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Escherichia coli LacZ β-galactosidase inhibition by monohydroxy acetylated glycopyranosides: Role of the acetyl groups

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ABSTRACT

Escherichia coli LacZ β-galactosidase is an extensively employed glycosidase for many different scientific purposes. Here, we describe how acetyl moieties protecting hydroxyl groups of the glycosides make these molecules better inhibitors to the enzyme activity. In particular, the presence of a unique hydroxyl group in the peracetylated glycosides still enhanced the inhibitory capacity of the molecule more. Molecular docking studies showed that the acetylation in the carbohydrate structure helps the substrate to accommodate into the active site. From a small biocatalytic synthesized library of different monohydroxy acetylated glycosides we can conclude that galactosidic structures are better for inhibition capacity. The best inhibitors were two monohydroxy lactal derivatives. The one with the OH free, in *C*-6 of the galactosidic part of the disaccharide, was a better inhibitor (K_i of 95 μM) than that with the OH free in *C*-3 in the glucosidic part of the molecule (K_i of 143 μM).

1. Introduction

Glycosidases are enzymes with a critical role in nature, relevant for understanding and controlling a variety of processes that are involved in carbohydrate-mediated essential biological events. The specific inhibition of their activity is of great interest and it has widespread application in the treatment of several diseases [1–4].

In the last years, a high number of different glycosidase competitive inhibitors have been synthesized using exquisite chemical routes, giving effective inhibition values in some cases [5,6]. Most of these synthesized glycosidase inhibitors are carbohydrate based-molecules. Iminosugars represent one of the most successful examples [7].

1-Deoxynojirimycin (DNJ) and 1-deoxygalactonojirimycin (DGJ) (Fig. 1) are two natural iminosugar drugs with efficacy in animal models for Pompe's and Fabry's diseases [8].

Considering the X-ray data available for glycosidases, in some cases in complexes with ligands, and the experimental data observed with the different inhibitors already synthesized, a universal structural feature of an ideal general glycosidases inactivator could not be found. The inhibition generally depends on the glycosidic nature of compounds or the configuration selectivity of the protein [9]. Indeed, in many cases, chemical structures with poor inhibitory activity toward β-galactosidases are excellent inhibitors of glucosidases [10–12].

In particular, among glycosidase enzymes, the *Escherichia coli LacZ* β-galactosidase has been extensively employed as a model, at different degrees of complexity, for many different scientific purposes [13]. The use of X-ray crystallography and site-directed mutagenesis has permitted elucidating a key aspect in the catalytic mechanism of this enzyme [14,15]. The enzymatic pocket of β-galactosidase from *E. coli* is well characterized and located at a defined cavity of the existing triosephosphate isomerase (TIM)-barrel structure of one of its domains [16].

The nucleophile is glutamic acid Glu537, although the glutamic acids Glu461 and Glu416, together with histidine His418 residues bound to the magnesium ion, are also intimately involved in the catalysis. Tryptophan Trp999 residue determines the complementary interaction surface between the enzyme and the nonpolar faces of the carbohydrate ligands, through CH–p-stacking interactions, as

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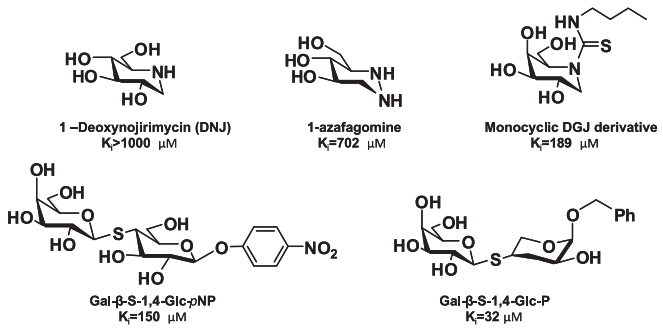


Fig. 1. Examples of different inhibitors of β-galactosidase from *E. coli*.

frequently found in carbohydrate-binding proteins. Thus Trp999 stacks with the properly oriented hydrogen atoms of the aglyconic moiety [17].

Chemical modification in the glycoside structures has permitted obtaining compounds with better inhibition values toward this enzyme [10,18–20]. Some examples of the β-galactosidase from *E. coli* inhibitors already known are shown in Fig. 1. In particular, two thiodisaccharide molecules have recently been found to show very interesting inhibition values for this glycosidase (Fig. 1) [21].

Historically, there has been a perception that glycosidase inhibitors must be polar structures, especially with the free hydroxyl groups in the molecules. However, for example, several competitive inhibitors of glycosyltransferases are partially or fully acetylated glycosyl structures, responsible for blocking the sialyl Lewis X formation in cancer cells [22–25]. Some of them are involved in slow tumor metastasis *in vivo* [24] or preventing leukocyte adhesion [25].

Therefore, the protection of the hydroxyl group of the saccharide molecule can lead to formation of H bridges with the residues in the active site of enzyme and cause its inhibition.

In the present work, we evaluated the inhibitory effect of the presence of acetyl groups in saccharides on the activity of *E. coli LacZ* β-galactosidase and a structure–activity correlation by using docking experiments on a small library of different glycosidic derivatives with different substitution in anomeric position.

In particular, we demonstrated for the first time that the presence of a free hydroxyl group on the peracetylated saccharide has a critical role in improving its inhibition ability. These monodeprotected-acetylated glycosides were synthesized by a biocatalytic approach in multigram scale [26–28], which is quite useful in order to synthesize large amounts of inhibitors required for testing *in vivo*.

2. Experimental

2.1. Materials

The lipases from *C. antarctica* B (Lipozyme CALBL) (CAL-B), *Thermomyces lanuginose* (Lipozyme TL100L) (TLL) and *Rhizomucor miehei* (Palatase 20000L) (RML) were kindly supplied by Novozymes, Denmark. Lipases from *Candida rugosa* (L1754) (CRL), *Aspergillus niger* (62301) (ANL) and *Pseudomonas fluorescens* (534730) (PFL), *E. coli* β-galactosidase (grade VIII, EC 3.2.1.23), D-glucopyranose, lactose, D-galactopyranose, 2-acetamido-2-deoxy-1,3,4,6-penta-*O*-acetyl-β-D-glucopyranose, triacetyl glucal, 1-*O*-methyl-2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) were from Sigma–Aldrich. Tri-*O*-acetyl-D-galactal, peracetylated lactose and hexa-acetyl lactal were from Iris Biotech. 1-Thioisopropyl-β-D-galactopyranoside was from Alfa Aesar. 1-(4-Nitrophenyl)-2,3,4-tri-*O*-acetyl-β-D-xylopyranoside was from BOC Sciences.

Octyl-sepharose (4BCL) and CNBr-activated sepharose (4BCL) were purchased from GE-Healthcare (Uppsala, Sweden). The immobilization of the different lipases on octyl-support or CNBr-activated sepharose was performed as previously described [26].

The preparations were called: octyl-CAL-B, octyl-TLL, octyl-CRL, octyl-RML, octyl-ANL, octyl-PFL, CNBr-CAL-B and CNBr-ANL. The immobilized preparation of acetyl xylan esterase (AXE) from *Bacillus pumilus* was from ACS Dobfar.

NMR spectra were recorded using a Varian Mercury 500 MHz spectrometer and calibrated according with solvent standard peaks. FAB measurements were taken with a VG Autospec apparatus by using a 3-nitrobenzyl alcohol (3-NBA) matrix. Elemental analysis was measured using LECO CHNS-932. Optical rotations were measured with a Perkin–Elmer 241 Polarimeter. Melting points were measured with a Büchi B-540 apparatus.

2.2. HPLC analysis

Generally, the regioselective hydrolyses were followed by HPLC, using a HPLC spectrum P100 (Thermo Separation products). The column was a Kromasil-C18 (250 × 4.6 mm and 5 μm Ø) from Analisis Vinicos (Spain). Analyses were run at 25 °C using an L-7300 column oven and UV detector L-7400 at 210 nm. The flow rate was 1.0 mL/min.

2.3. Synthesis and characterization of hydroxyl-free-acetylated glycosides (**1**–**13**)

Thirteen glycosides containing a unique free hydroxyl group in different positions were enzymatically synthesized with high regioselectivity by immobilized lipases at ambient temperature and were monitored by TLC and HPLC [26].

2.3.1. 1,2,3,4-Tetra-O-acetyl-α-D-glucopyranose (**1**)

Per-*O*-acetyl-glucopyranose (720 mg, 1.846 mmol) was hydrolyzed in 100 mL solution of sodium acetate (50 mM) (80%) and acetonitrile (20%) at pH 5 using 3 g of octyl-CRL preparation. After 24 h the substrate completely disappeared. The aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5× 50 mL). The collected organic layers were dried over anhydrous Na_2SO_4, which was then removed by filtration and concentrated under vacuum. Then, diethyl ether was added and removed under vacuum, to afford **1** as a white glassy solid (513 mg, 80%). TLC of **1**: hexane:ethyl acetate (1:1), R_f = 0.22. HPLC ($NH_4H_2PO_4$ 10 mM buffer:ACN 7:3 v/v, pH 4) R_t = 7.7 min. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.38 (d, J = 3.7 Hz, 1H, H-1), 5.56 (t, J = 9.9 Hz, 1H, H-3), 5.14 (t, J = 9.9 Hz, 1H, H-4), 5.10 (dd, J = 3.7, 9.9 Hz, 1H, H-2), 3.96 (m, 1H, H-5), 3.61–3.75 (dd, J = 4.2, 2.4, 12.9 Hz, 2H, H-6A, H-6B), 2.00–2.25 (4s, 12H, CH_3).

2.3.2. 1-Hydroxy-hepta-O-acetyl lactose (**2**)

Octa-*O*-acetyl lactose (360 mg, 0.526 mmol) was hydrolyzed in 50 mL solution of sodium acetate (50 mM) (80%) and acetonitrile (20%) at pH 5 using 1 g of the CNBr-ANL immobilized preparation. After 5 h, when the substrate disappeared, the aqueous solution was filtrated and then extracted with ethyl acetate (4× 50 mL). The collected organic layers were dried over anhydrous Na_2SO_4, which was then removed by filtration and concentrated under vacuum. Then, diethyl ether was added and removed under vacuum to afford **2** as a white glassy solid (323 mg, 96%). TLC of **2**: CH_2Cl_2:CH_3OH (9:1), R_f = 0.67. HPLC ($NH_4H_2PO_4$ 10 mM buffer:ACN 6:4 v/v, pH 4) R_t = 8.82 min (α-anomer), 9.33 (β anomer). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 5.51 (t, J = 9.7 Hz, 1H, H-3α), 5.36 (d, J = 3.4 Hz, 1H, H-1α), 5.34 (dd, J = 0.5, 1H, H-4′), 5.22 (t, J = 9.3 Hz, 1H, H-3β), 5.11 (dd, J = 10.5 Hz, 1H, H-2′α), 5.09 (dd, J = 10.6 Hz, 1H, H-2′β), 4.94 (dd, J = 3.2 Hz, 1H, H-3′), 4.81 (dd, 1H, H-2α), 4.76 (m, 2H, H-1β, H-2β),

4.49 (d, J = 7.9 Hz, 1H, H-1′α), 4.48 (dd, J = 3.4, 11.2 Hz, 1H, H-6B), 4.47 (d, J = 7.7 Hz, H-1′β), 4.22–4.00 (m, 4H, H-5, H-6A, H-6′A, H-6′B), 3.86 (dt, J = 6.3, 1H, 5′-H), 3.75 (dd, J = 9.3 Hz, H-4α), 2.15–1.96 (s, 21H, $7CH_3$).

*2.3.3. 2-Acetamido-2-deoxy-1,3,4-tri-O-acetyl-α-D-glucopyranose (**3**)*

2-Acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose (720 mg, 1.85 mmol) was hydrolyzed in 100 mL solution of sodium acetate (50 mM) (80%) and acetonitrile (20%) at pH 5, using 3 g of the octyl-CRL immobilized preparation. When the substrate disappeared, the aqueous solution was filtrated and then extracted with ethyl acetate (3× 50 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was purified by flash column chromatography (dichloromethane/methanol, 9.5:0.5) to provide **3** (white solid, 530 mg, 82% yield). TLC of **3**: CH_2Cl_2:CH_3OH (9:1), R_f = 0.52. HPLC ($NH_4H_2PO_4$ 10 mM buffer:ACN 8:2 v/v, pH 4) R_t = 5.70 min (α-anomer), 9.33 (β anomer). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.19 (d, J = 3.3 Hz, 1H, H-1), 5.61 (d, J = 9 Hz, 1H, NH), 5.30 (t, J = 9.9 Hz, 1H, H-3), 5.16 (t, J = 9.7 Hz, 1H, H-4), 4.46 (m, J = 10.4, 8.4, 3.7 Hz, 1H, H-2), 3.81 (m, 1H, H-5), 3.59–3.71 (2dd, J = 8.5, 3.9, 12.4 Hz, 2H, H-6A, H-6B), 2.20 (s, 3H, CH_3), 2.05–2.11 (2s, 6H, $2CH_3$), 1.96 (s, 3H, CH_3).

*2.3.4. 2,3,4-Tri-O-acetyl-1-O-methyl-β-D-glucopyranose (**4**)*

Tetra-*O*-acetyl-1-*O*-methyl-glucopyranose (727 mg, 2.01 mmol) was hydrolyzed in 100 mL solution of sodium acetate (50 mM) (80%) and acetonitrile (20%) at pH 5, using 3 g of octyl-CRL preparation. After 4 h, the substrate completely disappeared. The aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5× 50 mL). The collected organic layers were dried over anhydrous Na_2SO_4, which was then removed by filtration and concentrated under vacuum. Then, diethyl ether was added and removed under vacuum to afford **4** as a white glassy solid (529 mg, 82%). TLC of **4**: hexane:ethyl acetate (1:1), R_f = 0.23. HPLC ($NH_4H_2PO_4$ 10 mM buffer:ACN 7:3 v/v, pH 4) R_t = 6.7 min. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 5.04 (t, J = 9.5 Hz, 1H, H-3), 5.00 (t, J = 9.8 Hz, 1H-4), 4.94 (dd, J = 9.7, 8 Hz, 1H, H-2), 4.46 (d, J = 7.9 Hz, 1H, H-1), 3.6–3.8 (m, J = 7.9, 2, 12.5 Hz, 2H, H-6A, H-6B), 3.53 (m, 1H-5), 3.52 (s, 3H, CH_3), 1.92–2.01 (s, 9H, $3CH_3$).

*2.3.5. 3,4-Di-O-acyl glucal (**5**)*

Tri-*O*-acetyl-glucal (730 mg, 2.68 mmol) was hydrolyzed in 100 mL solution of sodium acetate (50 mM) (80%) and acetonitrile (20%) at pH 5 using 3 g of octyl-CRL preparation. After the substrate completely disappeared, the aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5× 50 mL). The collected organic layers were dried over anhydrous Na_2SO_4, which was then removed by filtration and concentrated under vacuum. Then, after purification on a flash column, the product was washed with diethyl ether and removed under vacuum to afford **5** as a white solid (430 mg, 70%). TLC of **5**: hexane:ethyl acetate (1:1), R_f = 0.29. HPLC ($NH_4H_2PO_4$ 10 mM buffer:ACN 7:3 v/v, pH 4) R_t = 6.7 min. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.49 (dd, J = 6.1 Hz, 1H, H-1), 5.41–5.50 (m, 1H, H-3), 5.22 (dd, J = 9.0, 6.5 Hz, 1H, H-4), 4.81 (dd, J = 5.9, 2.8 Hz, 1H, H-2), 3.98–4.09 (m, 1H, H-5), 3.66–3.86 (m, 2H, H-6A, H-6B), 2.07–2.13 (2s, 6H, $2CH_3$).

*2.3.6. 4,6-Di-O-acetyl-D-glucal (**6**)*

Tri-*O*-acetyl-glucal (727 mg, 2.68 mmol) was hydrolyzed in 100 mL solution of sodium acetate (50 mM) (80%) and acetonitrile (20%) at pH 5 using 1 g of CNBr-CAL-B immobilized preparation. After 18 h, the substrate completely disappeared. The aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5× 50 mL). The collected organic layers were dried over anhydrous Na_2SO_4, which was then removed by filtration and concentrated under vacuum. Then, diethyl ether was added and removed under vacuum to afford **6** as a white glassy solid (557 mg, 90%). TLC of **6**: hexane:AcOEt 5:5 v/v, R_f = 0.33, HPLC ($NH_4H_2PO_4$ 10 mM buffer:ACN 7:3 v/v, pH 4) R_t = 5.2 min. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.42 (dd, J = 6.1 Hz, 1H, H-1), 4.95 (dd, J = 6.2, 2.0 Hz, 1H, H-4), 4.84 (dd, J = 6.2, 3.2, 2.7 Hz, 1H, H-2), 4.43 (ddd, J = 6.7, 5.3 Hz, 1H, H-5), 4.22–4.38 (m, 2H, H-6A, H-6B), 4.20–4.11 (dd, J = 6.2, 2.2 Hz, 1H, H-3), 2.55 (bs, 1H, OH), 2.16 (s, 3H, CH_3), 2.11 (s, 3H, CH_3).

*2.3.7. 1,2,3,4-Tetra-O-acetyl-β-D-galactopyranose (**7**)*

Penta-*O*-acetyl-galactopyranose (320 mg, 0.82 mmol) was hydrolyzed in 50 mL solution of sodium acetate (50 mM) (80%) and acetonitrile (20%) at pH 5, using 3 g of octyl-TLL preparation. After 4 days, the substrate completely disappeared. The aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5× 50 mL). The collected organic layers were dried over anhydrous Na_2SO_4, which was then removed by filtration and concentrated under vacuum. Then, diethyl ether was added and removed under vacuum to afford **7** as a white glassy solid (243 mg, 85%). TLC of **7**: hexane:ethyl acetate (1:1), R_f = 0.23. HPLC ($NH_4H_2PO_4$ 10 mM buffer:ACN 7:3 v/v, pH4) R_t = 10.6 min. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 5.73 (d, J = 8.2 Hz, 1H, H-1), 5.44 (d, J = 3.3 Hz, 1H, H-4), 5.32 (t, J = 8.3 Hz, 1H, H-3), 5.13 (dd, J = 3.4, 10.4 Hz, 1H, H-2), 3.91 (dt, J = 6.4 Hz, 1H, H-5), 3.8–3.51 (m, 2H, H-6A, H-6B), 2.14–1.97 (s, 12H, $4CH_3$).

*2.3.8. 2,3,4-Tetra-O-acetyl-1-thioisopropyl-α-D-glucopyranose (**8**)*

1-Thioisopropyl-per-*O*-acetyl glucopyranose (681 mg, 1.67 mmol) was hydrolyzed in 100 mL solution of sodium acetate (50 mM) (80%) and acetonitrile (20%) at pH 5, using 3 g of the octyl-CRL immobilized preparation. When the substrate disappeared (checked by TLC and HPLC), the aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5× 50 mL). The collected organic layers were dried over anhydrous Na_2SO_4, which was then removed by filtration and concentrated under vacuum to afford **8** as a white glassy solid (583 mg, 96%). TLC of **8**: hexane: ethyl acetate (1:1), R_f = 0.29. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 5.40 (d, J = 2.9 Hz, 1H, H-4), 5.20 (t, J = 9.9 Hz, 1H, H-2), 5.08 (dd, J = 6.6, 3.1 Hz, 1H, H-3), 4.58 (d, J = 10.0 Hz, 1H, H-1), 3.78–3.68 (m, 2H, H-5, H-6A), 3.52–3.46 (m, 1H, H-6B), 3.23–3.14 (m, J = 6.6 Hz, 1H, CH), 2.16 (s, 3H, CH_3), 2.10 (s, 3H, CH_3), 1.99 (s, 3H, CH_3), 1.30 (m, 6H, $2CH_3$).

*2.3.9. 3,4-Di-O-acetyl-D-galactal (**9**)*

Tri-*O*-acetyl-galactal (720 mg, 2.64 mmol) was hydrolyzed in 100 mL solution of sodium acetate (50 mM) (80%) and acetonitrile (20%) at pH 5, using 3 g of octyl-CRL immobilized preparation. After 24 h, the substrate completely disappeared (checked by TLC and HPLC). The aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5× 50 mL). The collected organic layers were dried over anhydrous Na_2SO_4, which was then removed by filtration and concentrated under vacuum. Then, diethyl ether was added and removed under vacuum to afford **9** as a white glassy solid (603 mg, 99%). TLC of **9**: CH_2Cl_2:CH_3OH 9:1 v/v, R_f = 0.67, HPLC ($NH_4H_2PO_4$ 10 mM buffer:ACN 7:3 v/v, pH 4) R_t = 5.2 min. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.50 (dd, J = 6.2, 1.8 Hz, 1H, H-1), 5.60–5.55 (m, 1H, H-3), 5.50–5.44 (dt, J = 7.4, 3.6 Hz, 1H, H-4), 4.73 (dt, J = 5.9, 2.8 Hz, 1H, H-2), 4.22–4.16 (m, J = 7 Hz, 1H, H-5), 3.83–3.61 (dd, J = 11.6, 5.8 Hz, 2H, H-6A, H-6B), 2.31 (bs, 1H, OH), 2.16 (s, 3H, CH_3), 2.05 (s, 3H, CH_3).

*2.3.10. 4,6-Di-O-acetyl-D-galactal (**10**)*

Tri-*O*-acetyl-galactal (720 mg, 2.64 mmol) was hydrolyzed in 100 mL solution of sodium acetate (50 mM) (80%) and acetonitrile

(20%) at pH 5, using 3 g of octyl-PFL immobilized preparation. After 24 h, the substrate completely disappeared (checked by TLC and HPLC). The aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5× 50 mL). The collected organic layers were dried over anhydrous Na_2SO_4, which was then removed by filtration and concentrated under vacuum. Then, diethyl ether was added and removed under vacuum to afford **10** as a white glassy solid (522 mg, 86%). TLC of **10**: CH_2Cl_2:CH_3OH 9:1 v/v, R_f = 0.67, HPLC ($NH_4H_2PO_4$ 10 mM buffer:ACN 7:3 v/v, pH 4) R_t = 4.7 min. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.40 (dd, J = 6.2, 1.6 Hz, 1H, H-1), 5.48 (m, 1H, H-4), 4.75 (m, 1H, H-2), 4.30–4.15 (m, 1H, H-6A, H-6B), 4.14–4.10 (m, 1H, H-3), 2.11 (s, 3H, CH_3), 2.03 (s, 3H, CH_3).

2.3.11. 1-(4-Nitrophenyl)-2,3-di-O-acetyl-β-D-xylopyranoside (**11**)

1-(4-Nitrophenyl)-tri-*O*-acetyl-β-D-xylopyranoside (320 mg, 0.806 mmol) was hydrolyzed in 40 mL solution of sodium acetate (50 mM) (80%) and acetonitrile (20%) at pH 5, using 3 g of octyl-ANL immobilized preparation. Then, after the substrate completely disappeared (checked by TLC and HPLC) the aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5× 50 mL). The collected organic layers were dried over anhydrous Na_2SO_4, which was then removed by filtration and concentrated under vacuum. Then, diethyl ether was added and removed under vacuum to afford **10** as a white glassy solid (270 mg, 95%). TLC of **10**: CH_2Cl_2:CH_3OH 9:1 v/v, R_f = 0.59, HPLC ($NH_4H_2PO_4$ 10 mM buffer:ACN 6:4 v/v, pH 4) R_t = 8.3 min. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.10 (d, J = 8.4 Hz, 2H, H-3′, H-5′), 6.88 (d, J = 8.3 Hz, 2H, H-2′, H-6′), 5.21 (d, J = 5.3 Hz, 1H, H-1), 5.16 (m, 1H, H-2), 4.78 (m, 1H, H-3), 4.12 (m, 1H, H-5), 3.92 (m, 1H, H-4), 3.55 (m, 1H, H-5), 2.10 (s, 3H, CH_3), 2.05 (s, 3H, CH_3).

2.3.12. 3,6,2′,3′,4′-Penta-O-acetyl-D-lactal (**12**)

Hexa-*O*-acetyl lactal (300 mg, 0.526 mmol) was hydrolyzed in 50 mL solution of sodium acetate (50 mM) (80%) and acetonitrile (20%) at pH 5, using 3 g of the commercial immobilized preparation of acetyl xylan esterase (AXE) from *Bacillus pumilus*. When the substrate disappeared (checked by TLC and HPLC), the aqueous solution was filtrated and then extracted with ethyl acetate (3× 50 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was purified by a flash column chromatography (dichloromethane/ethyl acetate, 7:3) to provide **12** (white solid, 188 mg, 69% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.47 (dd, J = 0.9, 6.2 Hz, 1H, H-1), 5.46 (br, t, J = 4.2 Hz, 1H, H-3), 5.37 (d, J = 3.2 Hz, 1H, H-4′), 5.23 (dd, J = 7.9, 10.5 Hz, 1H, H-2′), 5.05 (dd, J = 3.5, 10.5 Hz, 1H, H-3′), 4.83 (dd, J = 4.2, 6.2 Hz, 1H, H-2), 4.68 (d, J = 7.9 Hz, 1H, H-1′), 4.40 (dd, J = 2.8, 11.2 Hz, 1H, H-6A), 4.19–4.28 (m, 2H, H-5, H-6B), 3.98 (br. t, J= 5.2 Hz, 1H, H-4), 3.71–3.79 (m, 2H, H-5′, H-6B′), 3.53 (dd, J = 3.8, 10.4 Hz, 1H, H-6A′), 2.01, 2.09, 2.10, 2.14, 2.18 (5s, 15H, $COCH_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 170.0, 170.7, 171.2, 171.4, 171.5 (*C*OCH3), 146.4 (CH), 102.2 (CH), 98.9 (CH), 75.1 (2CH), 74.8 (CH), 71.5 (CH), 69.8 (CH), 68.8 (CH), 68.5 (CH), 62.3 (CH_2), 61.9 (CH_2), 21.3, 21.5, 21.9 ($COCH_3$). HRMS (FAB): Calcd. for $C_{22}H_{30}O_{14} + Na^+$: 541.00; found [M+Na]: 541.1541.

2.3.13. 6, 2′,3′,4′,6′-Penta-O-acetyl-D-lactal (**13**)

Hexa-*O*-acetyl lactal (400 mg, 0.71 mmol) was hydrolyzed in 50 mL solution of sodium acetate (50 mM) (80%) and acetonitrile (20%) at pH 5, using 5 g of the octyl-RML immobilized preparation (ESI). When the substrate disappeared (checked by TLC and HPLC) in approximately 3 days, the aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5× 50 mL). The collected organic layers were dried over anhydrous Na_2SO_4, which was then removed by filtration and concentrated under vacuum to afford **13** as a white glassy solid (256 mg, 95%). TLC of **13**: CH_2Cl_2:ethyl acetate (7:3), R_f = 0.3. HPLC ($NH_4H_2PO_4$ 10 mM buffer:ACN 6:4 v/v, pH 4) R_t = 11.10 min. ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 6.33 (d, 1H, J = 5.90 Hz, H-1), 5.38 (d, 1H, J = 3.39 Hz, H-4′), 5.25 (t, 1H, J = 8.37 Hz, H-2′), 5.00 (dd, 1H, H-3′), 4.76 (dd, 1H, H-2), 4.59 (d, 1H, J = 8.0 Hz, H-1′), 4.43 (m, 1H, H-3), 4.20–4.10 (m, 2H, H-6a, 6b), 4.13–4.07 (m, 2H, H-6′a, 6′b), 4.08 (m, 1H, H-5′), 3.98 (m, 1H, H-5), 3.63 (m, 1H, H-4), 2.15–2.09 (5s, 15H, 5× CH_3). ^{13}C NMR (300 MHz, $CDCl_3$) δ (ppm): 170.58, 170.03, 169.60, 143.75, 102.66, 102.19, 81.96, 73.77, 71.51, 70.82, 68.70, 68.26, 66.98, 62.58, 62.10, 20.79, 20.59, 20.55, 20.45. $[\alpha]_D^{25}$ = +60 (c = 0.48, CH_2Cl_2). mp = 56.2–57.5 °C. MS (FAB): Calcd. for $C_{22}H_{30}O_{14}$: 518.1636; found [M+Na]: 541.1541 $C_{22}H_{30}O_{14}$ (518.16): calcd. C 50.96, H 5.83, O 43.20; found C 49.55, H 5.72, O 42.80.

2.4. In vitro hydrolytic activity assay with E. coli β-galactosidase in the presence of the different glycosides

Inhibition assays were performed in phosphate buffer (50 mM) containing acetonitrile 25% (v/v) at pH 7. Determination of the IC_{20} and IC_{50} values of the different glycosides was performed by UV-spectrophotometry, measuring the residual activity of the enzyme on the hydrolysis of OPNG, in the presence of a concentration range of carbohydrate derivatives.

An enzyme solution (10 mL) was prepared at a concentration of 47 μg of commercial enzyme powder per milliliter.

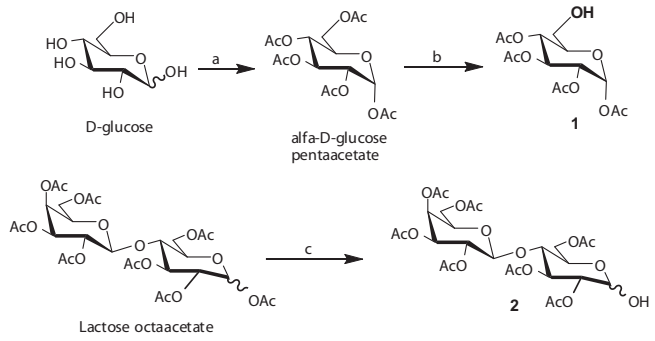
One milliliter of OPNG solution (8 mg/mL) was mixed with 1 mL of inhibitor solution (different amount of inhibitor dissolved in 1 mL of a acetonitrile/phosphate buffer 1:1 mixture) up to a final concentration of 0.5–200 mM in a 2.5 mL glass cuvette and then stirring for 2 min at 100 rpm. Then, to initialize the reaction, 0.02 μL of enzyme solution was added to the cuvette and the increase in absorbance of *o*-nitrophenol (*o*NP) was read at 405 nm in a kinetic mode (1 min) using a Shimazdu-UVmini1240 spectrophotometer. IC_{20} and IC_{50} values were determined as a concentration of the acetylated carbohydrates that inhibits 20% or 50% of the enzyme activity under the assay conditions, respectively.

2.5. Inhibition constant (K_i) calculation of β-Galactoside from E. coli

K_m value (0.16 mM) for the enzyme was obtained using Lineweaver–Burk plots using the different concentrations (from 0.5 to 13.3 mM) of ONPG, following the assay mentioned above. Then, considering the substrate concentration [S] and the previously calculated IC_{50} values, the K_i values were determined according to the Chi-Prusoff equation [29]: $K_i = [IC_{50}/(1 + [oNPG]/K_m)]$.

2.6. Docking experiments

Molecular docking was carried out using the GOLD (Genetic Optimization for Ligand Docking) software [30], which uses a genetic algorithm (GA) to perform the fitting of the ligand molecule. PDB code 1JYN was used because it is the unique crystallographic structure of the β-galactosidase in complex with lactose. This crystallographic structure contains a mutation Q537, instead of the salvage Glu, but no influence during the docking experiments was observed (after performing the docking with other PDB structures of β-galactosidase, no significant changes were observed). The active site was defined by a sphere of 6 Å radius around the lactose, and the lactose was used as template reference for the GA runs. Full flexibility of the ligand was set and partial flexibility for the residues at the active site. For each independent GA run, a maximum number of 100,000 GA operations were performed on a set of five groups with a population size of 100 individuals. Default cut-off values of 2.5 Å (dH-X) for hydrogen bonds and 4.0 Å for Van



Scheme 1. Synthesis of mono-hydroxy peracetylated carbohydrates (a) Ac_2O (1.1 eq.), DMAP (cat), pyridine, 95%; (b) *C. rugose* lipase immobilized on octyl-sepharose (Octyl-CRL), acetate buffer pH 5, 96%; (c) *A. niger* lipase immobilized on CNBr-sepharose (CNBr-ANL), acetate buffer pH 5, 96%.

der Waals distances were employed. When the top three solutions attained RMSD values for all ligand atoms within 1.5 Å, GA dockings were terminated. The RMSD values for each docking are based on the RMSD matrix of the ranked solutions. The best solutions were always among the first 5 GA runs. The best conformations of the docked molecules, based on the best GOLD fitness score (>50.00), were further analyzed to select the best fitted within the active site and compared with the crystallographic complex.

3. Results and discussion

Firstly, the effect of the presence of acetyls groups in the sugar moiety on the enzyme activity was evaluated with the natural sugars inhibitors, glucose, galactose and lactose in free and per-acetylated form. Monohydroxy peracetylated α-D-glucose **1** and D-lactose **2** were prepared by the biocatalytic approach (Scheme 1) previously described [26], in this case for the first time in multimilligram scale without any further purification.

Peracetylated α-D-glucose was prepared from D-glucose by treatment with acetic anhydride in 96% yield. Then, the regioselective hydrolysis in *C*-6 to synthesize **1** was catalyzed by the biocatalytic preparation octyl-CRL in aqueous media and room temperature at pH 5, with the aim of preventing the chemical acyl migration catalyzed by neutral or basic pH that can occur after the deprotection of saccharides on primary positions [26].

Commercial peracetylated lactose was regioselectively hydrolyzed in the anomeric position with 96% overall yield at pH 5, catalyzed by the CNBr-ANL preparation (Scheme 1).

The inhibition ability of **1** and **2** was determined and compared with the corresponding peracetylated and free carbohydrates. The IC_{20}, IC_{50} and K_i values are summarized in Table 1. Inhibition assays were performed in phosphate buffer containing 25% (v/v) acetonitrile at pH 6. Under these conditions both monodeprotected products were completely stable without any traces of possible migration subproducts. Determination of IC_{20} and IC_{50} values was performed by UV-spectrophotometry, measuring the residual hydrolytic activity of the enzyme with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) in the presence of varying concentration of each carbohydrate. Determination of K_i values was carried out based on the K_m calculated for the enzyme and the IC_{50} values [29].

Table 1
Inhibition values of β-galactosidase from *E. coli* by free, peracetylated and one-hydroxyl free acetylated glycosides.

Compound	IC_{20} [a]	IC_{50} [a]	K_i [b]
Galactose	91 ± 5.0	227 ± 10.0	2698
Glucose	100 ± 5.0	250 ± 10.0	2971
Peracetylated glucose	42 ± 0.9	106 ± 5.0	1273
1	12 ± 0.6	25 ± 0.9	297
Lactose	40 ± 2.0	131 ± 5	1550
Peracetylated lactose	38 ± 2.0	108 ± 5	1283
2	10 ± 0.5	20 ± 0.9	237

[a] The values are reported in mM and the averages have been obtained from triplicate analysis of each compound.
[b] The values are reported in μM.

The per-acetylated glucose was a stronger inhibitor (more than 2 fold) toward this enzyme, compared to glucose or galactose (Table 1). However, the simple monodeprotection of peracetylated glucose (compound **1**) increased the inhibition capacity of the molecule up to 6 fold (about 10 compared to glucose). The K_i value achieved with **1** was 297 μM, a moderate value, but similar to the results of several iminosugars and near the values obtained for the DNG derivative in Fig. 1. The results using galactose as glycosyl structures were similar to those obtained with glucose (data not shown).

Lactose is the natural substrate of this enzyme but it is a better inhibitor compared with glucose or galactose. The IC_{50} and K_i values achieved using lactose as substrate revealed that the inhibition power is similar to the peracetylated glucose (Table 1).

The peracetylation of the lactose did not change the inhibition capacity of the molecule too much, although the removing the acetyl group in the anomeric position (compound **2**) improved the inhibitory ability (5 fold) of the molecule (Table 1), with a slightly better inhibition value (K_i of 237 μM) than using **1**.

A molecular docking study was attempted using GOLD program [30] in order to allow a better understanding of the implication of the presence of acetyl groups in the decrease of enzymatic activity, and specially, the additional improvement after removing one of them in a specific position.

Initially, the different glucose molecules were docked into the active site of the β-galactosidase from *E. coli*, using the crystallographic complex of this enzyme with its natural substrate (lactose) as template (PDB code 1JYN) (Fig. 2) [14]. All molecules presented good docking scores (GOLD fitness score >50.00) except for the glucose. The presence of acetyl groups confers the sugar molecule a better arrangement in the active site. The Trp999 plays a key role packing the sugar rings of peracetylated glucose and **1**, but not of glucose (Fig. 2). Residue Glu461is involved in strong hydrogen bonds.

Peracetylated lactose followed a similar interaction pattern to that found in the crystal structure of the β-galactosidase:lactose complex (Fig. 3A), which corroborates the similar experimental inhibition ability of both molecules. However, a simple removing of the acetyl group in the anomeric position in **2** gave a completely different interaction pattern than the corresponding fully acetylated molecule, emphasized in the interaction between Trp999 with carboxyl group of acetyl in C_3 or Q537 with this in C_3, or a possible influence of the O in C_3' in the interaction between His418 and Mg^{2+} ion (Fig. 3B and C).

Interestingly, both molecules **1** and **2** presented a polar interaction with the Na^+ ion through their hydroxyl groups at the non-acetylated position (C-6 for **1** and anomeric position for molecule **2**) (Figs. 2B and 3C). This sodium atom presented a relevant role in the coordination of the lactose ligand in the β-galactosidase:lactose complex [14] and seems to also be relevant in the coordination of the enzyme with the acetylated sugar.

Thus, although the potencies of **1** and **2** were promising, structural variations considering the position of the OH free, the blocking group in anomeric position, and glucosidic or galactosidic structure, were introduced to study their influence on the inhibition ability of this enzyme activity. A small library of different monohydroxy-acetylated glycosides (**3**–**13**) (Fig. 4) was synthesized *via* a biotransformation using immobilized lipases in one step,

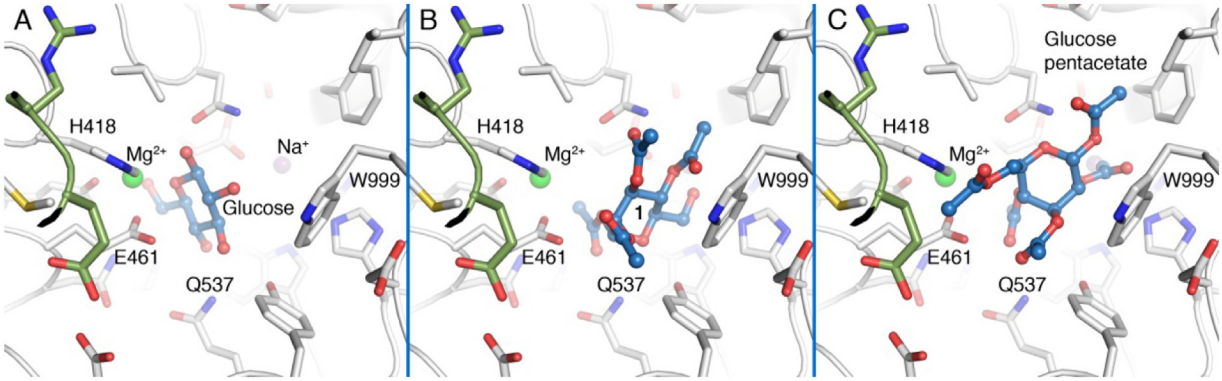


Fig. 2. Molecular docking of glucose, glucose pentaacetate and **1** on the β-galactosidase active site. (A) Glucose. (B) **1**. (C) Glucose pentaacetate. Each ligand molecule is drawn as blue ball-sticks. White and green sticks represent the protein monomers, which conforms the active site of the β-galactosidase. Green and violet spheres represent Mg^{2+} and Na^{+} ions respectively. Relevant residues involved in the coordination of Mg^{2+}, packing of the molecule and catalysis are labeled. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

similarly to **1** and **2**, previously described [26]. Most of the products were obtained in pure form with isolated yields between 80% and 95% (between 100 mg and 600 mg) without further purification or after a flash chromatography column.

Inhibition assays were performed in phosphate buffer containing 20% (v/v) acetonitrile at pH 6 conditions, both monodeprotected products were completely stable and no traces of possible migration subproducts were detected.

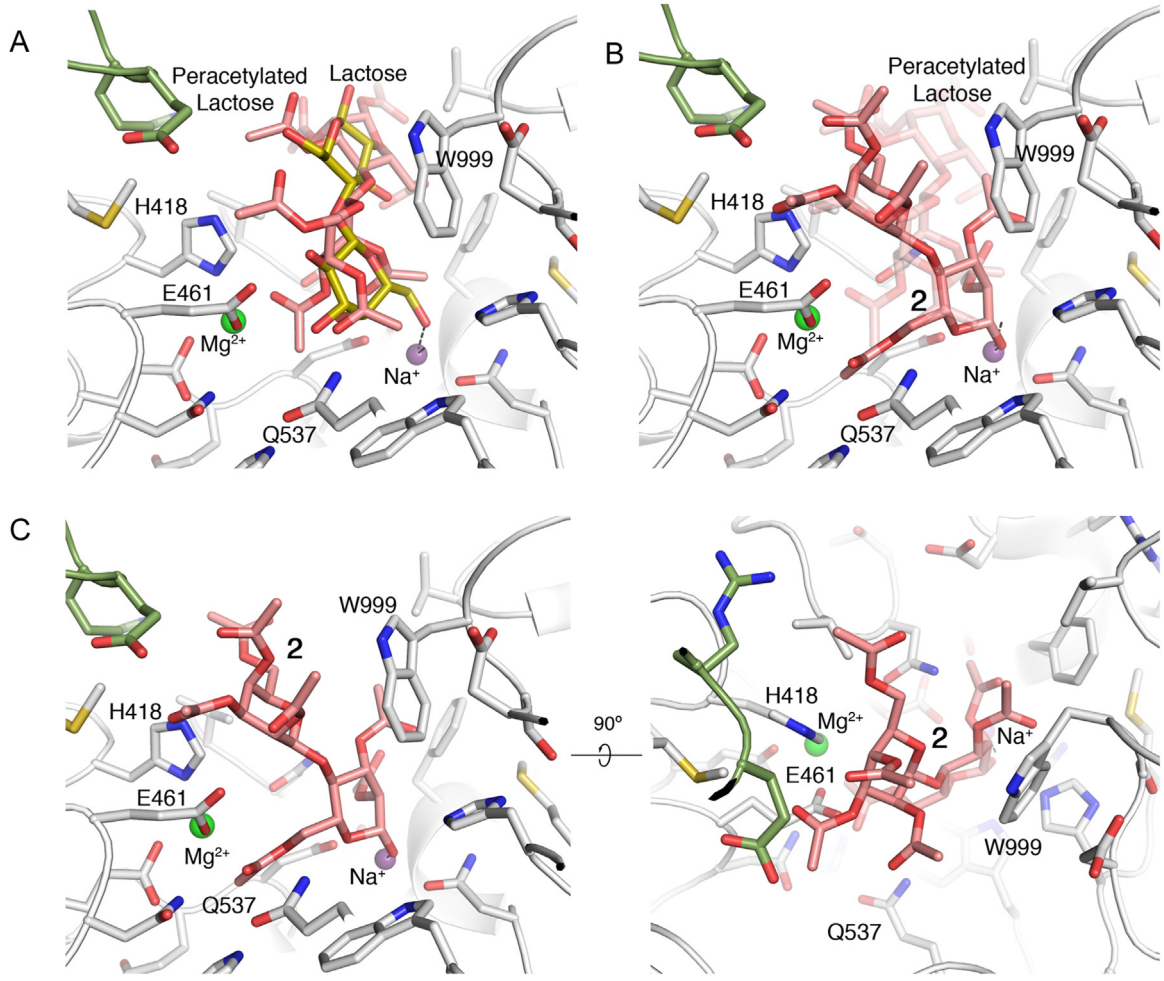


Fig. 3. Molecular docking of lactose, peracetylated lactose, and **2** on the β-galactosidase active site. (A) Comparison of the best-fitted peracetylated lactose and the crystallographic conformation of the lactose. (B) Comparison of the best-fitted **2** and the crystallographic conformation of the lactose. (C) **2** Lactose is represented as yellow sticks. Peracetylated lactose and **2** are represented as pink sticks. White and green sticks represent the protein monomers that conform the active site of the β-galactosidase. Mg^{2+} and Na^{+} ions are drawn as green and violet spheres, respectively. Relevant residues involved in the coordination of Mg^{2+}. Packing of the molecule and catalysis are labeled. The dotted lines indicate the interactions between the molecules (lactose or **1**) and the Na^{+} ion. **2** interacts with the Na^{+} ion through its hydroxyl group at the non-acetylated position 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

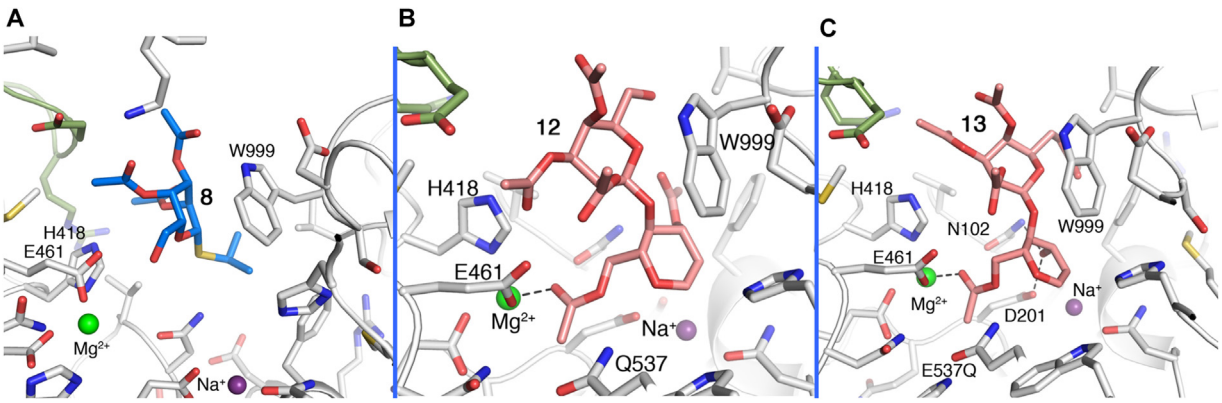


Fig. 5. Molecular docking studies on β-galactosidase crystal structure. (A) Conformation of **8**. (B) Conformation of **12**. (C) Conformation of **13**. **8** is represented as blue sticks. **12**, **13** are represented as pink sticks. White and green sticks represent the protein monomers that conforms the active site of the β-galactosidase. Mg^{2+} and Na^{+} ions are drawn as green and violet spheres respectively. Relevant residues involved in the coordination of Mg^{2+}, packing of the molecule and catalysis are labeled. The dotted line indicates the interaction between the lactose and the Mg^{2+} ion. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

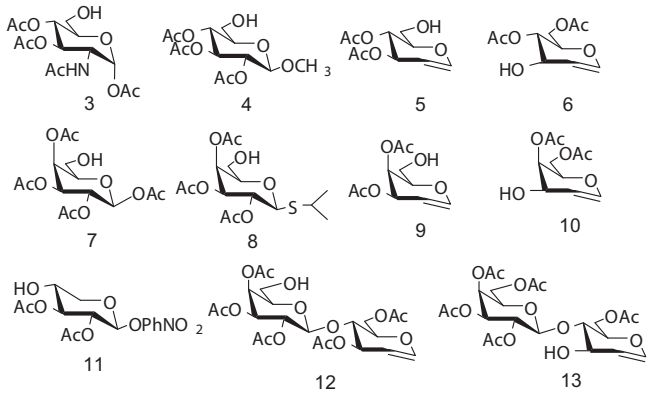


Fig. 4. Different monohydroxy peracetylated carbohydrates prepared to evaluate their enzyme inhibitor activity.

Table 2
Inhibition values of β-galactosidase from *E. coli* by the different synthesized monohydroxy peracetylated compounds.

Compound	IC_{20} [a]	IC_{50} [a]	K_i [b]
3	15 ± 0.7	25 ± 1.2	297
4	10 ± 0.5	25 ± 1.2	297
5	15 ± 0.7	25 ± 1.2	297
6	6 ± 0.3	25 ± 1.2	297
7	5 ± 0.3	25 ± 1.2	297
8	6 ± 0.3	12 ± 0.6	143
9	12 ± 0.6	20 ± 1.0	238
10	8 ± 0.4	15 ± 0.7	178
11	12 ± 0.6	25 ± 1.2	297
12	2 ± 0.1	8 ± 0.4	95
13	4 ± 0.2	12 ± 0.6	143

[a] The values are reported in mM and the averages were obtained from triplicate analysis of each compound.
[b] The values are reported in μM.

Evaluation of a series of compounds, as galactosidase inhibitors, revealed interesting variations (Table 2).

Several structural modifications of **1**, such as including an amino group in *C*-2 (**3**), blocking the anomeric position by a methyl group (**4**) or by *C*-1,2 double bond as glucal (**5**) did not cause any modification in the inhibition constants (Table 2). Nor did the use of a xylopyranose with *p*-nitrophenyl group in amoneric position (**11**) result in any improvement. However, the modification in the galactosidic structure gave some changes.

The introduction of a thiisopropyl moiety in the anomeric position (**8**) compared to the acetyl group (**7**) improved the inhibitory effect of the molecule on β-galactosidase activity from K_i of 297 μM to K_i of 143 μM (Table 2).

In the galactal, the position of the OH free has an influence on the final inhibition value, being the molecules with the OH free in *C*-3 (**10**) stronger inhibitors than in *C*-6 (**9**).

However, the best inhibition value of the library was achieved by using the disaccharide **12**, peracetylated lactal structure with the OH free in *C*-6 of the galactosidic part of the molecule (Fig. 4), with K_i of 95 μM. The compound **13**, with the OH free in *C*-3 in the glucosidic part of the molecule, displayed a higher K_i than **12** and the same inhibition values as **8**.

The molecular docking of **8** showed a strong interaction between the isopropyl group and the Trp999 to explain the inhibitory effect (Fig. 5A). In the case of disaccharides **12** and **13**, the docking revealed that both molecules display a strong polar interaction (2 Å) with the Mg^{2+} ion, not found in the β-galactosidase:lactose complex. The differences in the inhibitory capacity can be due to the different interactions of **12** or **13** with the protein. An interaction of acetyl group with the His418 or the OH free in 6 with the N in the Trp can be observed in **12** (Fig. 5B). Whereas in the case of **13**, we can emphasize the interaction of the OH free in **13** with the D201 or, the acetyl group with Trp999 (Fig. 5C). In fact, the best docking score was achieved with **12**.

4. Conclusion

Here and for the first time, we report the influence of the fully acetylation of a sugar molecule to improve its inhibition capacity against a β-galactosidase. More interesting is that the simple removing of one of these acetyl groups, which can be rapidly synthesized in multi-milligrams scale by an enzymatic protocol, introduces additional improvement generating new and better β-galactosidase inhibitors. The computational molecular docking studies revealed that the introduction of the acetyl groups in the carbohydrate structure helps the substrate to accommodate into the active site and even the presence of the free OH in the monodeprotected products intensify the interaction between the molecule and the protein. In this way, different sugars with structural variations considering the position of the OH free, the blocking group in anomeric position, and glucosidic or galactosidic structure were synthesized and evaluated as inhibitors of β-galactosidase from *E. coli.* From this small library, the monohydroxy lactal derivatives exhibited the best inhibition values. A small modification, such as

the position of the OH free in the molecule, in *C*-6 of the galactosidic part for **12** or in *C*-3 of the glucosidic part for **13**, was critical for the final inhibition value (K_i) of 95 μM and 143 μM respectively.

Therefore, this study has demonstrated that the presence of all hydroxyl groups in a sugar molecule is not necessary to inhibit a glycosidase.

These results could then open up a new concept in the synthetic approach to prepare glycosidase inhibitors. Several strategies could be further developed: for example, the application of protecting groups with different nature, the number and which hydroxyls groups are maintained free or the extension to other glycosidases (rational design of enzyme inhibition involved in cancer or Alzheimer).

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