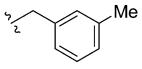
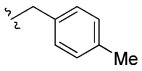
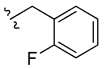
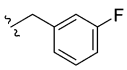
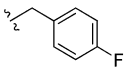
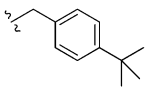
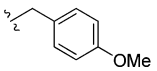
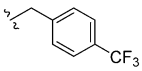
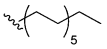
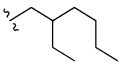
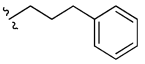
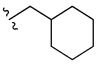
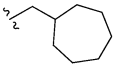
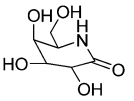
Table 1. Inhibition Constants of *N*-Substituted Galactonoamidines Obtained for Enzymatic Hydrolysis of 2-Chloro-4-nitrophenyl-β-D-galactopyranoside (5) by β-Galactosidase (*A. oryzae*)[a]



Entry	Compound	R	$K_i \pm \Delta K_i$ [nM]
Benzylamines:			
1	**1a**[17]		21.1 ± 1.2
2	**1b**[17]		23.6 ± 2.4
3	**1c**[17]		29.4 ± 5.2
4	**1d**[17]		8.0 ± 0.5
5	**1e**[17]		16.1 ± 0.3
6	**1f**[29]		20.3 ± 2.3
7	**1g**[17]		10.1 ± 2.5
8	**1h**		20.6 ± 2.5
9	**1i**		17.1 ± 3.4
10	**1j**		15.7 ± 0.1
Aliphatic linear amines:			
11	**1m**		149 ± 2
12	**1n**		10.8 ± 0.7
13	**1o**		48.4 ± 3.3
14	**1p**		7.8 ± 1.4
15	**1q**		8.6 ± 1.2
Aliphatic amines with cyclic substituent:			
16	**1r**		31.9 ± 1.8
17	**1s**		602 ± 52
18	**1t**		9.5 ± 1.1
19	**1u**		11.3 ± 0.9
20	**1v**		6.3 ± 0.6
No aglycon, control experiments:			
21	**1w**[27]	H	150 ± 23
22	**1x**[30]		615 ± 31

[a] k_{cat} = 2516 ± 53 min^{-1}; K_M = 0.70 ± 0.04 mM; k_{non} = 1.04 ± 0.31 × 10^{-5} min^{-1} M^{-1}; V_t = 100 μL; 50 mM acetate buffer at pH 5.00 ± 0.05; 30.0 ± 0.1 °C; $\varepsilon_{app,405} \times d$ = 1532 M^{-1}.

moiety upon interaction of the galactonoamidines with the active site of β-galactosidase (*A. oryzae*). The determined structure−activity relationship is described below.

Biology. *Substrate Choice and Kinetic Assay.* We previously established *N*-benzylgalactonoamidines **1a−g** as competitive inhibitors of β-galactosidase (*A. oryzae*)[18] and *p*-methyl-*N*-benzylgalactonoamidine **1d** as putative transition-state analog for hydrolysis of β-glycosidic bonds by β-galactosidase (*A. oryzae*).[29] We subsequently hypothesized a significant influence of the aglycon moiety on the high inhibition ability of **1d** ($K_i \approx 8$ nM) and its transition-state-like character that might be due to π−π stacking interactions of the aromatic ring of the aglycon moiety with aromatic side chains of amino acids in the active site of the β-galactosidase, by hydrophobic interaction of the methyl substituent with corresponding amino acids, or by a combination of both.

In order to verify or reject this hypothesis, we developed a fast screening tool based on a 96-well plate assay to evaluate our small library of *N*-substituted galactonoamidines in a timely manner. Along these lines, we replaced the previously used, commercially available substrate 2-nitrophenyl-β-D-galactopyranoside (**4**) by 2-chloro-4-nitrophenyl-β-D-galactopyranoside (**5**) synthesized for a different project.[29] The comparably low value for the extinction coefficient of **4** in acetate buffer at pH 5 ($\varepsilon_{400} \approx 820 \pm 60$ cm^{-1} M^{-1}; V_t = 1000 μL)[18] translates into an even smaller apparent extinction coefficient in a kinetic assay

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under these conditions in a 96-well plate ($\varepsilon_{app,405} \approx 125$ cm^{-1} M^{-1}; $V_t = 100$ μL), increases the error associated with the kinetic data, and prompted this change. While mathematical corrections of an apparent extinction coefficient can be done to account for the reduced optical path length and the smaller total volume in a 96-well plate assay, the thereby initially obtained kinetic data showed a larger error than data obtained from standard UV−vis cells and were consequently discarded. To circumvent any uncertainty of kinetic data while relying on a fast screening method, substrate **5** was used throughout the kinetic structure−activity study described below.[29]

The hydrolytic activity of β-galactosidase (*A. oryzae*) was determined in the presence and absence of the synthesized *N*-substituted galactonoamidines **1** in 50 mM acetate buffer at pH 5 and 30 °C under steady state conditions. The absorbance recorded for formation of 2-chloro-4-nitrophenolate was transformed into the product concentration using the uncorrected apparent extinction coefficient of 2-chloro-4-nitrophenolate obtained separately from a calibration curve under identical conditions ($\varepsilon_{app,405} \times d = 1532$ M^{-1}). Initial rates of the hydrolysis were obtained from plots of product formation over time. The 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 (0−2 mM) and fitting the hyperbolic data by nonlinear regression applying the Michaelis−Menten model. All experiments were conducted in triplicate, and the resulting data were averaged.

In the presence of three different concentrations of inhibitors **1a−w**, the value of the apparent Michaelis−Menten constant (K'_M) increases while the apparent rate constant remains unaffected. Competitive inhibition constants (K_i) were estimated from the apparent Michaelis−Menten constants in the presence and absence of **1a−w** and are given as an average of three independent experiments for each concentration (Table 1).

Structure−Activity Relationship. Initially, we re-evaluated already available inhibitors **1a−g** containing methyl- or fluoro-substituted benzyl aglycons with substrate **5** instead of previously used substrate **4** in 96-well plate format under otherwise identical conditions. We determined competitive inhibition constants for those 7 inhibitors (K_i = 8−30 nM) that reflect the same stereoelectronic effects as described previously.[18,29] In short, the fluoride substituent on the *N*-benzyl aglycon mimics a hydrogen atom in size but has electron-withdrawing properties that vary depending on the position on the aromatic ring, while a methyl group substituent is larger in size than the H atom and has electron-donating properties. The unsubstituted *N*-benzylgalactonoamidine **1a** shows a smaller inhibition constant than the 2- or- 3-methyl-*N*-benzylgalactonoamidines **1b−c** and the 2- or 3-fluoro-*N*-benzylgalactonoamidines **1e−f** (Table 1, entries 1−3, 5−6). In contrast, a methyl or fluoro substituent in the para position of the benzyl substituent favor the interactions of inhibitors **1d** and **1g** over those of **1a** with the amino acids in the active site of β-galactosidase (*A. oryzae*) (Table 1, entry 4 and 7). This result indicates favorable stereoelectronic effects of substituents in the para position of the benzyl aglycon upon interactions of the inhibitors, like **1d** or **1g**, for inhibition of β-galactosidase (*A. oryzae*) and possible hydrophobic interactions with the methyl group in the active site ($K_{i,1d} < K_{i,1g}$).

To further explore the supporting effects of para substituents on the *N*-benzyl aglycon, we synthesized inhibitors **1h−j** (Table 1, entries 8−10) with *tert*-butyl, methoxy, and trifluoromethyl groups and compared their inhibition ability to **1d**. The increased size of the *p*-*tert*-butyl group decreases the inhibition ability of **1h** compared to the smaller *p*-methyl group in **1d**, indicating steric constraints for large substituents. Likewise, the inhibition ability of **1i** with a *p*-methoxy substituent is smaller than that determined for **1d** ($K_{i,1d} < K_{i,1i}$) but of the same order of magnitude as for **1h**, indicating that H-bonding interactions toward the oxygen atom in the aglycon are absent ($K_{i,1h} < K_{i,1i}$). A similar lack of supporting H-bonding interactions is apparent when comparing the effect of the *p*-methyl substituent in **1d** to the *p*-trifluoromethyl group in **1j**, which is also considerably larger in size than the *p*-methyl group in **1d**. The study of para-substituted *N*-benzylgalactonoamidines revealed size constraints and supportive hydrophobic interactions between the inhibitors and the active site in β-galactosidase (*A. oryzae*) but a lack of supporting H-bonding interactions with the para substituent of the aglycon.

Galactonoamidines with *p*-chloro and *p*-bromo substituents are not accessible with the current synthetic procedure described above and resulted in partial or complete dehalogenation of the corresponding precursor compounds depending on the catalyst amount. However, given the size constraints and lack of supporting H-bonding interactions observed by comparing **1a**, **1d**, and **1h**, we did not improve the synthetic strategy toward these target compounds for comparison to **1g** and **1j** and focused on evaluating hydrophobic and $\pi-\pi$ stacking interactions instead.

Along these lines we synthesized a selection of aliphatic galactonoamidines with linear aliphatic, branched aliphatic, and aliphatic cyclic amines (Table 1, entries 11−20). The distribution of the inhibition ability determined for galactonoamidines **1m−o** with linear aliphatic side chains (Table 1, entry 11−13) disclosed that a side chain consisting of 3 C atoms has only a small contribution on the inhibition ability of the compound, while a linear side chain of 7 C atoms promotes almost the same interaction as the aromatic *N*-benzyl groups ($K_{i,1d} \approx K_{i,1n}$). A carbon side chain of 12 carbon atoms reveals lower inhibition ability and points at steric constraints due to the side chain length.

Galactonoamidines with aglycons consisting of cyclic amines directly attached to the amidine group show insufficient interactions with the active site for both a 3-membered ring (**1r**) and a 6-membered ring (**1s**). Interestingly, galactonoamidines with a methylene spacer and aliphatic cyclic aglycon (**1t−v**) show remarkable high inhibition ability toward the β-galactosidase (Table 1, entries 18−20). This observation indicates strong hydrophobic interactions of the active site with the methylene spacer and also with the aliphatic cyclic moiety of the aglycon in **1t−v**. Most pronounced is a comparison of the inhibition ability between **1r** and **1t** and between **1s** and **1u**, respectively, revealing the importance of the aliphatic methylene spacer in the aglyon, while a comparison of **1a** with *N*-benzyl aglycon to **1u** with aliphatic methylcyclohexyl aglycon clearly discloses a lack of favorable $\pi-\pi$ stacking interactions to the galactonoamidines in the active site of the β-galactosidase ($K_{i,1u} < K_{i,1a}$).

These findings prompted the synthesis of galactonoamidines **1p** with branched aliphatic and of galactonoamidines **1q** with a methylene spacer consisting of 3 C atoms attached to an aromatic ring (Table 1, entries 14 and 15). Both compounds were indeed found to inhibit the β-galactosidase in the same order of magnitude as **1d** and **1t−u** ($K_i \approx 8 \pm 1$ nM). A similar

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phenomenon on the inhibition ability has been previously observed by others after introduction of flexible and hydrophobic substituents.[18,32] *N*-Ethylphenylgluconoamidine and C(2)-phenylethyl-substituted glucoimidazole were identified as very potent inhibitors with very low inhibition constants ($K_i \leq 1.2$ nM) toward selected β-glucosidases (*Almonds*, *A. niger*, and *Caldocellum saccharolyticum.*).[19,32]

Consequently, the previously observed high inhibition ability of **1d** is not based on $\pi-\pi$ stacking interactions with the aromatic *N*-benzyl aglycon but rather on stabilizing hydrophobic interactions with the *p*-methyl substituent on the aromatic aglycon. For comparison and control experiments, we resynthesized already described galactonoamidine **1w** and galactono-δ-lactam **1x** (Table 1, entries 21 and 22).[27,30] The inhibition constant of **1w** is about 1 order of magnitude above the inhibition constant of galactonoamidines with an aglycon, while the exchange of the amidine group in **1w** against the lactam group in **1x** reveals the contribution of the exocyclic N atom on the inhibition ability of the galactonoamidines **1a–v**. The lack of an aglyon diminishes the inhibitory activity of the investigated compounds, thereby revealing its significant contribution.

We then determined the efficacy of selected inhibitors to characterize the concentrations needed to cause inhibition effects for hydrolysis of **5** by β-galactosidase (*A. oryzae*). Toward this goal, we varied the concentration of inhibitors **1d**, **1n**, **1p-q**, **1s-v**, and **1x** between 5×10^{-4} and 50 μM at constant substrate concentration (Figure 1).

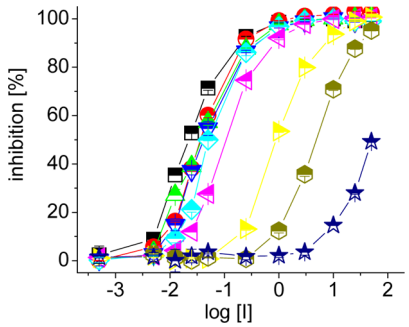

Figure 1. Efficacy of selected inhibitors during enzymatic hydrolysis of **5**: black square, **1u**; red circle, **1v**; green up triangle, **1p**; dark blue down triangle, **1d**; blue diamond, **1q**; pink left side triangle, **1n**; yellow right side triangle, **1t**; dark yellow hexagon, **1s**; blue star, **1x**.

Compounds **1d**, **1n**, **1p–q**, and **1t–v** were identified as very potent inhibitors with inhibition constants equal to or less than 12 nM, while **1s** and **1x** have higher inhibition constants and were selected for control experiments (Table 1). In general, the apparent inhibition efficacy of **1d**, **1n**, **1p–q**, and **1t–v** is very similar, showing high inhibition efficacy. All of the selected inhibitors support hydrophobic interactions between the active site of the enzyme and their aglycon as described above. A significant difference in the overall inhibitor efficacy related to the respective aglycon structure in **1d**, **1p–q**, and **1u–v** is not apparent. The maximal apparent inhibition effect for these inhibitors is achieved at concentrations between 3 and 10 μM. The concentration required to reach the half-maximal inhibition (IC_{50}) of the enzymatic hydrolysis of **5** was determined to be as low as 22 (**1u**) and 36 nM (**1d**, **1p–q**, and **1v**). Although the inhibition constant for compound **1n** is on the same order of magnitude, a somewhat diminished inhibition efficacy for **1n** (IC_{50} = 107 nM) is noted that is attributed to insufficient hydrophobic interactions of the heptyl aglycon in the active site. An further diminished inhibition efficacy is noted for inhibitor **1t** (IC_{50} = 0.86 μM) that contains an even smaller methylenecyclohexyl aglycon.

Furthermore, the importance of the methylene spacer relative to the size of the remaining algycon for the inhibitory ability of the galactonoamidines becomes apparent by comparing the inhibitory efficacy of **1t** to that of **1s**. Control compound **1s** lacks the methylene spacer that is contained in **1t** but contains a larger cyclohexyl aglycon instead of a methylenecyclopropyl rest and shows a considerably lower inhibitory efficacy (IC_{50} = 4.8 μM). Even more pronounced is the difference in efficacy for galactonolactam **1x** that was found to require a 49 μM concentration to achieve 50% inhibition of the reaction. Both control compounds, **1s** and **1x**, have inhibition constants on the same order of magnitude (Table 1), but compound **1x** lacks an aglycon moiety and has a 10-fold lower inhibitory efficacy than **1s**. Overall, the efficacy studies provide further evidence for the important contributions of the aglycon to the performance of the galactonoamidines as inhibitors and reveal high inhibitory efficacy for **1u**, **1d**, **1p–q**, and **1v** (IC_{50} = 22–36 nM).

CONCLUSION

A small library of 22 *N*-substituted galactonoamidines (**1a–v**) was synthesized and evaluated for their ability to inhibit the hydrolytic activity of β-galactosidase (*A. oryzae*). Along these lines, a fast screening assay based on UV–vis spectroscopy was developed suitable for small volumes in acidic conditions employing a 96-well plate format to follow hydrolysis of 2-chloro-4-nitrophenyl-β-D-galactopyranoside. The *N*-substituted galactonoamidines were synthesized from a selection of aliphatic and benzylic amines representing steric, electronic, hydrophobic, and spacer effects, $\pi-\pi$ stacking, and van der Waals interactions. The aglycon moiety of all compounds was found to have a profound effect on their inhibitory ability in comparison to control compounds lacking aglycons, namely, galactonolactam (**1w**) and galactonoamidines (**1x**). In general, galactonoamidines derived from cyclic aliphatic and linear amines were found to show higher inhibition activity than those derived from benzylamines. Finally, hydrophobic interactions of the methyl group rather than $\pi-\pi$ stacking interactions of the aromatic ring in *p*-methylbenzyl-D-galactonoamidine (**1d**) were identified to cause its transition-state-like character and the remarkably high inhibitory ability (K_i = 8 nM) and efficacy (IC_{50} = 36 nM).

EXPERIMENTAL SECTION

Instrumentation. ^{1}H and ^{13}C NMR spectra were recorded on a 400 MHz Bruker magnet with *Z* gradient and 5 mm broad-band head using Topspin 2.1 software. High-resolution mass spectrometry data were obtained in the state-wide mass spectrometry facility at the University of Arkansas on a Bruker ultrOTOF-Q quadrupole time-of-flight (qQ-TOF) mass spectrometer equipped with an electrospray ionization source or the Mass Spectrometry Facility at Georgia State University, Atlanta, GA. UV–vis data were recorded on a FilterMax F5 Multi-Mode Microplate Reader from Molecular Devices using 96-well, medium-binding microlon Elisa plates from Greiner Bio-one. Lyophilization was performed on a FreeZone 1 L benchtop freeze-dry system from Labconco. Melting points were recorded on a Mel-Temp melting point apparatus, and values are uncorrected. Nanopure water at a resistance of 18.2 mΩ was obtained from a ThermoScientific Barnstead E-pure water purification system. Purity assays were

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performed on an HPLC system from Shimadzu equipped with a SCL-10Avp system controller, 2 LC-20AD analytical pumps, DGU-20A3R three-channel online degassers, a SIL-20A UFLC autosampler with 96-well capability, a CTO-20A/prominence column oven, and ELSD-90LT light scattering and LC solution software, version 1.25 from Shimadzu for data recording and analysis.

Materials and Methods. Chemical shifts (δ) in NMR data are expressed in parts per million (ppm) and coupling constants (J) in Hertz. Signal multiplicities are denoted as s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Deuterated chloroform, deuterated dichloromethane, DMSO-d_6, acetone-d_6, MeOH-d_4, and deuterium oxide were used as solvents. Chemical shift values are reported relative to the residual signals of these solvents ($CDCl_3$ δ_H 7.29, δ_C 77.0; CD_2Cl_2 δ_H 5.32, δ_C 54.0; DMSO-d_6 δ_H 2.50, δ_C 39.5; acetone-d_6 δ_H 2.05, δ_C 29.8; MeOH-d_4 δ_H 3.35, δ_C 49.0; D_2O δ_H 4.80, δ_C 49.0 after addition of a few drops of MeOH-d_4). High-resolution mass spectrometry data were obtained from samples that were mixed with acetonitrile containing 0.1% formic acid and injected into the source via a syringe pump operating at 3 μL/h. The dry gas temperature was 180 °C, the dry gas flow was 5 L/min, and the nebulizing gas pressure was 1 bar. The remaining instrument parameters were optimized to obtain maximum signal for ions between 100 and 1000 amu. Column chromatography was carried out using silica gel 60 from Silicycle (40−63 μm, 230−240 mesh). Thin layer chromatography (TLC) was performed using silica gel TLC plates from SORBENT Technologies, 200 μm, 4 × 8 cm, aluminum backed, with fluorescence indicator F254 and detection by UV light or by charring with an ethanolic vanillin−sulfuric acid reagent and subsequent heating of the TLC plate. Compound purity was established on a Rezex RMN−Na^+ column (Phenomenex) using nanopure water as eluent with a flow rate of 0.4 mL/min at 80 °C and electric light scattering detection (ELSD) at 50 °C (GAIN 5). In a typical experiment, a 10 μL aliquot of a solution prepared from 5 mg of compound in 1 mL of nanopure water was injected, and the isocratic elution was followed over 30 min. All elution peaks were integrated, and purity data were obtained as area percent values to confirm a purity of all compounds above 95%. All pH values of the buffer solutions were obtained using a Beckman Φ 250 pH meter equipped with a refillable ROSS combination pH electrode from Orion with epoxy body and an 8 mm semimicrotip. The pH meter was calibrated before each set of readings (3-point calibration).

Chemicals. All commercially obtained chemicals had reagent-grade quality or better and were used as received, if not noted otherwise. 4-Trifluoromethylbenzylamine, 4-methoxybenzylamine, 4-*tert*-butylbenzylamine, 4-chlorobenzylamine, 4-bromobenzylamine, propylamine, *n*-heptylamine, 2-ethylhexylamine, cyclopropylamine, cyclohexylamine, cyclopropylmethylamine, cyclohexylmethylamine, benzylamine, cyclohexane, and ethyl acetate were distilled prior to use; dichloromethane was dried over molecular sieves 4 Å; β-galactosidase [3.2.1.23] from *A. oryzae* was obtained from Sigma-Aldrich as lyophilized powder stabilized on dextrin and stored at −18 °C. A BCA assay confirmed that the supplied batch contains 10% of protein.[18] The molar weight of the tetrameric protein was determined as 347.2 kDa (86.8 kDa per unit) by microchip electrophoresis.[18]

Synthetic Procedures. *Synthesis of Galactonoamidines.* 2,3,4,6-Tetra-*O*-benzyl-D-galactothionolactam (**2**),[17,20,30] *N*-benzyl-D-galactonoamidine (**1a**),[17] *N*-2-methylbenzyl-D-galactonoamidine (**1b**),[17] *N*-3-methylbenzyl-D-galactonoamidine (**1c**),[17] *N*-4-methylbenzyl-D-galactonoamidine (**1d**),[17] *N*-2-fluorobenzyl-D-galactonoamidine (**1e**),[17] *N*-3-fluorobenzyl-D-galactonoamidine (**1f**),[29] *N*-4-fluorobenzyl-D-galactonoamidine (**1g**),[17] D-galactonoamidine (**1w**),[27] and D-galactono-δ-lactam (**1x**)[30] were prepared as described. Quantitative HPLC analysis for all galactonoamidines used in structure−activity relationship studies was performed to verify a compound purity above 95% (see Supporting Information).

*General Procedure for Synthesis of N-Aryl- and N-Alkyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidines (**3h−v**).* Meerwein's salt was added to a solution of 2,3,4,6-tetra-*O*-benzyl-D-galactothionolactam (**2**)[17,20,30] in dry dichloromethane under nitrogen atmosphere at 0 °C. After 2 h, freshly distilled amine was added and the solution was allowed to warm to ambient temperature. After 12 h to 8 days, all volatile material was removed under reduced pressure. The resulting residue was purified by column chromatography over silica gel, yielding the title compounds **3h−v** in fair to moderate yields.

*N-4-tert-Butylbenzyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidine (**3h**).* Reaction: Meerwein's reagent (0.54 g, 2.820 mmol, 1.5 equiv) and **2** (1.05 g, 1.950 mmol) in 10 mL of dichloromethane; amine 4-*tert*-butylbenzylamine (0.62 g, 3.800 mmol, 2.0 equiv); reaction time 8 days; eluent for chromatography cyclohexane/ethyl acetate = 6/1−3/1, v/v; compound **3h** (0.53 g, 0.776 mmol, 41%); colorless oil. R_f 0.27 (SiO_2, cyclohexane/ethyl acetate = 3/1, v/v). δ_H (CD_2Cl_2) 7.21−7.49 (m, 20H), 7.14 (d, 8.3, 2H), 5.01 (br s, 1H), 4.94 (d, 11.0, 1H), 4.85 (t, 12.0, 2H), 4.63−4.69 (m, 2H), 4.62 (d, 3.8, 1H), 4.56 (s, 2H), 4.51 (d, 9.5, 1H), 4.32 (s, 1H), 4.21 (quind, 14.3, 4.3, 2H), 3.99 (dd, 9.7, 1.9, 1H), 3.67−3.76 (m, 3H), 3.59−3.66 (m, 2H), 1.31 (s, 9H). δ_C (CD_2Cl_2) 156.2, 150.4, 139.7, 139.4, 138.9, 138.7, 137.3, 129.0, 129.0, 128.9, 128.7, 128.6, 128.5, 128.4, 128.4, 128.3, 128.2, 128.1, 128.0, 127.9, 125.8, 82.5, 76.1, 75.2, 74.7, 74.2, 73.8, 72.0, 71.6, 60.1, 44.9, 34.9, 31.7. δ_C ($CDCl_3$) 156.0, 149.8, 139.0, 138.4, 138.1, 137.7, 136.1, 128.5, 128.4, 128.3, 128.1, 128.1, 128.0, 127.9, 127.8, 127.7, 127.6, 127.6, 127.6, 127.4, 125.3, 81.8, 75.3, 74.5, 73.9, 73.7, 73.3, 71.1, 71.0, 59.5, 45.0, 34.4, 31.3. HRMS (ESI) calcd for $C_{45}H_{51}N_2O_4$ $[M + H]^+$ 683.3843; found, 683.3843.

*N-4-Methoxybenzyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidine (**3i**).* Reaction: Meerwein's reagent (0.62 g, 3.260 mmol, 2.0 equiv) and **2** (0.90 g, 1.630 mmol) in 6 mL of dichloromethane; amine 4-methoxybenzylamine (0.45 g, 3.260 mmol, 2.0 equiv); reaction time 60 h; eluent for chromatography cyclohexane/ethyl acetate = 6/1−3/1, v/v; compound **3i** (0.64 g, 0.976 mmol, 63%); colorless oil. R_f 0.21 (SiO_2, cyclohexane/ethyl acetate = 3/1, v/v). δ_H (CD_2Cl_2) 7.19−7.48 (m, 20H), 7.11 (d, 8.8, 2H), 6.80 (d, 8.8, 2H), 4.76−5.00 (m, 4H), 4.62 (dd, 11.4, 7.2, 4H), 4.55 (s, 2H), 4.50 (br s, 2H), 4.30 (br s, 1H), 4.17 (br s, 2H), 3.96 (d, 9.8, 1H), 3.77 (s, 3H), 3.54−3.74 (m, 3H). δ_C (CD_2Cl_2) 159.3, 156.2, 139.7, 139.4, 138.9, 138.7, 132.3, 129.5, 129.0, 129.0, 128.9, 128.8, 128.6, 128.5, 128.4, 128.3, 128.2, 128.1, 128.0, 114.2, 82.4, 76.0, 75.2, 74.7, 74.2, 73.8, 72.0, 71.6, 60.1, 55.7, 44.8. δ_C ($CDCl_3$) 158.6, 155.8, 139.0, 138.4, 138.0, 137.7, 131.3, 129.0, 128.5, 128.4, 128.3, 128.1, 128.1, 127.9, 127.8, 127.8, 127.6, 127.6, 127.5, 127.3, 113.7, 81.8, 75.2, 74.4, 73.9, 73.7, 73.3, 71.1, 70.9, 59.5, 55.2, 44.5. HRMS (ESI) calcd for $C_{42}H_{45}N_2O_5$ $[M + H]^+$ 657.3323; found, 657.3321.

*N-4-Trifluoromethylbenzyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidine (**3j**).* Reaction: Meerwein's reagent (0.25 g, 1.355 mmol, 1.5 equiv) and **2** (0.50 g, 0.906 mmol) in 6 mL of dichloromethane; amine 4-trifluoromethylbenzylamine (0.31 g, 1.812 mmol, 2.0 equiv); reaction time 48 h; eluent for chromatography cyclohexane/ethyl acetate = 6/1−3/1, v/v; compound **3j** as a colorless oil (0.23 g, 0.331 mmol, 33%). R_f 0.20 (SiO_2, cyclohexane/ethyl acetate = 2/1, v/v). δ_H (CD_2Cl_2) 7.53 (d, 7.8, 2H), 7.41−7.46 (m, 2H), 7.23−7.40 (m, 20H), 5.09 (br s, 1H), 4.95 (d, 11.0, 1H), 4.92 (d, 11.5, 1H), 4.85 (d, 11.5, 1H), 4.62−4.71 (m, 3H), 4.55 (s, 3H), 4.52 (d, 1.3, 1H), 4.35 (s, 3H), 4.32 (t, 2.0, 1H), 3.99 (dd, 9.7, 1.9, 1H), 3.65−3.73 (m, 3H), 3.60 (dd, 11.3, 9.8, 1H). δ_C (CD_2Cl_2) 156.1, 144.9, 139.5 (d, $J^2_{C,F}$ =40.3 Hz), 138.8 (d, $J^3_{C,F}$ =22.7 Hz), 129.1, 129.1, 129.0, 128.9, 128.8, 128.7, 128.5, 128.5, 128.4, 128.3, 128.3, 128.2, 128.1, 128.1, 125.7 (q, $J^4_{C,F}$ = 3.7 Hz), 125.0 (q, $J^1_{C,F}$ =270.7 Hz), 82.6, 76.1, 75.3, 74.6, 74.5, 73.8, 71.9, 71.6, 60.1, 44.7. HRMS (ESI) calcd for $C_{42}H_{43}F_3N_2O_4$ $[M + H]^+$ 695.3097; found 695.3092.

*N-4-Chlorobenzyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidine (**3k**).* Reaction: Meerwein's reagent (0.51 g, 2.72 mmol, 2.0 equiv) and **2** (0.75 g, 1.36 mmol) in 10 mL of dichloromethane; amine 4-chlorobenzylamine (0.38 g, 2.72 mmol, 2.0 equiv); reaction time 5 days; eluent for chromatography cyclohexane/ethyl acetate = 8/1−4/1, v/v; compound **3k** (0.40 g, 0.608 mmol, 45%); colorless oil. R_f 0.25 (SiO_2, cyclohexane/ethyl acetate = 2/1, v/v). δ_H (CD_2Cl_2) 7.18−7.45 (m, 22H), 7.14 (d, 8.5, 2H), 5.00 (br s, 1H), 4.94 (d, 11.0, 1H), 4.88 (d, 11.5, 1H), 4.84 (d, 11.8, 1H), 4.59−4.68 (m, 3H), 4.55 (s, 2H), 4.51 (d, 9.8, 1H), 4.31 (t, 2.0, 1H), 4.24 (br s, 2H), 3.97 (dd, 9.5, 1.8, 1H), 3.64−3.74 (m, 2H), 3.56−3.64 (m, 1H). δ_C (CD_2Cl_2) 156.1, 139.7, 139.3, 138.9, 138.6, 132.9, 129.6, 129.1, 129.0, 128.9, 128.9,

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128.8, 128.7, 128.5, 128.5, 128.4, 128.3, 128.2, 128.1, 128.0, 82.5, 76.0, 75.2, 74.6, 74.4, 73.8, 71.9, 71.6, 60.0, 44.5. δ_C ($CDCl_3$) 155.7, 139.0, 138.3, 138.0, 137.8, 137.7, 132.5, 129.0, 128.6, 128.4, 128.4, 128.3, 128.1, 128.0, 127.9, 127.8, 127.8, 127.7, 127.6, 127.6, 127.4, 81.9, 77.2, 75.3, 74.5, 74.1, 73.6, 73.3, 71.0, 71.0, 59.5, 44.2. HRMS (ESI) calcd for $C_{41}H_{42}ClN_2O_4$ $[M + H]^+$, 661.2828 and 662.2861; found 661.2825 and 662.2845.

N-4-Bromobenzyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidine ***(3l)***. Reaction: Meerwein's reagent (0.54 g, 2.820 mmol, 1.5 equiv) and **2** (1.05 g, 1.900 mmol) in 10 mL of dichloromethane; amine 4-bromobenzylamine (0.71 g, 3.817 mmol, 2.0 equiv); reaction time 8 days; eluent for chromatography cyclohexane/ethyl acetate = 6/1−3/1, v/v; compound **3l** (0.73 g, 1.035 mmol, 55%); colorless oil. R_f 0.20 (SiO_2, cyclohexane/ethyl acetate = 3/1, v/v). δ_H (CD_2Cl_2) 7.19−7.45 (m, 22H), 7.08 (d, 8.5, 2H), 4.99 (br s, 1H), 4.92 (d, 10.8, 1H), 4.87 (d, 11.3, 1H), 4.83 (d, 11.8z, 1H), 4.58−4.67 (m, 3H), 4.54 (s, 2H), 4.49 (d, 9.5, 1H), 4.30 (t, 1.8, 1H), 4.22 (br s, 2H), 3.96 (dd, 9.7, 1.9, 1H), 3.63−3.71 (m, 2H), 3.54−3.62 (m, 1H). δ_C (CD_2Cl_2) 156.1, 139.7, 139.3, 138.9, 138.6, 131.8, 129.9, 129.2, 129.1, 129.0, 128.9, 128.8, 128.6, 128.6, 128.5, 128.5, 128.4, 128.3, 128.2, 128.1, 128.0, 120.9, 82.5, 76.0, 75.2, 74.6, 74.4, 73.8, 71.8, 71.6, 60.0, 44.6. HRMS (ESI) calcd for $C_{41}H_{42}BrN_2O_4$ $[M + H]^+$, 705.2322 and 707.2302; found 705.2322 and 707.2309.

N-Propyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidine ***(3m)***. Reaction: Meerwein's reagent (0.25 g, 1.355 mmol, 1.5 equiv) and **2** (0.50 g, 0.906 mmol) in 6 mL of dry dichloromethane; amine propylamine (0.11 g, 1.812 mmol, 2.0 equiv); reaction time 36 h; eluent for chromatography cyclohexane/ethyl acetate = 3/1−1/2, v/v; compound **3m** (0.30 g, 0.520 mmol, 58%); colorless oil. R_f 0.23 (SiO_2, cyclohexane/ethyl acetate = 1/2, v/v). δ_H (CD_2Cl_2) 7.25−7.44 (m, 20H), 6.56−6.94 (m, 1H), 5.04 (d, 11.8, 1H), 4.88 (d, 10.8, 1H), 4.67−4.84 (m, 4H), 4.50−4.65 (m, 3H), 4.33 (t, 2.3, 1H), 4.04 (dd, 9.9, 1.9, 1H), 3.90−3.98 (m, 1H), 3.84 (dd, 8.9, 6.4, 1H), 3.70 (dd, 9.0, 8.0, 1H), 3.16 (dquin, 45.7, 7.0, 2H), 1.50 (qd, 7.3, 1.3, 2H), 0.85 (t, 7.4, 3H). δ_C (CD_2Cl_2) 162.9, 138.2, 138.1, 137.7, 136.9, 129.4, 129.3, 129.2, 129.1, 128.9, 128.9, 128.9, 128.7, 128.6, 128.6, 128.5, 128.5, 128.4, 79.9, 75.9, 75.7, 73.9, 72.7, 72.4, 71.9, 67.9, 56.7, 54.5, 54.3, 53.7, 53.5, 44.1, 21.3, 11.2. HRMS (ESI) calcd for $C_{37}H_{43}N_2O_4$ $[M + H]^+$ 579.3223; found, 579.3200.

N-n-Heptyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidine ***(3n)***. Reaction: Meerwein's reagent (0.51 g, 2.717 mmol, 1.5 equiv) and **2** (1.00 g, 1.82 mmol) in 10 mL of dichloromethane; amine *n*-heptylamine (0.42 g, 3.665 mmol, 2.0 equiv); reaction time 41 h; eluent for chromatography cyclohexane/ethyl acetate = 4/1−2/1, v/v; compound **3n** (0.67 g, 1.055 mmol, 58%); colorless oil. R_f 0.25 (SiO_2, cyclohexane/ethyl acetate = 3/2, v/v). δ_H 7.18−7.49 (m, 20H), 6.65 (br s, 1H), 5.04 (d, 11.5, 1H), 4.88 (d, 10.8, 1H), 4.78−4.83 (m, 1H), 4.75 (d, 12.0, 1H), 4.68−4.72 (m, 1H), 4.53 (d, 11.3, 1H), 4.33 (t, 2.0, 1H), 4.03 (dd, 9.9, 1.9, 1H), 3.94 (d, 5.5, 1H), 3.80−3.90 (m, 1H), 3.69 (t, 8.5, 1H), 3.04−3.33 (m, 2H), 1.38−1.54 (m, 2H), 1.11−1.33 (m, 9H), 0.88 (t, 7.0, 3H). δ_C 162.5, 138.3, 138.2, 137.8, 137.0, 129.6, 129.5, 129.3, 129.2, 129.0, 129.0, 128.8, 128.7, 128.6, 128.6, 128.5, 128.5, 79.9, 76.1, 75.8, 74.1, 72.8, 72.1, 68.1, 57.1, 54.4, 42.7, 32.1, 29.2, 28.1, 27.0, 23.1, 14.4. HRMS (ESI) calcd for $C_{41}H_{51}N_2O_4$ $[M + H]^+$ 635.3849; found 635.3848.

N-n-Dodecyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidine ***(3o)***. Reaction: Meerwein's reagent (0.54 g, 2.82 mmol, 1.5 equiv) and **2** (1.05 g, 1.90 mmol) in 10 mL of dichloromethane; amine *n*-dodecylamine (0.70 g, 3.80 mmol, 2.0 equiv); reaction time 72 h; eluent for chromatography; cyclohexane/ethyl acetate = 8/1−4/1, v/v, compound **3o** (0.70 g, 0.993 mmol, 52%); colorless oil. R_f 0.27 (SiO_2, cyclohexane/ethyl acetate = 2/1, v/v). δ_H (CD_2Cl_2) 7.12−7.59 (m, 20H), 4.86 (td, 19.7, 11.3, 3H), 4.57−4.67 (m, 4H), 4.54 (s, 2H), 4.42−4.42 (m, 1H), 4.43 (d, 9.8, 1H), 4.22−4.31 (m, 1H), 3.92 (dd, 9.8, 1.8, 1H), 3.48−3.73 (m, 3H), 3.01 (dd, 12.5, 6.8, 2H), 1.13−1.48 (m, 20H), 0.84−0.91 (m, 3H). δ_C (CD_2Cl_2) 156.3, 139.8, 139.4, 139.0, 138.9, 129.1, 129.0, 128.9, 128.8, 128.7, 128.5, 128.5, 128.4, 128.3, 128.2, 128.1, 128.0, 82.4, 76.2, 75.3, 74.7, 74.2, 73.8, 72.1, 71.6, 60.1, 41.3, 32.6, 30.3, 30.3, 30.2, 30.2, 30.1, 30.0, 29.8, 27.8, 23.3, 14.6. HRMS (ESI) calcd for $C_{46}H_{61}N_2O_4$ $[M + H]^+$ 705.4626; found 705.4632.

N-2-Ethylhexyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidine ***(3p)***. Reaction: Meerwein's reagent (0.51 g, 2.717 mmol, 1.5 equiv) and **2** (1.00 g, 1.82 mmol) in 10 mL of dichloromethane; amine 2-ethylhexylamine (0.47 g, 3.623 mmol, 2.0 equiv); reaction time 41 h; eluent for chromatography cyclohexane/ethyl acetate = 4/1−2/1, v/v; compound **3p** (0.47 g, 0.724 mmol, 40%); colorless oil. R_f 0.21 (SiO_2, cyclohexane/ethyl acetate = 3/2, v/v). δ_H (CD_2Cl_2) 7.25−7.45 (m, 20H), 6.30 (br s, 1H), 5.01 (d, 11.5, 1H), 4.90 (d, 10.5, 1H), 4.82 (d, 11.5, 1H), 4.75 (d, 12.5, 1H), 4.67−4.72 (m, 2H), 4.63 (d, 10.8, 1H), 4.59 (d, 11.8, 1H), 4.54 (d, 11.8, 1H), 4.33 (d, 2.0, 1H), 4.03 (dd, 9.8, 1.8, 1H), 3.88−3.95 (m, 1H), 3.83 (dd, 8.8, 6.3, 1H), 3.69 (t, 8.5, 1H), 3.15 (dd, 14.1, 6.5, 1H), 3.05 (dd, 13.3, 6.3, 1H), 1.35−1.47 (m, 1H), 1.07−1.31 (m, 8H), 0.84 (dt, 19.0, 7.2, 6H). δ_C (CD_2Cl_2) 161.8, 138.5, 138.4, 138.0, 137.2, 129.5, 129.4, 129.3, 129.2, 129.2, 129.1, 129.0, 128.9, 128.9, 128.8, 128.8, 128.7, 128.6, 128.6, 128.5, 128.5, 128.4, 128.3, 128.3, 128.2, 80.4, 75.7, 75.6, 73.9, 73.2, 72.5, 68.8, 57.5, 54.4, 45.4, 38.7, 38.6, 31.2, 31.1, 29.1, 29.0, 24.5, 23.4, 14.3, 11.0, 11.0. HRMS (ESI) calcd for $C_{42}H_{53}N_2O_4$ $[M + H]^+$ 649.4005; found 649.4003.

N-3-Phenylpropyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidine ***(3q)***. Reaction: Meerwein's reagent (0.54 g, 2.820 mmol, 1.5 equiv) and **2** (1.05 g, 1.950 mmol) in 10 mL of dichloromethane; amine 3-phenylpropylamine (0.51 g, 3.800 mmol, 2.0 equiv); reaction time 8 days; eluent for chromatography cyclohexane/ethyl acetate = 4/1−1/1, v/v; compound **3q** (0.64 g, 0.977 mmol, 51%); colorless oil. R_f 0.20 (SiO_2, cyclohexane/ethyl acetate = 1/1, v/v). δ_H (CD_2Cl_2) 7.46−7.07 (m, 25H), 4.99−4.78 (m, 3H), 4.59−4.69 (m, 4H), 4.56 (s, 2H), 4.47 (d, 9.5, 1H), 4.30 (br s, 1H), 3.95 (d, 9.5, 1H), 3.77−3.52 (m, 3H), 3.09 (br s, 2H), 2.56 (t, 7.3, 2H), 1.73 (quin, 7.0, 2H); δ_C (CD_2Cl_2) 156.4, 142.6, 139.7, 139.3, 138.9, 138.8, 129.1, 128.9, 128.9, 128.8, 128.8, 128.7, 128.6, 128.5, 128.5, 128.4, 128.3, 128.2, 128.1, 128.0, 126.2, 82.4, 76.1, 75.2, 74.6, 74.3, 73.8, 71.9, 71.6, 60.0, 40.8, 33.8, 31.4. HRMS (ESI) calcd for $C_{43}H_{47}N_2O_4$ $[M + H]^+$ 655.3530; found 655.3535.

N-Cyclopropyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidine ***(3r)***. Reaction: Meerwein's reagent (0.51 g, 2.72 mmol, 1.5 equiv) and **2** (1.00 g, 1.82 mmol) in 10 mL of dichloromethane; amine cyclopropylamine (0.22 g, 3.62 mmol, 2.0 equiv) was added, and the solution was allowed to warm to ambient temperature. After 3 days, all volatiles were removed; reaction time 3 days; eluent for chromatography cyclohexane/ethyl acetate = 2/1−1/2, v/v; compound **3r** (0.54 g, 0.938 mmol, 52%); colorless solid; mp 128−130 °C. R_f 0.14 (SiO_2, cyclohexane/ethyl acetate = 1/2, v/v). δ_H ($CDCl_3$) 7.72 (br s, 1H), 7.28−7.44 (m, 17H), 7.19−7.26 (m, 3H), 4.98 (d, 11.5, 1H), 4.73−4.83 (m, 2H), 4.65−4.73 (m, 3H), 4.53−4.60 (m, 3H), 4.26 (t, 2.3, 1H), 4.10 (dd, 7.0, 3.5, 1H), 4.06 (dd, 9.3, 2.0, 1H), 3.89 (dd, 9.2, 7.4, 1H), 3.69 (dd, 9.3, 7.3, 1H), 2.66 (dd, 6.5, 3.5, 1H), 1.60 (s, 1H), 0.88−1.00 (m, 2H), 0.53−0.64 (m, 1H), 0.42−0.50 (m, 1H). δ_C ($CDCl_3$) 164.8, 137.3, 137.2, 136.9, 136.3, 128.8, 128.7, 128.5, 128.5, 128.4, 128.4, 128.1, 128.0, 128.0, 127.9, 127.8, 78.8, 75.4, 74.8, 73.4, 72.3, 71.7, 70.9, 67.4, 55.8, 22.9, 6.6. HRMS (ESI) calcd for $C_{40}H_{41}N_2O_4$ $[M + H]^+$ 577.3061; found 577.3067.

N-4-Cyclohexyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidine ***(3s)***. Reaction: Meerwein's reagent (0.52 g, 2.720 mmol, 1.5 equiv) and **2** (1.00 g, 1.820 mmol) in 10 mL of dichloromethane; amine cyclohexylamine (0.36 g, 3.620 mmol, 2.0 equiv); reaction time 4 days; eluent for chromatography cyclohexane/ethyl acetate = 8/1−4/1, v/v; compound **3s** (0.30 g, 0.485 mmol, 27%); colorless oil. R_f 0.20 (SiO_2, cyclohexane/ethyl acetate = 1/1, v/v). δ_H (CD_2Cl_2) 7.04−7.66 (m, 20H), 6.36 (br s, 1H), 5.02 (d, 11.8, 1H), 4.89 (d, 10.8, 1H), 4.81 (d, 11.5, 1H), 4.62−4.75 (m, 4H), 4.57 (dd, 19.1, 11.8, 2H), 4.34 (t, 2.1, 1H), 4.02 (dd, 9.9, 1.9, 1H), 3.80−3.96 (m, 2H), 3.70 (t, 8.5, 1H), 3.52 (br s, 1H), 1.89 (d, 9.5, 1H), 1.49−1.73 (m, 4H), 1.24−1.48 (m, 2H), 1.05−1.22 (m, 2H), 0.95 (q, 11.0, 1H). δ_C (CD_2Cl_2) 160.9, 138.3, 138.3, 137.9, 137.1, 129.6, 129.3, 129.3, 129.1, 128.9, 128.9, 128.7, 128.6, 128.5, 128.5, 128.4, 79.8, 75.8, 75.7, 73.9, 72.9, 72.6, 72.2, 68.2, 57.0, 51.3, 32.1, 31.6, 25.4, 24.4. HRMS (ESI) calcd for $C_{40}H_{47}N_2O_4$ $[M + H]^+$ 619.3536; found 619.3531.

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*N-Cyclopropylmethyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidine (**3t**).* Reaction: Meerwein's reagent (0.51 g, 2.717 mmol, 1.5 equiv) and **2** (1.00 g, 1.82 mmol) in 10 mL of dichloromethane; amine cyclopropylmethylamine (0.22 g, 3.62 mmol, 2.0 equiv); reaction time 17 h; eluent for chromatography cyclohexane/ethyl acetate = 4/1−1/1, v/v; compound **3t** (0.86 g, 1.692 mmol, 80%); colorless oil. R_f 0.17 (SiO_2, cyclohexane/ethyl acetate = 3/2, v/v). δ_H (CD_2Cl_2) 7.12−7.55 (m, 20H), 6.76 (br s, 1H), 5.05 (d, 11.5, 2H), 4.90 (d, 10.8, 3H), 4.82 (d, 11.8, 1H), 4.77 (d, 11.5, 1H), 4.72 (dd, 11.5, 9.8, 2H), 4.61 (t, 11.8, 2H), 4.53 (d, 11.5, 1H), 4.32 (t, 2.3, 1H), 4.05 (dd, 10.2, 1.6, 1H), 3.88−3.98 (m, 1H), 3.82 (dd, 8.9, 6.7, 1H), 3.68 (t, 8.5, 1H), 3.13 (dd, 14.1, 7.8, 1H), 2.99 (dd, 13.7, 7.4, 1H), 0.87 (td, 8.0, 4.1, 1H), 0.51−0.62 (m, 2H), 0.14−0.31 (m, 2H). δ_C (CD_2Cl_2) 162.5, 138.2, 138.1, 137.8, 136.9, 129.5, 129.4, 129.3, 129.1, 128.9, 128.9, 128.7, 128.6, 128.5, 128.5, 128.4, 79.8, 75.8, 75.7, 73.9, 72.7, 72.5, 72.1, 68.2, 56.9, 47.4, 9.3, 4.2, 4.0. HRMS (ESI) calcd for $C_{38}H_{43}N_2O_4$ $[M + H]^+$ 591.3223; found 591.3222.

*N-Cyclohexylmethyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidine (**3u**).* Reaction: Meerwein's reagent (0.25 g, 1.355 mmol, 1.5 equiv) and **2** (0.50 g, 0.906 mmol, 1 equiv) in 6 mL of dichloromethane; amine cyclohexylmethylamine (0.21 g, 1.812 mmol, 2.0 equiv); reaction time 72 h; eluent for chromatography cyclohexane/ethyl acetate = 4/1−1/1, v/v; compound **3u** (0.34 g, 0.538 mmol, 59%); colorless oil. R_f 0.27 (SiO_2, cyclohexane/ethyl acetate = 1/2, v/v). δ_H (CD_2Cl_2) 7.10−7.55 (m, 20H), 6.50 (br s, 1H), 5.04 (d, 11.8, 1H), 4.88 (d, 10.5, 1H), 4.81 (d, 11.5, 1H), 4.67−4.77 (m, 3H), 4.47−4.66 (m, 4H), 4.35 (br s, 1H), 4.03 (dd, 9.9, 1.9, 1H), 3.83−3.98 (m, 2H), 3.72 (t, 8.8, 1H), 3.53 (br s, 1H), 1.89 (d, 10.0, 1H), 1.51−1.73 (m, 4H), 1.38 (sxt, 12.8, 2H), 1.05−1.24 (m, 2H), 0.95 (q, 11.5, 1H); δ_H (CD_2Cl_2) 161.4, 138.3, 138.2, 137.8, 136.9, 129.7, 129.5, 129.4, 129.1, 129.0, 128.9, 128.8, 128.8, 128.6, 128.6, 128.6, 128.5, 128.4, 128.2, 79.6, 76.0, 75.8, 74.0, 72.8, 72.7, 72.0, 67.8, 56.8, 51.4, 32.0, 31.6, 25.3, 24.3. HRMS (ESI) calcd for $C_{41}H_{49}N_2O_4$ $[M + H]^+$ 633.3692; found, 633.3674.

*N-Cycloheptylmethyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidine (**3v**).* Reaction: Meerwein's reagent (0.46 g, 2.446 mmol, 1.5 equiv) and **2** (0.90 g, 1.630 mmol) in 10 mL of dichloromethane; amine cycloheptylmethylamine (0.41 g, 3.260 mmol, 2.0 equiv); reaction time 17 h; eluent for chromatography cyclohexane/ethyl acetate = 4/1−2/1, v/v; compound **3v** (0.63 g, 0.974 mmol, 60%); colorless oil. R_f 0.20 (SiO_2, cyclohexane/ethyl acetate = 3/2, v/v). δ_H 7.10−7.68 (m, 20H), 6.33 (br s, 1H), 5.00 (d, 11.8, 1H), 4.90 (d, 10.8, 1H), 4.79−4.85 (m, 1H), 4.74 (d, 11.5, 1H), 4.66−4.71 (m, 2H), 4.63 (d, 10.8, 1H), 4.59 (d, 11.8, 1H), 4.54 (d, 11.5, 1H), 4.33 (t, 1.8, 1H), 4.02 (dd, 9.8, 1.8, 2H), 3.86−3.94 (m, 1H), 3.82 (dd, 8.8, 6.3, 1H), 3.68 (t, 8.5, 1H), 3.06 (dd, 12.8, 6.8, 1H), 2.97 (dd, 13.1, 6.3, 1H), 1.28−1.71 (m, 13H), 1.01−1.19 (m, 2H). δ_C 161.6, 138.5, 138.4, 138.0, 137.3, 129.4, 129.3, 129.2, 129.1, 129.0, 129.0, 128.9, 128.9, 128.6, 128.5, 128.4, 128.3, 80.4, 75.6, 73.9, 73.2, 72.6, 72.5, 68.9, 57.5, 48.6, 38.7, 32.3, 32.2, 28.7, 26.6, 26.5. HRMS (ESI) calcd for $C_{42}H_{51}N_2O_4$ $[M + H]^+$ 647.3849; found 647.3846

*General Description for Synthesis of N-Aryl- and N-Alkyl-D-galactonoamidines (**1h−v**) from N-Aryl- and N-Alkyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidine (**3h−v**).* Compounds **3h−v** were suspended with Pd/C (10% Pd and 50% wet with water) and 0.1−6 mL of trifluoroacetic acid in 5−30 mL of ethanol and stirred under hydrogen atmosphere at room temperature. After 8−24 h, the mixture was filtered through a pad of Celite. The pad was rinsed with 2−3 mL of ethanol three times. The combined filtrates were concentrated under reduced pressure, and the resulting residues were lyophilized, yielding compounds **1h−v** in excellent yields.

*N-4-tert-Butylbenzyl-D-galactonoamidine (**1h**).* Suspension: compound **3h** (0.27 g, 0.395 mmol), Pd/C (0.27 g, 0.127 mmol, 0.322 equiv) in 0.75 mL of trifluoroacetic acid and 7.5 mL of ethanol; reaction time 24 h; ethanol amount for rinsing 3 mL each; compound **1h** (0.12 g, 0.373 mmol, 95%), pale gray foam. R_f 0.19 (SiO_2, ethyl acetate/methanol = 3/1, v/v). δ_H (D_2O) 7.47 (d, 8.5, 2H), 7.26 (d, 8.3, 2H), 4.62−4.50 (m, 3H), 4.24 (t, 2.3, 1H), 3.92 (dd, 10.2, 2.4, 1H), 3.77−3.62 (m, 3H), 1.23 (s, 9H). δ_C (D_2O + MeOH-d_4) 165.1, 153.0, 131.9, 128.3, 127.1, 71.5, 67.9, 67.4, 61.0, 58.3, 45.7, 35.0, 31.4. HRMS (ESI) calcd for $C_{17}H_{27}N_2O_4$ $[M + H]^+$ 323.1965; found 323.1971.

*N-4-Methoxybenzyl-D-galactonoamidine (**1i**).* Suspension: compound **3i** (0.32 g, 0.488 mmol), Pd/C (0.64 g, 0.301 mmol, 0.617 equiv) in 1.5 mL of trifluoroacetic acid and 7.5 mL of ethanol; reaction time 24 h; ethanol amount for rinsing 3 mL each; compound **1i** (0.14 g, 0.473 mmol, 92%), pale yellow foam. R_f 0.12 (SiO_2, ethyl acetate/methanol = 3/1, v/v). δ_H (D_2O) 7.21 (d, 8.8, 2H), 6.88 (d, 8.8, 2H), 4.54 (d, 10.3, 1H), 4.46 (d, 3.5, 2H), 4.20 (t, 2.3, 1H), 3.88 (dd, 10.0, 2.3, 1H). δ_C (D_2O/MeOH-d_4) 164.9, 159.8, 130.0, 127.1, 115.4, 71.5, 67.9, 67.4, 61.0, 58.3, 56.3, 45.7. HRMS (ESI) calcd for $C_{14}H_{21}N_2O_5$ $[M + H]^+$ 297.1445; found 297.1441.

*N-4-Trifluoromethylbenzyl-D-galactonoamidine (**1j**).* Suspension: compound **3j** (0.24 g, 0.346 mmol), Pd/C (0.48 g, 0.226 mmol, 0.653 equiv) in 2 mL of trifluoroacetic acid and 10 mL of ethanol reaction time 20 h; ethanol amount for rinsing 2 mL each; compound **1j** (0.10 g, 0.299 mmol, 87%); colorless oil. R_f 0.27 (SiO_2, ethyl acetate/methanol = 3/1, v/v). δ_H (D_2O) 7.66 (d, 8.0, 2H), 7.42 (d, 8.0, 2H), 4.67 (d, 2.5, 2H), 4.62 (d, 10.0, 1H), 4.25 (t, 2.3, 1H), 3.94 (dd, 10.2, 2.4, 1H), 3.69−3.77 (m, 2H), 3.65 (dd, 3.3, 9.5, 1H). δ_C (D_2O/MeOH-d_4) 165.6, 139.0 (q, 1.5), 130.7 (q, 32.3), 128.5, 126.9 (q, 3.7), 125.0 (q, 271.0), 71.5, 68.0, 67.5, 60.9, 58.4, 45.6. HRMS (ESI) calcd for $C_{14}H_{17}F_3N_2O_4$ $[M + H]^+$ 335.1213; found 335.1211.

*N-Benzyl-D-galactonoamidine (**1a**) from N-4-Chlorobenzyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidine (**3k**).* Suspension: compound **3k** (0.16 g, 0.242 mmol), Pd/C (0.16 g, 0.0751 mmol, 0.310 equiv) in 1 mL of trifluoroacetic acid and 5 mL of ethanol; reaction time 9 h; ethanol amount for rinsing 2 mL each; compound **1a** (0.06 g, 0.223 mmol, 92%); colorless oil. R_f 0.21 (SiO_2, ethyl acetate/methanol = 3/1, v/v). δ_H (D_2O) 7.44 (d, 8.3, 7H), 7.25 (d, 8.0, 2H), 4.49−4.63 (m, 3H), 4.24 (t, 1.8, 1H), 3.92 (dd, 10.0, 2.3z, 1H), 3.60−3.78 (m, 3H). δ_C (D_2O + MeOH-d_4) 165.2, 134.7, 130.1, 129.4, 128.3, 71.5, 67.9, 67.4, 60.9, 58.3, 46.2.

*N-Benzyl-D-galactonoamidine (**1a**) from N-4-Bromobenzyl-2,3,4,6-tetra-O-benzyl-D-galactonoamidine (**3l**).* Suspension: compound **3l** (0.10 g, 0.142 mmol), Pd/C (0.10 g, 0.0469 mmol, 0.330 equiv) in 0.5 mL of trifluoroacetic acid and 5 mL of ethanol; reaction time 8 h; ethanol amount for rinsing 2 mL each; compound **1a** (37.70 mg, 0.142 mmol, quantitative); colorless foam. R_f 0.21 (SiO_2, ethyl acetate/methanol = 3/1, v/v). δ_H (D_2O) 7.44 (d, 8.3, 7H), 7.25 (d, 8.0, 2H), 4.49−4.63 (m, 3H), 4.24 (t, 1.8, 1H), 3.92 (dd, 10.0, 2.3z, 1H), 3.60−3.78 (m, 3H). δ_C (D_2O + MeOH-d_4) 165.2, 134.7, 130.1, 129.4, 128.3, 71.5, 67.9, 67.4, 60.9, 58.3, 46.2.

*N-Propyl-D-galactonoamidine (**1m**).* Suspension: compound **3m** (0.32 g, 0.554 mmol), Pd/C (0.64 g, 0.301 mmol, 0.543 equiv) in 4 mL of trifluoroacetic acid and 20 mL of ethanol; reaction time 24 h; ethanol amount for rinsing 3 mL each; compound **1m** (0.119 g, 0.505 mmol, 92%) as a pale white foam. R_f 0.25 (SiO_2, ethyl acetate/methanol = 2/1, v/v). δ_H (D_2O) 4.48 (d, 10.0, 1H), 4.23 (t, 2.5, 1H), 3.87 (dd, 10.2, 2.4, 1H), 3.74−3.79 (m, 2H), 3.67−3.73 (m, 1H), 3.24 (t, 7.0, 2H), 1.55 (sxt, 7.3, 2H), 0.84 (t, 7.5, 3H). δ_C (D_2O/MeOH-d_4) 164.8, 71.6, 68.1, 67.3, 61.0, 58.1, 44.4, 21.4, 11.3. HRMS (ESI) calcd for $C_9H_{19}N_2O_4$ $[M + H]^+$ 219.1339; found 219.1338.

*N-n-Heptyl-D-galactonoamidine (**1n**).* Suspension: compound **3n** (0.45 g, 0.709 mmol), Pd/C (0.45 g, 0.211 mmol, 0.298 equiv) in 0.2 mL of trifluoroacetic acid and 10 mL of ethanol; reaction time 19 h, ethanol amount for rinsing 2 mL each; compound **1n** (0.19 g, 0.693 mmol, 97%); pale white foam. R_f 0.25 (SiO_2, ethyl acetate/methanol = 4/1, v/v). δ_H (D_2O) 4.53 (d, 10.0, 1H), 4.28 (t, 2.3, 1H), 3.92 (dd, 10.2, 2.1, 1H), 3.69−3.86 (m, 3H), 3.33 (t, 6.9, 2H), 1.60 (quin, 6.8, 2H), 1.14−1.37 (m, 8H), 0.75−0.87 (m, 3H). δ_C (D_2O + MeOH-d_4) 164.8, 71.5, 68.0, 67.2, 61.0, 58.1, 42.8, 32.1, 29.2, 27.9, 27.0, 23.0, 14.4. HRMS (ESI) calcd for $C_{13}H_{27}N_2O_4$ $[M + H]^+$ 275.1971; found 275.1968.

*N-n-Dodecyl-D-galactonoamidine (**1o**).* Suspension: compound **3o** (0.36 g, 0.511 mmol), Pd/C (0.36 g, 0.169 mmol, 0.330 equiv) in 0.8 mL of trifluoroacetic acid and 8 mL of ethanol; reaction time 21 h; ethanol for rinsing 2 mL each; compound **1o** (0.17 g, 0.494 mmol, 97%); pale white solid; mp 69−70 °C. R_f 0.14 (SiO_2, ethyl acetate/methanol = 3/1, v/v). δ_H (acetone-d_6) 9.36 (br s, 1H), 8.32 (br s, 1H),

5.05 (br s, 1H), 4.63 (d, 9.0, 1H), 4.31 (br s, 1H), 3.76−4.01 (m, 4H), 3.49 (dd, 11.8, 6.3, 2H), 1.73 (quin, 7.3, 2H), 1.15−1.49 (m, 20H), 0.87 (t, 6.8, 3H). δ_C (acetone-d_6) 166.8, 72.9, 69.6, 68.7, 62.2, 59.5, 43.3, 33.1, 30.8, 30.8, 30.8, 30.5, 30.4, 28.9, 27.9, 23.8, 14.8. HRMS (ESI) calcd for $C_{18}H_{37}N_2O_4$ $[M + H]^+$ 345.2748; found 345.2752.

*N-2-Ethylhexyl-D-galactonoamidine (**1p**).* A suspension of compound **3p** (0.17 g, 0.262 mmol), Pd/C (0.17 g, 0.0798 mol, 0.305 equiv) in 0.2 mL of trifluoroacetic acid and 6 mL of ethanol; reaction time 24 h; ethanol for rinsing 2 mL each; compound **1p** (0.07 g, 0.244 mmol, 93%); colorless foam. R_f 0.21 (SiO_2, ethyl acetate/methanol = 4/1, v/v). δ_H (D_2O) 4.54 (d, 10.0, 1H), 4.27 (s, 1H), 3.93 (dd, 10.0, 1.8, 1H), 3.72−3.87 (m, 3H), 3.25 (d, 4.3, 2H), 1.68 (quin, 5.5, 1H), 1.12−1.42 (m, 9H), 0.82 (t, 7.0, 6H). δ_C (D_2O + MeOH-d_4) 165.0, 71.6, 68.1, 67.4, 61.2, 58.2, 46.3, 38.3, 38.3, 30.8, 30.7, 28.8, 24.3, 23.2, 14.3, 10.7, 10.7. HRMS (ESI) calcd for $C_{14}H_{29}N_2O_4$ $[M + H]^+$ 289.2127; found 289.2125.

*N-3-Phenylpropyl-D-galactonoamidine (**1q**).* Suspension: compound **3q** (0.21 g, 0.320 mmol), Pd/C (0.21 g, 0.0986 mmol, 0.308 equiv) in 0.1 mL of trifluoroacetic acid and 5 mL of ethanol; reaction time 24 h; ethanol for rinsing 2 mL each; compound **1q** (0.09g, 0.320 mmol, quantitative); colorless foam. R_f 0.17 (SiO_2, ethyl acetate/methanol = 3/1, v/v). δ_H (D_2O) 7.15−7.38 (m, 5H), 4.42 (d, 10.3, 1H), 4.24 (t, 2.5, 1H), 3.86 (dd, 10.3, 2.5, 1H), 3.72−3.81 (m, 2H), 3.65−3.71 (m, 1H), 3.32 (t, 7.0, 2H), 2.67 (t, 7.5, 2H), 1.95 (quin, 7.3, 2H). δ_C (D_2O + MeOH-d_4) 164.7, 142.0, 129.6, 129.4, 127.2, 71.5, 68.0, 67.2, 61.0, 58.1, 42.1, 32.9, 29.1. HRMS (ESI) calcd for $C_{15}H_{23}N_2O_4$ $[M + H]^+$ 295.1652; found 295.1650.

*N-Cyclopropyl-D-galactonoamidine (**1r**).* Suspension: compound **3r** (0.28 g, 0.486 mmol), Pd/C (0.12 g, 0.132 mmol, 0.272 equiv) in 0.5 mL of trifluoroacetic acid in 5 mL of ethanol; reaction time 24 h; ethanol for rinsing 2 mL each; compound **1r** (0.11 g, 0.486 mmol, quantitative); pale gray foam. R_f 0.12 (SiO_2, ethyl acetate/methanol = 3/1, v/v). δ_H (D_2O) 4.50 (d, 10.0, 1H), 4.27 (s, 1H), 3.92 (dd, 10.2, 2.1, 1H), 3.77−3.86 (m, 3H), 2.53−2.66 (m, 1H), 0.85−1.02 (m, 2H), 0.63−0.82 (m, 2H). δ_H (D_2O + MeOH-d_4) 167.0, 71.5, 68.0, 67.0, 61.0, 58.1, 23.3, 7.2, 6.7. HRMS (ESI) calcd for $C_9H_{17}N_2O_4$ $[M + H]^+$ 217.1188; found 217.1179.

*N-Cyclohexyl-D-galactonoamidine (**1s**).* Suspension: compound **3s** (0.25 g, 0.404 mmol), Pd/C (0.25 g, 0.117 mmol, 0.290 equiv) in 0.5 mL of trifluoroacetic acid and 5 mL of ethanol; reaction time 17 h; ethanol for rinsing 2 mL; compound **1s** (0.10 g, 0.387 mmol, 96%); pale gray foam. R_f 0.14 (SiO_2, ethyl acetate/methanol = 3/1, v/v). δ_H (D_2O) 4.49 (d, 10.0, 1H), 4.26 (br s, 1H), 3.90 (dd, 10.2, 1.9, 1H), 3.69−3.85 (m, 3H), 3.50−3.68 (m, 1H), 1.80−1.93 (m, 2H), 1.72 (d, 4.8, 2H), 1.58 (d, 12.5, 1H), 1.21−1.46 (m, 4H), 1.03−1.20 (m, 1H). δ_H (D_2O + MeOH-d_4) 163.4, 71.6, 68.1, 67.3, 61.2, 58.2, 52.3, 32.0, 31.5, 25.4, 25.0, 25.0. HRMS (ESI) calcd for $C_{12}H_{23}N_2O_4$ $[M + H]^+$ 259.1652; found 259.1650.

*N-Cyclopropylmethyl-D-galactonoamidine (**1t**).* Suspension: compound **3t** (0.45 g, 0.763 mmol), Pd/C (0.45 g, 0.211 mmol, 0.277 equiv) in 0.2 mL of trifluoroacetic acid and 10 mL of ethanol; reaction time 24 h; ethanol for rinsing 2 mL each; compound **1t** (0.17 g, 0.760 mmol, quantitative); pale gray foam. R_f 0.21 (SiO_2, ethyl acetate/methanol = 3/1, v/v). δ_H (D_2O) 4.55 (d, 10.3, 1H), 4.28 (s, 1H), 3.93 (dd, 10.0, 2.3, 1H), 3.71−3.86 (m, 3H), 3.20 (d, 5.8, 2H), 0.99−1.13 (m, 1H), 0.53−0.60 (m, 2H), 0.25 (ddd, 9.5, 4.8, 1.5, 2H). δ_C (D_2O + MeOH-d_4) 164.7, 71.6, 68.1, 67.3, 61.1, 58.1, 47.5, 9.2, 4.0, 3.8. HRMS (ESI) calcd for $C_{10}H_{19}N_2O_4$ $[M + H]^+$ 231.1345; found 231.1343.

*N-Cyclohexylmethyl-D-galactonoamidine (**1u**).* Suspension: compound **3u** (0.47 g, 0.741 mmol), Pd/C (0.94 g, 0.442 mmol, 0.596 equiv) in 6 mL of trifluoroacetic acid and 30 mL of ethanol; reaction time 24 h; ethanol for rinsing 2 mL each; compound **1u** (0.26 g, 0.698 mol, 93%); white foam. R_f 0.30 (SiO_2, ethyl acetate/methanol = 3/1, v/v). δ_H (D_2O) 4.44 (d, 10.0, 1H), 4.18 (t, 2.1, 1H), 3.83 (dd, 10.0, 2.3, 1H), 3.69−3.75 (m, 2H), 3.62−3.68 (m, 1H), 3.01−3.16 (m, 2H), 1.42−1.65 (m, 6H), 0.93−1.18 (m, 3H), 0.72−0.90 (m, 2H). δ_C (D_2O/MeOH-d_4) 164.9, 71.5, 68.0, 67.3, 61.1, 58.1, 48.6, 37.0, 30.9, 30.9, 26.6, 26.1, 26.1. HRMS (ESI) calcd for $C_{13}H_{25}N_2O_4$ $[M + H]^+$ 273.1814; found 273.1804.

*N-Cycloheptylmethyl-D-galactonoamidine (**1v**).* A suspension of compound **3v** (0.47 g, 0.726 mmol), Pd/C (0.47 g, 0.221 mmol, 0.304 equiv) in 0.2 mL of trifluoroacetic acid and 10 mL of ethanol; reaction time 24 h; ethanol for rinsing 2 mL each; compound **1v** (0.21 g, 0.726 mol, quantitative); pale white foam. R_f 0.20 (SiO_2, ethyl acetate/methanol = 4/1, v/v). δ_H (D_2O) 4.53 (d, 10.3, 1H), 4.26 (br s, 1H), 3.91 (dd, 10.2, 2.4, 1H), 3.67−3.85 (m, 3H), 3.17 (dd, 7.2, 2.9, 2H), 1.74−1.93 (m, 1H), 1.54−1.71 (m, 4H), 1.31−1.53 (m, 6H), 1.15 (qd, 10.3, 2.0, 2H). δ_C (D_2O + acetone-d_6) 164.4, 71.1, 67.5, 66.8, 60.6, 57.6, 48.2, 37.8, 31.7, 31.7, 28.3, 25.9, 25.9. HRMS (ESI) calcd for $C_{14}H_{27}N_2O_4$ $[M + H]^+$ 287.1971; found 287.1963.

*D-Galactonoamidine (**1w**).*[27] A suspension of perbenzylated galactonoamidine (0.18 g, 0.336 mmol), Pd/C (0.18 g, 0.0845 mmol, 0.251 equiv), 0.12 mL of trifluoroacetic acid, and 6 mL of ethanol; reaction time 12 h; ethanol for rinsing 2 mL; compound **1w** (0.06 g, 0.336 mol, quantitative); pale yellow foam. R_f 0.27 (SiO_2, ethyl acetate/methanol = 4/1, v/v). δ_H (D_2O) 4.55 (d, 10.3, 1H), 4.24 (t, 2.3, 1H), 3.96 (dd, 10.2, 2.4, 1H), 3.79 (dd, 9.8, 4.3, 1H), 3.64−3.76 (m, 2H). δ_C (D_2O + MeOH-d_4) 176.9, 168.4, 80.9, 74.6, 73.8, 71.6, 69.8, 68.2, 67.0, 62.9, 61.3, 57.9; the compound matches the characterization data described by Ganem et al. and consists of two resonance isomers.[27]

*D-Galactono-δ-lactam (**1x**).*[30] The title compound was prepared from perbenzylated galactonolactam (2.50 g, 4.655 mmol) and $Pd(OH)_2$/C (0.48 g, 0.343 mmol, 0.137 equiv) in a solvent mix of methanol, ethanol, and 1 M aqueous HCl (67.5 mL, 27.0 mL, 45 μL) as described by Overkleeft et al.,[30] yielding compound **1x** (0.16 g, 0.090 mmol, 20%) as a pale gray solid; mp 190−192 (lit.[30] mp 192−195 °C). δ_H (D_2O) 4.11−4.19 (m, 2H), 3.88 (dd, 10.0, 2.3, 1H), 3.67−3.76 (m, 1H), 3.55−3.65 (m, 2H). δ_C (D_2O + MeOH-d_4) 174.5, 73.2, 70.2, 68.8, 61.8, 55.8; the compound matches the characterization data described.[30]

Kinetic Assays. *General Remarks.* All kinetic experiments were performed in 50 mM acetate buffer at pH 5.00 ± 0.05 at 30 °C with β-galactosidase (*A. oryzae*) as catalyst and 2-chloro-4-nitrophenyl-β-D-galactopyranoside (**5**) as substrate in a 96-well plate format and a total volume of 100 μL. All experiments were performed at least in triplicate, and obtained data were averaged.

Enzyme Stock Solution. In a typical experiment, 4−5 mg of commercially available β-galactosidase (*A. oryzae*) was dissolved in 5 mL of acetate buffer in a volumetric flask. Then 250.0 μL of this solution was diluted into 5.0 mL of acetate buffer in a similar fashion to obtain the enzyme stock solution. For each experiment, 20 μL of this stock solution was used to initiate hydrolysis immediately prior to data recording. Using a BCA assay, the protein concentration of the corresponding protein batch was determined as 10%.[18] The molar weight of β-galactosidase in this enzyme batch was previously determined as 86 800 g mol^{-1}.[18]

Substrate Stock Solution. In a typical experiment, 5.0 mg (0.015 mmol) of 2-chloro-4-nitrophenyl-β-D-galactopyranoside was dissolved in 5.0 mL of acetate buffer in a volumetric flask immediately prior to use. For determination of the substrate decomposition in the absence of catalyst, 5.0 mg of substrate was dissolved in 10 mL of acetate buffer under otherwise identical conditions.

Inhibitor Stock Solution. In a typical experiment, 50 μM stock solutions of the inhibitors **1a**−**v** in nanopure water were diluted into 0.25−10.0 μM stock solutions using volumetric flasks. For each experiment with inhibitors, 10 μL of the respective stock solution was used.

Assay To Determine Uncatalyzed Substrate Decomposition. The substrate stock solution was pipetted in 0−70 μL aliquots and adjusted to a total volume of 100 μL with buffer solution, thoroughly mixed and thermostated at 30 °C for 60 min prior to data recording. Formation of 2-chloro-4-nitrophenolate was followed at 405 nm over 2 h, and the change in absorbances was recorded every 6 min. Product concentration was derived from these values by correction for the apparent molar extinction coefficient ($\varepsilon_{app} \times d$ = 1532 M^{-1}) that was previously determined from purified 2-chloro-4-nitrophenol by a calibration curve.[29] The product concentration was then plotted over

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time and the resulting data fitted linearly to derive the rate constant of the uncatalyzed reaction from the slope.

Assay To Determine Uninhibited Enzyme Activity. The substrate stock solution was pipetted in 0−70 μL aliquots and adjusted to a total volume of 80 μL with buffer solution, thermostated at 30 °C for 30 min prior to addition of a 20 μL aliquot of enzyme stock solution, thorough mixing, and data recording. Formation of 2-chloro-4-nitrophenolate was followed at 405 nm for 5−15 min, and the change in absorbances was recorded every 27 s. Product formation in dependence of time was derived from these values by correction for the apparent molar extinction coefficient ($\varepsilon_{app} \times d$ =1532 M^{-1}).

A plot of the initial rates corrected for the enzyme concentration over substrate concentration resulted in hyperbolic data that were analyzed by the Michaelis−Menten model as described previously to derive the rate constant (k_{cat}) and the Michaelis−Menten constant (K_M) for noninhibited enzyme activity.[18,29]

Assay To Determine Inhibition of Enzyme Activity. Substrate stock solution was pipetted in 0−70 μL aliquots, 10 μL of inhibitor stock solution was added for each inhibitor stock solution, and the total volume of the solutions was then adjusted to 80 μL with buffer solution. Resulting solutions were thermostated at 30 °C for 30 min prior to addition of 20 μL of enzyme stock solution, thorough mixing, and data recording. Formation of 2-chloro-4-nitrophenolate was followed at 405 nm for 5−15 min, and the change in absorbances was recorded every 27 s as described for the assay in the absence of inhibitor.

Data Analysis. A plot of the initial rates corrected for the enzyme concentration over substrate concentration resulted in hyperbolic data that were analyzed by the Michaelis−Menten model as described previously to derive the apparent rate constant (k'_{cat}) and the Michaelis−Menten constant (K'_M) for the inhibited enzyme activity.[18,29] The competitive inhibition constants K_i (6−602 nM) were calculated from the kinetic parameters obtained in the presence and absence of inhibitor solutions.

Inhibitor Efficacy. *General Remarks.* All experiments were performed in 50 mM acetate buffer at pH 5.00 ± 0.05 at 30 °C with β-galactosidase (*A. oryzae*) as catalyst and 2-chloro-4-nitrophenyl-β-D-galactopyranoside (**5**) as substrate in a 96-well plate using a total volume of 100 μL in the cells. All experiments were performed in duplicate, and obtained data were averaged. The enzyme stock solution was prepared as described for kinetic assays.

Substrate Stock Solution. In a typical experiment, 5.0 mg (0.015 mmol) of 2-chloro-4-nitrophenyl-β-D-galactopyranoside was dissolved in 20.0 mL of acetate buffer in a volumetric flask immediately prior to use.

Inhibitor Stock Solution. In a typical experiment, 100 μM solutions of the inhibitors **1d**, **1n**, **1p-q**, **1s−v**, and **1x** in nanopure water were diluted into 5 × 10^{-3} and 30 μM stock solutions using 50 mM acetate buffer and volumetric flasks.

Assay To Determine Inhibitor Efficacy. A 30 μL aliquot of the substrate stock solution was pipetted in each cell of a 96-well plate, and then 10 μL of the diluted inhibitor solutions or appropriate amounts of the 100 μM inhibitor stock solution, respectively, were added. The total volume of the solutions was subsequently adjusted to 80 μL with acetate buffer. Blanks were prepared for each plate by adding 50 μL of buffer to 30 μL of substrate aliquots. All resulting solutions were thermostated at 30 °C for 30 min prior to addition of 20 μL of the enzyme stock solution and thorough mixing. Maximal product formation was determined as a change in absorbance at 405 nm after 60 min. All experiments were conducted in duplicate, and obtained data were averaged.

Data Analysis. Recorded absorbances were transformed into percentage using the blank experiments of each plate as reference and then plotted against inhibitor concentrations in a logarithmic scale.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Analytical data for intermediates **3h−v** and final galactonoamidines **1h−x**; HPLC traces of **1a−x**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Gloster, T. M.; Davies, G. J. Glycosidase inhibition: assessing mimicry of the transition state. *Org. Biomol. Chem.* **2010**, *8*, 305−320.

(2) Pereira-Rodriguez, A.; Fernandez-Leiro, R.; Gonzalez-Siso, M. I.; Cerdan, M. E.; Becerra, M.; Sanz-Aparicio, J. Structural basis of specificity in tetrameric Kluyveromyces lactis β-galactosidase. *J. Struct. Biol.* **2012**, *177*, 392−401.

(3) Boeris, V.; Balce, I.; Vennapusa, R. R.; Rodriguez, M. A.; Pico, G.; Lahore, M. F. Production, recovery and purification of a recombinant β-galactosidase by expanded bed anion exchange adsorption. *J. Chromatogr. B* **2012**, *900*, 32−37.

(4) Kumari, S.; Panesar, P. S.; Panesar, R. Production of β-galactosidase using novel yeast isolate from whey. *Int. J. Dairy Sci.* **2011**, *6*, 150−157.

(5) Suzuki, Y.; Ichinomiya, S.; Kurosawa, M.; Ohkubo, M.; Watanabe, H.; Iwasaki, H.; Matsuda, J.; Noguchi, Y.; Takimoto, K.; Itoh, M.; Tabe, M.; Iida, M.; Kubo, T.; Ogawa, S.; Nanba, E.; Higaki, K.; Ohno, K.; Brady, R. Chemical chaperone therapy: clinical effect in murine G(M1)-gangliosidosis. *Ann. Neurol.* **2007**, *62*, 671−5.

(6) Suzuki, Y. Chemical chaperone therapy for GM1-gangliosidosis. *Cell. Mol. Life Sci.* **2008**, *65*, 351−353.

(7) Cheng, W.; Wang, L.; Jiang, Y.-L.; Bai, X.-H.; Chu, J.; Li, Q.; Yu, G.; Liang, Q.-L.; Zhou, C.-Z.; Chen, Y. Structural insights into the substrate specificity of streptococcus pneumoniae β(1,3)-galactosidase BgaC. *J. Biol. Chem.* **2012**, *287*, 22910−22918.

(8) Maksimainen, M.; Hakulinen, N.; Kallio, J. M.; Timoharju, T.; Turunen, O.; Rouvinen, J. Crystal structures of Trichoderma reesei β-galactosidase reveal conformational changes in the active site. *J. Struct. Biol.* **2011**, *174*, 156−163.

(9) Maksimainen, M.; Paavilainen, S.; Hakulinen, N.; Rouvinen, J. Structural analysis, enzymatic characterization, and catalytic mechanisms of β-galactosidase from Bacillus circulans sp. alkalophilus. *FEBS J.* **2012**, *279*, 1788−1798.

(10) Maksimainen, M. M.; Lampio, A.; Mertanen, M.; Turunen, O.; Rouvinen, J. The crystal structure of acidic β-galactosidase from Aspergillus oryzae. *Int. J. Biol. Macromol.* **2013**, *60*, 109−115.

(11) Morita, M.; Saito, S.; Ikeda, K.; Ohno, K.; Sugawara, K.; Suzuki, T.; Togawa, T.; Sakuraba, H. Structural bases of GM1 gangliosidosis and Morquio B disease. *J. Hum. Genet.* **2009**, *54*, 510−515.

(12) Ohto, U.; Usui, K.; Ochi, T.; Yuki, K.; Satow, Y.; Shimizu, T. Crystal structure of human β-galactosidase: structural basis of GM1 gangliosidosis and Morquio B diseases. *J. Biol. Chem.* **2012**, *287*, 1801−1812.

(13) Rojas, A. L.; Nagem, R. A. P.; Neustroev, K. N.; Arand, M.; Adamska, M.; Eneyskaya, E. V.; Kulminskaya, A. A.; Garratt, R. C.; Golubev, A. M.; Polikarpov, I. Crystal structures of β-galactosidase from penicillium sp. and its complex with galactose. *J. Mol. Biol.* **2004**, *343*, 1281−1292.
(14) Froehlich, R. F. G.; Furneaux, R. H.; Mahuran, D. J.; Saf, R.; Stuetz, A. E.; Tropak, M. B.; Wicki, J.; Withers, S. G.; Wrodnigg, T. M. 1-Deoxy-D-galactonojirimycins with dansyl capped N-substituents as β-galactosidase inhibitors and potential probes for GM1 gangliosidosis affected cell lines. *Carbohydr. Res.* **2011**, *346*, 1592−1598.
(15) Froehlich, R. F. G.; Fantur, K.; Furneaux, R. H.; Paschke, E.; Stuetz, A. E.; Wicki, J.; Withers, S. G.; Wrodnigg, T. M. A fluorescent probe for GM1 gangliosidosis related β-galactosidase: N-(dansylamino)hexylaminocarbonylpentyl-1,5-dideoxy-1,5-imino-D-galactitol. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 6872−6875.
(16) Kim, Y.-W.; Chen, H.-M.; Kim, J. H.; Müllegger, J.; Mahuran, D.; Withers, S. G. Thioglycoligase-based assembly of thiodisaccharides: screening as β-galactosidase inhibitors. *ChemBioChem* **2007**, *8*, 1495−1499.
(17) Kanso, R.; Striegler, S. Multi gram-scale synthesis of galactothionolactam and its transformation into a galactonoamidine. *Carbohydr. Res.* **2011**, *346*, 897−904.
(18) Kanso, R.; Yancey, E. A.; Striegler, S. N-Benzylgalactonoamidines as potent β-galactosidase inhibitors. *Tetrahedron* **2012**, *68*, 47−52.
(19) Kato, E.; Kumagai, T.; Ueda, M. Concise synthesis of glyconoamidines as affinity ligands for the purification of β-glucosidase involved in control of some biological events including plant leaf movement. *Tetrahedron Lett.* **2005**, *46*, 4865−4869.
(20) Heck, M.-P.; Vincent, S. P.; Murray, B. W.; Bellamy, F.; Wong, C.-H.; Mioskowski, C. Cyclic amidine sugars as transition state analogue inhibitors of glycosidases: potent competitive inhibitors of mannosidases. *J. Am. Chem. Soc.* **2004**, *126*, 1971−1979.
(21) Guo, W.; Hiratake, J.; Ogawa, K.; Yamamoto, M.; Ma, S. J.; Sakata, K. β-D-Glycosylamidines potent, selective, and easily accessible β-glycosidase inhibitors. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 467−470.
(22) von Hoff, S.; Heightman, T. D.; Vasella, A. Inhibition of glycosidases by lactam oximes: Influence of the aglycon in disaccharide analogues. *Helv. Chim. Acta* **1998**, *81*, 1710−1725.
(23) Legler, G.; Finken, M.-T. N1-alkyl-D-gluconamidines: are they 'perfect' mimics of the first transition state of glucosidase action? *Carbohydr. Res.* **1996**, *292*, 103−115.
(24) Bleriot, Y.; Dintinger, T.; Guillo, N.; Tellier, C. Synthesis of an amidine pseudo-(1->6)-dimannoside and evaluation as a glycosidase inhibitor. *Tetrahedron Lett.* **1995**, *36*, 5175−5178.
(25) Bleriot, Y.; Dintinger, T.; Genre-Grandpierre, A.; Padrines, M.; Tellier, C. Inhibition of glycosidases by substituted amidines. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 2655−2660.
(26) Bleriot, Y.; Genre-Grandpierre, A.; Tellier, C. Synthesis of a benzylamidine derived from D-mannose. A potent mannosidase inhibitor. *Tetrahedron Lett.* **1994**, *35*, 1867−1870.
(27) Papandreou, G.; Tong, M. K.; Ganem, B. Amidine, amidrazone, and amidoxime derivatives of monosaccharide aldonolactams: synthesis and evaluation as glycosidase inhibitors. *J. Am. Chem. Soc.* **1993**, *115*, 11682−11690.
(28) Bartlett, P. A.; Marlowe, C. K. Phosphonamidates as transition-state analogue inhibitors of thermolysin. *Biochemistry* **1983**, *22*, 4618−4624.
(29) Fan, Q.-H.; Striegler, S.; Langston, R. G.; Barnett, J. D. Evaluating *N*-benzylgalactonoamidines as putative transition state analogs for β-galactoside hydrolysis. *Org. Biomol. Chem.* **2014**, *12*, 2792−2800.
(30) Overkleeft, H. S.; van Wiltenburg, J.; Pandit, U. K. A facile transformation of sugar lactones to azasugars. *Tetrahedron* **1994**, *50*, 4215−4224.
(31) Ramanathan, A.; Jimenez, L. S. Reductive dehalogenation of aryl bromides and chlorides and their use as aryl blocking groups. *Synthesis* **2010**, *2*, 217−220.
(32) Panday, N.; Canac, Y.; Vasella, A. Very strong inhibition of glucosidases by c(2)-substituted tetrahydroimidazopyridines. *Helv. Chim. Acta* **2000**, *83*, 58−79.