Available online at www.sciencedirect.com
ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 52–53 (2008) 153–157
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Synthesis of novel glycoconjugates and evaluation as inhibitors against β-glucosidase from almond

María Pérez [a], F. Javier Muñoz [a], Eva Muñoz [a,b], María Fernández [a], José Vicente Sinisterra [a,c], María J. Hernáiz [a,b,c,*]

[a] *Biotransformations Group, Organic and Pharmaceutical Chemistry Department, Pharmacy Faculty, Complutense University, 28040 Madrid, Spain*
[b] *Biomolecular Interactions Unit, Pharmacy Faculty, Complutense University, 28040 Madrid, Spain*
[c] *Industrial Biotransformations Unit, Scientific Park, 28760-Tres Cantos-Madrid, Spain*

Available online 16 January 2008

Abstract

Hydrolytic activity of glycosidases can be influenced by inhibitors of glycosidic nature. We have synthetized a serie of glycoconjugates consisting of galactose, glucosaminide or lactose 1-*O*-linked with allyl alcohol, pentenol or tetraethyleneglycol moieties. These compounds were found to be inhibitors of the hydrolase activity of the β-glucosidase from almond, with K_i in the range of 7.7–107 mM and presenting different types of inhibition. Based on the structure of the functionalized glycosides and the inhibition studies performed, we suggest that the nature of the carbohydrate is determinant in the inhibitory strength of these glycoconjugates.

Keywords: β-Glucosidase; Almond; Glycoconjugate; Inhibitor

1. Introduction

In recent years, glycobiology has received an enormous interest due to important roles that carbohydrates play in biological processes. Oligosaccharides and glycoconjugates (e.g. glycoproteins, proteoglycans or glycolipids), present on the cellular surface, play key biological roles through molecular recognition events [1,2]. The detailed investigation of these phenomena requires the synthesis of oligosaccharides with specific structural features that will be used in posterior studies.

To date, the chemical synthesis of oligosaccharides has been extensively used but it has several disadvantages related with the need of numerous protection–deprotection steps [3]. In this sense, the enzymatic synthesis of oligosaccharides employing glycosyltransferases and glycosidases is advantageous [4,5]. It avoids the use of protecting groups and provides regio- and steroselectivity, reducing time and effort.

However, glycosidases could be seen influencing their activity by the presence of glycoconjugates and glycomimetics, which can act as inhibitors of these enzymes, having a tremendous potential as lead for therapeutic reagents. The most important class are the family of azasugars [6–9]. As an example, 1-deoxynojirimycin, first synthetized by Paulsen and Todt [10] is a potential inhibitor of glycosidases in vitro. A second group of inhibitors are glycosylamines, where the introduction of substituents on the amino functionality can modulate the inhibitory potency of the molecule, presumably through binding between the substituent and specific amino acid residues immediately adjacent to the active site of the enzyme [11–13]. Other types of inhibitors include the glycopeptides and their mimetics [14] and the spiro derivatives. The latest were shown to be weak inhibitors of the β-glucosidase from almond (K_i 4.5–5.9 mM) [15].

In an attempt to find novel inhibitors we have synthesized a series of functionalized mono- and disaccharides and carried out studies on its inhibitory hydrolytic activity against β-glucosidase from almond.

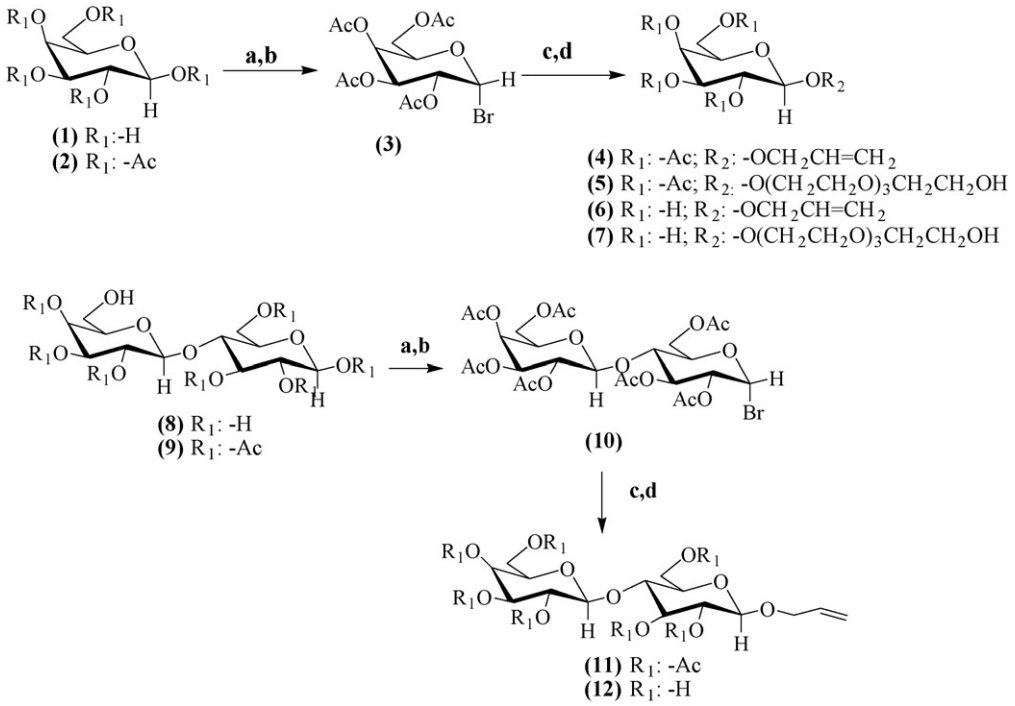
2. Results and discussion

2.1. Synthesis of the functionalized mono- and disaccharides

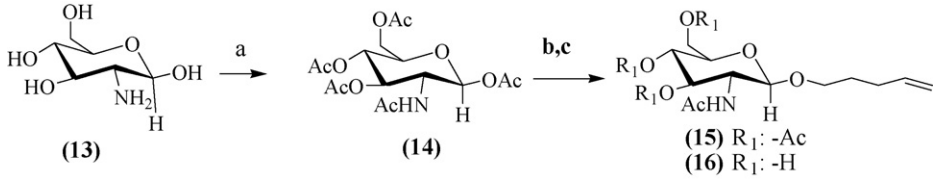
We carried out the synthesis of five functionalized saccharides using galactose, glucosaminide and lactose as starting

* Corresponding author at: Biotransformations Group, Organic and Pharmaceutical Chemistry Department, Pharmacy Faculty, Complutense University, Pz/ Ramon y Cajal, s/n, 28040 Madrid, Spain.
E-mail address: mjhernai@farm.ucm.es (M.J. Hernáiz).

1381-1177/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2007.11.021



Scheme 1. Reagents and conditions: (a) Ac_2O, py, (b) 30% HBr in AcOH, (c) alcohol, HgO, $HgBr_2$ and (d) MeONa/MeOH.



Scheme 2. Reagents and conditions: (a) Ac_2O, py, (b) TMSOTf, 1-pentenol and (c) NaOMe, MeOH.

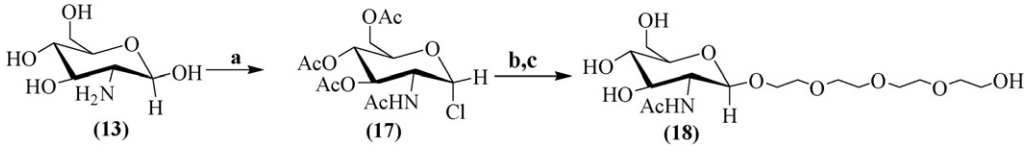
materials and allyl alcohol, tetraethylene glycol and 1-pentenol as linkers. Three different methodologies were used.

For the synthesis of the protected glycoconjugates **4**, **5** and **10** the method of Dasgupta and Anderson [16] was followed consisting of peracetylation of the carbohydrate followed by bromination of the anomeric center and posterior reaction with the linker in the presence of mercury(II) oxide (Scheme 1). In all cases the reaction yields calculated are in the range of those expected for this methodology (65%).

The synthesis of the protected glycoconjugate **15** was achieved with the method of de Paz et al. [17], starting from peracetylated 2-acetamido-2-deoxy-D-glucosaminide **14**, and employing TMSOTf at 40 °C to incorporate the pentenyl linker (Scheme 2). Here, compound **15** was obtained with a slightly lower yield as expected from published data (60%).

The deprotection of **4**, **5**, **10** and **15** was carried out in basic media giving rise to the final functionalized saccharides allyl-β-D-galactopyranoside **6**, 1-tetraethylene glycol-β-D-galactopyranoside **7**, 1-allyl-β-D-lactose **12** and 1-pentenyl 2-acetamido-2-deoxy-β-D-glucopyranoside **16** in yields ranging from 80 to 95%.

For the synthesis of **18**, the intermediate *N*-acetyl-tri-*O*-acetyl-2-amino-2-deoxy-galactopyranosyl chloride **17** was synthesized in one step following the Horton's methodology [18] and using **13** as substrate (Scheme 3). Compound **17** reacted with tetraethylene glycol in the presence of mercury cyanide and the



Scheme 3. Reagents and conditions: (a) CH_3COCl, (b) $Hg(CN)_2$, tetraethyleneglycol and (c) NaOMe, MeOH.

Table 1
Inhibition studies of -glucosidase from almond with glycoconjugates

Compounds	K_i (mM)	Inhibition type
6	7.96 ± 0.07	NC
7	9.1 ± 0.9	UC
12	9.7 ± 0.25	NC
16	107 ± 8	UC
18	36.8 ± 0.65	C

Inhibition type: (C) competitive; (NC) non-competitive; (UC) uncompetitive.

resulting intermediate was immediately treated with NaOMe in MeOH to obtain the unprotected compound **18** with 40% yield.

2.2. Inhibition studies

We investigated the capacity of **6**, **7**, **12**, **16** and **18** to inhibit the enzymatic activity of the β-glucosidase from almond (24 μU/mg prot) using UV spectroscopic methodology. The hydrolysis of the substrate *p*-nitrophenyl-β-D-glucoside, *p*NP-β-Glc (0.5–4 mM) was monitored, measuring the rate of released *p*-nitrophenol (UV Abs. at λ = 410 nm) at 37 °C. The experiment was repeated in the absence and presence of two different concentrations of each functionalized saccharide. The Michaelis–Menten constant in the absence of inhibitor (K_m = 7.94 mM), inhibition constants (K_i) and the type of inhibition (competitive, non-competitive and uncompetitive) were determined from Lineweaver–Burk plots [19].

The synthesized compounds **6**, **7**, **12**, **16** and **18** were found to be inhibitors of the β-glucosidase from almond with K_i values in the millimolar range. Thus, they showed different types of inhibition (Table 1). Compound **6** resulted to be the most potent inhibitor with a K_i of 7.96 mM, and a non-competitive mode of action. However, inhibition was only observed at low concentrations, possibly due to its limited solubility in buffer. The same type of inhibition was presented by compound **12**, whose K_i is 9.7 mM (Fig. 1). Compounds **7** and **16** showed uncompetitive inhibition with a K_i of 9.1 and 107 mM, respectively (Fig. 2) and only compound **18** exhibited competitive inhibition with a K_i of 37 mM (Fig. 3). These K_i values calculated are in the same order as the values described for the *C*-(D-glycopyranosyl)ethylamines [11] and D-glucopyranosylidene-spiro-oxathiazoles [15] under similar experimental conditions (Table 2).

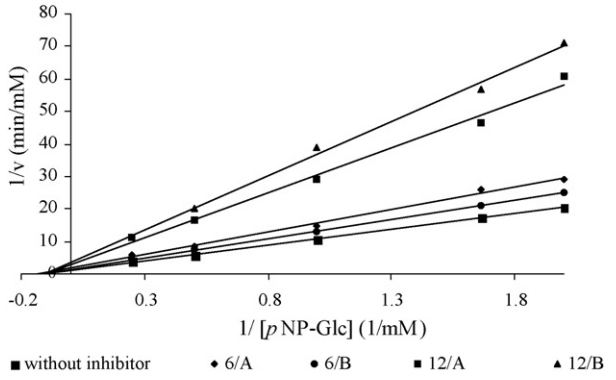


Fig. 1. Lineweaver–Burk plot. Hydrolysis of *p*NP-β-Glc by β-glucosidase from almond with different concentrations of **6** (A: 3 mM and B: 6 mM) and **12** (A: 1.7 mM and B: 3.3 mm). The common point of intersection at *x* axis indicates a non-competitive inhibition.

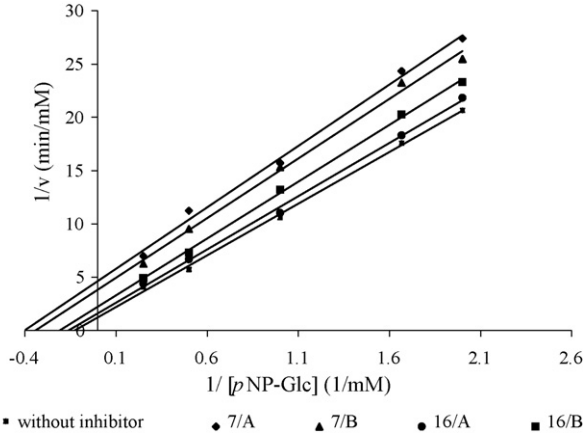
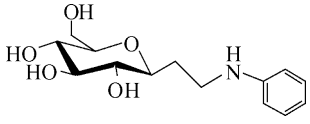
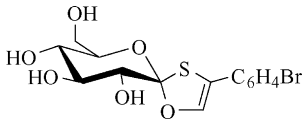
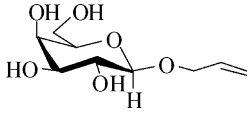


Fig. 2. Lineweaver–Burk plot. Hydrolysis of *p*NP-β-Glc by β-glucosidase from almond with different concentrations of **7** (A: 14 mM and B: 28 mM) and **16** (A: 32 mM and B: 64 mM). The absence to common point of intersection indicates an uncompetitive inhibition.

Table 2
Inhibition constants obtained with β-glucosidase from almond at 37 °C

Type of sugar		Buffer	K_i (mM)	Reference
Glycosilamines		Phosphate 70 mM pH 6.8	0.15	[11]
Spirooxathiazoles		Citrate-phosphate 0.2 M pH 5.2	6.7	[15]
Glycoconjugates		Phosphate 50 mM pH 6.8	7.9	

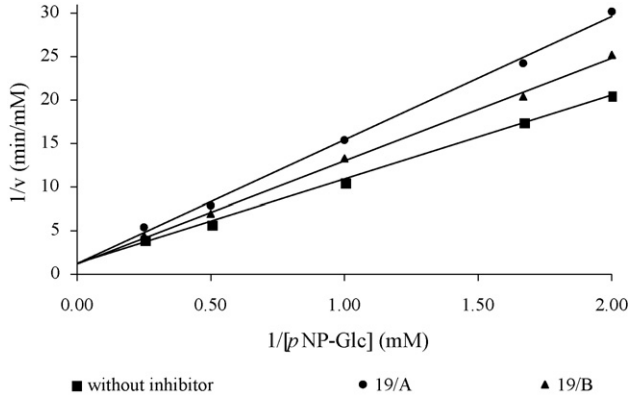


Fig. 3. Lineweaver–Burk plot. Hydrolysis of *p*NP-β-Glc by β-glucosidase from almond with different concentrations of **19** (A: 7.3 mM and B: 14.6 mM). The common point of intersection at *y* axis indicates a competitive inhibition.

3. Conclusions

A serie of glycoconjugates has been synthesized and evaluated as inhibitors of the β-glucosidase from almond. All the compounds showed inhibitory activity at certain extent. The analysis of the K_i values demonstrated that compounds **6**, **7** and **12** are the best inhibitors among the five compounds tested while **16** and **18** are weaker inhibitors. **6**, **7** and **12** have linkers of different nature but share a common structural motif, that is the presence of the galactose residue. **7** and **18** with galactose and *N*-acetylglucosamine, respectively, share the same tetraethylene glycol linker; however, **7** is a better inhibitor of the hydrolysis. These data suggest that the nature of the carbohydrate is determinant in the inhibitory ability meanwhile the nature of the linker is less relevant. On the other hand, galactose itself is a galactosyl acceptor of the β-glucosidase from almond but inhibits the hydrolysis when it is modified with allyl and tetraethylene glycol linkers. Consequently, the presence of the linker moiety is essential for the inhibition to occur.

To conclude, the functionalized mono- and disaccharides synthetized can be used as a starting point for the rational design of new glycosidase inhibitors based on carbohydrates.

4. Experimental

4.1. General

The commercially available β-glucosidase from almond was purchased by Sigma. All other chemicals were obtained from commercial sources. TLC on Kieselgel Plates 60 F_{254} (SDS) was used to follow the evolution of the reactions. TLC plates were revealed with phosphomolybdic acid in MeOH and 10% H_2SO_4 and heating. Column chromatography was carried out, if necessary, on silicagel 60, AC, 40–63 μm (purchased from SDS). UV–visible spectra were recorded on a UV-2401 PC Shimadzu. Elemental analysis and mass spectrometry were carried out by the Support Services (CAI) of the UCM. NMR spectra of samples in $CDCl_3$, CD_3OD or D_2O were recorded on a Bruker Avance 250 (250 MHz) spectrometer. The assignment of the ^1H NMR spectra was based on COSY, TOCSY and ^1H,^{13}C-heteronuclear HMQC and HMBC NMR experiments.

4.2. Experimental procedures

4.2.1. Synthesis of the functionalized mono- and disaccharides

Synthesis of 1-allyl-β-D-O-tetracetylgalactopyranoside **4**. This compound was synthesized as previously described by Dasgupta and Anderson [16]. The acetylated compound, 2,3,4,6-tetracetyl-α-D-*O*-galactopyranose **2** (15 mmol) was dissolved in CH_2Cl_2 (27 mL) and treated with 30% HBr in AcOH (27 mL) to give the compound 1-bromo-2,3,4,6-α-D-*O*-tetracetylgalactopyranose **3** readily isolated as a solid after washing with water, extraction and evaporation of the organic solvent in vacuo. Next, allyl alcohol (0.11 mmol) was dissolved in CH_2Cl_2 in the presence of HgO (2.5 g), $HgBr_2$ (0.19 g) and 3 Å molecular sieves. After 30 min, the bromide **3** (12 mmol) was dissolved in the minimum amount of CH_2Cl_2 and added to the mixture that was left stirring overnight. The mixture was filtered and the filtrate washed with water, dried ($MgSO_4$) and the solvent was evaporated in vacuo. The residue was purified by flash chromatography (petroleum ether/EtOAc 2:1) to yield **4** (65%). TLC 0.48 (petroleum ether/EtOAc 2:1). ^1H NMR [20].

Synthesis of 1-tetraethyleneglycol-β-D-O-tetracetylgalactopyranoside **5**. This compound was obtained similarly to compound **3** starting from tetraethylene glycol as linker and purified by flash chromatography (CH_2Cl_2/acetone 3:2) to yield **5** (67%) TLC 3.1 (CH_2Cl_2/acetone 3:2).

^1H NMR (250 MHz, $CDCl_3$): 5.32 (d, 1H, $J = 3.3$ Hz, H-4); 5.15 (dd, 1H, $J = 7.8$ Hz, $J = 10.5$ Hz, H-2); 4.95 (dd, 1H, $J = 3.4$ Hz, $J = 7.8$ Hz, H-3); 4.52 (d, 1H, $J = 7.9$ Hz, H-1); 4.14 (m, 2H, H-6a, H-6b); 3.86 (m, 1H, H-5); 3.62–3.82 (m, 16H, $-(CH_2)_2-$); 2.17, 2.08, 2.07, 2.00 (4s, 12H, $-COCH_3$).

Synthesis of 1-allyl-2,3,6,12′,3′,4′,6′-β-D-O-octaacetyllactose **10**. This compound was obtained similarly to compound **3** using lactose as the starting saccharide and was purified by flash chromatography (toluene/acetone 3:1) to yield **10** (50%) TLC 0.4 (toluene/acetone 3:1). ^1H NMR [16].

Synthesis of 1-pentenyl-2-acetamido-3,4,6,-tri-O-acetyl-2-deoxy-β-D-glucopyranoside **15**. This compound was synthesized as previously described by de Paz et al. [17]. To a solution of 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-D-glucopyranoside **14** (1 g, 2.5 mmol) in dry CH_2Cl_2 (15 mL), TMSOTf (3.63 mmol) was added and the mixture stirred at 40 °C for 72 h. Then, 4-pentenol (7.5 mmol) was added and stirred at that temperature for an additional 3 h. The suspension was neutralized with Et_3N, filtered and the solvent was evaporated to dryness. The residue was purified by flash chromatography (CH_2Cl_2/acetone 3:1) to yield **15** (60%). TLC 0.75 (CH_2Cl_2/acetone 3:1).

^1H NMR (250 MHz, $CDCl_3$) 5.75 (m, 1H, $-CH{=}CH_2$); 5.61 (d, 1H, $J = 8.6$ Hz, –NH–); 5.27 (t, 1H, $J = 9.9$ Hz, H-3); 5.03 (t, 1H, $J = 9.6$ Hz, H-4); 4.98–4.92 (m, 2H, $-CH{=}CH_2$); 4.64 (d, 1H, $J = 8.3$ Hz, H-1); 4.22 (dd, 1H, $J = 4.8$, 12.2 Hz, H-6a); 4.09 (dd, 1H, $J = 2.3$, 12.2 Hz, H-6b); 3.86–3.77 (m, 2H, H-2, $-CH_2-O-$); 3.67 (m, 1H, $-CH_2-O-$); 2.17 (m, 4H, $J = 7.5$ Hz, $-(CH_2)_2-$); 2.04, 1.99, 1.98, 1.91 (4s, 12H, $-COCH_3$); 2.05–1.59 (m, 4H, $-(CH_2)_2-$).

Synthesis of 1-tetraethylenglycol-2-acetamido-2-deoxy-β-D-glucopyranoside **18**. Starting to *N*-acetyl-tri-*O*-acetyl-2-amino-2-deoxy-galactopyranosyl chloride **17** (8.2 mmol) was dissolved in a 1:1 mixture of toluene and nitrometane (12 mL) and mercury cyanide (8.2 mmol), $MgSO_4$ and tetraethylene glycol (321 mmol) were added. The mixture was stirred at room temperature overnight. After addition of 20 mL CH_2Cl_2 the reaction mixture was filtered over celite and the solvent was evaporated under reduced pressure. To the resulting syrup, 8.2 mmol of NaOMe in 30 mL MeOH were added and the mixture was allowed to react for 3 h at room temperature. The reaction was neutralized with Amberlite, the mixture was filtered and the solvent evaporated in vacuo. The final product **18** (29%) was purified by flash chromatography (CH_2Cl_2/MeOH 3:1). TLC 0.32 (CH_2Cl_2/MeOH 3:1). ESI-MS: [M + Na] Estimated: 420.4. Found: 420.0.

^{1}H NMR (250 MHz, $CDCl_3$) 5.32 (d, 1H, J = 5.3 Hz, H-4); 5.15 (dd, J = 10.5 Hz, J = 7.8 Hz, H-2); 4.95 (dd, 1H, J = 10.3 Hz, J = 3.3 Hz, H-3); 4.52 (d, 1H, J = 7.8 Hz, H-1); 4.14 (m, 2H, H-6a, H-6b); 3.86 (m, 2H, H-5, $-(CH_2)_2-$); 3.50–3.80 (m, 15H, $-(CH_2)_2-$); 2.07 (s, 3H, $-COCH_3$).

Synthesis of 1-allyl-β-O-galactopiranoside **6**, *1-tetraethylene glycol-β-O-galactopiranoside* **7**, *1-allyl-β-O-lactose* **12** *and 1-pentenyl 2-acetamido-2-deoxy-β-D-glucopyranoside* **16**. These compounds were obtained starting from **4**, **5**, **11** and **15**, respectively. Each compound (2 g) was dissolved in dry MeOH (2 mL) and NaOMe (2 g) was added. After stirring overnight at room temperature the reaction was neutralized with Amberlite IR-120H$^+$. The mixture was filtered and the solvent was evaporated in vacuo to obtain **6**, **7** and **12** in quantitative yield. TLC **6** 0.55, **7** 0.4, **12** 0.38, **16** 0.62 (isopropanol/nitrometane/water 10:9:2). ESI-MS (7): [M + Na] Estimated: 379.1. Found: 378.9.

6: ^{1}H NMR [16]; **12:** ^{1}H NMR [16]. **7:** ^{1}H NMR (250 MHz, $CDCl_3$) 4.28 (d, 1H, J = 7.6 Hz, H-1); 3.95 (m, 1H, H-5); 3.78 (d, 1H, J = 3.4 Hz, H-4); 3.60 (m, 18H, $-(CH_2)_2-$, H-6a, H-6b); 3.51 (m, 1H, H-3); 3.39 (dd, 1H, J = 7.6 Hz, J = 9.7 Hz, H-2). **16:** ^{1}H NMR (250 MHz, MeOD) 5.78 (m, 1H, $-CH=CH_2$); 4.99–4.83 (m, 2H, $-CH=CH_2$); 4.35 (d, 1H, J = 8.4 Hz, H-1); 3.88–3.82 (m, 2H, H-6a, $-CH_2-O-$); 3.67–3.58 (m, 2H, H-2, H-6b); 3.46–3.42 (m, 2H, H-4, CH_2-O-); 3.30–3.26 (m, 2H, H-3, H-5); 2.07–1.94 (m, 4H, $-(CH_2)_2-$); 1.94 (s, 3H,$-COCH_3$).

4.2.2. Enzyme activity assay

Protein concentration was determined by the Bradford method [21] using bovine serum albumin as standard. The activity of the β-Glucosidase from almond was assayed spectrophotometrically as follows: a sample of enzyme solution (20 μL) was added to 80 μL of 50 mM buffer phosphate pH 6.8 containing 4 mM *p*NP-β-Glc. The reaction mixture was incubated for 3 min at 37 °C. Absorbance was measured at 410 nm.

The K_m value for the hydrolysis of *p*NP-β-Glc catalysed by β-glucosidase from almond was determined by measurements of enzyme activity with various concentrations of substrate (0.5–4 mM). K_m was calculated by using Lineweaver–Burk double reciprocal plots [19].

One unit of enzyme activity is defined as that quantity of enzyme hydrolyzing 1 mmol of *p*NP-β-Glc under the conditions stated above.

4.2.3. Kinetics of enzyme inhibition

The enzyme activity for β-glucosidase from almond was measured according to the foregoing reaction conditions with substrate concentrations in the range of 0.5–4 mM and two inhibitor concentrations: **6** (3 and 6 mM), **7** (14 and 28 mM), **12** (1.7 and 3.3 mM), **16** (32 and 64 mM) and **18** (7.3 and 14.6 mM). The type of inhibition and K_i were determined using Lineweaver–Burk double reciprocal plots [19].

Acknowledgment

This work was supported by Spanish MEC (CTQ2006-09052/BQU), Post-doctoral contract Juan de la Cierva programme 2006 and MEC-FPU predoctoral fellowship (AP2003-4820).

References

[1] A. Varki, Glycobiology 3 (1993) 97–130.
[2] C.R. Bertozzi, L.L. Kiessling, Science 291 (2001) 2357–2364.
[3] B.G. Davis, J. Chem. Soc., Perkin Trans. 1 (2000) 2137–2160.
[4] G.J. Davies, T.M. Gloster, B. Henrissat, Curr. Opin. Struct. Biol. 15 (2005) 637–645.
[5] N. Wymer, E.J. Toone, Curr. Opin. Struct. Biol. 4 (2000) 110–119.
[6] B.A. Jhons, Y.T. Pan, A.D. Elbein, C.R. Johnson, J. Am. Chem. Soc. 119 (1997) 4856–4865.
[7] Y. Nishimura, Studies in Natural Products Chemistry, Atta-ur-Rahman, Amsterdam, 1992, p. 495.
[8] B. Winchester, G.W. Fleet, Glycobiology 2 (1992) 199–210.
[9] L. Gao, R.I. Hollingsworth, Tetrahedron 61 (2005) 3801–3811.
[10] H. Paulsen, K. Todt, Adv. Carbohydr. Chem. 23 (1968) 115–232.
[11] A.A.H. Abdel-Rahman, E.S.H. El Asir, R.R. Schmidt, Carbohydr. Res. 315 (1999) 106–116.
[12] T. Oku, A. Kimura, S. Chiba, A. Takatsuki, Bioorg. Med. Chem. Lett. 15 (2005) 1489–1492.
[13] L.L. Rossi, A. Basu, Bioorg. Med. Chem. Lett. 15 (2005) 3596–3599.
[14] L. Kröger, D. Henkensmeir, A. Schäfer, J. Thiem, J. Bioorg. Med. Chem. Lett. 14 (2004) 73–75.
[15] J.P. Praly, R. Faure, B. Joseph, L. Kiss, P. Rollin, Tetrahedron 50 (1994) 6559–6568.
[16] F. Dasgupta, L. Anderson, Carbohydr. Res. 264 (1994) 155–160.
[17] J.L. de Paz, R. Ojeda, A.G. Barrientos, S. Penadés, M. Martín Lomas, Tetrahedron Asymmetry 16 (2005) 149–158.
[18] D. Horton, Methodos in Carbohydrate. Chemistry, Academic Press, New York, 1972, p. 282.
[19] H. Lineweaver, D. Buró, J. Am. Chem. Soc. 56 (1934) 658–666.
[20] H.J. Vermee, K.H. Halkes, J.A. van Kuik, J.P. Kamerling, J.F.G. Vliegenthart, Perkin Trans. 1 (2000) 2249–2263.
[21] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.