Supporting Information for:

Orthoester Functionalized N-Guanidino Derivatives of 1,5dideoxy-1,5-imino-D-xylitol as pH-Responsive Inhibitors of β -Glucocerebrosidase

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General Procedures

Reagents, solvents and solutions. Unless stated otherwise, chemicals were obtained from commercial sources and used without further purification. All solvents were purchased from Biosolve (Valkenswaard, The Netherlands) and were stored on molecular sieves (4 Å). 2,3,4-Tri-O-benzyl-5-des(hydroxymethyl)-1-deoxynojirimycin¹ (12), Cbz-NCS² and compounds 18^3 and 19^3 were prepared as previously described.

Purification Techniques. All reactions and fractions from column chromatography were monitored by thin layer chromatography (TLC) using plates with a UV fluorescent indicator (normal SiO₂, Merck 60 F254). One or more of the following methods were used for visualization: 10% H₂SO₄ in MeOH, molybdenum blue, KMnO₄ or ninhydrine followed by warming until spots could be visible detected under UV light. Flash chromatography was performed using Merck type 60, 230–400 mesh silica gel. Removal of solvent was performed under reduced pressure using a rotary evaporator.

Instrumentation for Compound Characterization. For LC-MS analysis, an HPLC system (detection simultaneously at *l* 214, 254 nm and evaporative light detection) equipped with an analytical C_{18} column (100 Å pore size, 4.6 mm (\emptyset) x 250 mm (1), 10 µm particle size) or C₈ column (100 Å pore size, 4.6 mm (\emptyset) x 250 mm (1), 10 µm particle size) in combination with buffers A: H₂O, B: MeCN, A and B with 1.0% aqueous trifluoroacetic acid (TFA), in some cases - coupled with an electrospray interface (ESI) was used. Formic acid (FA; LC-MS grade), acetic acid, sodium acetate and ammonium acetate were acquired from Sigma-Aldrich (St. Louis, MA, USA). Ultra-pure water was obtained from a Synergy UV water delivery system from Millipore (Billerica, MA, USA). For RP-HPLC purifications (detection simultaneously at l 213, 254 nm), an automated HPLC system equipped with a preparative C_{18} column (20 mm (\emptyset) x 250 mm (1), 5 µm particle size) or C_8 column $(20 \text{ mm} (\emptyset) \times 250 \text{ mm} (1), 5 \text{ µm} \text{ particle size})$ in combination with buffers A: H₂O, B: MeCN, A and B with 1.0% aqueous trifluoroacetic acid (TFA). High-resolution mass spectra were recorded by direct injection (2 µL of a 2 µM solution in H₂O/MeCN 50:50 v/v and 0.1% formic acid) on a mass spectrometer. ¹H and ¹³C NMR spectra were recorded on 500–125 MHz or 400–100 MHz spectrometers. Chemical shifts (d) are given in ppm relative to tetramethylsilane (TMS) as internal standard. All ¹³C NMR spectra are proton decoupled. ¹H NMR data are reported in the following order: number of protons, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet and m, multiplet) and coupling constant (J) in Hertz (Hz). When appropriate, the multiplicity is preceded by br, indicating that the signal was broad. ¹¹³C NMR spectra were recorded at 75.5 MHz with chemical shifts reported relative to CDCl₃ d 77.0. ¹³C NMR spectra were recorded using the attached proton test (APT) sequence. All literature compounds had ¹H NMR and mass spectra consistent with the assigned structures.

Experimental Details

Overview synthetic route towards acetylated-DIX 16.

Benzyl protected 1,5-dideoxy-1,5-imino-D-xylitol **12** was synthesized according to the literature procedure¹ and used as a starting material for the preparation of acetylated 1,5-dideoxy-1,5-imino-D-xylitol **16** used in this work. This was done to avoid acidic environment needed to deprotect the benzyl protection groups, which would simultaneously affect the orthoester precursor, resulting in a prematurely hydrolysed species (Scheme S1). Acetylated species, on the other hand, needs basic conditions in the deprotection step and would not promote the hydrolysis.



Scheme S1. Synthetic route towards acetylated iminosugar DIX 16.

2,3,4-Tri-O-benzyl-5-des(hydroxymethyl)-1-deoxynojirimycin (12).¹



Benzyl protected 1,5-dideoxy-1,5-imino-D-xylitol **12** was synthesized according to the literature procedure.¹ Characterization data for compound **12** is in accordance to

previously published data. R_f (EtOAc + 2% NH₄OH) = 0.72. ¹H NMR (400 MHz, CD₃OD) δ 1.51 (s, 1H), 2.48 (dd, J = 9.8, 12.3 Hz, 2H), 3.20 (dd, J = 4.8, 12.3 Hz, 2H), 3.35 – 3.58 (m, 3H), 4.60 – 4.76 (m, 4H), 4.88 (s, 2H), 7.16 – 7.44 (m, 15H). ¹³C NMR (100 MHz, CD₃OD) δ 139.0, 138.6, 128.4, 128.3, 128.0, 127.7, 127.6, 127.5, 85.8, 79.8, 77.3, 77.0, 76.7, 75.5, 72.9, 49.2. HRMS (ESI, [M+H]⁺), calculated for C₂₆H₂₉NO₃, 404.2226; found 404.2226.

Tert-butyl 3,4,5-tris(benzyloxy)piperidine-1-carboxylate (13).



To a previously synthesised benzyl protected DIX **12** (2.00 g, 8.57 mmol) in DCM (50 mL), the mixture was cooled to 0 $^{\circ}$ C under Ar atmosphere. Next, Et₃N (1.25 mL, 9.00 mmol) and di-

tert-butoxycarbonyl dicarbonate (Boc₂O, 2.42 g, 11.06 mmol) were added. The mixture was stirred until it reached the room temperature and additionally for 15 h under Ar atmosphere. The next day, the solvent was evaporated and the resulting residue washed with 2M HCl (2 x 30 mL) and saturated aqueous solution of NaHCO₃ (2 x 40 mL). The organic fractions were dried with Na₂SO₄ and the solvent was concentrated to give the residue, which was coevaporated several times with chloroform and purified by column chromatography. Yield: 4.13 g (96%). ¹H NMR (400 MHz, CDCl₃) δ 1.41 (s, 9H), 2.51 – 2.71 (m, 2H), 3.33 – 3.60 (m, 2H), 3.91 – 4.44 (m, 3H), 4.69 (s, 4H), 4.88 (s, 2H). ¹³C NMR (101 MHz, CDCl₃) δ 154.4, 146.7, 138.2, 128.4, 128.3, 127.9, 127.7, 127.5, 85.7, 85.1, 80.2, 75.5, 72.8, 28.3, 27.4. HRMS (ESI, [M+Na]⁺), calculated for C₃₁H₃₇NO₅, 526.2569; found 526.2564.

Tert-butyl 3,4,5-trihydroxypiperidine-1-carboxylate (14).



The benzylated boc protected iminosugar **13** was dissolved in a mixture of glacial AcOH in MeOH (1/1, v/v) and transferred to a Parr high-pressure hydrogenation flask. A catalytic amount of 10%

palladium on carbon was added to the flask (ca. 10 mg catalyst/mg of benzylated starting material). The flask was put under reduced pressure and ventilated with hydrogen gas. This procedure was repeated twice after which the pressure was adjusted to 4.5-5.0 bar hydrogen pressure. The reaction was allowed to proceed for 12 h while mechanically shaken and the pressure maintained at the value initially set. The mixture was filtered over Celite on a glass microfiber filter, followed by rinsing the filter with MeOH. The mixture was concentrated under reduced pressure. The crude product thus obtained was immediately used in the next reaction. Yield: 1.91 g (quant.). ¹H NMR (400 MHz, DMSO) δ 1.35 (s, 9H), 2.36 – 2.45 (m, 2H), 2.96 (t, *J* = 8.4 Hz, 1H), 3.02 – 3.13 (m, 2H), 3.71 – 3.88 (m, 2H), 4.94 (s, 1H), 5.00 (s, 2H). ¹³C NMR (101 MHz, DMSO) δ 154.3, 105.0, 79.3, 78.8, 69.9, 48.9, 28.4. HRMS (ESI, [M+Na]⁺), calculated for C₁₀H₁₉NO₅, 256.1161; found 256.1162.

Tert-butoxycarbonyl-piperidine-3,4,5-triyl triacetate (15).



To a solution of **14** (1.91 g, 8.20 mmol), a mixture of Ac_2O and pyridine (1:1, 40 mL) was added and the resulting mixture was stirred at room temperature for 24 h. The reaction mixture was

quenched with H₂O (30 mL), diluted with CH₂Cl₂ (100 mL) and washed with 2M HCl (30 mL) and saturated aqueous solution of NaHCO₃ (40 mL). The organic fractions were dried with Na₂SO₄ and the solvent was concentrated to give the residue, which was coevaporated several times with toluene and purified by flash column chromatography (EtOAc:PE = 1:2). Yield: 2.95 g (quant.). ¹H NMR (400 MHz, CDCl₃) δ 1.47 (s, 9H), 2.05 (2x s, 9H), 3.06 (dd, *J* = 8.8, 13.6 Hz, 2H), 4.06 (dd, *J* = 8.8, 13.6 Hz, 2H), 4.82 (br s, 2H), 5.08 (t, *J* = 8.0 Hz, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 169.8, 154.2, 80.8, 72.4, 28.2, 20.74, 20.70. HRMS (ESI, [M+Na]⁺), calculated for C₁₆H₂₅NO₈, 382.1478; found 382.1476.

3,4,5-Tri-O-acetyl piperidin trifluoroacetate (16).



Compound **15** (2.95 g, 8.20 mmol) was treated with TFA-CH₂Cl₂ (9:1, 50 mL) at 0 °C for 30 min. After, the mixture was concentrated and coevaporated several times with

CH₂Cl₂. The resulting residue was purified by column chromatography (CH₂Cl₂:MeOH 2:1). Yield: 3.01 g (quant.). ¹H NMR (400 MHz, CDCl₃) δ 2.13 (s, 6H), 2.15 (s, 3H), 3.39 – 3.54 (m, 4H), 5.05 (td, *J* = 3.6 Hz, 2H), 5.17 (t, *J* = 4.0 Hz, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 169.3, 168.4, 64.6, 64.5, 43.3, 20.5, 20.3. HRMS (ESI, [M+H-TFA]⁺), calculated for C₁₁H₁₇NO₆, 260.1134; found 260.1133.

Overview Synthetic Route Towards Orthoester Armed Guanidines 10 and 11.

A series of orthoester amines **18** and **19**, comprised of simple alkyl chains with four and eight carbon atoms in length, were synthesized according to a literature procedure step (Scheme S2).³ Treatment of the corresponding amines **18** and **19** with CbzNCS generated appropriate thioureas **20** and **21**, which served as an integral part of our approach to incorporate orthoester moiety through subsequent guanidine formation step (Scheme S2). Activation of the Cbz-protected thioureas **20** and **21** with EDCI followed by addition of previously prepared acetyl protected DIX **16**, led to clean formation of protected guanidines **22** and **23**. Interestingly, we were able to simultaneously remove Cbz and acetyl groups *via* hydrogenation under basic conditions to yield fully deprotected orthoester-armed guanidine products **10** and **11** in high yields. It should be taken into account that the Cbz-deprotection step occurred in the presence of TEA and aprotic THF solvent that was not dried prior to use, which might be a plausible explanation for immediate deprotection of the acetyls.



Scheme S2. Synthetic route towards orthoester rich DIX-guanidines 10 and 11.

2,2,2-Trifluoro-N-(2,3-dihydroxy-propyl)-acetamide (24).³



To a stirred solution of 3-amino-1,2-propanediol (17, 8.4 g, 0.090 mol) in THF (70 mL), ethyl trifluoroacetate (13 mL, 0.110 mol) was added dropwise at 0 $^{\circ}$ C. After 2 h, the mixture was evaporated and the residue dissolved in ethyl

acetate (70 mL). It was washed with aqueous potassium hydrogen sulfate (0.5 M, 2 x 10 mL) and brine (30 mL), then dried over Na₂SO₄ and concentrated to obtain a colourless viscous liquid. Characterization data for compound **24** is in accordance to previously published data. Yield: 16 g (95%). ¹H NMR (CDCl₃, 300 MHz): δ 2.12 (t, J = 5.5 Hz, 1H), 2.61 (d, J = 5.0 Hz, 1H), 3.25–3.85 (m, 4H,), 3.92 (m, 1H), 6.88 (m, 1H). HRMS (ESI, [M+H]⁺), calculated for C₅H₈F₃NO₃, 188.0535; found 188.0537.

2,2,2-Trifluoro-N-(2-methoxy-[1,3]dioxolan-4-ylmethyl)-acetamide (25).³



The diol **24** (14.5 g, 0.08 mol) was dissolved in dichloromethane (40 ml) and trimethyl orthoformate (38 ml, 0.70 mol); p-toluene sulfonic acid (PTSA; 0.3 g, 0.0017 mol) was then added. The solution was stirred at room temperature for 2 h. The solution was then diluted with

dichloromethane (150 ml), washed successively with saturated aqueous sodium hydrogen carbonate (3 x 100 ml) and brine (100 ml). The organic phase was then dried over Na₂SO₄, filtered and concentrated to yield colourless oil. Characterization data for compound **25** is in accordance to previously published data. Yield: 15 g (86%). ¹H NMR (CDCl₃, 300 MHz): mixture of two diastereomers 50/50: δ 3.33 and 3.37 (s, 3H), 3.35–3.80 (m, 3H), 4.10–4.25 (m, 1H), 4.50 (m, 1H), 5.73 and 5.78 (s, 1H), 6.66 and 7.55 (s, 1H). HRMS (ESI, [M+H]⁺), calculated for C₇H₁₀F₃NO₄, 230.0640; found 230.0642.

N-(2'-Butyloxy-[1',3']dioxolan-4'-ylmethyl)trifluoroacetamide (26).³



To a solution of **25** (2.5 g, 10.9 mmol) in toluene (20 mL), 1-butanol (0.81 g, 10.9 mmol) and pyridinium p-toluenesulfonate (PPTS, 28 mg, 0.11 mmol) were added. The resulting solution was stirred under reflux for 2 h. After addition of cyclohexane (100 mL), the organic layer was washed with saturated aqueous

NaHCO₃ (3 x 30 mL) and brine (30 mL), dried (Na₂SO₄), filtered and the solvent was eliminated. The residue was purified by column chromatography (EtOAc:PE = 1:6). Yield: (2.46 g, 83%). Characterization data for compound **26** is in accordance to previously published data. $R_f(EtOAc:PE = 1:4) = 0.40$. ¹H NMR (400 MHz, CDCl₃) δ 0.89 (t, *J* = 7.4 Hz, 3 H), 1.39 (m, 2 H), 1.55 (m, 2 H), 3.37-3.73 (m, 4 H), 3.71-3.82 (m, 1 H), 4.03-4.16 (m, 1 H), 4.47 (m, 1 H), 5.79 and 5.83 (s, 1 H), 6.67 and 7.47 (br s, 1 H). ¹³C NMR (100.6 MHz, CDCl₃) δ 14.0, 19.2, 19.4, 31.4, 31.5, 41.1, 42.2, 64.6, 65.8, 65.1, 65.9, 73.4, 73.9, 115.6, 115.71, 115.73, 157.8, 158.2. HRMS (ESI, [M+H]⁺), calculated for C₁₀H₁₆F₃NO₄, 272.1110; found 272.1113.

2,2,2-Trifluoro-N-(2-octyloxy-[1,3]dioxolan-4-ylmethyl)-acetamide (27).³



To a solution of **25** (2.5 g, 10.9 mmol) in toluene (17 mL), 1-octanol (1.42 g, 10.9 mmol) and pyridinium p-toluenesulfonate (PPTS, 28 mg, 0.11 mmol) were added. The resulting solution was stirred under reflux for 2 h. After addition of cyclohexane (100 mL), the organic layer was washed with saturated

aqueous NaHCO₃ (3 x 30 mL) and brine (30 mL), dried (Na₂SO₄), filtered and the solvent was eliminated. The residue was purified by column chromatography (EtOAc:PE = 1:3). Yield: 2.63 g (74%). Characterization data for compound **27** is in accordance to previously published data. R_f (EtOAc:PE = 1:3) =0.56. ¹H NMR (500 MHz, CDCl₃) δ 0.89 (t, *J* = 6.9 Hz, 3 H), 1.27 (m, 10 H), 1.59 (m, 2 H), 3.37-3.70 (m, 4 H), 3.73-3.80 (m, 1 H), 4.03-4.21 (m, 1 H), 4.48 (m, 1 H), 5.79 and 5.84 (s, 1 H), 6.68 and 7.47 (br s, 1 H). ¹³C NMR (125.7 MHz, CDCl₃) δ 13.9, 22.6, 25.8, 25.9, 26.1, 26.3, 29.2, 29.4, 29.6, 31.6, 32.6, 41.0, 42.1, 64.5, 65.7, 65.1, 65.9, 73.4, 74.1,

115.6, 115.72, 115.73, 157.9, 158.1. HRMS (ESI, $[M+H]^+$), calculated for $C_{14}H_{24}F_3NO_4$, 328.1736; found 328.1741.

(2-Butyloxy-[1,3]dioxolan-4-yl)methylamine (18).³



To a solution of **26** (1.9 g, 7.0 mmol) in THF (15 mL), a solution of 4% aqueous NaOH (16 mL) was added at 0 °C and the mixture reaction was stirred for 4 h at rt. The aqueous layer was extracted with CH_2Cl_2 (3 x 70 mL) and the combined organic layers were washed with brine (50

mL), dried (Na₂SO₄), then filtered and evaporated the solvent. The residue was purified by column chromatography (CH₂Cl₂:MeOH = 9:1). Characterization data for compound **18** is in accordance to previously published data. Yield: 0.99 g (81%). R_f (CH₂Cl₂:MeOH = 9:1) =0.54. ¹H NMR (500 MHz, CDCl₃) δ 0.89 (m, 3 H), 1.31 (m, 2 H), 1.59 (m, 2 H), 2.73-2.95 (m, 2 H), 3.54 (m, 2 H), 3.71 and 3.79 (m, 1 H), 4.06 and 4.14 (m, 1 H), 4.19 and 4.35, (m, 1 H), 5.78 and 5.81 (s, 1 H). ¹³C NMR (125.7 MHz, CDCl₃) δ 13.8, 19.3, 19.6, 31.1, 31.6, 44.5, 44.9, 64.82, 64.9, 66.1, 66.2, 78.3, 115.6, 115.9. HRMS (ESI, [M+H]⁺), calculated for C₈H₁₇NO₃, 176.1287; found 176.1282.

(2-Octyloxy-[1,3]dioxolan-4-yl)methylamine (19).³



To a solution of **27** (2.4 g, 7.0 mmol) in THF (15 mL), a solution of 4% aqueous NaOH (16 mL) was added at 0 °C and the mixture reaction was stirred for 4 h at rt. The aqueous layer was extracted with CH_2Cl_2 (5 x 100 mL) and the combined organic layers were washed with brine

(50 mL), dried (Na₂SO₄), then filtered and evaporated the solvent. The residue was purified by column chromatography (CH₂Cl₂:MeOH = 9:1). Characterization data for compound **19** is in accordance to previously published data. Yield: 1.28 g (79%). R_f (CH₂Cl₂:MeOH = 9:1) =0.28. ¹H NMR (500 MHz, CDCl₃) δ 0.89 (m, 3 H), 1.31 (m, 10 H), 1.63 (m, 2 H), 2.72-2.96 (m, 2 H), 3.53 (m, 2 H), 3.70 and 3.79 (m, 1 H), 4.04 and 4.14 (m, 1 H), 4.26 and 4.34, (m, 1 H), 5.78 and 5.81 (s, 1 H). ¹³C NMR (125.7 MHz, CDCl₃) δ 13.9, 22.6, 26.4, 29.4, 29.5, 31.1, 31.6, 44.5, 44.9, 64.82, 64.9, 66.1,

66.2, 78.3, 115.6, 115.9. HRMS (ESI, $[M+H]^+$), calculated for $C_{12}H_{25}NO_3$, 232.1913; found 232.1911.

N-(Benzyloxycarbonyl)-N'-(2'-Butyloxy-[1',3']dioxolan-4'-ylmethyl) thiourea (20).



The amine **18** (250 mg, 1.4 mmol, 1eq) was dissolved in CH_2Cl_2 (100 mL) and treated with a 0.5 M solution of CbzNCS in CH_2Cl_2 (2.85 mL, 1 eq) and NEt₃ (10 eq). After stirring for 12

h at room temperature, TLC analysis indicated complete conversion to thiourea species. The CH₂Cl₂ was then removed under reduced pressure, and the residue dissolved in chloroform was applied directly to a silica column, eluting with EtOAc/PE mixture. Yield: 423 mg, 80%. R_f(EtOAc:PE = 1:4) = 0.32. ¹H NMR (400 MHz, CDCl₃) δ 0.90 (t, *J* = 7.4 Hz, 3H), 1.27 – 1.45 (m, 2H), 1.47 – 1.61 (m, 2H), 3.43 – 3.59 (m, 2H), 3.72 – 3.95 (m, 2H), 4.04 – 4.20 (m, 2H), 4.39 – 4.65 (m, 1H), 5.17 (s, 2H), 5.83 (2 x s, 1H), 7.28 – 7.45 (m, 2H), 8.02 (s, 1H), 9.90 (2 x s, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 179.7, 152.1, 134.4, 128.9, 128.9, 128.73, 128.71, 128.4, 115.9, 74.1, 73.1, 68.3, 68.2, 65.8, 65.7, 65.0, 64.8, 48.0, 47.5, 31.5, 31.4, 19.2, 19.2, 13.8, 13.7. HRMS (ESI, [M+Na]⁺), calculated for C₁₇H₂₄N₂O₅S, 391.1304; found, 391.1287.

N-(Benzyloxycarbonyl)-N'-(2'-Octyloxy-[1',3']dioxolan-4'-ylmethyl) thiourea (21).



The amine **19** (300 mg, 1.3 mmol, 1eq) was dissolved in CH_2Cl_2 (100 mL) and treated with a 0.5 M solution of CbzNCS in CH_2Cl_2 (2.59 mL, 1 eq)

and NEt₃ (10 eq). After stirring for 12 h at room temperature, TLC analysis indicated complete conversion to thiourea species. The CH_2Cl_2 was then removed under reduced pressure, and the residue dissolved in chloroform was applied directly to a silica column, eluting with EtOAc/PE mixture. Yield: 452 mg, 82%. R_f (EtOAc:PE = 1:4) = 0.28. ¹H NMR (400 MHz, CDCl₃) δ 0.78 – 0.96 (m, 3H), 1.17 – 1.42 (m, 10H), 1.49 – 1.69 (m, 2H), 3.48 – 3.69 (m, 2H), 3.74 – 3.98 (m, 3H), 4.07 – 4.21 (m, 2H),

4.39 – 4.70 (m, 1H), 5.19 (s, 2H), 5.85 (2 x s, 1H), 7.31 – 7.49 (m, 5H), 8.04 (s, 1H), 9.92 (2 x s, 1H). ¹³C NMR (101 MHz, CDCl3) δ 179.9, 179.7, 152.1, 134.4, 128.94, 128.93, 128.73, 128.70, 128.4, 115.9, 77.3, 77.0, 76.7, 74.1, 73.1, 68.3, 68.2, 65.8, 65.7, 65.3, 65.1, 48.1, 47.5, 31.82, 31.80, 29.41, 29.40, 29.33, 29.31, 29.22, 29.21, 26.1, 26.0, 22.6, 14.1. HRMS (ESI, [M+Na]⁺), calculated for C₂₁H₃₂N₂O₅S, 447.1930; found, 447.1921.

Synthesis of Acetyl protected N-Substituted Guanidine Compounds 22 and 23.

N'-((benzyloxy)carbonyl)-N-((2-(butoxy)-1,3-dioxolan-4-yl)methyl)carbamimidoyl)piperidine-3,4,5-triyl triacetate (22).



The thiourea **20** (150 mg, 0.41 mmol, 1 eq), OAc-DIX (**16**, 110 mg, 0.43 mmol, 1.05 eq) and EDCI (95 mg, 0.61 mmol, 1.5 eq) were dissolved in CH₂Cl₂ (40 mL),

followed by addition of NEt₃ (0.17 mL, 1.2 mmol, 3 eq). Reaction mixture was stirred for 18h at room temperature, after which TLC analysis confirmed total consumption of starting material. The solvent was then removed under reduced pressure, and the residue, dissolved in CHCl₃, was applied directly to a silica column, eluting with hexanes chaser and subsequently with EtOAc/PE. Yield: 220 mg, 91%. R_r (EtOAc:PE = 1:1) = 0.31. ¹H NMR (400 MHz, CDCl₃) δ 0.88 – 0.96 (m, 3H), 1.31 – 1.44 (m, 2H), 1.50 – 1.62 (m, 2H), 2.04 (s, 9H), 2.82 – 3.02 (m, 2H), 3.26 – 3.49 (2 x m, 2H), 3.49 – 3.59 (m, 2H), 3.74 (2 x dd, *J* = 6.0, 8.2 Hz, 1H), 3.92 – 4.03 (m, 2H), 4.13 (2 x dd, *J* = 6.9, 8.2 Hz, 1H), 4.25 – 4.51 (2 x m, 1H), 4.81 – 4.94 (m, 2H), 5.12 (s, 2H), 5.14 – 5.22 (m, 1H), 5.83 (2 x s, 1H), 7.19 – 7.48 (m, 5H), 8.28 (2 x s, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 169.93, 169.91, 169.83, 169.82, 169.81, 169.7, 128.32, 128.31, 128.24, 128.22, 127.8, 127.7, 116.0, 115.5, 75.0, 73.8, 72.6, 72.5, 69.02, 69.01, 68.9, 68.8, 67.1, 67.0, 65.82, 65.81, 65.3, 64.8, 48.6, 48.4, 48.3, 48.2, 47.4, 46.9, 31.42, 31.40, 20.7, 19.23, 19.21, 13.8. HRMS (ESI, [M+H]⁺), calculated for C₂₈H₃₉N₃O₁₁, 594.2663; found, 594.2657.

N'-((benzyloxy)carbonyl)-N-((2-(octyloxy)-1,3-dioxolan-4-yl)methyl)carbamimidoyl)piperidine-3,4,5-triyl triacetate (23).



The thiourea **21** (240 mg, 0.57 mmol, 1 eq), OAc-DIX (**16**, 154 mg, 0.59 mmol, 1.05 eq) and EDCI (132 mg, 0.85 mmol, 1.5

eq) were dissolved in CH₂Cl₂ (40 mL), followed by addition of NEt₃ (0.24 mL, 1.7 mmol, 3 eq). Reaction mixture was stirred for 18h at room temperature, after which TLC analysis confirmed total consumption of starting material. The solvent was then removed under reduced pressure, and the residue, dissolved in CHCl₃, was applied directly to a silica column, eluting with hexanes chaser and subsequently with EtOAc/PE. Yield: 326 mg, 89%. R_f (EtOAc/PE = 1:1) = 0.27. ¹H NMR (400 MHz, CDCl₃) δ 0.79 – 0.99 (m, 3H), 1.18 – 1.42 (m, 10H), 1.49 – 1.68 (m, 2H), 2.04 (3 x s, 9H), 2.81 – 3.06 (m, 2H), 3.28 – 3.60 (2 x m, 4H), 3.74 (2 x dd, *J* = 6.1, 8.2 Hz, 1H), 3.90 – 4.03 (m, 2H), 4.13 (2 x dd, *J* = 6.1, 8.2 Hz, 1H), 4.24 – 4.52 (m, 1H), 4.82 – 4.93 (m, 2H), 5.12 (s, 2H), 5.13 – 5.23 (m, 1H), 5.83 (2 x s, 1H), 7.27 – 7.44 (m, 5H), 8.28 (2 x s, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 169.93, 169.91, 169.84, 169.83, 169.81, 169.7, 137.0, 128.32, 128.31, 128.2, 127.8, 127.7, 116.0, 75.0, 72.6, 72.5, 69.0, 68.83, 68.80, 67.0, 65.83, 65.82, 65.6, 65.2, 48.6, 48.3, 48.2, 47.4, 31.8, 29.43, 29.42, 29.3, 29.24, 29.21, 26.03, 26.01, 22.6, 20.7, 14.1, 14.0. HRMS (ESI, [M+H]⁺), calculated for C₃₂H₄₇N₃O₁₁, 650.3289; found, 650.3283.

General procedure for one-pot Pd/C catalyzed hydrogenolysis and deacetylation of 22 and 23 for the synthesis of N-Substituted Guanidine Compounds 10 and 11.

The triacetylated iminosugars **22** and **23** were dissolved in a mixture of Et₃N in THF (1/5, v/v), and transferred to a Parr high-pressure hydrogenation flask. A catalytic amount of 10% palladium on carbon was added to the flask (ca. 10 mg catalyst per 100 mg of starting material). The flask was put under reduced pressure and ventilated with hydrogen gas. This procedure was repeated twice after which the pressure was adjusted to 1.5-2.0 bar hydrogen pressure. The reaction was allowed to proceed for 6-12 h while mechanically shaken and the pressure maintained at the value initially set. The mixture was filtered over Celite on a glass microfiber filter, followed by rinsing the filter with THF. The mixture was concentrated under reduced pressure. The crude product thus obtained was purified using RP-HPLC employing a preparative C8 column and a gradient moving from 95% to 5% eluent A (50 mM NH₄HCO₃:5 mM Et₃N, pH = 8.3) over 60 min (flow rate, 18.0 mL/min, eluent B: MeOH). Fractions containing the desired product were combined and lyophilized to yield the pure compounds as amorphous white powders. Analytical data for compounds **10** and **11** and in-depth characterization data for both final compounds are given below.

N-((2-butoxy-1,3-dioxolan-4-yl)methyl)-3,4,5-trihydroxypiperidine-1carboximidamide (10).



Yield: 32 mg, 92%. ¹H NMR (400 MHz, D_2O) δ 0.93 (br t, 3H), 1.29 – 1.48 (m, 2H), 1.52 – 1.67 (m, 2H), 3.09 (dd, J = 10.3, 13.4 Hz, 2H), 3.41 – 3.73 (m, 7H), 3.83 –

3.99 (m, 3H), 4.18 - 4.31 (m, 1H), 4.43 - 4.67 (2 x m, 1H), 6.00 (2 x s, 1H). ¹³C NMR (101 MHz, D₂O) δ 157.13, 157.11, 115.6, 114.8, 76.9, 76.8, 75.1, 74.3, 68.54, 68.51, 68.50, 68.4, 65.9, 65.4, 65.3, 49.63, 49.61, 49.60, 44.5, 44.2, 30.7, 30.6, 18.6, 18.5, 12.93, 12.91. HRMS (ESI, [M+H]⁺), calculated for C₁₄H₂₇N₃O₆, 334.1978; found, 334.1977.

N-((2-octyloxy-1,3-dioxolan-4-yl)methyl)-3,4,5-trihydroxypiperidine-1carboximidamide (11).



Yield: 26 mg, 89%. ¹H NMR (400 MHz, D₂O) δ 0.90 (br t, 3H), 1.22 - 1.47 (m, 10H), 1.52 -1.72 (m, 2H), 3.10 (dd, J = 10.2,

13.4 Hz, 2H), 3.42 - 3.72 (m, 7H), 3.84 - 3.99 (m, 3H), 4.18 - 4.32 (m, 1H), 4.44 - 4.66 (2 x m, 1H), 6.01 (2 x s, 1H). ¹³C NMR (101 MHz, D₂O) δ 157.1, 115.6, 76.9, 76.8, 75.1, 74.4, 68.55, 68.53, 68.52, 68.50, 66.2, 65.7, 65.33, 65.31, 49.62, 49.60, 44.6, 44.3, 31.02, 31.01, 28.5, 28.4, 28.33, 28.31, 28.22, 28.21, 25.2, 25.1, 21.92, 21.91, 13.33, 13.30. HRMS (ESI, [M+H]⁺), calculated for C₁₈H₃₅N₃O₆, 390.2604; found, 390.2607.

Hydrolysis of compounds 10 and 11.

Next, we studied the hydrolysis process of compounds **10** and **11** to their corresponding species **A** and **B** (Scheme S3).



Scheme S3. Hydrolysis of 10 and 11 towards compounds A and B.

Mechanism for the hydrolysis process is presented in Scheme S4 and Scheme S5 to rational the formation of the product with molecular weight 278 and 250. In the case of hydrolysis to compound \mathbf{A} , the oxygen is protonated, followed by immediate loss of the corresponding alcohol with subsequent addition of the water molecule and final deprotonation, which results in compound \mathbf{A} .



Scheme S4. Mechanism for the hydrolysis of 10 and 11 towards compound A.

In Scheme S5 is the proposed mechanism of the occurring hydrolysis, resulting in a fully hydrolysed compound **B**. In this case, one of the oxygens in the ring is protonated and water acts as a nucleophile on the newly formed electrophilic carbon, eliminating the hemiacetal, which subsequently results in the corresponding alcohol **B** and CO_2 .



Scheme S5. Mechanism for the hydrolysis of 10 and 11 towards compound B.

Collision-induced dissociation mass spectrometry (CID MS-MS) studies.

Accurate mass measurements of the degradation compounds showed a mass of m/z 278.1347 ($[M+H]^+$; compound **A**) and m/z of 250.1395 ($[M+H]^+$; compound **B**), corresponding to compounds with monoisotopic mass 277.1266 and 249.1325 and a molecular formula $C_{10}H_{19}N_3O_6$ and $C_9H_{19}N_3O_5$, respectively.

With this molecular formulas the following structures were predicted and confirmed:



To fully confirm structures **A** and **B**, precursor ions corresponding to the compounds with m/z 278 and 250 were subjected to collision induced dissociation fragmentation to obtain fragments that could approve the structures. Purified samples **10** and **11** were dissolved in water:acetonitrile 1:1 (% v/v) with 0.1% formic acid and infused on an Agilent 6560 ion mobility-quadrupole time-off-flight mass spectrometer (Agilent technologies, Waldbronn, Germany). Spectra were acquired in positive ion mode with a capillary voltage of 3.5 kV, nebulizer pressure of 0.8 bar, drying gas flow of 5 L/min, temperature of 320 °C, and CID energies of 20 and 30 V for compounds **A** and **B**, respectively. Several fragments belonging to the guanidine iminosugar moiety were observed in both spectra (Figure S1, Figure S2 and Table S1), confirming the presence of the same moiety in both compounds. The unique fragment ions m/z 120 and 102 (loss of water from m/z 120) for compound **A** and m/z 92 for compound **B**

also indicated a similar fragmentation pathway for both compounds and confirmed the different branches attached to the guanidine iminosugar moiety.



Figure S1. CID MS-MS spectrum of compound A with precursor ion m/z 278.



Figure S2. CID MS-MS spectrum of compound B with precursor ion m/z 250.

In this respect, the collected data are consistent with the proposed orthoester hydrolysis mechanism of compounds **10** and **11** to their corresponding hydrolysed products **A** and **B**.

Measured <i>m/z</i> of compound A (278) fragment, proposed fragment structure and mass accurracy	Measured <i>m/z</i> of compound B (250) fragment, proposed fragment structure and mass accuracy	Molecular formula	Exact mass (Da)	
		$C_{10}H_{20}N_3O_6$ +H	278.1347	
[M+H] ⁺ =278.1347 (0 ppm)	NH			
		$C_9H_{19}N_3O_5$ +H	250.1397	
NH	[MI+H] =250.1395 (-0.8 ppin)			
HO N HO OH		$C_{10}H_{16}N_2O_6$ +H	261.1081	
[M+H] ⁺ =261.1084 (1.1 ppm)	NILI			
	HO OH HO OH	C ₉ H ₁₆ N ₂ O ₅ +H	233.1132	
NH	[M+H] ⁺ =233.1132 (0 ppm)			
HO NH OH	HO OH OH	C ₅ H ₁₁ NO ₃ +H	134.0812	
[M+H] ⁺ =134.0812 (0 ppm)	[M+H] ⁺ =134.0810 (-1.5 ppm)			
		C ₄ H ₉ NO ₃ +H	120.0655	
[M+H]*=120.0657 (1.7 ppm)	NH			
HO CH HO CH	HO CH HO OH	C ₅ H ₉ NO ₂ +H	116.0706	
[M+H] ⁺ =116.0708 (1.7 ppm)	[M+H] ⁺ =116.0705 (-0.9 ppm)			
HO OH HO OH		C ₄ H ₇ NO ₂ +H	102.0550	
[M+H] [*] =102.0551 (1.0 ppm)	NH			
HO CH HO OH	HO CH NOH	C₅H ₇ NO+H	98.0600	
[M+H] ⁺ =98.0598 (-2.0 ppm)	[M+H] ⁺ =98.0598 (-2.0 ppm)			
		C ₃ H ₉ NO ₂ +H	92.0706	
	[M+H] ⁺ =92.0702 (-4.3 ppm)			

Table S1: Fragments and proposed structures as observed in the MS-MS spectra of compounds A and B.

UHPLC-MS method for kinetic hydrolysis experiments of orthoesters 10 and 11.

The kinetic evaluation of the in-house synthesized guanidine compounds **10** and **11** in aqueous solutions was studied by ultra-high performance liquid chromatography – mass spectrometry (UHPLC-MS). Buffer solutions of 200 mM ammonium acetate (pH 7.0) and 21 mM acetic acid with 85 mM sodium acetate (pH 5.2) were prepared in ultra-pure water and standard solutions of 1 mM guanidine compounds **10** and **11** were prepared in buffer solutions with pH 5.2 and pH 7.0.

Chromatography

Separation of guanidine compounds and their degradation products was achieved on a Zorbax Eclipse plus C₁₈ column (4.6 x 50 mm, 1.8 μ m particles). A 2- μ L injection volume was used of all samples. UHPLC was performed on a 1290 Infinity UHPLC system (Agilent Technologies, Wald-bronn, BW, Germany) consisting of a binary pump and an autosampler. The optimal separation was achieved with a binary gradient with 0.5% FA (% v/v) in water (eluent A) and ACN (eluent B) at a flow rate of 0.5 mL/min. Detection was performed on a quadrupole-time-off-flight mass spectrometer, equipped with an electrospray ionization source (Bruker Daltonics, Bremen, HB, Germany). Masses were acquired from *m*/*z* 50-700 at a spectra rate of 1.5 Hz, nebulizer pressure was 3.8 bar, gas flow was 9.4 L/min, gas temperature was 200 °C and capillary voltage was 4.5 kV. Guanidine compounds **10** and **11** and their degradation products **A** and **B** (Figure S3 and Figure S4), were detected as positive ions ([M+H]⁺).

Thus prepared samples degraded in buffer solution (pH 5.2) at 21 °C for 6 hours, were used to develop the stability indicating method. The separation of compounds **10** and **11** and the degradation products **A** and **B** was optimized by adjusting the amount and type of acidifier and organic modifier and the gradient time and slope. Good selectivity for all high and low polar compounds was achieved in one run with 0.5% FA/ACN gradients. The optimized gradient started with a composition of 97% eluent A and 3% eluent B (% v/v) for 3 min, increased linearly to 100% B in 3.5 min and remained at 100% B for 2 min. MS setting were optimized to obtain maximum detector response for all compounds.



Figure S3. Extracted ion chromatogram for compound 10 obtained with the optimized UHPLC-MS method of a 1mM solution (pH 5.2), degraded at 21 °C for 6 hours. The following compounds were identified by their m/z value: A) m/z 278; B) m/z 250 and 10) m/z 334.



Figure S4. Extracted ion chromatogram for compound 11 obtained with the optimized UHPLC-MS method of a 1mM solution (pH 5.2), degraded at 21 °C for 6 hours. The following compounds were identified by their m/z value: A) m/z 278; B) m/z 250 and 11) m/z 390.

The optimized method was used to study the time dependent degradation of the synthesized guanidine compounds. Samples were dissolved in buffer solutions with pH 5.2 or pH 7.0, kept at 21 °C and analysed at regular time intervals.

The compounds A and B were identified by their m/z value as the main hydrolysed products. The sum of peak areas in the chromatograms of the guanidines 10 and 11 and the hydrolysed compounds A and B remained constant in time in the samples

analysed at different time points, which indicates that their detector responses are similar. This allows for the application of relative responses for quantitative analysis of these compounds to determine the degradation process. Figures S5 and S6 show the first-order degradation data of **10** and **11** and the formation of **A** and **B** at pH 7.0, both expressed as % area. The half-life time, where the concentration of **10** and **11** decreased by 50%, was 157 min for compound **10** and 277 min for compound **11**, respectively. By using the logarithm (to base-e) of the concentration, a linear graph was obtained, confirming that the degradation of studied compounds was a first-order reaction with equation:

$$[10, 11(t)] = [10, 11]_0 e^{-k.t}$$

The slopes of the Ln-linearized curves of all orthoester guanidine compounds were used to calculate the degradation rate constants (k) and the half-life times $(t_{0.5}=(Ln2/k))$ for each compound.



Figure S5: First-order kinetics of 10 and formation of A and B in pH 5.2 buffer solution at 21 °C.



Figure S6: First-order kinetics of 11 and formation of A and B in pH 5.2 buffer solution at 21 $^{\circ}$ C.

The half-life time, where the concentration of **10** and **11** decreased by 50%, was 157 min for compound **10** and 277 min for compound **11**, respectively. Table S2 shows the $t_{0.5}$ values for compounds **10** and **11** for the degradation at pH 5.2 and pH 7.0. Degradation was fast at pH 5.2, compared to pH 7.0. Compounds **10** and **11** showed no degradation at pH 7.0 during at least 15h.

Table S2: t_{0.5} values for guanidine compounds 10 and 11 at pH 7.0 and 5.2.

	$t_{0.5}$ in buffer pH 7.0 (h)	$t_{0.5}$ in buffer pH 5.2 (h)
Compound		
10 11	>15 >15	157 277

Experimental details and analytical data for compounds 28-32 and compound B.

In order to confirm the structure of the orthoester adduct \mathbf{B} we report a synthesis of the compound \mathbf{B} in Scheme S6 and evaluate its structure by NMR and HRMS studies.



Scheme S6. Synthetic route towards compound B.

1-N-(tert-Butoxycarbonylamino)propane-2,3-diol (28).³



To a solution of commercial (±)-3-aminopropane-1,2-diol (**17**, 420 mg, 4.61 mmol) in DMF (5 mL) at 0 °C, Et₃N (704 μ L, 5.07 mmol) and di-*tert*-butoxycarbonyl dicarbonate (Boc₂O)

(1.21 g, 5.53 mmol) were added. The mixture was stirred at first at 0 °C and then at rt for 15 h, under Ar atmosphere. The solvent was evaporated and the resulting residue was purified by column chromatography (CH₂Cl₂:MeOH gradient 9:1 to 5:1). Characterization data for compound **28** is in accordance to previously published data. Yield: 835 mg (95%). R_f (CH₂Cl₂:MeOH = 5:1) = 0.63. ¹H NMR (300 MHz, CD₃OD) δ 1.44 (s, 9 H), 3.06 (m, 1 H), 3.21 (dd, 1 H), 3.49 (m, 2 H), 3.65 (m, 1 H). ¹³C NMR (75.5 MHz, CD₃OD) δ 158.8, 80.1, 72.3, 65.1, 44.0, 28.7. HRMS (ESI, [M+H]⁺), calculated for C₈H₁₇NO₄, 192.1236; found 192.1234.

2,3-Di-O-acetyl-1-N-((tert-butoxycarbonyl)amino)propane (29).³



To a solution of **28** (783 mg, 4.10 mmol), a mixture of Ac_2O and pyridine (1:1, 12 mL) was added and the resulting mixture was stirred at rt for 24 h. The reaction mixture was

quenched with H₂O (10 mL), diluted with CH₂Cl₂ (30 mL) and washed with 2M HCl (15 mL) and saturated aqueous solution of NaHCO₃ (20 mL). The organic fractions were dried with Na₂SO₄ and the solvent was concentrated to give the residue, which was coevaporated several times with toluene and purified by column chromatography (EtOAc:PE = 1:2). Characterization data for compound **29** is in accordance to previously published data. Yield: 1.15 g (quant.). R_f (EtOAc:PE = 1:2) = 0.48. ¹H NMR (300 MHz, CD₃OD) δ 1.43 (s, 9 H), 2.04 (2 x s, 6 H), 3.20 (dd, J_{gem} = 14.4 Hz, J = 6.7 Hz, 1 H), 3.36 (d, J = 4.9 Hz, 1 H), 4.06 (dd, J = 3.0 Hz, 1 H), 4.27 (dd, J = 12.0 Hz, J = 4.0 Hz, 1H), 5.06 (m, 1 H). ¹³C NMR (75.5 MHz, CD₃OD) δ 172.3, 172.2 (CO_{ester}), 158.7 (CO_{carbamate}), 80.4, 72.1, 64.1, 41.3, 28.9, 21.2, 20.5. HRMS (ESI, [M+H]⁺), calculated for C₁₂H₂₁NO₆, 276.1447; found 276.1445.

1,2-Di-O-acetyl-3-aminopropane trifluoroacetic acid salt (30).³

A solution of **29** (829 mg, 3.01 mmol) was treated with TFA-CH₂Cl₂ (9:1, 40 mL) at 0 °C for 20 min. After, the mixture was concentrated and coevaporated several times

with CH₂Cl₂. The resulting residue was purified by column chromatography (CH₂Cl₂:MeOH gradient 15:1 to 9:1). Characterization data for compound **30** is in accordance to previously published data. Yield: 527 mg (quant.). R_f (CH₂Cl₂:MeOH = 9:1) = 0.39. ¹H NMR (300 MHz, CD₃OD) δ 2.04-2.11 (2 x s, 6 H), 3.25 (m, 2 H), 4.11 (dd, *J* = 4.7 Hz, 1 H), 4.41 (dd, *J* = 12.3 Hz, J = 4.5 Hz, 1 H), 5.26 (m, 1 H). ¹³C NMR (75.5 MHz, CD₃OD) δ 172.2, 171.9, 69.7, 63.6, 41.1, 20.6, 20.4. HRMS (ESI, [M+H]⁺), calculated for C₇H₁₃NO₄, 176.0923; found 176.0922.

3-(3-((benzyloxy)carbonyl)thioureido)propane-1,2-diyl diacetate (31).



The amine salt **30** (440 mg, 1.52 mmol, 1eq) was dissolved in a mixture of NEt_3 (1.1 mL, 8 mmol) in CHCl₃ (100 mL) and treated with a 0.5 M solution of

CbzNCS in CH₂Cl₂ (3.04 mL, 1 eq). After stirring for 12 h at room temperature, TLC analysis indicated complete conversion to thiourea species. The solvent was then removed under reduced pressure, and the residue dissolved in chloroform was applied directly to a silica column, eluting with EtOAc/PE mixture. Yield: 526 mg, 94%. ¹H NMR (400 MHz, CDCl₃) δ 2.10 (2 x s, 6H), 3.85 (ddd, *J* = 7.1, 13.2 Hz, 1H), 4.05 – 4.13 (m, 1H), 4.16 (dd, *J* = 4.4, 12.1 Hz, 1H), 4.34 (dd, *J* = 4.4, 12.1 Hz, 1H), 5.19 (s, 2H), 5.26 – 5.34 (m, 1H), 7.30 – 7.44 (m, 5H), 8.14 (s, 1H), 9.87 (s, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 180.0, 170.5, 170.1, 152.3, 134.3, 128.9, 128.7, 128.3, 69.3, 68.3, 62.9, 45.8, 20.9, 20.7. HRMS (ESI, [M+Na]⁺), calculated for C₁₆H₂₀N₂O₆S, 391.094; found, 391.097.

N'-((benzyloxy)carbonyl)-N-(2,3-diacetoxypropyl)carbamimidoyl)piperidine-3,4,5-triyl triacetate (32).



The thiourea **31** (210 mg, 0.57 mmol, 1 eq), OAc-DIX (**16**, 162 mg, 0.62 mmol, 1.1 eq) and EDCI (133 mg, 0.86 mmol, 1.5 eq) were dissolved in CH_2Cl_2 (40 mL), followed by addition of NEt₃

(0.24 mL, 1.7 mmol, 3 eq). Reaction mixture was stirred for 18h at room temperature, after which TLC analysis confirmed total consumption of starting material. The solvent was then removed under reduced pressure, and the residue, dissolved in CHCl₃, was applied directly to a silica column, eluting with hexanes chaser and subsequently with CH₂Cl₂:MeOH mixture (CH₂Cl₂:MeOH gradient 2:1 to 4:1). Yield: 296 mg, 87%. R_f(CH₂Cl₂:MeOH = 4:1) = 0.34. ¹H NMR (400 MHz, CDCl₃) δ 2.05 (5 x s, 15H), 2.78 – 2.91 (m, 2H), 3.39 – 3.56 (m, 2H), 3.91 – 4.05 (m, 2H), 4.12 – 4.31 (m, 2H), 4.77 – 4.96 (m, 2H), 5.03 – 5.13 (m, 3H), 5.12 – 5.25 (m, 1H), 7.22 – 7.42 (m, 5H), 8.52 (s, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 170.5, 170.3, 169.93, 169.91, 169.90, 136.8, 128.3, 128.2, 127.8, 72.8, 69.9, 69.0, 68.9, 67.1, 62.7, 48.6, 48.5, 45.2, 20.72, 20.71, 20.6. HRMS (ESI, [M+H]⁺), calculated for C₂₇H₃₅N₃O₁₂, 594.2299; found, 594.2302.

Deprotection of compound 32 and experimental details for compound B.

The pentacetylated iminosugar 32 was dissolved in a mixture of Et_3N in THF (1/5, v/v), and transferred to a Parr high-pressure hydrogenation flask. A catalytic amount of 10% palladium on carbon was added to the flask (ca. 10 mg catalyst per 100 mg of starting material). The flask was put under reduced pressure and ventilated with hydrogen gas. This procedure was repeated twice after which the pressure was adjusted to 1.5-2.0 bar hydrogen pressure. It should be taken into account, that the Cbz-deprotection step occurred in the presence of aprotic THF solvent that was not dried prior to use, and might be a plausible explanation for simultaneous deprotection of the acetyls. The reaction was allowed to proceed for 12 h while mechanically shaken and the pressure maintained at the value initially set. The mixture was filtered over Celite on a glass microfiber filter, followed by rinsing the filter with THF. The mixture was concentrated under reduced pressure. The crude product thus obtained was first analysed using LCMS and resulted in a fully deprotected species \mathbf{B} , which were further purified using C₈ column (100 Å pore size, 4.6 mm (\emptyset) x 250 mm (l), 10 µm particle size) in combination with buffers A: H₂O, B: MeCN, A and B with 1.0% aqueous trifluoroacetic acid (TFA). Fractions containing the desired product **B** were combined and lyophilized to yield the pure compound as amorphous white powder. Analytical data and in-depth characterization data for final compound **B** are given below.

N-(2,3-dihydroxypropyl)-3,4,5-trihydroxypiperidine-1 carboximidamide (B).



Yield: 3 mg, 84%. ¹H NMR (400 MHz, D₂O) δ ¹H NMR (400 MHz, CDCl₃) δ 3.10 (dd, J = 10.1, 13.4 Hz, 2H), 3.36 – 3.44 (m, 1H), 3.45 – 3.54 (m, 2H), 3.59 – 3.72 (m, 4H), 3.88 – 3.99 (m, 3H). ¹³C NMR

(101 MHz, D_2O) δ 171.0, 76.9, 70.2, 68.5, 62.8, 49.6, 49.6, 45.1.HRMS (ESI, [M+H]⁺), calculated for $C_9H_{19}N_3O_5$, 250.1403; found, 250.1395.

Biochemical Assays

Inhibition Assays against Commercial Glycosidases and Human Recombinant GBA (R&D 7410-GH) and Human Recombinant GALC (R&D 7310-GH)

Inhibition assays against commercial glycosidases were performed in either phosphate or acetate buffer at the optimum pH for each enzyme (See below for enzyme specific data). Determination of the IC50 values of the iminosugars was carried out by spectrophotometrically measuring the residual hydrolytic activities of the glycosidases on the corresponding p-nitrophenyl glycoside substrates in the presence of a concentration range of iminosugar derivatives. The incubation mixture consisted of 50 μ L of inhibitor solution in buffer (0.1 U mL⁻¹) and 50 μ L of enzyme solution. The concentrations of the enzyme were adjusted so that the reading for the final absorbance was in the range of 0.5-1.5 units. Inhibitor and enzyme solutions were mixed in a disposable 96-well microtiter plate and then incubated at room temperature for 5 minutes. Next, the reactions were initiated by addition of 50 μ L of a solution of the corresponding p-nitrophenyl glycoside substrates solution in the appropriate buffer at the optimum pH for the enzyme. After the reaction mixture was incubated at 37 0 C for 30 min, the reaction was quenched with 0.5 M Na₂CO₃ (150 µL) and the absorbance of 4-nitrophenol released from the substrate was read immediately at 405 nm using a BioTek mQuant Microplate Spectrophotometer.

 IC_{50} values were determined graphically with GraphPad Prism (version 6.0) by making a plot of percentage inhibition versus the log of inhibitor concentration, using at least 8 different inhibitor concentrations. IC_{50} values were presented as a concentration of the iminosugars that inhibits 50% of the enzyme activity under the assay conditions. NN-DNJ was used as a reference compound. All materials were purchased from Sigma-Aldrich. All data are reported in Table S3.

The commercial glycosidase solutions were prepared as following:

^bFor α -glucosidase (from *baker's yeast*, Sigma G5003, 0.05 U/mL) the activity was determined with p-nitrophenyl- α -D-glucopyranoside (0.7 mM final conc. in well) in sodium phosphate buffer (100 mM, pH 7.2).

°For α -galactosidase (from *green coffee beans*, Sigma G8507, 0.05 U/mL) activity was determined with p-nitrophenyl- α -D-galactopyranoside (0.7 mM final conc. in well) in sodium phosphate buffer (100 mM, pH 6.8).

^dFor **\beta-glucosidase** (from *almond*, Sigma G4511, 0.05 U/mL) the activity was determined with p-nitrophenyl- β -D-glucopyranoside (0.7 mM final conc. in well) in sodium acetate buffer (100 mM, pH 5.0).

^eFor **\beta-galactosidase** (from *bovine liver*, Sigma G1875, 0.05 U/mL) activity was determined with p-nitrophenyl- β -D-galactopyranoside (0.7 mM final conc. in well) in sodium phosphate buffer (100 mM, pH 7.2).

^tFor **Naringinase** (from *penicillium decumbens*, Sigma N1385, 0.06 U/mL) the activity was determined with p-nitrophenyl- β -D-glucopyranoside (0.7 mM final conc. in well) in sodium acetate buffer (100 mM, pH 5.0).

^g**Recombinant Human Glucosylceramidase/β-glucocerebrosidase/GBA** (7410-GH), purchased from R&D was used in the inhibition studies. The used substrate 4methylumbelliferyl-β-D-glucopyranoside was purchased by Sigma-Aldrich. GBA activity was determined with 4-methylumbelliferyl-β-D-glucopyranoside as reported in (A. Trapero, J. Med. Chem. 2012, 55, 4479-4488).⁴ Briefly, enzyme solutions (25 µL from a stock solution containing 0.6 µg/mL) in the presence of 0.25% (w/v) sodium taurocholate and 0.1% (v/v) Triton X-100 in McIlvaine buffer (100 mM sodium citrate and 200 mM sodium phosphate buffer, pH 5.2 or pH 7.0) were incubated at 37°C without (control) or with inhibitor at a final volume of 50 µL for 30 min. After addition of 25 µL 4-methylumbelliferyl-β-D-glucopyranoside (7.2 mM, McIlvaine buffer pH 5.2 or pH 7.0), the samples were incubated at 37°C for 10 min. Enzymatic reactions were stopped by the addition of aliquots (100 µL) of Glycine/NaOH buffer (100 mM, pH 10.6). The amount of 4-methylumbelliferone formed was determined with a FLUOstar microplate reader (BMG Labtech) at 355 nm (excitation) and 460 nm (emission).

Samples for GBA inhibition studies were dissolved in buffers 24 hours prior to the start of the experiment. This was done to determine the inhibitory potency of fully hydrolyzed species **10** and **11** at pH 5.2. Experiments at pH 7.0 were done in the same

fashion knowing that no difference in inhibitory potency would be obtained, since minor hydrolysis would occur in such time period.

^h**Recombinant Human Galactosylceramidase/GALC** (7310-GH), purchased from R&D was also used in the inhibition studies. The used substrate 4methylumbelliferyl-β-D-galactopyranoside was purchased by Sigma-Aldrich. GALC activity was determined with 4-methylumbelliferyl-β-D-galactopyranoside as reported in assay procedure R&D product 7310-GH. Briefly, enzyme solutions (25 µL from a stock solution containing 60 ng/mL) in the presence of 0.5% (v/v) Triton X-100 in Assay buffer (50 mM sodium citrate and 125 mM NaCL, pH 4.5) were incubated at 37°C without (control) or with inhibitor at a final volume of 50 µL for 10 min. After addition of 25 µL 4-methylumbelliferyl-β-D-galactopyranoside (0.75mM, Assay buffer), the samples were incubated at 37°C for 20 min. Enzymatic reactions were stopped by the addition of aliquots (50 µL) of Glycine/NaOH buffer (100 mM, pH 10.6). The amount of 4-methylumbelliferone formed was determined with a FLUOstar microplate reader (BMG Labtech) at 355 nm (excitation) and 460 nm (emission).

Enzyme	10	11	NNDNJ
α -glu ^b	> 30	> 30	> 30
α -gal ^c	> 30	> 30	> 30
β-glu ^d	19.150 ± 0.536	14.570 ± 0.573	> 30
β-gal ^e	> 30	> 30	> 30
Nar ^f	> 30	> 30	0.085 ± 0.004
GBA ^g pH 7.0	2.561 ± 0.233	0.025 ± 0.003	0.532 ± 0.059
GBA ^g pH 5.2	> 30	> 30	5.584 ± 0.731
GALC ^h	> 30	> 30	> 30

Table S3. Glycosidase inhibition values obtained for orthoester-armed guanidines 10 and 11.^a

^aIC₅₀ values are reported in µM and are averages obtained from triple independent duplicate analysis of each compound. For ease of comparison, the IC₅₀ values obtained for all compounds shown in Table 1 are compared to a reference compound NNDNJ. ^bα-glucosidase (from *baker's yeast*, Sigma G5003): 0.05 U/mL, the activity was determined with p-nitrophenyl- α -D-glucopyranoside (0.7 mM final conc. in well) in sodium phosphate buffer (100 mM, pH 7.2). °a-galactosidase (from green coffee beans, Sigma G8507): 0.05 U/mL; α-galactosidase activity was determined with pnitrophenyl- α -D-galactopyranoside (0.7 mM final conc. in well) in sodium phosphate buffer (100 mM, pH 6.8). ^dβ-glucosidase (from *almond*, Sigma G4511): 0.05 U/mL; the activity was determined with p-nitrophenyl- β -D-glucopyranoside (0.7 mM final conc. in well) in sodium acetate buffer (100 mM, pH 5.0). ^e\beta-galactosidase (from bovine liver, Sigma G1875): 0.05 U/mL; activity was determined with p-nitrophenyl- β -D-galactopyranoside (0.7 mM final conc. in well) in sodium phosphate buffer (100 mM, pH 7.2). ¹Naringinase (from *penicillium decumbens*, Sigma N1385): 0.06 U/mL. The activity was determined with p-nitrophenyl-β-D-glucopyranoside (0.7 mM final conc. in well) in sodium acetate buffer (100 mM, pH 5.0). ^gβ-glucocerebrosidase (GBA) activities were determined using 4-methylumbelliferyl-β-D-glucopyranoside respectively using assay conditions based on those previously reported⁴ and samples were preincubated in corresponding buffer for 24 hours before assay was performed. ^hβ-galactocerebrosidase 4-methylumbelliferyl-β-D-galactopyranoside (GALC) respectively using assay conditions based on those previously reported.⁴

Inhibition Assays against Human Recombinant GBA (R&D 7410-GH) in a time dependent fashion for compounds 10 and 11.

Due to hydrolysis stability studies done for our compounds **10** and **11** we hypothesized no difference in inhibitory potency for samples left in solution at pH 7.0 and a complete inactivation of the samples that were left overnight at pH 5.2. Since such claims were made, we found it necessary to determine whether such statements could be backed up with an additional, independent experiment.

For **pH 7.0**, samples were dissolved in buffers 24 hours prior to or right before the biological evaluation for GBA inhibition. As envisioned, only a minor difference for all compounds were obtained (approx. 10% in both cases), proving that these compounds did not underwent hydrolysis process under neutral conditions and pertained the inhibition properties (Table S4).

It should be taken in consideration that the difference in inhibition at 0 min and 24 min at pH 7.0 for compounds **10**, **11** and NNDNJ (**2**) occurs due to experiments fluctuations and is in the same order of magnitude as determined in regular GBA biological evaluations.

For **pH 5.2**, samples were dissolved in buffers 24 hours prior to or right before the biological evaluation for GBA inhibition. The inhibitory difference between time dependent hydrolysis is noticeable in both cases as presented in Table S4. A big jump in IC_{50} value was determined for compound **11** from 1934 nM to more than 30000 nM when left in the solution overnight.

t (h)	t (h)	10	11	NNDNJ
GBA (pH 7.0)	0	3394 ± 372	29.6 ± 2.4	775 ± 58
GBA (pH 7.0)	24	2561 ± 233	25.2 ± 2.6	532 ± 59
GBA (pH 5.2)	0	> 30000	1934 ± 178	3730 ± 424
GBA (pH 5.2)	24	> 30000	> 30000	5584 ± 731

Table S4: IC₅₀ values in nM for GBA inhibition of compounds **10**, **11** and NNDNJ at pH 7.0 and pH 5.2 after immediate evaluation (t = 0 hours) and 24-hour incubation (t = 24 hours) in the corresponding buffer.

To extend our studies, we prepared a series of samples of compound **11** preincubated in the acidic buffer every two hours to illustrate the inactivation process of the enzyme during the time of 20 hours. Thus, 11 data points were plotted in a Figure S7 for a better graphical representation of the inactivation process as the hydrolysis of the orthoester occurs.



Figure S7: IC_{50} values for GBA inhibition for the orthoester-armed DIX derivative **11** at pH 5.2 determined immediately after addition and mixing and after every 2 hours incubation time, for 20 hours, at the acidic pH. The data reflect the inactivation of the chaperone **11**, thereby losing the inhibitory capacity, following hydrolysis to result in a completely inactive hydrolyzed species with IC_{50} of more than 30000 nM.



IC₅₀ = 2.561e-006 ± 2.334e-007





NNDNJ - pH 7



IC₅₀ = $5.322e-007 \pm 5.888e-008$

Measurement of N370S and L444P GBA Activity in Fibroblasts Derived from Patients with GD.

Observations of the same cell confluence for both 0mM and 10mM concentration of the compounds tested during a longer period of time indicate that all compounds tested in this study seem to be non-toxic as previously observed in the preceding study involving a series of DIX derivatives that were non-toxic, although no additional cytotoxicity assays were performed for this study.5 The chaperone assay was performed according to a modified version as previously described.⁴ Fibroblasts were plated into 24-well assay plates and incubated at 37°C under 5% CO₂ atmosphere until a monolayer of at least 50% confluency was reached. The media were then replaced with fresh media with or without various concentrations of test compounds and incubated at 37°C in 5% CO₂ for 4 days. The enzyme activity assay was performed after removing media supplemented with the corresponding compound. The monolayers were washed twice with phosphate buffered saline (PBS) solution. Then, 80 µl of PBS and 80 µl of 200 mM acetate buffer (pH 4.0) were added to each well. The reaction was started by addition of 100 µl of 7.2 mM 4methylumbelliferyl-\beta-D-glucopyranoside (200 mM acetate buffer pH 4.0) to each well, followed by incubation at 37°C for 2h. Enzymatic reactions were stopped by the addition of 0.9 ml glycine/NaOH buffer (100 mM pH 10.6). Liberated 4methylumbelliferone was measured (excitation 355 nm, emission at 460 nm) with the Fluoroskan Ascent FL plate reader (Labsystems) in 96-well format. Data are reported in Figure S8 and Figure S9.



Figure S8. The effect of compounds 10, 11, NNDNJ and IFG on GBA activity in N370S fibroblasts (GM00372) from Gaucher patients. Cells were cultured for 4 days in the absence or presence of increasing concentrations (nM) of the compounds before GBA activity was measured. Experiments were performed in triplicate, and each bar represents the mean \pm SD. Enzyme activity is normalized to untreated cells, assigned a relative activity of 1.



Figure S9. The effect of compounds **10**, **11**, **NNDNJ** and **IFG** on GBA activity in L444P fibroblasts (GM00877) from Gaucher patients. Cells were cultured for 4 days in the absence or presence of increasing concentrations (nM) of the compounds before GBA activity was measured. Experiments were performed in triplicate, and each bar represents the mean \pm SD. Enzyme activity is normalized to untreated cells, assigned a relative activity of 1.

¹H, ¹³C NMR, ¹H – ¹H COSY and ¹H – ¹³C HSQC NMR spectra for all new compounds.



Compound 12: ¹H NMR (400 MHz, CDCl₃)

Compound 12: ¹³C NMR (101 MHz, CDCl₃)



Compound 13: ¹H NMR (400 MHz, CDCl₃)



Compound 13: ¹³C NMR (101 MHz, CDCl₃)



Compound 14: ¹H NMR (400 MHz, DMSO)



Compound 14: ¹³C NMR (101 MHz, DMSO)



Compound 15: ¹H NMR (400 MHz, CDCl₃)



Compound 15: ¹³C NMR (101 MHz, CDCl₃)



Compound 16: ¹H NMR (400 MHz, CDCl₃)



Compound 16: ¹³C NMR (101 MHz, CDCl₃)



Compound 20: ¹H NMR (400 MHz, CDCl₃)



Compound 20: ¹³C NMR (101 MHz, CDCl₃)



Compound 20: ¹H – ¹H COSY NMR (400 MHz, CDCl₃)



Compound 20: ¹H – ¹³C HSQC NMR (101 MHz, CDCl₃)



Compound 21: ¹H NMR (400 MHz, CDCl₃)



Compound 21: ¹³C NMR (101 MHz, CDCl₃)



Compound 21: ¹H – ¹H COSY NMR (400 MHz, CDCl₃)



Compound 21: ¹H – ¹³C HSQC NMR (101 MHz, CDCl₃)



Compound 22: ¹H NMR (400 MHz, CDCl₃)



Compound 22: ¹³C NMR (101 MHz, CDCl₃)



Compound 22: ¹H – ¹H COSY NMR (400 MHz, CDCl₃)



Compound 22: ¹H – ¹³C HSQC NMR (101 MHz, CDCl₃)



Compound 23: ¹H NMR (400 MHz, CDCl₃)



Compound 23: ¹³C NMR (101 MHz, CDCl₃)



Compound 23: ¹H – ¹H COSY NMR (400 MHz, CDCl₃)



Compound 23: ¹H – ¹³C HSQC NMR (101 MHz, CDCl₃)





Compound 10: ¹H NMR (400 MHz, CDCl₃)

Compound 10: ¹³C NMR (101 MHz, CDCl₃)



Compound 10: ¹H – ¹H COSY NMR (400 MHz, CDCl₃)



Compound 10: ¹H – ¹³C HSQC NMR (101 MHz, CDCl₃)



Compound 11: ¹H NMR (400 MHz, D₂O)



Compound 11: ¹³C NMR (101 MHz, D₂O)



Compound 11: ¹H – ¹H COSY NMR (400 MHz, D₂O)



Compound 11: ¹H – ¹³C HSQC NMR (101 MHz, D₂O)



Compound 31: ¹H NMR (400 MHz, CDCl₃)



Compound 31: ¹³C NMR (101 MHz, CDCl₃)



Compound 31: ¹H – ¹H COSY NMR (400 MHz, CDCl₃)



Compound 31: ¹H – ¹³C HSQC NMR (101 MHz, CDCl₃)





Compound 32: ¹H NMR (400 MHz, CDCl₃)

Compound 32: ¹³C NMR (101 MHz, CDCl₃)



Compound 32: ¹H – ¹H COSY NMR (400 MHz, CDCl₃)



Compound 32: ¹H – ¹³C HSQC NMR (101 MHz, CDCl₃)



Compound B: ¹H NMR (400 MHz, CDCl₃)



Compound B: ¹³C NMR (101 MHz, CDCl₃)



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