ELSEVIER

Carbohydrate Research 304 (1997) 111–115

CARBOHYDRATE RESEARCH

An efficient chemical-enzymatic synthesis of 4-nitrophenyl β-xylobioside: a chromogenic substrate for xylanases

Adva Mechaly [a], Valery Belakhov [b], Yuval Shoham [a,*] Timor Baasov [b,*]

[a] *Department of Food Engineering and Biotechnology, Technion — Israel Institute of Technology, Haifa 32000, Israel*

[b] *Department of Chemistry, Technion — Israel Institute of Technology, Haifa 32000, Israel*

Received 12 March 1997; accepted in revised form 10 June 1997

Abstract

4-Nitrophenyl-β-xylobioside was synthesized by an improved short chemical-enzymatic method, based on the use of xylobiose as a starting material. Xylobiose was prepared following extensive enzymatic digestion of birchwood xylan with xylanase T-6. The resulting digest, containing mainly xylobiose and xylose, was directly subjected to an acetylation step, which after silica gel chromatography, provided highly pure hexaacetate of xylobiose. Bromination with HBr in acetic acid gave in quantitative yields the corresponding bromide, which after the coupling and deprotection steps, afforded the target 4-nitrophenyl-β-xylobioside.

Keywords: 4-Nitrophenyl-β-D-xylobioside; β-D-Xylobiosides; Xylan; 2,3,4,2′,3′,4′-Hexa-*O*-acetyl-xylobioside; Xylanase T-6; Xylanases (1,4-β-D-xylan xylanohydrolases; EC 3.2.1.8)

Xylanases (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) are hemicellulases that hydrolyze xylan, a major constituent of the hemicellulose complex. Xylan is composed of β-(1 → 4)-linked xylopyranose units that are acetylated or glycosylated on the free hydroxyls with α-D-glucuronic acid, α-L-arabinose, or β-D-galactose. The complete degradation of xylan requires a variety of enzymes including esterases and glycosidases. The most important enzymes are the endo-xylanases which cleave the major backbone. Xylanases have many biotechnological uses and potential applications, including bioconversion of lignocellulase material to fermentation products, clarification of juices, and improvement of the consistency of beer and the digestibility of animal feedstock [1]. Recently, the utilization of hemicellulases in bleaching of Kraft pulp has become one of the most important new large scale industrial applications of enzymes [2,3]. In fact, the large-scale use of xylanases for bio-bleaching is currently being considered by various paper manufacturers [2–4].

The biochemistry of xylanases is of major interest for both applied and basic science. However, good substrates for these enzymes are not available commercially. To date, the standard assay for xylanase

* Corresponding authors.

0008-6215/97/$17.00
PII S0008-6215(97)00209-7

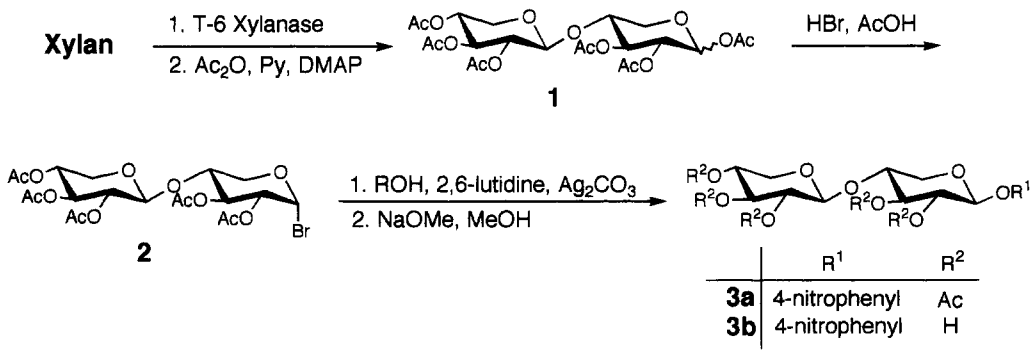
has been based on the accumulation of reducing sugars during the degradation of partially soluble xylan. Since the average molecular weight of the substrate changes during the reaction, this assay is highly variable and true kinetic constants cannot be obtained [5]. In addition, the use of different xylan substrates with different types and degrees of substitution hinders accurate comparison of reported activities.

Aryl glycosides of xylobioside have been recognized previously as superior substrates for xylanases. However, the direct method of making significant amounts of xylobiosides has been hampered by the very limited accessibility of β-(1 → 4)-linked *xylo*-oligosaccharides. Recently, Ziser and Withers [6] have developed a synthetic pathway for the preparation of nitrophenyl-β-xylobiosides via coupling of a suitably partially protected nitrophenyl-β-D-xylopyranoside with α-D-xylopyranosyl trichloroacetimidate. Although this synthetic route was successful, the procedure is very long and gives poor overall yields. An alternative approach for the synthesis of these nitrophenyl derivatives is to use xylobiose as a starting material. However, the high price of this commercially available compound limits its use.

In the present note, we describe an efficient, combined enzymatic and chemical synthesis for the preparation of 4-nitrophenyl-β-xylobioside that utilizes xylobiose as the starting sugar. Known procedures for the preparation of xylobiose include the enzymatic degradation of xylan [7,8], followed by tedious extraction and chromatographic steps which usually yield xylobiose in about 80–85% purity [8–10]. We have improved significantly the above procedure in the following ways: (1) Birchwood xylan was used as the starting material. Birchwood xylan is more homogeneous and has less substituents and branching points on the main xylose chain than other commercially available xylans (for example, oat spelts xylan [8]). (2) For the complete degradation of xylan, high quantities of recombinant xylanase T-6 were used. Xylanase T-6 is capable of completely degrading xylan to a mixture of xylobiose (target-product) and xylose (side-product). (3) This mixture, without any purification, was then directly subjected to acetylation (Ac_2O, pyridine, DMAP) which, after chromatography on silica gel, provided highly pure (> 95%) hexaacetate **1** (Scheme 1) as a mixture of anomers (β:α 1.2:1). The pure β anomer could be separated from this mixture by recrystallization from ethanol; however, the subsequent bromination reactions were successfully conducted on the anomeric mixture as described in the Experimental section. It is noteworthy that deacetylation of hexaacetate **1** is an efficient route for obtaining high amounts of pure xylobiose.

The bromination of glycosyl acetates is also a problematic step, since the resulting glycosyl bromides are unstable and difficult to obtain in large quantities. Consequently, various methods for bromination of sugars have been developed over the past years [11–15].

The target penta-*O*-acetylxylobiosyl bromide (**2**) is a known compound [15], but is difficult to obtain in large quantities and no data on this compound is available. In addition, **2** is usually obtained by a dangerous procedure which involves treatment of the reactant sugar with red phosphorus and bromine [11]. In the current study, compound **1** is treated with hydrogen bromide in acetic acid to produce the corresponding α-bromide **2** in near-quantitative yield. This reaction can be performed on a relatively large scale (up to 10 g) and the product bromide can be stored for long periods at −20 °C in ethyl acetate solution. Condensation of **2** with 4-nitrophenol in the presence of 2,6-lutidine and silver carbonate [9] affords the



Scheme 1. Chemical-enzymatic synthesis of 4-nitrophenyl-β-xylobioside (**3b**).

crystalline **3a** (67%), which is O-deacetylated to provide the nitrophenyl xylobioside **3b** in 36% overall yield from hexaacetate **1**.

In conclusion, a straightforward five-step chemical-enzymatic transformation has been devised to convert commercial xylan into 4-nitrophenyl-β-xylobioside in high overall yield. The synthesis has been used to provide several grams of the desired product in the course of a few days.

1. Experimental

General methods.—^{1}H NMR spectra were recorded on a Bruker AM-400 spectrometer, and chemical shifts reported (in ppm) relative to internal Me_4Si (δ 0.0) with $CDCl_3$ as the solvent and to HOD (δ 4.63) with D_2O as the solvent. ^{13}C NMR spectra were recorded on a Bruker AM-400 spectrometer at 100.61 MHz and the chemical shifts reported (in ppm) relative to the residual solvent signal for $CDCl_3$ (δ 77.00) or to external sodium 2,2-dimethyl-2-silapentane sulfonate (δ 0.0) for D_2O as the solvent. Mass spectra were obtained on a TSQ-70B mass spectrometer (Finnigan Mat) under fast-atom bombardment (FAB) condition in glycerol matrices or by negative chemical ionization (NCI) in isobutane.

Reactions were monitored by TLC on Silica Gel 60 F_{254} (0.25 mm, Merck) and spots were visualized by charring with a yellow solution containing $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (120 g) and $(NH_4)_2Ce(NO_3)_6$ (5 g) in 10% H_2SO_4 (800 mL). Flash column chromatography was performed on Silica Gel 60 (70–230 mesh). Melting points were determined using a Electrothermal IA-9300 melting point apparatus in open capillary tubes and are uncorrected. Optical rotations were measured at 20 °C using a Jasco DIP-370 digital polarimeter. Xylanase T-6 (E.C. 3.2.1.8) [16,17] (specific catalytic activity 400 U/mg) was overproduced and isolated from strain *E. coli* BL21(DE3) (pET9d) according to a recently published procedure [18].

2,3,4,2′,3′,4′-hexa-O-acetyl-xylobioside (**1**).—Birchwood xylan (3 g, Sigma, St. Louis) was suspended in 100 mL of 0.25% MOPS (3-[*N*-morpholino]propanesulfonic acid) buffer, pH 7.0. The enzymatic digestion was performed at 60 °C by portional addition of recombinant xylanase T-6 (2.8 mg in each addition) and the reaction progress was monitored by the reducing-sugar assay [19]. The complete digestion of xylan (12 h, and 3 enzyme additions) produced 122 μmol xylose equivalent per mL, which corresponds to a molar ratio of about 1:1 of xylose and xylobiose. The reaction mixture was precipitated by addition of cold acetone (to a final concentration of 70%) and centrifuged (20 min, 4 °C, 15,000 rpm). The supernatant was first rotary-evaporated under reduced pressure followed by lyophilization to give a mixture (2.3 g) of xylobiose (R_f 0.54, 5:4:1 acetone–*n*-butanol–water) and xylose (R_f 0.65) as a white foam.

The above mixture (2.3 g) was dissolved in freshly distilled dry pyridine (50 mL) and concentrated to dryness. This procedure was repeated twice in order to remove residual water. The residue obtained was dissolved in dry pyridine (50 mL) and 4-dimethylaminopyridine (1 g) was added. The mixture was cooled to 4 °C and freshly distilled acetic anhydride (30 mL) was introduced dropwise. The reaction progress was monitored by TLC (1:1 EtOAc–hexane), and the mixture was stirred at room temperature until all the starting material had been consumed (5 h). The reaction mixture was diluted with EtOAc (300 mL) and ice cold water (200 mL) was added. After thorough extraction of the water fraction with EtOAc, the organic fractions were combined and sequentially washed with dilute aq H_2SO_4, water and saturated NaCl. The dried ($MgSO_4$) organic layer was concentrated under reduced pressure, and the residue was purified by chromatography on a silica gel column (1:9 EtOAc–hexane) to give **1** (2.16 g, 4.05 mmol) as a mixture of anomers (β:α 1.2:1), and 1.30 g (4.0 mmol) of the peracetylated xylose, also as a mixture of anomers (β:α 1.3:1). The pure β-**1** could be obtained from the above mixture by recrystallizing twice from ethanol; mp 154–155 °C, lit 153–155 °C [20]; $[\alpha]_D^{20}$ −74.0° (*c* 1, $CHCl_3$), lit. −75° [21]; R_f 0.54 (6:4 EtOAc–hexane); ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.61 (d, 1H, $J_{1,2}$ 7.4 Hz, H-1), 4.94 (dd, 1H, $J_{2,3}$ 8.6 Hz, H-2), 5.12 (t, 1H, $J_{3,4}$ 8.6 Hz, H-3), 3.83 (ddd, 1H, $J_{4,5a}$ 9.3, $J_{4,5b}$ 5.1 Hz, H-4), 3.44 (dd, 1H, $J_{5a,5b}$ 12.1 Hz, H-5a), 3.98 (dd, 1H, H-5b), 4.53 (d, 1H, $J_{1',2'}$ 6.06 Hz, H-1′), 4.77 (dd, 1H, $J_{2',3'}$ 7.9 Hz, H-2′), 5.06 (t, 1H, $J_{3',4'}$ 7.9 Hz, H-3′), 4.85 (ddd, 1H, $J_{4',5'a}$ 7.8, $J_{4',5'b}$ 4.7 Hz, H-4′), 3.36 (dd, 1H, $J_{5'a,5'b}$ 12.0 Hz, H-5′), 4.06 (dd, 1H, H-5′b), 1.99, 2.00, 2.01, 2.02, 2.03, and 2.06 (6s, 18H, Ac); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.55 (3 OAc), 20.67 (3 OAc), 61.62 (C-5′), 63.32 (C-5), 68.32 (C-2), 69.87 (C-2′), 70.35 (C-3), 70.51 (C-3′), 71.96 (C-4′), 74.18 (C-4), 92.19 (C-1), 99.56 (C-1′), 168.84, 168.97, 169.40, 169.48, 169.66, and 169.81 (6 OAc); negative CIMS *m/z* 533.2 [$(M-H)^-$, $C_{22}H_{29}O_{15}$, Calcd 533.0].

2,3,2′,3′,4′-penta-O-acetyl-β-xylobiosyl bromide (**2**).—To a solution of **1** (2.53 g, 4.75 mmol) in dry 1,2-dichloroethane (25 mL) a solution of hydrogen bromide in 30% acetic acid (1.32 mL, 16.53 mmol) was added at room temperature. The reaction progress was monitored by TLC (3:2 EtOAc–hexane). After stirring for 2 h at room temperature, the reaction mixture was diluted with cold $CHCl_3$ (100 mL) and then ice cold 5% $NaHCO_3$ (30 mL) was introduced. The aqueous layer was washed twice with $CHCl_3$ and the combined organic layer was washed with water (100 mL), dried ($MgSO_4$) and concentrated under reduced pressure to afford the bromide **2** (2.63 g, 100% yield) as a yellow oil. This material was not purified by flash chromatography without decomposition and typically was used fresh for the next step. However, we found that the solution of bromide **2** in dry ethyl acetate at −20 °C can be stored for a few weeks without significant decomposition. Data for **2**: $[\alpha]_D^{20}$ +39.2° (*c* 10, $CHCl_3$); R_f 0.62 (6:4 EtOAc–hexane); ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.48 (d, 1H, $J_{1,2}$ 4.0 Hz, H-1), 4.65 (dd, 1H, $J_{2,3}$ 10.0 Hz, H-2), 5.42 (t, 1H, $J_{3,4}$ 9.8 Hz, H-3), 3.74–3.90 (m, 3H, H-4, H-5a, H-5b), 4.54 (d, 1H, $J_{1',2'}$ 5.8 Hz, H-1′), 4.74, (dd, 1H, $J_{2',3'}$ 7.53 Hz, H-2′), 5.04 (t, 1H, $J_{3',4'}$ 7.55 Hz, H-3′), 4.83 (ddd, 1H, $J_{4',5'a}$ 7.53, $J_{4',5'b}$ 4.5 Hz, H-4′), 3.36 (dd, 1H, $J_{5'a,5'b}$ 12.04 Hz, H-5′a), 4.05 (dd, 1H, H-5′b), 1.98, 1.99, 2.00, and 2.01 (4s, 15H, Ac). ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.57 (2 OAc), 20.67 (OAc), 20.73 (OAc), 20.94 (OAc), 61.41 (C-5′), 63.11 (C-5), 68.20 (C-2), 69.93 (C-2′), 70.12 (C-3), 70.21 (C-3′), 70.91 (C-4′), 74.05 (C-4), 87.49 (C-1), 99.42 (C-1′), 169.25, 169.27, 169.69, 169.84, and 169.85 (5 OAc).

4-Nitrophenyl-2,3,2′,3′,4′-penta-O-acetyl-β-xylobioside (**3a**).—Compound **2** (1.38 g, 2.49 mmol) was condensed with 4-nitrophenol (0.69 g, 4.97 mmol) according to a published procedure [9]. The product was purified by column chromatography (1:4 EtOAc–hexane) and then crystallized from the same solvents to give **3a** (1.15 g, 67%): mp 183–184 °C, lit. 182–184 °C [6]; $[\alpha]_D^{20}$ −83.4° (*c* 1, $CHCl_3$); R_f 0.38 (1:1 EtOAc–hexane), lit. 0.38 [6]; ^{1}H NMR data of **3a** were in agreement with the literature [6]. ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.59 (3 OAc), 20.67 (OAc), 20.74 (OAc), 61.67 (C-5′), 62.5 (C-5), 68.29 (C-2), 70.05 (C-2′), 70.41 (C-3), 70.53 (C-3′), 71.21 (C-4′), 73.94 (C-4), 97.90 (C-1), 99.69 (C-1′), 116.43, 125.71, 142.97, and 161.02 (Ar), 169.08, 169.45, 169.57, 169.72, and 169.84 (5 OAc). FABMS: *m/z* 613.4 (M^+ + H, $C_{26}H_{31}NO_{16}$, Calcd 613.0).

4-Nitrophenyl-β-D-xylobiose (**3b**).—Compound **3a** (1.15 g, 1.88 mmol) was O-deacetylated according to a published procedure [6]. The crude product was purified by column chromatography (7:3 $CHCl_3$–MeOH) to give **3b** (632 mg, 84%) as a foamy solid: $[\alpha]_D^{20}$ −88.5° (*c* 1, MeOH), lit. −88.8° [6]; R_f 0.32 (17:2:1 EtOAc–MeOH–H_2O), lit. 0.32 [6]. ^{1}H NMR data of **3b** were in agreement with the literature [6]. ^{13}C NMR (100 MHz, D_2O): δ 65.50 (C-5′), 67.69 (C-5), 71.61 (C-2), 74.90 (C-2′), 75.18 (C-3), 75.84 (C-3′), 78.04 (C-4′), 78.51 (C-4), 102.25 (C-1), 104.30 (C-1′), 118.76, 128.36, 144.73, and 163.96 (Ar). FABMS: *m/z* 402.6 (M^+ + H, $C_{16}H_{21}O_{11}$, Calcd 403.0).

Acknowledgements

This work was funded by grants 4935 and 5932 from the Israeli Ministry of Science and the Arts to Y.S. and T.B. V.B. acknowledges the financial support by the Center for Absorption in Science, Ministry of Immigration Absorption and the Ministry of Science and Arts, State of Israel (Gilady Program). Technical support was provided by the Technion Otto Meyerhof Biotechnological Laboratories established by the Minerva Foundation, Germany.

References

[1] K.Y. Wong, L.U.L. Tan, and J.N. Saddler, *Microbiol. Rev.*, 52 (1988) 305–317.
[2] L. Viikari, A. Kantelinen, J. Sundquist, and M. Linko, *FEMS Microbiol. Rev.*, 13 (1994) 335–350.
[3] P. Bajpai and P.K. Bajpai, *Adv. Biochem. Eng.*, 56 (1997) 1–31.
[4] K.R. Lundgren, L. Bergkvist, S. Hogman, H. Joves, G. Eriksson, T. Bartfai, J.V.D. Laan, E. Rosenberg, and Y. Shoham, *FEMS Microbiol. Rev.*, 13 (1994) 365–368.
[5] M.J. Bailey, P. Biely, and K. Poutanen, *J. Biotechnol.*, 23 (1992) 257–270.
[6] L. Ziser and S.G. Withers, *Carbohydr. Res.*, 265 (1994) 9–17.
[7] D. Kluepfel, S. Vats-Mehta, F. Aumont, F. Shareck, and R. Morosoli, *Biochem. J.*, 267 (1990) 45–50.
[8] J. Puls, A. Borchmann, D. Gottschalk, and J. Wiegel, *Methods Enzymol.*, 160 (1988) 528–536.
[9] L. Ziser, I. Setyawati, and S.G. Withers, *Carbohydr. Res.*, 274 (1995) 137–153.
[10] K. Takeo, Y. Ohguchi, R. Hasegawa, and S. Kitamura, *Carbohydr. Res.*, 277 (1995) 231–244.
[11] F.G. Loontiens and C.K. Bruyne, *Naturwissenchaften*, 51 (1964) 359.
[12] J. Conchie and G.A. Levvy, *Methods Carbohydr. Chem.*, 2 (1963) 335–337.

[13] L.R. Schroeder, K.M. Counts, and F.C. Haigh, *Carbohydr. Res.*, 27 (1974) 368–372.
[14] B. Capon, P.M. Collins, A.A. Levy, and W.G. Overend, *J. Chem. Soc.*, (1964) 3242–3254.
[15] P. Kovac, *Chem. Zvesti*, 34 (1980) 234–240; J. Hirsch and P. Kovac, *Chem. Zvesti*, 36 (1982) 125–131.
[16] A. Khasin, I. Alchanati, and Y. Shoham, *Appl. Environ. Microbiol.*, 59 (1993) 1725–1730.
[17] O. Gat, A. Lapidot, I. Alchanati, C. Regueros, and Y. Shoham, *Appl. Environ. Microbiol.*, 60 (1994) 1889–1896.
[18] A. Lapidot, A. Mechaly, and Y. Shoham, *J. Biotechnol.*, 51 (1996) 259–264.
[19] G.L. Miller, *Anal. Chem.*, 31 (1959) 426–428.
[20] P. Kovac, *Chem. Zvesti.*, 33 (1979) 365–370.
[21] R.L. Whistler and C.-C. Tu, *J. Am. Chem. Soc.*, 74 (1952) 4334–4335.