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Illuminating the binding interactions of galactonoamidines during the inhibition of β-galactosidase (*E. coli*)

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ABSTRACT

Several galactonoamidines were previously identified as very potent competitive inhibitors that exhibit stabilizing hydrophobic interactions of the aglycon in the active site of β-galactosidase (*Aspergillus oryzae*). To elucidate the contributions of the glycon to the overall inhibition ability of the compounds, three glyconoamidine derivatives with alteration in the glycon at C-2 and C-4 were synthesized and evaluated herein. All amidines are competitive inhibitors of β-galactosidase (*Escherichia coli*) and show significantly reduced inhibition ability when compared to the parent. The results highlight strong hydrogen-bonding interactions between the hydroxyl group at C-2 of the amidine glycon and the active site of the enzyme. Slightly weaker H-bonds are promoted through the hydroxyl group at C-4. The inhibition constants were determined to be picomolar for the parent galactonoamidine, and nanomolar for the designed derivatives rendering all glyconoamidines very potent inhibitors of glycosidases albeit the derivatized amidines show up to 700-fold lower inhibition activity than the parent.

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1. Introduction

Glycosidases are enzymes that catalyze the hydrolysis of glycosidic bonds.[1] The reaction proceeds through mechanisms that retain or invert the stereochemistry on the anomeric center of the substrates. Both pathways are hypothesized to involve an oxocarbenium ion-like transition state that encompasses a flattened chair configuration of the sugar ring, a positive partial charge at the endocyclic oxygen atom, sp^2-like character of the anomeric carbon atom and lengthening of the exocyclic glycosidic bond upon its cleavage (Chart 1).[2–5]

We previously demonstrated that galactonoamidines competitively inhibit β-galactosidase (*Aspergillus oryzae*).[6] We elaborated a gram-scale synthesis toward the galactoamidine precursor compounds,[7] and evaluated the contributions of the respective aglycon to the overall compound inhibition ability in structure–activity relationship studies with a small library of 22 amidines.[8] Hydrophobic interactions of the aglycon were identified as driving force for the inhibition ability of the most competitive inhibitors during the enzymatic hydrolysis of model substrates (K_i = 8–12 nM).[8] *p*-Methylbenzyl-D-galactonoamidine (**1**) was additionally characterized as putative transition state analog for the enzymatically catalyzed glycoside hydrolysis.[9] While efforts to co-crystallize **1** with β-galactosidase (*Escherichia coli*) are ongoing, we designed derivatives of **1** with modifications of the hydroxyl groups at C-2 and C-4 in the glycon and preserved aglycon structure (Chart 2).

The alterations will diminish or completely hamper the inhibition of the enzymatically catalyzed glycoside hydrolysis and thus illuminate the binding interactions of the glycon of **1** in the active site of the enzyme. We report here the synthesis of derivatized glyconoamidines **1a–c**, and the evaluation of their inhibitory ability of β-galactosidase (*E. coli*) in comparison to **1**.

2. Results and discussion

The synthetic strategy toward the derivatives of **1** consists of a series of transformations resembling the following key steps toward benchmark compounds: firstly, oxidation of the anomeric hydroxyl group yielding glyconolactones after installation of selective protecting groups and methylation of commercially available carbohydrates; secondly, transformation of glyconolactones into glycothionolactams via glyconolactams and glyconamides, and thirdly, activation of the glycothionolactams with Meerwein's salt and coupling with *p*-methylbenzylamine yielding the targeted glyconoamidines after exhaustive hydrogenation. This strategy builds on synthetic methodology previously described by us and others.[10,7] The corresponding efforts are summarized below and sorted by benchmark compounds to streamline the discussion.

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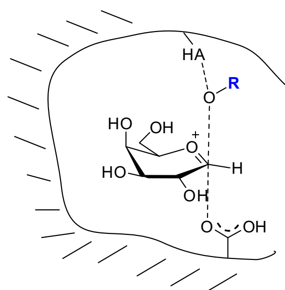


Chart 1. Putative transition state of the enzymatic glycoside hydrolysis.

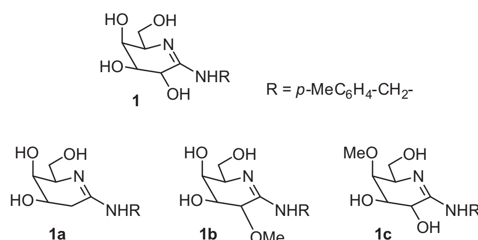


Chart 2. Structures of glyconoamidines **1** and **1a**–**c**.

2.1. Synthesis of perbenzylated glyconolactones

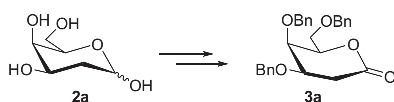
For the first amidine derivative, commercially available 2-deoxy-*lyxo*-hexose (**2a**) was transformed in 6 synthetic steps into perbenzylated lactone (**3a**) as described yielding the first benchmark compounds in this compound series (Scheme 1).[11–16]

For the second amidine derivative, galactose was transformed into galactopyranoside **4** as described.[17,18] After protection of the primary hydroxyl group at C-6 of **4** as triphenylmethyl ether yielding **5** (Scheme 2),[19,20] the hydroxyl group at C-2 was methylated affording **6**.[21] All temporary protecting groups of **6** were then removed with aqueous acetic acid affording **7** and, after perbenzylation, **8**. Treatment of **8** with *N*-bromosuccinimide in aqueous acetone removed the anomeric thiotolyl group yielding **9** as an intermediate that was typically not isolated, but immediately subjected to Swern oxidation with acetic anhydride in DMSO affording galactonolactone (**3b**).

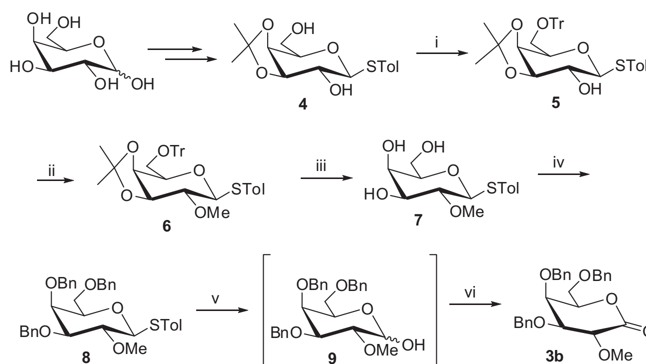
For the third amidine derivative, literature-known galactopyranoside (**10**) was derived in four steps from peracetylated galactose by selective introduction of a thiotolyl group at C-1, exhaustive deacetylation, installation of a benzylidene group in positions 4 and 6, and benzylation of the hydroxyl groups at C-2 and C-3 (Scheme 3).[22–24] Selective opening of the benzylidene ring in **10** with borane trimethylamine complex and aluminium(III) chloride yielded **11**, followed by methylation of the hydroxyl group at C-4 to give **12**. Removal of the anomeric thiotolyl group with *N*-bromosuccinimide in aqueous acetone provided **13**. Swern oxidation of **13** with acetic anhydride in DMSO afforded galactonolactone (**3c**).

2.2. Synthesis of glyconoamidines

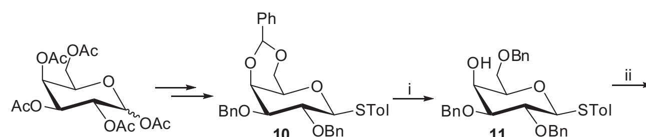
With perbenzylated glyconolactones **3a**–**c** on hand, we followed a synthetic strategy by Overkleeft and our previously elaborated protocols for the transformation of glyconolactones into glyconamides (**14a**–**c**) and glyconolactams (**15a**–**c**) (Scheme 4).[7,10,13] Treatment of the glyconolactams with Lawesson's reagent gave glycothionolactams (**16a**–**c**) in good to very good yields.[25–27] Activation of **16a**–**c** with Meerwein's salt and subsequent coupling with *p*-methylbenzylamine yielded perbenzylated glyconoamidines **17a**–**c**.[25,10,7]



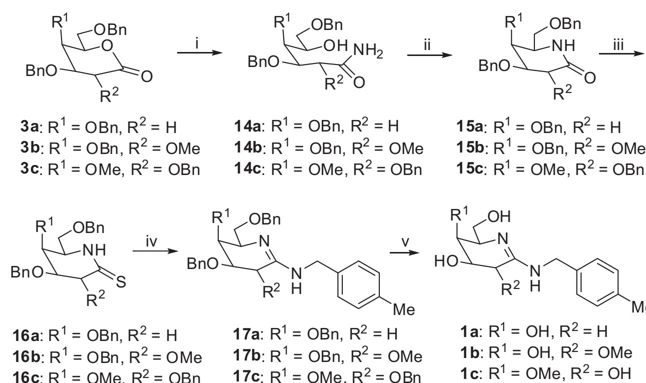
Scheme 1. Synthesis of 2-deoxy-D-*lyxo*-hexono-1,5-lactone (**3a**).[11–16]



Scheme 2. Synthetic strategy toward perbenzylated 2-*O*-methyl-D-galactonolactone (**3b**); reagents and conditions: (i) TrCl, C_5H_5N, 80 °C, 2 h, 91%; (ii) NaH, MeI, THF, 0 → 50 °C, 2 h, 85%; (iii) HOAc, H_2O, 60 °C, 3 h, 87%; (iv) NaH, BnBr, DMF, 0 °C → rt, 84%; (v) NBS, acetone, H_2O, 0 °C, 30 min, 96%; (vi) Ac_2O, DMSO, rt, 12 h, 76%.



Scheme 3. Synthetic strategy toward perbenzylated 4-*O*-methyl-D-galactonolactone (**3c**); reagents and conditions: (i) $Me_3N{\cdot}BH_3$, $AlCl_3$, H_2O, THF, 7 h, rt, 56%; (ii) NaH, MeI, THF, 50 °C, 1 h, 86%; (iii) NBS, acetone/water (9/1, v/v), rt, 5 min, 87%; (iv) Ac_2O, DMSO, 10 h, rt, 48%.



Scheme 4. Synthesis of glyconoamidines **1a**–**c**; reagents and conditions: (i) NH_3/MeOH, rt, 9–17 h, 85–96%; (ii) Ac_2O, DMSO, rt, 11–24 h; $NaBH_3CN$, HCOOH, CH_3CN, reflux, 2 h, 71–95%; (iii) Lawesson's reagent, C_6H_6, rt or reflux, 1 h, 85–87%; (iv) Et_3OBF_4, CH_2Cl_2, 0 °C, 2 h; *p*-methylbenzylamine, 0 °C → rt, 72 h, 75%; (v) H_2, Pd/C (10%), CF_3COOH, EtOH (1/10, v/v), rt, 24 h, quantitative.

$^{1}H^{1}H$ COSY NMR experiments with **17c** revealed a correlation between the proton of a NH group at 5.59 ppm and the protons of the methylene group of the aglycon at 4.33 and 4.23 ppm. Such correlation is only possible for a proton at the exocyclic nitrogen atom. Consequently, the double bond of the amidine **17c** is endocyclic as proposed by Ganem and Papandreou for related compounds.[28] Our data do not support an exocyclic double bond in **17c** in contrast to computational, NMR and X-ray diffraction data discussed by Vasella and Withers for related gluconoamidines.[25] Debenzylation of glyconoamidines **17a–c** with hydrogen gas in the presence of Pd on charcoal and trifluoroacetic acid in ethanol afforded the derivatized glyconoamidines **1a–c** in excellent yields. The compound purity was documented to exceed 95% by HPLC analysis.

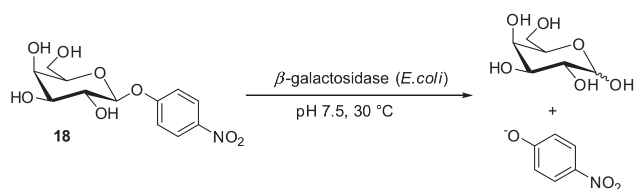
2.3. Evaluation of galactonoamidines derivatives as inhibitors of β-galactosidase (*E. coli*)

With galactonoamidine **1** and its derivatives **1a-c** on hand, we evaluated their interaction with β-galactosidase (*E. coli*) during the enzymatic hydrolysis of *p*-nitrophenyl-β-D-galactopyranoside (**18**) (Scheme 5). The study was designed to determine which modifications in the glycon of **1** lead to significant changes in its inhibitory ability. The hydrophobic interactions between the aglycon of the amidines and the active site of the enzyme are identical for all compounds, and are consequently negligible here.

A 96-well plate assay based on UV/Vis spectroscopy was used to assess the enzymatic hydrolysis of **18** as a change in absorbance due to *p*-nitrophenolate formation in 5 mM HEPES buffer at pH 7.50 ± 0.05 and 30.0 ± 0.1 °C. The kinetic parameters were deduced by application of the Michaelis–Menten model as described for related studies.[6,9] The catalytic rate constant (k_{cat}) of the hydrolysis of **18** by β-galactosidase (*E. coli*) was determined to be 91 ± 2 s^{-1} and the substrate affinity (K_M) was found to be 0.040 ± 0.004 mM confirming previous results of others under comparable conditions (k_{cat} 90 ± 1 s^{-1}; K_M = 0.040 ± 0.002 mM, 30 mM TES buffer, 145 mM NaCl, pH 7.0, 25 °C).[29,30] The apparent rate constant (k'_{cat}) and the apparent Michaelis–Menten constant (K'_M) were then determined in presence of at least three different inhibitor concentrations for each amidine.[6,9] The corresponding inhibition constants (K_i) were calculated from the recorded data, and the obtained values were averaged (Table 1).

All galactonoamidines **1** and **1a–c** were identified as competitive inhibitors of β-galactosidase (*E. coli*) as visualized by Lineweaver–Burk plots (Fig. 1a–d) verifying the applicability of the Michaelis–Menten model.

Galactonoamidine **1** inhibits β-galactosidase (*E. coli*) in the picomolar concentration range and thereby classifies as one of the most potent inhibitors of glycosidases known today (Table 1, entry 1). In contrast to **1a–b**, galactonoamidine **1** possesses a hydroxyl group at C-2 that allows the formation of H-bonding donor and acceptor interactions in the active site of the enzyme. The lack of these interactions causes a significant increase in the inhibition constant (Table 1, entry 2) and corresponds to a 22-fold weaker ability of **1a** compared to **1** to inhibit the enzymatic hydrolysis of **18**. When methylating the hydroxyl group at C-2 yielding galactonoamidine **1b** (Table 1, entry 3), the ability of the resulting inhibitor to form H-bond donating interactions is further diminished due to the steric demands of the methyl group in **1b** in place of the previous H-atom in **1**. In comparison with the underivatized parent **1**, galactonoamidine **1b** shows a 700-fold reduced ability to inhibit the enzymatic hydrolysis of **18**. Likewise, methylating the hydroxyl group at C-4 in **1** affording galactonoamidine **1c** (Table 1, entry 4) diminishes the hydrogen bonding ability of the galactonoamidine **1** and introduced steric constraints, but to a somewhat lesser extent than **1b** accounting for the preserved free hydroxyl group at C-2 in **1c**. Galactonoamidine **1c** decreases the enzyme activity during the hydrolysis of the model compound more than 530-fold in



Scheme 5. Enzymatic hydrolysis of **18** in 5 mM HEPES buffer at pH 7.50 ± 0.05 and 30.0 ± 0.1 °C.

Table 1
Inhibition constants K_i of galactonoamidines **1**, **1a–c**

Entry	Inhibitor	Inhibitor structure	$K_i \pm \Delta K_i^*$ [nM]
1	**1**		0.06 ± 0.01
2	**1a**		1.3 ± 0.1
3	**1b**		42 ± 1.0
4	**1c**		32 ± 1.4

* Calculated from kinetic parameters obtained during the hydrolysis of **18** by β-galactosidase (*E. coli*) in the presence and absence of **1**, **1a–c**.

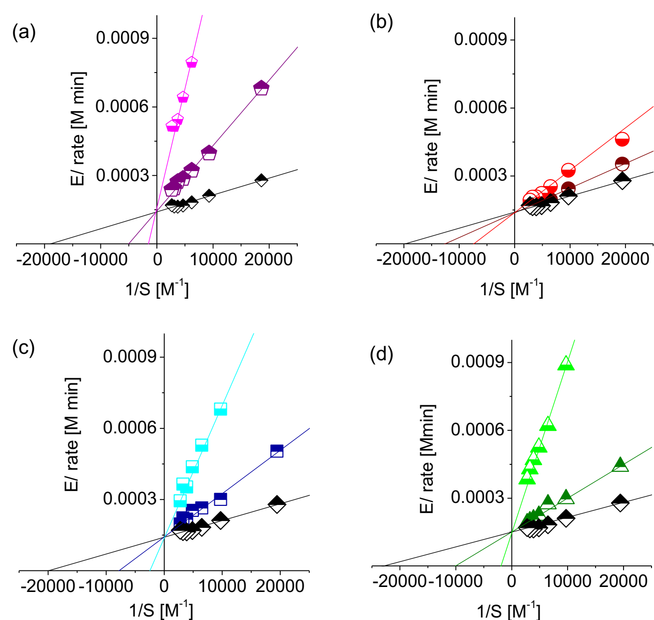


Figure 1. Lineweaver–Burk plots to visualize competitive inhibition of the enzymatic hydrolysis of **18** by galactonoamidines (a) **1** at 0.15 nM and 0.2 nM; (b) **1a** at 1.0 nM and 1.5 nM; (c) **1b** at 100 nM and 250 nM; (d) **1c** at 100 nM and 200 nM; *I* = 0 nM.

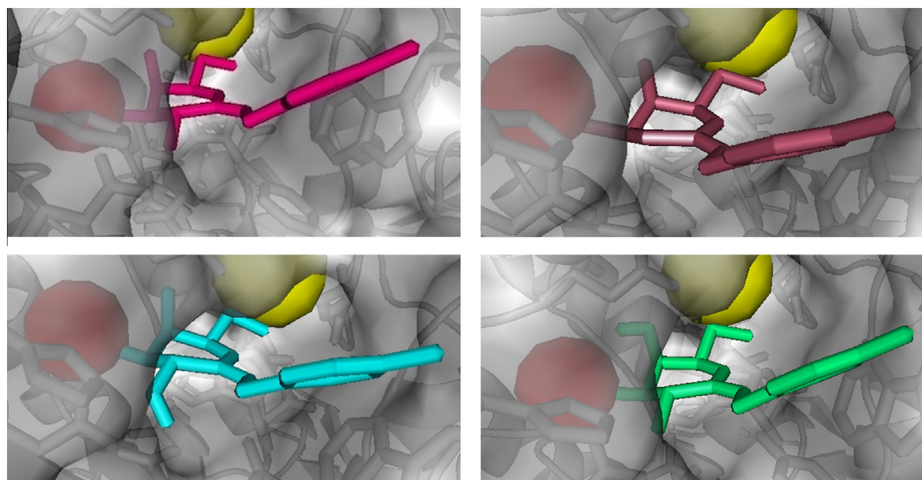


Figure 2. Visualization of the inhibitors **1** (pink), **1a** (wine), **1b** (cyan), and **1c** (green) in the active site of β-galactosidase (*E. coli*), 1JZ8; Mg^{2+} ions in red, Na^{+} ions in yellow.[31]

comparison to **1** pointing at a loss of stabilizing H-bonding interactions and increased steric constraints upon binding of the inhibitor in the active site (see Fig. 2).

Docking experiments of the amidines **1** and **1a**–**c** in the active site of β-galactosidase (*E. coli*) using the structure of 1JZ8 from the PDB data base reveal a significantly higher number of interactions for amidine **1** in comparison to **1a**–**c**.[32,33] While limited interactions of **1a** are noted and assigned to the lack of a hydroxyl group at C-2, the *O*-methyl group at C-2 in **1b** hampers the interaction of the inhibitor with the active site sterically, and prevents its full inclusion. Thus, only a single point interaction of **1b** over the hydroxyl group at C-6 is observed. To a smaller extent, the *O*-methyl group at C-4 in **1c** causes likewise limited access of the inhibitor to the active site, but does still allow interactions with the catalytically active GLU537. Overall, the docking experiments confirm the already noted importance of the hydroxyl group at C-2 for the interactions of the selected amidines within the active site of β-galactosidase (*E. coli*).

Similar observations were made by others in elegant studies elucidating the hydrogen bonding ability of β-galactosidase (*E. coli*) with fluorinated substrates to elaborate the contributions of the amino acids in its active site and the mechanism of the hydrolysis.[34–37] Thus, our results may be likewise attributed to stabilization of transition state binding promoted through strong interactions of the hydroxyl group at C-2 and slightly weaker interactions of the hydroxyl group at C-4.

3. Conclusions

Three glyconoamidines **1a**–**c** were synthesized with derivatizations in their glycon at C-2 and C-4 and compared to their underivatized parent **1** by kinetic studies in their inhibitory ability toward β-galactosidase (*E. coli*).[7,6] All glyconoamidines were shown to inhibit the selected enzyme competitively; parent amidine **1** shows inhibition ability in the picomolar and derivatized amidines **1a**–**c** in the low nanomolar concentration range rendering **1** one of the most potent glycosidase inhibitor known today. Removal of the hydroxyl group at C-2, as accomplished in **1a,** or derivatizations at the hydroxyl groups of C-2 and C-4, as accomplished in **1b** and **1c**, respectively, diminish the hydrogen bonding and inhibition ability of the glyconoamidines. This observation points at hydrogen bonding interactions of the hydroxyl group at C-2 and C-4 in the underivatized parent **1** in the active site of β-galactosidase (*E. coli*) to stabilize the transition state of the glycoside hydrolysis.

4. Experimental

4.1. Instrumentation

^{1}H and ^{13}C NMR spectra were recorded on a 400 MHz Bruker magnet with Z gradient and 5 mm broadband 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. Combustion data were obtained from Atlantic Microlab, 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. The purity of the amidines was established on an HPLC system from Shimadzu equipped with SCL-10Avp system controller, 2 LC-20AD analytical pumps, DGU-20A3R three channel online degassers, SIL-20A UFLC autosampler with 96 well capability, CTO-20A/prominence column oven and ELSD-90LT light scattering and LC solution software, version 1.25 from Shimadzu for data recording and analysis. A 300 × 7.5 mm (5μ) RNM-Carbohydrate Na^{+} (8%) column from Phenomenex was used as stationary phase for the HPLC assays to demonstrate compound purity; nanopure water was used as isocratic eluent at a flow rate of 0.4 mL/min at 80 °C. Apparent optical rotations were measured at 589 nm on an Autopol III polarimeter from Rudolph Research Analytical at ambient temperature in a 1 mL cell glass center fill stainless steel jacketed cell with an optical path length *l* of 100 mm. Melting points were recorded on a Mel-Temp melting point apparatus, and the values are uncorrected. All pH values 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 semi-micro tip. Nanopure water at a resistance of 18.2 mΩ was obtained from a ThermoScientific Barnstead E-pure™ water purification system. Lyophilization was performed on a FreeZone 1 L benchtop freeze dry system from Labconco.

4.2. Materials and methods

Chemical shifts (δ) in NMR data are expressed in parts per million (ppm) and coupling constants (J) in Hz. Signal multiplicities are denoted as s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet). Deuterated chloroform, deuterated dichloromethane, acetone-d_6, DMSO-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; acetone-d_6, δ_H 2.05, δ_C 29.8; DMSO-d_6, δ_H 2.50, δ_C 39.5; D_2O: δ_H 4.80, δ_C 29.8 after addition of a few drops of acetone-d_6 or δ_C 49.0 after addition of a few drops of MeOH-d_4. The specific optical rotation is given as average of three independent experiments, and calculated as $[\alpha]^T_D = \alpha \times 100/c \times l$, where the value for the concentration c is expressed in g/100 mL solvent and the length l is 1 dm. Column chromatography was carried out using silica gel 60 from Silicycle® (40–63 μm, 230–240 mesh) and basic aluminium oxide (50–200 μm, 70–270 mesh) from Acros. Thin layer chromatography (TLC) was performed using silica gel TLC plates from SORBENT Technologies, 200 μm, 4 × 8 cm, aluminium backed, with fluorescence indicator F_{254} and detection by UV light or by charring with an ethanol–vanillin–sulfuric acid reagent and subsequent heating of the TLC plate. All commercially obtained chemicals had reagent grade quality or better and were used as received, if not noted otherwise. Ethyl acetate, cyclohexane, hexane and 4-methylbenzylamine were distilled prior to use, THF, dichloromethane, DMSO and DMF were dried over neutral aluminium oxide and stored over molecular sieves prior to use; 2-deoxy-D-*lyxo*hexono-1,5-lactone (**3a**),[11–16] *p*-tolyl-3,4-*O*-isopropylidene-1-thio-β-D-galactopyranoside (**4**),[11,12] and *p*-methylphenyl-4,6-*O*-benzylidene-1-thio-β-D-galactopyranoside (**10**)[22–24] were synthesized as described. β-Galactosidase [3.2.1.23] from *Escherichia coli* was obtained from Sigma–Aldrich as lyophilized powder and used as received.

4.3. Synthesis of perbenzylated glyconolactones

4.3.1. 3,4,6-Tri-O-benzyl-2-deoxy-D-*lyxo*-hexono-1,5-lactone (3a)[11–16]

The compound was prepared according to literature procedures;[11–16] yielding **3a** as a colorless oil (7.50 g, 63%); R_f 0.42 (SiO_2, cyclohexane/ethyl acetate, 2/1 v/v); δ_H (acetone-d_6) 7.47–7.17 (m, 15H), 4.96 (d, 11.3, 1H), 4.72 (dd, 12.0, 25.3, 2H), 4.33 (d, 1.8, 1H), 4.21 (ddd, 2.1, 6.7, 11.0, 1H), 3.76 (dd, 6.5, 9.5, 1H), 3.67 (dd, 6.3, 9.5, 1H), 2.90 (ddd, 1.0, 6.3, 17.8, 1H), 2.85–2.71 (m, 3H); δ_C (acetone-d_6) 169.1, 139.6, 139.4, 139.2, 129.2, 129.2, 129.1, 128.8, 128.7, 128.5, 128.4, 128.4, 78.7, 75.6, 75.0, 73.8, 72.1, 71.3, 69.7, 34.0; the spectral data of **3a** match those previously reported.[15]

4.3.2. *p*-Tolyl 3,4-*O*-isopropylidene-1-thio-β-D-galactopyranoside (4)[38]

The compound was synthesized by adapting a protocol for a related compound with different aglycon.[17] Colorless oil; δ_H ($CDCl_3$) 7.44 (dt, 8.0, 1.8, 2H), 7.13 (d, 7.8, 2H), 4.41 (d, 10.3, 1H), 4.18 (dd, 5.5, 2.3, 1H), 4.11 (dd, 7.0, 5.5, 1H), 3.98 (dd, 11.3, 7.0, 1H), 3.84–3.89 (m, 1H), 3.81 (dd, 11.5, 4.3, 1H), 3.55 (dd, 10.2, 6.9, 4H), 2.34 (s, 3H), 1.43 (s, 3H), 1.34 (s, 3H); δ_C ($CDCl_3$) 138.2, 132.8, 129.7, 128.0, 110.3, 87.9, 79.2, 77.0, 73.8, 71.4, 62.4, 27.9, 26.3, 21.0; the spectral data of **4** match those previously reported.[38]

4.3.3. *p*-Tolyl 3,4-*O*-isopropylidene-6-*O*-trityl-1-thio-β-D-galactopyranoside (5)

The synthesis was performed according to a protocol for closely related compound described by Kakarla et al.[39] Triphenylmethyl chloride (18.72 g, 0.0673 mol, 1.46 equiv) was added to the solution of **4** (15.00 g, 0.0460 mol) in 60 mL pyridine and heated to 80 °C for 2 h. The reaction was performed in this set-up in duplicate. The two batches were combined after cooling of the reaction mixtures, 50 mL of water were added, and the resulting solution was extracted three times with 200 mL ethyl acetate each. The combined organic layers were washed with 80 mL water, 80 mL brine and dried over anhydrous sodium sulfate. After filtration, the filtrate was concentrated to dryness in vacuum, and the obtained residue was purified by column chromatography over silica gel (hexane/ethyl acetate, 6/1–3/1, v/v) to afford compound **5** (31.1 g, 55.78 mmol, 91%) as a colorless solid; mp 71–73 °C; R_f 0.16 (SiO_2, cyclohexane/ethyl acetate, 4/1, v/v); δ_H (CD_2Cl_2) 7.52–7.43 (m, 8H), 7.37–7.23 (m, 9H), 7.11 (d, 8.0, 2H), 4.39 (d, 10.3, 1H), 4.14 (dd, 2.1, 5.4, 1H), 3.99 (dd, 5.5, 6.8, 1H), 3.80 (ddd, 2.0, 4.9, 7.2, 1H), 3.47 (td, 7.3, 9.9, 2H), 3.29 (dd, 5.0, 9.8, 1H), 2.37 (br s, 1H), 2.32 (s, 3H), 1.57 (br s, 1H), 1.35 (s, 3H), 1.29 (s, 3H); δ_C (CD_2Cl_2) 144.6, 138.7, 133.1, 130.3, 129.3, 128.4, 127.6, 110.5, 88.8, 87.3, 79.7, 76.6, 74.5, 72.0, 63.9, 28.4, 26.6, 21.4; HRMS (ESI) calcd for $C_{35}H_{36}NaO_5S$ $[M+Na]^+$: 591.2181; found: 591.2188.

4.3.4. *p*-Tolyl 3,4-*O*-isopropylidene-2-*O*-methyl-6-*O*-trityl-1-thio-β-D-galactopyranoside (6)

The synthesis was achieved by adapting a protocol for the methylation of a similarly protected galactose.[21] Sodium hydride (0.14 g, 3.600 mmol, 2 equiv) was added to a solution of 5 (1.00 g, 1.800 mmol) in 10 mL THF at 0 °C. After 5 min, methyl iodide (0.51 g, 3.600 mmol, 2.00 equiv) was added to the suspension in the cold. Then, the reaction mixture was warmed to 50 °C. After 2 h, the mixture was cooled again to 0 °C prior to addition of 2 mL of methanol, then 3 mL water. The resulting mixture was then extracted three times with 80 mL ethyl acetate each. The combined organic layers were washed with water (50 mL), brine (50 mL) and dried over anhydrous sodium sulfate. After filtration and concentration of the filtrate to dryness in vacuum, a residue was obtained that was purified by column chromatography over silica gel (hexane/ethyl acetate, 8/1–6/1, v/v) to afford compound **6** (0.91 g, 1.594 mmol, 85%) as a colorless solid; R_f 0.20 (SiO_2, cyclohexane/ethyl acetate, 5/1, v/v); mp 149–151 °C; δ_H (CD_2Cl_2) 7.37–7.52 (m, 8H), 7.21–7.36 (m, 9H), 7.03–7.11 (m, 2H), 4.47 (d, 9.8, 1H), 4.04–4.13 (m, 2H), 3.70 (ddd, 7.0, 4.8, 1.8, 1H), 3.49 (s, 3H), 3.41–3.47 (m, 1H), 3.27 (dd, 10.0, 4.5, 1H), 3.17 (dd, 9.7, 5.9, 1H), 2.30 (s, 3H), 1.40 (s, 3H), 1.29 (s, 3H); δ_C (CD_2Cl_2) 144.6, 138.0, 132.5, 130.8, 130.2, 129.3, 128.4, 127.6, 110.4, 87.4, 86.9, 81.1, 80.2, 76.3, 74.6, 64.1, 59.9, 28.2, 26.6, 21.4; Anal. Calcd for $C_{36}H_{38}O_5S$: C, 74.20; H, 6.57. Found: C, 74.16; H, 6.60.

4.3.5. *p*-Tolyl 2-*O*-methyl-1-thio-β-D-galactopyranoside (7)

The synthesis was performed by adapting protocols for removal of acid-labile protecting groups including isopropylidene as described for similarly protected galactopyranosides.[40–42] The solution of **6** (1.00 g, 1.752 mmol) in 8 mL acetic acid and 2 mL water was stirred at 60 °C. After 3 h, the solution was cooled to ambient temperature and neutralized with saturated aqueous sodium bicarbonate solution. Subsequently, 100 mL ethyl acetate were added. After separation, the organic layer was washed once with 20 mL water, 20 mL brine and dried over anhydrous sodium sulfate. After filtration, the filtrate was concentrated in vacuum yielding residue that was purified by column chromatography over silica gel (hexane/ethyl acetate, 2/1–1/3, v/v) to afford compound **7** (0.44 g, 87%) as a colorless solid; (R_f 0.25 (SiO_2, ethyl acetate); mp 145–147 °C; δ_H (DMSO-d_6) 7.39–7.34 (m, 2H), 7.12 (d, 8.0, 2H), 5.00 (d, 6.5, 1H), 4.64 (t, 5.5, 1H), 4.60 (d, 4.5, 1H), 4.53 (d, 9.8, 1H), 3.68 (t, 3.6, 1H), 3.55–3.42 (m, 4H), 3.41 (s, 3H), 3.33 (s, 2H), 3.14 (t, 9.4, 1H), 2.27 (s, 3H); δ_C (DMSO-d_6) 136.0, 131.2, 130.2, 129.5, 86.3, 79.4, 78.9, 74.3, 68.5, 60.4, 60.0, 20.6; Anal. Calcd for $C_{14}H_{20}O_5S$: C, 55.98; H, 6.71. Found: C, 55.72; H, 6.61.

4.3.6. *p*-Tolyl 3,4,6-tri-*O*-benzyl-2-*O*-methyl-1-thio-β-D-galactopyranoside (8)

The synthesis was performed by adapting a benzylation protocol for related compounds.[12] Sodium hydride (0.14 g, 3.600 mmol, 2 equiv) was added at 0 °C to a solution of **6** (1.00 g, 1.800 mmol) in

10 mL THF. After 5 min, methyl iodide (0.51 g, 3.600 mmol, 2.00 equiv) was added to the suspension in the cold. The reaction mixture was then warmed to 50 °C. After 2 h, the mixture was cooled again to 0 °C prior to addition of 2 mL of methanol and 3 mL of water. After 5 min, the resulting mixture was extracted three times with 50 mL ethyl acetate each. The combined organic layers were washed once with 30 mL water, 30 mL brine and dried over anhydrous sodium sulfate. After filtration and concentration of the filtrate to dryness, a residue was obtained that was purified by column chromatography over silica gel (hexane/ethyl acetate, 8/1–6/1, (v/v) to give compound **8** (0.91 g, 1.594 mmol, 85%) as a colorless solid; R_f 0.20 (SiO$_2$, cyclohexane/ethyl acetate—5/1 (v/v); mp: 63–64 °C; δ_H (CD$_2$Cl$_2$) 7.53–7.17 (m, 17H), 7.07–7.02 (m, 2H), 4.91 (d, 11.3, 1H), 4.74 (dd, 11.5, 14.3, 2H), 4.56 (d, 11.3, 1H), 4.51–4.39 (m, 3H), 3.95 (d, 2.0, 1H), 3.66–3.59 (m, 3H), 3.58 (s, 3H), 3.55–3.46 (m, 2H), 2.30 (s, 3H); δ_H (CD$_2$Cl$_2$) 139.4, 139.2, 138.8, 137.9, 132.4, 130.9, 130.1, 128.9, 128.9, 128.7, 128.4, 128.2, 128.1, 128.0, 88.3, 84.5, 79.8, 77.7, 75.2, 74.7, 73.9, 73.1, 69.6, 61.5, 21.4; Anal. Calcd for $C_{35}H_{38}O_5S$: C, 73.65; H, 6.71. Found: C, 73.67; H, 6.69.

4.3.7. 3,4,6-Tri-*O*-benzyl-2-*O*-methyl-D-galactopyranose (9)

The selective removal of the thiotolyl group was achieved by adapting protocols for related compounds with slight modifications.[11,17] *N*-Bromosuccinimide (3.75 g, 21.1 mmol, 1.2 equiv) was added to a solution of **8** (10.00 g, 17.5 mmol) in 100 mL acetone/H$_2$O (9/1, v/v) at 0 °C. After 30 min, 20 mL of a saturated aqueous sodium bicarbonate solution were added. The reaction mixture was then extracted three times with 150 mL ethyl acetate each. The combined organic layer was washed once with 50 mL water, 50 mL brine and dried over anhydrous sodium sulfate. After filtration and concentration, a residue was obtained that was purified by column chromatography over silica gel (hexane/ethyl acetate, 4/1–2/1, v/v) affording compound **9** as a colorless oil (7.90 g, 17.0 mmol, 96%); R_f 0.25 (SiO$_2$, cyclohexane/ethyl acetate—3/1, v/v); HRMS (ESI) calcd for $C_{28}H_{32}NaO_6$ [M+Na]$^+$: 487.2097; found: 487.2097; The product was used in the next step without further characterization.

4.3.8. 3,4,6 Tri-*O*-benzyl-2-*O*-methyl-D-galactonolactone (3b)

The compound was synthesized from **9** after adapting a literature protocol for related compounds.[11] A solution of **9** (7.90 g, 17.0 mmol) in 48 mL DMSO and 32 mL acetic anhydride was stirred at ambient temperature for 12 h. Then, 400 mL acetyl acetate and 100 mL water were added, the organic layer was separated and washed once with 50 mL water, 50 mL brine and dried over anhydrous sodium sulfate. After filtration and concentration, a residue was obtained that was purified by column chromatography over silica gel (hexane/ethyl acetate, 4/1–2/1, v/v) yielding **3c** (6.00 g, 12.9 mmol, 76%) as a colorless oil; R_f 0.30 (SiO$_2$, cyclohexane/ethyl acetate, 4/1, v/v). δ_H (CD$_2$Cl$_2$) 7.47–7.21 (m, 15H), 4.95 (d, 11.3, 1H), 4.77 (d, 11.8, 1H), 4.72 (d, 12.0, 1H), 4.63 (d, 11.3, 1H), 4.52 (d, 11.0, 1H), 4.46 (d, 12.0, 1H), 4.35 (dt, 1.5, 6.7, 1H), 4.21–4.15 (m, 1H), 3.84 (dd, 2.1, 9.7, 1H), 3.71–3.63 (m, 5H); δ_C (CD$_2$Cl$_2$) 170.3, 138.7, 138.5, 138.3, 129.0, 128.9, 128.5, 128.5, 128.4, 128.4, 128.3, 128.1, 80.5, 80.1, 77.9, 75.4, 74.1, 73.4, 73.3, 68.6, 61.4, 54.5, 54.3, 53.7, 53.5; HRMS (ESI) calcd for $C_{28}H_{30}NaO_6$ [M+Na]$^+$: 485.1940; found: 485.1917.

4.3.9. *p*-Tolyl 2,3-di-*O*-benzyl-4,6-*O*-benzylidene-1-thio-β-D-galactopyranoside (10)

The synthesis of the title compound was achieved after modification of a protocol for a related compound.[43] Under inert atmosphere, *p*-tolyl 4,6-*O*-benzylidene-1-thio-β-D-galactopyranoside (7.26 g, 19.41 mmol) was dissolved in 200 mL dry DMF. After cooling of the solution to 0 °C, 2.32 g (0.0582 mol, 3 equiv) of sodium hydride were added. After 1 h, 6.8 mL (0.059 mol, 3 equiv) benzyl chloride were added in the cold and the reaction mixture allowed to warm to ambient temperature. After 19 h, all volatile material was removed yielding a yellow residue. The residue was taken up in 100 mL water, and the resulting mixture was extracted four times with 100 mL dichloromethane each. The organic layers were combined, dried over anhydrous sodium sulfate, filtered and concentrated to dryness yielding a yellow solid. The solid was triturated with 50 mL hexane, filtered off and dried to yield **10** (8.57 g, 15.44 mmol 79%) as a yellow solid; mp 151–154 °C (mp[44] 129–131 °C); δ_H (CDCl$_3$) 7.68–7.28 (m, 17H), 7.03 (dd, 1.0, 9.0, 2H), 5.50 (s, 1H), 4.79–4.69 (m, 4H), 4.59 (d, 9.5, 1H), 4.39 (dd, 1.5, 12.3, 1H), 4.16 (d, 3.0, 1H), 4.00 (dd, 1.8, 12.3, 1H), 3.87 (t, 9.3, 1H), 3.64 (dd, 3.4, 9.2, 1H), 3.42 (d, 1.0, 1H), 2.33 (s, 3H); δ_C (CDCl$_3$) 138.5, 138.1, 137.9, 137.6, 133.4, 129.6, 129.0, 128.6, 128.3, 128.3, 128.1, 128.0, 127.7, 127.7, 127.6, 126.6, 101.2, 86.5, 81.4, 75.3, 75.3, 73.6, 71.7, 69.7, 69.4, 21.1; Anal. Calcd for $C_{27}H_{27}O_5$: C, 73.62; H, 6.18. Found: C, 73.76; H, 6.17. The spectral data match those in the literature,[44] the melting point does not.[44]

4.3.10. *p*-Tolyl 2,3,6-tri-*O*-benzyl-1-thio-β-D-galactopyranoside (11)

The synthesis was achieved after modifying a protocol for related compounds.[45] Compound **10** (6.03 g, 10.9 mmol) was dissolved in 220 mL THF at ambient temperature, followed by addition of 5.00 g (0.0435 mol, 4 equiv) borane trimethylamine complex, 8.67 g (0.0652 mol, 6 equiv) aluminium(III) chloride and 0.39 mL (0.022 mol, 2 equiv) water. After 7 h, 120 mL water and 120 mL 1 M aqueous hydrochloride acid solution were added to quench the reaction. The reaction mixture was then extracted three times with 200 mL ethyl acetate each. The organic layers were combined and washed with 200 mL brine. The organic layer was separated, dried over sodium sulfate, filtered and concentrated to dryness yielding a solid residue. Trituration of the residue with 50 mL hexane yielded **11** (3.57 g, 6.409 mmol, 56%) as colorless needles. An analytical sample (100 mg) was recrystallized from 10 mL ethyl acetate and 5 mL hexane; mp 123–125 °C; R_f 0.55 (SiO$_2$, hexane/ethyl acetate, 2/1, v/v); δ_H (CDCl$_3$) 7.59–7.28 (m, 17H), 7.09 (d, 8.0, 2H), 4.87 (d, 10.3, 1H), 4.81–4.69 (m, 3H), 4.65–4.55 (m, 3H), 4.13 (d, 2.8, 1H), 3.89–3.70 (m, 3H), 3.65–3.56 (m, 2H), 2.47 (br s, 1H), 2.34 (s, 3H); δ_C (CDCl$_3$) 138.2, 137.9, 137.6, 137.5, 132.5, 129.8, 129.6, 128.5, 128.4, 128.3, 128.2, 127.9, 127.8, 127.7, 127.7, 87.9, 82.6, 77.0, 76.9, 75.7, 73.7, 72.0, 69.4, 66.8, 21.1; Anal. Calcd for $C_{34}H_{36}O_5S$: C, 73.35; H, 6.52. Found: C, 73.15; H, 6.58.

4.3.11. *p*-Tolyl 2,3,6-tri-*O*-benzyl-4-*O*-methyl-1-thio-β-D-galactopyranoside (12)

The synthesis was performed after slight modifications of a protocol for a closely related compound.[21] Under atmosphere inert, **11** (3.00 g, 5.39 mmol, 1 equiv) was dissolved in 30 mL dry THF. The solution was cooled to 0 °C and 0.65 g (0.016 mol, 3 equiv) sodium hydride (60% in oil) added. The solution was allowed to warm to ambient temperature. After 30 min, 1.21 mL (2.28 mmol, 3.6 equiv) methyl iodide were added and the solution warmed to 50 °C. After 1 h, the solution was cooled to 0 °C and 5 mL methanol and 50 mL water were added. The aqueous layer was extracted four times with 50 mL diethyl ether each. The organic layers were combined, dried over sodium sulfate, filtered and concentrated to dryness under reduced pressure. The obtained residue was triturated with 50 mL hexane, filtered off and dried to yield **12** (2.65 g, 4.641 mmol 86%) as a colorless solid; mp 80–81 °C; R_f 0.50 (SiO$_2$, hexane/ethyl acetate, 4/1, v/v); δ_H (CDCl$_3$) 7.55–7.29 (m, 17H), 7.09 (d, 8.0, 2H), 4.91–4.70 (m, 4H), 4.61 (d, 9.8, 1H), 4.55 (dd, 11.8, 16.8, 2H), 3.86–3.66 (m, 4H), 3.61 (s, 3H), 3.60–3.54 (m, 2H), 2.33 (s, 3H); δ_C (CDCl$_3$) 138.3, 138.1, 137.8, 137.3,

132.3, 130.5, 129.5, 128.4, 128.3, 127.9, 127.8, 127.7, 127.7, 88.5, 83.8, 77.8, 77.1, 75.8, 75.7, 73.6, 72.6, 68.5, 61.3, 21.1; Anal. Calcd for $C_{34}H_{38}O_5S$: C, 73.65; H, 6.71. Found: C, 73.66; H, 6.71.

4.3.12. 2,3,6-Tri-*O*-benzyl-4-*O*-methyl-D-galactopyranose (13)

The synthesis was performed after slight modifications of a protocol for a closely related compound.[46] Compound **12** (2.00 g, 3.50 mmol) was dissolved in 18 mL acetone and 2 mL water at ambient temperature followed by addition of 1.31 g (7.36 mmol, 2 equiv) of *N*-bromosuccinimide. After 5 min, 6.11 g (0.0727 mol) of sodium bicarbonate were added and the solution concentrated to dryness under reduced pressure. The obtained residue was dissolved in 150 mL ethyl acetate and 50 mL water. The organic layer was separated and washed three times with 50 mL saturated aqueous sodium bicarbonate solution each, three times with 50 mL water each and once with 25 mL brine, separated and dried over anhydrous sodium sulfate, filtered and concentrated to dryness under reduced pressure. The obtained residue was purified by column chromatography over silica gel (hexane/ethyl acetate, 9/1–1/1, v/v) affording **13** (1.42 g, 3.054 mmol 87%) as a colorless, translucent oil consisting of a mix of α/β-isomers in a 10/4 molar ratio; R_f 0.37 (SiO_2, hexane/ethyl acetate, 3/2, v/v); δ_H 7.47–7.28 (m, 26H), 7.48–7.27 (m, 26H), 5.28 (d, 3.5, 1H), 4.97–4.48 (m, 11H), 4.99–4.46 (m, 11H), 4.20 (t, 6.8, 1H), 3.96 (dd, 3.5, 9.8, 1H), 3.90 (dd, 2.5, 9.5, 1H), 3.75–3.60 (m, 6H), 3.59 (s, 2H), 3.58 (s, 3H), 3.51 (dd, 3.0, 9.8, 1H); δ_C (CDCl3) 138.5, 138.2, 137.8, 137.7, 128.4, 128.4, 128.3, 128.3, 128.2, 128.0, 127.9, 127.8, 127.8, 127.7, 127.7, 127.6, 127.6, 127.5, 97.6, 91.8, 81.8, 80.8, 78.4, 77.0, 76.5, 75.9, 75.1, 73.5, 73.5, 73.4, 73.3, 72.7, 72.7, 69.1, 68.6, 68.4, 61.3, 61.3; HRMS (ESI) calcd for $C_{28}H_{32}NaO_6$ $[M+Na]^+$: 487.2097; found: 487.2090; calcd for $C_{21}H_{26}NaO_6$ $[M-Bn+Na]^+$: 397.1627; found: 397.1620; compound **13** was typically used without characterization directly in the next step.

4.3.13. 2,3,6-Tri-*O*-benzyl-4-*O*-methyl-D-galactonolactone (3c)

The compound was synthesized following a protocol by Overkleeft for a related compound.[13] Under inert atmosphere, **13** (3.87 g, 8.323 mmol) was dissolved in 22.5 mL dry DMSO and 13.5 mL acetic anhydride at ambient temperature. After 10 h, additional 13.5 mL acetic anhydride were added. After further 3 h, 25 mL of ice water were added. After another 30 min, the organic layer was separated from the aqueous layer by centrifugation at 6000 rpm for 2 min. The remaining oil was dissolved in 10 mL dichloromethane, and the resulting organic layer was extracted four times with 5 mL water each. The separation of the organic and aqueous layers was achieved by centrifugation; the aqueous layer was discarded, the organic layer was dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The obtained residue was purified by column chromatography over silica gel (hexane/ethyl acetate, 4/1, v/v) to give **3c** (1.84 g, 3.974 mmol, 48%) as a translucent, colorless oil; R_f 0.35 (SiO_2, hexane/ethyl acetate, 4/1, v/v); δ_C ($CDCl_3$) 7.49–7.28 (m, 15H), 5.20 (d, 11.0, 1H), 4.82–4.70 (m, 3H), 4.57 (dd, 11.5, 19.6, 2H), 4.41 (d, 9.5, 1H), 4.33 (ddd, 1.6, 5.5, 8.4, 1H), 3.92 (t, 1.9, 1H), 3.86 (dd, 2.3, 9.5, 1H), 3.76 (dd, 8.3, 9.3, 1H), 3.69 (dd, 5.5, 8.8, 1H), 3.59 (s, 3H); δ_C ($CDCl_3$) 169.9, 137.7, 137.6, 137.4, 128.5, 128.4, 128.3, 128.3, 128.0, 127.9, 127.9, 127.8, 127.5, 80.0, 77.4, 77.1, 75.4, 74.6, 73.7, 72.7, 67.2, 61.3; HRMS (ESI) calcd for $C_{28}H_{30}NaO_6$ $[M+Na]^+$: 485.1940; found: 487.1936.

4.4. General procedure for the synthesis of perbenzylated glyconamides 14a–c from glyconolactones 3a–c[13]

Typically, glyconolactone **3** was dissolved in 5–18 mL of 7 N ammonia in methanol under inert atmosphere and stirred at ambient temperature. After 4–17 h, the solution was concentrated to dryness. The obtained residue was purified by column chromatography over silica gel to afford the title compounds as colorless oils or solids.

4.4.1. 3,4,6-Tri-*O*-benzyl-2-deoxy-D-*lyxo*-hexonamide (14a)

Compound **3a** (1.10 g, 2.546 mmol), 6.0 mL 7 N ammonia in methanol; reaction time: 9 h; eluent for chromatography: cyclohexane/ethyl acetate, 2/1–1/2, v/v; colorless oil; yield: 0.97 g (2.546 mmol, 85%); R_f 0.20 (SiO_2, cyclohexane/ethyl acetate = 1/2, v/v); δ_H ($CDCl_3$) 7.45–7.28 (m, 12H), 5.97 (br s, 1H), 5.33 (br s, 1H), 4.78 (d, 11.0, 1H), 4.64 (dd, 11.3, 24.6, 2H), 4.56–4.44 (m, 3H), 4.15 (td, 4.5, 7.0, 1H), 3.96 (dt, 3.3, 5.8, 1H), 3.76 (dd, 3.3, 4.8, 1H), 3.57 (dd, 5.3, 9.5, 1H), 3.50 (dd, 6.3, 9.5, 1H), 2.64 (dd, 4.0, 15.1, 1H), 2.56 (dd, 6.8, 14.6, 1H), 1.72 (br s, 2H); δ_C ($CDCl_3$) 174.0, 137.6, 137.6, 137.6, 128.2, 128.2, 128.0, 127.7, 127.7, 127.6, 127.6, 127.5, 78.6, 77.2, 73.7, 73.1, 72.6, 70.9, 69.7, 37.5; HRMS (ESI) calcd for $C_{27}H_{32}NO_5$ $[M+H]^+$: 450.2280; found: 450.2287.

4.4.2. 3,4,6-Tri-*O*-benzyl-2-*O*-methyl-D-galactonamide (14b)

Compound **3b** (6.00 g, 12.9 mmol); 70 mL 7 N ammonia in methanol; reaction time: 12 h; eluent for column chromatography: hexane/ethyl acetate, 3/1–1/1, v/v) to give compound **14b** (5.87 g, 12.2 mmol, 95%) as a colorless solid; R_f 0.33 (SiO_2, cyclohexane/ethyl acetate, 2/1, v/v); mp 85–87 °C; δ_H (CD_2Cl_2) 7.52–7.15 (m, 15H), 6.59 (br s, 1H), 5.61 (br s, 1H), 4.63 (d, 10.8, 1H), 4.57 (s, 3H), 4.55–4.46 (m, 2H), 4.10–4.03 (m, 2H), 3.88 (d, 2.0, 1H), 3.80 (dd, 1.5, 8.8, 1H), 3.63–3.50 (m, 2H), 3.40 (s, 3H); δ_C (CD_2Cl_2) 174.6, 138.7, 138.6, 138.6, 128.9, 128.9, 128.8, 128.6, 128.5, 128.3, 128.3, 128.2, 128.2, 82.2, 79.6, 77.7, 75.3, 74.7, 73.8, 72.1, 69.7, 59.4; Anal. Calcd for $C_{28}H_{33}NO_5$: C, 70.13; H, 6.94. Found: C, 69.95; H, 6.87.

4.4.3. 2,3,6-Tri-*O*-benzyl-4-*O*-methyl-D-galactonamide (14c)

Compound **3c** (2.70 g, 5.832 mmol,), 18 mL of 7 N ammonia in methanol; reaction time: 4 h. The solvent was evaporated under reduced pressure, and the resulting residue was triturated in hexane and filtered off to give compound **14c** (2.48 g, 5.167 mmol, 89%) as an off-white solid; mp: 78–80 °C; R_f 0.39 (SiO_2, hexane/ethyl acetate, 1/4, v/v); δ_H (CD_2Cl_2) 7.43–7.21 (m, 15H), 6.66 (d, 2.8, 1H), 6.01 (d, 2.8, 1H), 4.69 (d, 11.3, 1H), 4.62 (d, 10.3, 1H), 4.59–4.47 (m, 4H), 4.11 (d, 2.0, 1H), 4.06 (dt, 1.3, 6.5, 1H), 4.02 (dd, 2.0, 8.8, 1H), 3.65–3.51 (m, 3H), 3.30 (s, 3H), 2.56 (br s, 1H); δ_C (CD_2Cl_2) 174.8, 138.8, 137.6, 129.1, 128.9, 128.8, 128.7, 128.7, 128.3, 128.2, 128.1, 80.3, 79.5, 75.4, 74.0, 73.7, 72.3, 60.6; HRMS (ESI) calcd for $C_{28}H_{34}NO_6$ $[M+H]^+$: 480.2386; found: 480.2378; calcd for $C_{28}H_{33}NNaO_6$ $[M+Na]^+$: 502.2206; found: 502.2194.

4.5. General procedure for the synthesis of perbenzylated glyconolactams 15a–c[13]

Typically, the glyconamides **14** were treated with acetic anhydride in DMSO at ambient temperature. After 24 h, 50 mL water were added. After stirring for additional 10 min, ethyl acetate was added, and stirring continued. After further 5 min, the organic layer was separated, and the aqueous layer was extracted three times with 200 mL ethyl acetate each. The combined organic layers were washed with water (50 mL), brine (50 mL) and dried over anhydrous sodium sulfate. After filtration, the filtrate was concentrated under reduced pressure. The obtained residue was purified by column chromatography over silica gel yielding an intermediate that was typically used without any further purification or characterization in the next step.

Sodium cyanoborohydride was added to a solution of the intermediate in acetonitrile and formic acid at ambient temperature. The solution was kept under reflux for 2 h and cooled. Then, 1 N

aqueous hydrochloric acid was added and stirring continued at ambient temperature. After 15 min, the mixture was poured into 200 mL of ethyl acetate and saturated aqueous sodium bicarbonate solution (1/1, v/v). The organic layer was separated, and the aqueous layer was extracted three times with 100 mL ethyl acetate each. The combined organic layers were washed once with 50 mL water and 50 mL brine, and dried over anhydrous Na_2SO_4. After filtration, the filtrate was concentrated in vacuum, and the obtained residue was purified by column chromatography over silica gel using cyclohexane/ethyl acetate as eluent yielding the title compounds **15**.

4.5.1. 3,4,6-Tri-*O*-benzyl-2-deoxy-D-*lyxo*-hexono-1,5-lactam (15a)

Compound **14a** (4.0 g, 8.949 mmol), 20.0 mL acetic anhydride, 30.0 mL DMSO; reaction time: 24 h; eluent for column chromatography: cyclohexane/ethyl acetate, 2/1–1/1, v/v; yielding 3,4,6-tri-*O*-benzyl-5-dehydro-5-oxo-2-deoxy-D-lyxo-hexonamide as a colorless oil; 3.40 g (7.640 mmol, 85%); R_f 0.24 (SiO_2, cyclohexane/ethyl acetate, 1/2, v/v); HRMS (ESI) calcd for $C_{27}H_{29}NNaO_5$ $[M+Na]^+$: 470.1943; found: 470.1933.

Sodium cyanoborohydride (1.69 g, 0.0269 mol, 3.0 equiv), 3,4,6-tri-*O*-benzyl-5-dehydro-5-oxo-2-deoxy-D-lyxo-hexonamide (3.40g, 7.640 mmol), 80 mL acetonitrile, 16 mL formic acid; 2 mL 1 N aqueous hydrochloric acid; eluent for chromatography: cyclohexane/ethyl acetate, 2/1—1/1 (v/v); yielding **15a** as a colorless oil (2.70 g, 6.323 mmol, 71% over two steps); R_f 0.20 (SiO_2, cyclohexane/ethyl acetate = 1/1, v/v); δ_H (CD_2Cl_2) 7.45–7.18 (m, 15H), 5.96 (s, 1H), 4.94 (d, 11.5, 1H), 4.62 (s, 2H), 4.59 (s, 1H), 4.55–4.43 (m, 3H), 4.04 (s, 1H), 3.88 (ddd, 1.8, 6.1, 10.9, 1H), 3.65–3.51 (m, 4H), 2.74 (ddd, 1.0, 10.8, 16.8, 1H), 2.65 (ddd, 0.5, 5.3, 16.8, 1H); δ_C (CD_2Cl_2) 170.5, 139.0, 138.7, 138.4, 128.9, 128.9, 128.8, 128.4, 128.4, 128.3, 128.2, 128.1, 128.0, 76.3, 74.5, 73.8, 72.3, 71.2, 70.8, 54.6, 34.4; HRMS (ESI) calcd for $C_{27}H_{30}NO_4$ $[M+H]^+$: 432.2175; found: 432.2171.

4.5.2. 3,4,6-Tri-*O*-benzyl-2-*O*-methyl-D-galactonolactam (15b)

Compound **14b** (1.60 g, 3.340 mmol), 5.0 mL acetic anhydride, 7.5 mL DMSO; reaction time: 48 h; eluent for column chromatography: cyclohexane/ethyl acetate, 2/1–1/1; yielding 3,4,6-tri-*O*-benzyl-5-dehydro-5-oxo-2-*O*-methyl-D-galactonamide as a colorless oil (0.90 g, 1.887 mmol, 56%); R_f 0.33 (SiO_2, cyclohexane/ethyl acetate, 2/1, v/v); HRMS (ESI) calcd for $C_{28}H_{31}NNaO_6$ $[M+Na]^+$: 500.2049; found: 500.2045.

Sodium cyanoborohydride (0.36 g, 5.71 mol, 3 equiv); 3,4,6-tri-*O*-benzyl-5-dehydro-5-oxo-2-*O*-methyl-D-galactonamide (0.90 g, 1.887 mmol); 15 mL acetonitrile, 3.0 mL formic acid; 2 mL 1 N aqueous hydrochloric acid; eluent for column chromatography: cyclohexane/ethyl acetate, 2/1–1/1 (v/v), yielding **15b** as a colorless oil (0.80 g, 0.174 mmol, 52% over two steps); R_f 0.35 (SiO_2, cyclohexane/ethyl acetate = 2/1, v/v); δ_H (CD_2Cl_2) 7.17–7.51 (m, 15H), 5.83 (br s, 1H), 4.94 (d, 11.5, 1H), 4.79 (d, 12.8, 1H), 4.68–4.75 (m, 1H), 4.59 (d, 11.3, 1H), 4.50 (d, 11.5, 1H), 4.45 (d, 11.8, 1H), 4.00–4.07 (m, 2H), 3.79 (dd, 9.3, 2.0, 1H), 3.67 (s, 3H), 3.58 (ddd, 12.3, 3.9, 2.6, 1H), 3.51–3.57 (m, 1H), 3.48 (dd, 8.0, 3.8, 1H); δ_C (CD_2Cl_2) 170.8, 139.0, 138.7, 138.2, 128.9, 128.9, 128.9, 128.5, 128.4, 128.4, 128.3, 128.2, 128.1, 81.1, 80.0, 74.8, 73.9, 73.4, 71.0, 61.3; HRMS (ESI) calcd for $C_{28}H_{32}NO_5$ $[M+H]^+$: 462.2280, found: 462.2267.

4.5.3. 2,3,6-Tri-*O*-benzyl-4-*O*-methyl-D-galactonolactam (15c)

Compound **14c** (1.21 g, 2.707 mmol), 6.75 mL acetic anhydride, 10 mL DMSO; reaction time: 18 h; trituration with hexane yielded 2,3,6-tri-*O*-benzyl-5-dehydro-5-oxo-4-*O*-methyl-D-galactonamide (2.02 g, 4.226 mmol, 87%) as an off-white waxy solid; HRMS (ESI) calcd for $C_{28}H_{32}NO_6$ $[M+H]^+$: 478.2224; found: 478.2226.

Sodium cyanoborohydride (0.97 g, 15.4 mmol, 4 equiv); 2,3,6-tri-*O*-benzyl-5-dehydro-5-oxo-4-*O*-methyl-D-galactonamide (1.84 g, 3.849 mmol); 40 mL acetonitrile, 16 mL formic acid; 2 mL 1 N aqueous hydrochloric acid; eluent for column chromatography: cyclohexane/ethyl acetate, 1/0–1/1 (v/v), yielding **15c** as yellow oil (0.84 g, 1.883 mmol, 39% on 2 steps); R_f 0.79 (SiO_2, hexane/ethyl acetate = 1/4, v/v); δ_H (CD_2Cl_2) 7.51–7.24 (m, 15H), 6.08 (br s, 1H), 5.17 (d, 11.0, 1H), 4.79 (d, 11.3, 1H), 4.75–4.70 (m, 2H), 4.55 (dd, 11.0, 18.8, 2H), 4.23 (d, 9.0, 1H), 3.86–3.80 (m, 2H), 3.68–3.61 (m, 2H), 3.59 (d, 10.0, 1H), 3.55 (s, 3H); δ_C (CD_2Cl_2) 171.1, 139.2, 138.9, 138.4, 129.0, 128.9, 128.7, 128.6, 128.4, 128.2, 128.0, 81.2, 78.0, 76.0, 75.6, 73.9, 73.3, 70.7, 61.2, 54.0; HRMS (ESI) calcd for $C_{28}H_{32}NO_5$ $[M+H]^+$: 462.2280; found: 462.2278.

4.6. General procedure for the synthesis of perbenzylated glycothionolactams[7,10,25]

Treatment of glyconolactams **15** with Lawesson's reagent in dry benzene under reflux for 1 h yielded glycothionolactams **16**. The resulting solution was cooled and concentrated under reduced pressure. The obtained residue was purified by column chromatography over silica gel yielding the title compounds in very good yields.

4.6.1. 3,4,6-Tri-*O*-benzyl-2-deoxy-D-*lyxo*-hexo-thionolactam (16a)

Lawesson's reagent (0.28 g, 0.693 mmol, 0.70 equiv); compound **15a** (0.43 g, 0.001 mol), 15 mL benzene; eluent for column chromatography: cyclohexane/ethyl acetate, 10/1–8/1 (v/v); yielding compound **16a** as a colorless oil (0.39 g, 0.892 mmol, 87%); R_f 0.30 (SiO_2, cyclohexane/ethyl acetate, 4/1, v/v); the compound is a mixture of two resonance isomers in a 1 to 1.7 molar ratio; δ_H (CD_2Cl_2) 8.09 (br s, 1H), 7.44–7.21 (m, 15H), 4.92 (d, 11.3, 1H), 4.68–4.39 (m, 6H), 4.02 (s, 1H), 3.84 (ddd, 1.8, 5.5, 10.8, 1H), 3.72–3.62 (m, 3H), 3.57–3.47 (m, 1H), 3.30 (ddd, 1.0, 5.5, 18.3, 1H), 3.14 (dd, 10.5, 18.1, 1H); δ_C (CD_2Cl_2) 200.8, 138.5, 138.4, 138.0, 128.9, 128.9, 128.8, 128.5, 128.4, 128.3, 128.2, 128.2, 127.9, 75.4, 74.4, 73.8, 71.3, 71.2, 69.7, 59.0, 54.4, 42.0; HRMS (ESI) calcd for $C_{27}H_{30}NO_3S$ $[M+H]^+$: 448.1941; found: 448.1936.

4.6.2. 3,4,6-Tri-*O*-benzyl-2-*O*-methyl-D-galactothionolactam (16b)

Lawesson's reagent (0.06 g, 0.152 mmol, 0.7 equiv); compound **15b** (0.200 g, 0.217 mmol), 10 mL benzene; ambient temperature 24 h, eluent for column chromatography: hexane/ethyl acetate, 10/1–8/1, (v/v); yielding compound **16b** as a colorless oil (0.08 g, 0.168 mmol, 77%); R_f 0.28 (SiO_2, cyclohexane/ethyl acetate, 5/1, v/v); δ_H (CD_2Cl_2) 8.06 (br s, 1H), 7.48–7.15 (m, 15H), 4.87 (d, 11.5, 1H), 4.78 (d, 12.0, 1H), 4.71 (d, 12.0, 1H), 4.57 (d, 11.5, 1H), 4.54 (d, 11.5, 1H), 4.45 (d, 12.0, 1H), 4.14 (d, 8.3, 1H), 4.08–4.04 (m, 1H), 3.79 (dd, 2.0, 8.3, 1H), 3.73 (s, 3H), 3.70–3.62 (m, 2H), 3.55–3.47 (m, 1H); δ_C (CD_2Cl_2) 202.3, 138.8, 138.4, 138.1, 129.0, 128.9, 128.9, 128.5, 128.4, 128.4, 128.3, 128.2, 84.1, 79.7, 74.4, 74.0, 73.4, 73.3, 70.1, 61.6, 58.1; HRMS (ESI) calcd for $C_{28}H_{32}NO_4S$ $[M+H]^+$: 478.2052; found: 478.2041; calcd for $C_{28}H_{32}NNaO_4S$ $[M+H]^+$: 500.1871; found: 500.1858.

4.6.3. 2,3,6-Tri-*O*-benzyl-4-*O*-methyl-D-galactothionolactam (16c)

Lawesson's reagent (0.27 g, 0.658 mmol, 1.6 equiv); compound **15c** (0.19 g, 0.411 mmol); 6 mL benzene; 90 °C for 3 h; filtration over celite; eluent for column chromatography: hexane/ethyl acetate (97/3–4/1, v/v) with the addition of 1% (v/v) triethylamine; yielding compound **16c** as a yellowish oil (0.04 g, 0.084 mmol, 20%); R_f 0.77 (SiO_2, cyclohexane/ethyl acetate, 3/2, v/v); δ_H (acetone-d_6) 7.52–7.43 (m, 2H), 7.42–7.21 (m, 13H), 5.33 (d, 10.8,

1H), 4.82 (s, 1H), 4.72 (dd, 12.3, 21.8, 2H), 4.59 (dd, 11.8, 15.3, 2H), 4.30 (d, 8.3, 1H), 4.13–4.07 (m, 1H), 3.92 (dd, 2.3, 8.3, 1H), 3.90–3.84 (m, 1H), 3.82–3.70 (m, 2H), 3.53 (s, 3H); δ_C (acetone-d_6) 202.7, 139.8, 139.5, 139.1, 129.2, 129.1, 129.1, 128.9, 128.6, 128.5, 128.5, 128.3, 128.3, 82.5, 80.6, 76.0, 75.4, 73.8, 73.0, 69.5, 60.6, 58.1; HRMS (ESI) calcd for $C_{28}H_{32}NO_4S$ $[M+H]^+$: 478.2052; found: 478.2048.

4.7. Synthesis of glyconoamidines

4.7.1. *N-p*-Methylbenzyl-3,4,6-tri-*O*-benzyl-2-deoxy-D-*lyxo*-hexonoamidine (17a)

Meerwein's salt (0.17 g, 0.899 mmol, 1.5 equiv) was added to a solution of **16a** (0.27 g, 0.604 mmol) in 6 mL of dry dichloromethane at 0 °C under nitrogen atmosphere. After 2 h, freshly distilled *p*-methylbenzylamine (0.15 g, 1.240 mmol, 2.0 equiv) was added at 0 °C, and the solution was allowed to warm to ambient temperature. After 72 h, the solution was concentrated under reduced pressure. The obtained residue was purified by column chromatography over silica gel (cyclohexane/ethyl acetate = 2/1–1/2, v/v) yielding compound **17a** as a colorless foam (0.25 g, 0.468 mmol, 75%); R_f 0.30 (SiO_2, cyclohexane/ethyl acetate = 1/2, v/v); the compound exists as a mixture of two resonance isomers in a 1 to 1.4 molar ratio; δ_H (CD_2Cl_2) 8.50 (t, 5.0, 1H), 8.32 (t, 5.0, 1H), 8.02 (s, 1H), 7.52–6.98 (m, 30H), 6.67 (s, 1H), 4.86 (dd, 11.3, 14.1, 2H), 4.72–4.49 (m, 7H), 4.48–4.29 (m, 7H), 4.11 (s, 1H), 4.00 (br s, 1H), 3.88 (dddd, 1.5, 6.0, 10.5, 16.7, 2H), 3.76–3.64 (m, 2H), 3.61 (d, 7.0, 2H), 3.55–3.44 (m, 2H), 3.24 (dd, 5.8, 17.8, 1H), 3.12 (dd, 10.5, 17.6, 1H), 2.91 (dd, 6.3, 17.6, 1H), 2.80 (dd, 10.3, 17.6, 1H); δ_C (CD_2Cl_2) 165.6, 163.3, 139.3, 138.9, 138.1, 138.1, 138.0, 137.9, 137.9, 137.7, 132.1, 130.5, 130.3, 130.3, 129.1, 129.0, 128.9, 128.9, 128.8, 128.7, 128.6, 128.6, 128.6, 128.5, 128.5, 128.5, 128.4, 128.2, 128.2, 128.1, 127.7, 74.9, 74.8, 74.1, 74.1, 74.0, 73.5, 72.0, 71.7, 71.1, 70.7, 68.9, 57.0, 57.0, 47.2, 46.5, 29.5, 27.8, 21.4; HRMS (ESI) calcd for $C_{35}H_{39}N_2O_3$ $[M+H]^+$: 535.2961, found: 535.2957.

4.7.2. *N-p*-Methylbenzyl-3,4,6-tri-*O*-benzyl-2-*O*-methyl-D-galactonoamidine (17b)

Meerwein's salt (0.60 g, 3.145 mmol, 1.5 equiv) was added to a solution of **16b** (1.00 g, 2.096 mmol) in 10 mL dry dichloromethane at 0 °C under nitrogen atmosphere. After 2 h, freshly distilled 4-methyl benzylamine (0.51 g, 4.19 mmol, 2.0 equiv) was added at 0 °C, and the resulting solution was allowed to warm to ambient temperature while stirring for additional 13 h. The solution was then concentrated under reduced pressure yielding a residue that was purified by column chromatography over silica gel dried at 80 °C using freshly distilled solvents (cyclohexane/ethyl acetate = 1/1–0/1, v/v) as eluents to afford **17b** as a colorless oil (0.90 g, 1.596 mmol, 76%); R_f 0.24 (SiO_2, ethyl acetate); δ_H (CD_2Cl_2) 7.23–7.49 (m, 15H), 7.15 (dd, 31.4, 7.8, 4H), 4.88 (d, 11.3, 1H), 4.77 (d, 11.5, 1H), 4.69 (d, 11.5, 1H), 4.39–4.62 (m, 6H), 4.19–4.24 (m, 1H), 3.99 (dd, 9.8, 1.8, 1H), 3.82–3.90 (m, 1H), 3.69 (s, 3H), 3.50–3.67 (m, 3H), 2.30 (s, 3H); δ_C (CD_2Cl_2) 162.6, 139.3, 138.0, 137.8, 131.0, 130.4, 129.1, 129.0, 129.0, 128.7, 128.6, 128.5, 128.5, 128.4, 79.6, 75.7, 75.6, 74.0, 72.9, 72.1, 68.8, 62.0, 57.2, 46.5, 21.4; HRMS (ESI) calcd for $C_{36}H_{41}N_2O_3$ $[M+H]^+$: 565.3066, found: 565.3059.

4.7.3. *N-p*-Methylbenzyl-3,4,6-tri-*O*-benzyl-4-*O*-methyl-D-galactonoamidine (17c)

Meerwein's salt (0.62 g, 3.26 mmol, 1.5 equiv) was added to a solution of **16c** (1.01 g, 2.11 mmol) in 20 mL of dry dichloromethane at 0 °C under nitrogen atmosphere. After 2.5 h, freshly distilled 4-methylbenzylamine (0.60 mL, 4.65 mmol, 2.2 equiv) was added at 0 °C, and the solution was allowed to warm to ambient temperature. After 19 h, the solution was concentrated under reduced pressure. The obtained residue was purified by column chromatography over silica gel (cyclohexane/ethyl acetate = 1/0, 10/1–1/1, 0/1–1/2, v/v) and filtered over deactivated basic alumina using ethyl acetate yielding compound **17c** as a colorless oil (0.12 g, 0.212 mmol, 10%); R_f 0.73 (hexane/ethyl acetate, 1/1 (v/v) containing 1% (v/v) triethylamine); δ_H (acetone-d_6) 7.49–7.23 (m, 15H), 7.11 (dd, 8.0, 28.1, 4H), 5.48 (br s, 1H), 4.88 (dd, 2.3, 11.5, 2H), 4.69 (dd, 11.4, 13.7, 2H), 4.59 (dd, 12.0, 17.8, 2H), 4.43 (dd, 1.3, 9.5, 1H), 4.33 (d, 14.6, 1H), 4.23 (d, 14.6, 1H), 4.08 (t, 1.9, 1H), 4.01 (dd, 1.9, 9.4, 1H), 3.72–3.63 (m, 2H), 3.62–3.57 (m, 1H), 3.57 (s, 3H), 2.28 (s, 3H); 156.2, 140.1, 139.7, 139.6, 138.4, 136.7, 129.6, 129.2, 129.2, 129.1, 128.8, 128.7, 128.5, 128.4, 128.4, 128.2, 82.9, 76.6, 76.5, 74.1, 73.6, 72.2, 71.5, 61.0, 60.1, 44.8, 21.2; HRMS (ESI) calcd for $C_{36}H_{41}N_2O_4$ $[M+H]^+$: 565.3066, found: 565.3067.

4.8. Synthesis of glyconoamidines

4.8.1. *N-p*-Methylbenzyl-2-deoxy-D-*lyxo*-hexonoamidine (1a)

The synthesis of **1a** was achieved after modifying our previous synthetic strategy toward galactonoamidines by lowering the catalyst amount compared to perbenzylated amidine from 2/1 to 1/1 (w/w) and reducing the trifluoroacetic acid amount compared to ethanol from 1/5 to 1/10 or 1/50 (v/v).[7,10] A mixture of compound **17a** (0.12 g, 0.225 mmol) and Pd/C (0.12 g, 10% Pd on C) in 0.5 mL trifluoroacetic acid and 5 mL ethanol were stirred under hydrogen atmosphere at ambient temperature. After 24 h, the mixture was filtered through a pad of celite, and the celite was washed three times with ethanol 2 mL each. The combined filtrates were concentrated yielding a residue that was lyophilized to give **1a** as a pale white foam (0.08 g, 0.225 mmol, quantitative); $[\alpha]^{22.0}_D = +42.3$ (*c* 0.0756, H_2O); R_f 0.12 (SiO_2, ethyl acetate/methanol, 3/1, v/v); the compound is a mixture of two resonance isomers in a 1 to 5.4 molar ratio; δ_H (D_2O) 7.38–7.06 (m, 5H), 4.52–4.48 (m, 1H), 4.44 (s, 2H), 4.23–3.99 (m, 2H), 3.79 (dd, 9.3, 14.3, 1H), 3.73–3.59 (m, 1H), 2.90 (dd, 5.8, 17.8, 1H), 2.75 (dd, 10.3, 17.6, 1H), 2.29 (s, 3H); δ_C ($D_2O + CD_3OD$) 163.2, 139.8, 131.7, 130.6, 130.6, 128.8, 128.4, 66.7, 65.7, 61.1, 58.3, 46.3, 30.4, 21.1; MS (HR-ESI) calcd for $C_{14}H_{21}N_2O_3$ $[M+H]^+$: 265.1552, found: 265.1546.

4.8.2. *N-p*-Methylbenzyl-2-*O*-methyl-D-galactonoamidine (1b)

The suspension of compound **17b** (0.10 g, 0.17 mmol), Pd/C (0.06 g, 30% Pd on charcoal) and 0.06 mL trifluoroacetic acid in 6 mL ethanol was stirred under hydrogen atmosphere at ambient temperature. After 14 h, the mixture was filtered, and the filtrate was concentrated under reduced pressure to give a residue that was lyophilized yielding **1b** as a colorless foam (0.05m 0.170 mmol, 96%); R_f 0.20 (SiO_2, ethyl acetate/methanol = 16/1, v/v); $[\alpha]^{22.0}_D = +51.9$ (*c* 0.0964, H_2O); δ_H (D_2O) 7.23 (dd, 12.8, 8.3, 4H), 4.49–4.62 (m, 3H), 4.25 (t, 2.3, 1H), 4.19 (dd, 10.0, 2.5, 1H), 3.62–3.78 (m, 4H), 3.54 (s, 3H), 2.29 (s, 3H); δ_C ($D_2O + CD_3OD$) 163.4, 139.8, 131.6, 130.7, 128.4, 75.8, 69.8, 68.3, 60.9, 59.5, 58.3, 46.0, 21.1; MS (HR-ESI) calcd for $C_{15}H_{23}N_2O_4$ $[M+H]^+$: 295.1658, found: 295.1651.

4.8.3. *N-p*-Methylbenzyl-4-*O*-methyl-D-galactonoamidine (1c)

The suspension of compound **17c** (0.07 g, 0.124 mmol), Pd/C (0.07 g, 30% Pd on charcoal) and 0.06 mL trifluoroacetic acid in 6 mL ethanol was stirred under hydrogen atmosphere at ambient temperature. After 12 h, the mixture was filtered, the filtrate concentrated under reduced pressure, and the resulting residue lyophilized to yield **1c** as colorless foam (0.03 g, 0.102 mmol, 83%); R_f 0.18 (ethyl acetate/methanol = 20/1, v/v); $[\alpha]^{22.0}_D = +64.6$ (*c* 0.0758, H_2O); δ_H (D_2O) 7.22 (dd, 14.8, 8.5, 4H), 4.59 (d, 10.0, 1H), 4.54 (d, 2.0, 2H), 4.01 (dd, 10.0, 2.5, 1H), 3.97 (t, 2.4, 1H), 3.73–

3.81 (m, 2H), 3.67 (dd, 13.3, 9.5, 1H), 3.54 (s, 3H), 2.29 (s, 3H); δ_C ($D_2O + CD_3OD$) 165.0, 139.8, 131.6, 130.7, 128.4, 78.1, 72.0, 67.6, 62.6, 60.5, 58.4, 46.0, 21.1; MS (HR-ESI) calcd for $C_{15}H_{23}N_2O_4$ $[M+H]^+$: 295.1658, found: 295.1647.

4.9. Kinetic studies

All kinetic experiments were conducted as 96-well plate assays using UV/Vis spectroscopy in 5 mM HEPES buffer at pH 7.50 ± 0.05 and 30.0 ± 0.1 °C.

4.9.1. Enzyme stock solution

The commercially obtained β-galactosidase (*E. coli*) was obtained as a 5 mg sample, dissolved in 1 mL degassed nanopure water and stored in 10 μL aliquots at −80 °C over several months without noticeable loss in activity. A BCA assay confirmed a protein content of 77.1 ± 3.8% in the commercial sample. For the kinetic assays, a 10 μL aliquot was thawed immediately prior to use and diluted to 5 mL with buffer. An 80 μL aliquot of this solution was then further diluted into 10 mL with buffer solution to obtain a stock solution of the tetrameric enzyme with an active site concentration of 528 pM that was kept on ice and used in 20 μL aliquots for all experiments described herein; a molecular weight of 464 kDa was used for all calculations.[47]

4.9.2. Inhibitor stock solutions

A 100 μM inhibitor solution of **1a**, and 200 μM inhibitor solutions of **1b** and **1c** were diluted by addition of to appropriate amounts of nanopure water yielding inhibitor stock solutions with a nominal value of 1.5 and 2.0 nM for **1**, 10 and 15 nM for **1a**, and 1.0 and 2.5 μM for **1b**, and 1 and 2 μM for **1c**. All inhibitor stock solutions were kept at ambient temperature, and used in 10 μL aliquots in a total volume of 100 μL for the kinetic assays.

4.9.3. Substrate stock solution

Typically, 6.20 mg (0.0206 mmol) *p*-nitrophenyl-β-D-galactopyranoside were dissolved in 20 mL of 5 mM buffer in a volumetric flask and diluted 1:1 with buffer solution to obtain a 0.5 mM solution. This substrate stock solution was prepared immediately prior to use and kept at ambient temperature.

4.9.4. Kinetic hydrolysis assay

Typically, the 96-well plate assay based on UV/Vis spectroscopy was prepared with a total volume of 100 μL by using the substrate stock solution in 10 μL increasing aliquot increments between 0 and 70 μL, constant 10 μL inhibitor solution aliquots, and appropriate amounts of buffer solution to obtain a solution with 80 μL volume in all wells. The plates were covered and equilibrated for 30 min at 30 °C prior to addition of 20 μL of enzyme stock solution to initiate the substrate hydrolysis. The change of absorbance due to release of *p*-nitrophenolate was then recorded at 405 nm over 30 min in 30 s intervals. All experiments were performed in duplicate. Control experiments were performed in the same manner after substituting inhibitor by buffer solution.

4.9.5. Data analysis

The absorbance recorded was plotted versus time in minutes. The initial rate of the reaction was determined at each substrate concentration from the slope of the linear fit of the data after conversion of the absorbance into product concentration using the apparent extinction coefficient for *p*-nitrophenolate under identical conditions ($\varepsilon_{app} \times d = 3780 \pm 140\ M^{-1}$ for $V_t = 100$ μL, 30.0 ± 0.1 °C, 5 mM HEPES buffer at pH 7.50 ± 0.05). The rate of the reaction was corrected for the enzyme concentration, and then plotted versus the substrate concentration. By applying a non-linear fit of the resulting hyperbolic data, the apparent catalytic rate constants k_{cat} [min^{-1}], and k'_{cat} [min^{-1}], respectively, and the apparent substrate affinity K_M [M^{-1}], and K'_M [M^{-1}], were discerned utilizing the Michaelis–Menten model in absence, and presence of inhibitor. 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 inhibitor:

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

All experiments were conducted at least in duplicate for each inhibitor concentration, and the value of the inhibition constants are given as average from the obtained data.

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Supplementary data

Supplementary data (^1H and ^{13}C NMR data of all new compounds, and HPLC traces of amidines **1a–c**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.12.034.

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