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Note

An alternative approach for the synthesis of fluorogenic substrates of *endo*-β-(1→4)-xylanases and some applications

Mária Vršanská,[a] Wim Nerinckx,[b] Marc Claeyssens[b] and Peter Biely[a,*]

[a]*Institute of Chemistry, Slovak Academy of Sciences, Dúbravská cesta 9, SK-84538 Bratislava, Slovakia*
[b]*Laboratory of Glycobiology, Department of Biochemistry, Physiology and Microbiology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium*

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Abstract—Fluorogenic substrates of *endo*-β-(1→4)-xylanases (EXs), 4-methylumbelliferyl β-glycosides of xylobiose and xylotriose were synthesized from fully acetylated oligosaccharides using the α-trichloroacetimidate procedure. A commercially available syrup containing xylose and xylo-oligosaccharides was used as the starting material. Both fluorogenic glycosides were found to be suitable substrates for EXs, particularly for sensitive detection of the enzymes in electrophoretic gels and their in situ localization on sections of fruiting bodies of some plants, such as tomato, potato and eggplant, all of the family *Solanaceae*.

Keywords: *endo*-β-(1→4)-Xylanase; Fluorogenic substrates; 4-Methylumbelliferyl β-xylobioside; 4-Methylumbelliferyl β-xylotrioside; Activity stain

Microbial *endo*-β-(1→4)-xylanases (EXs, EC 3.2.1.8) represent important industrial enzymes.[1] EXs also play important physiological roles in plant tissues.[2] They seem to be involved in fruit softening, seed germination, pollinization and possibly in cell wall extension.[3–5] These enzymes have been reported in germinating wheat,[6,7] and have been characterized more extensively in germinating barley.[8,9] An EX was identified as the predominant protein on the surface of maize pollen.[10]

Due to low levels of EXs in plants, their studies require sensitive substrates such as 4-methylumbelliferyl β-glycosides of xylobiose (Umb-Xyl_2) and xylotriose (Umb-Xyl_3). The first synthesis of these artificial substrates, carried out in our group,[11] was based on the Koenigs–Knorr synthesis. The principal xylosyl acceptor for the synthesis was 4-methylumbelliferyl 2,3-di-*O*-acetyl-β-D-xylopyranoside isolated from a mixture of products obtained on partial deacetylation of the per-*O*-acetylglycoside by hydrazine. The acceptor was then condensed with 2,3,4-tri-*O*-acetyl-α-D-xylopyranosyl bromide or 2,3,2′,3′,4′-penta-*O*-acetyl-α-xylobiosyl bromide to give the acetylated forms of the desired substrates. Umb-Xyl_2 and Umb-Xyl_3 were then obtained on deacetylation.[11] A bromosugar route towards Umb-Xyl_2 from xylobiose was also reported by Kaneko et al.[12] Similar strategies were used for preparation of other types of aryl β-glycosides of xylobiose.[13–16] 4-Methylumbelliferyl glycosides of higher xylo-oligosaccharides, including Umb-Xyl_3, were obtained from Umb-Xyl_2 in transglycosylation reactions catalyzed by a β-xylosidase from *Aspergillus* sp.[17] The glycosyl donor of this reaction, Umb-Xyl_2, was obtained by the condensation of protected Umb-Xyl with ethyl 2,3,4-tri-*O*-acetyl-1-thio-β-D-xylopyranoside.[17] In this paper we describe an alternative route leading with relatively high yields to Umb-Xyl_2 and Umb-Xyl_3, starting from an inexpensive commercially available syrupy mixture of Xyl, Xyl_2 and Xyl_3 as a source of oligosaccharides.

The mixture was deprived of water and subsequently acetylated. The per-*O*-acetylated xylo-oligosaccharides

Abbreviations: EX, *endo*-β-(1→4)-xylanase; Xyl, D-xylose; Xyl_2–Xyl_5, xylobiose up to xylopentaose; Umb-Xyl, 4-methylumbelliferyl β-D-xyloside; Umb-Xyl_2, 4-methylumbelliferyl β-xylobioside; Umb-Xyl_3, 4-methylumbelliferyl β-xylotrioside; Umb-Cel, 4-methylumbelliferyl β-cellobioside

* Corresponding author. Tel.: +421 259410275; fax: +421 259410222; e-mail: chempbsa@savba.sk

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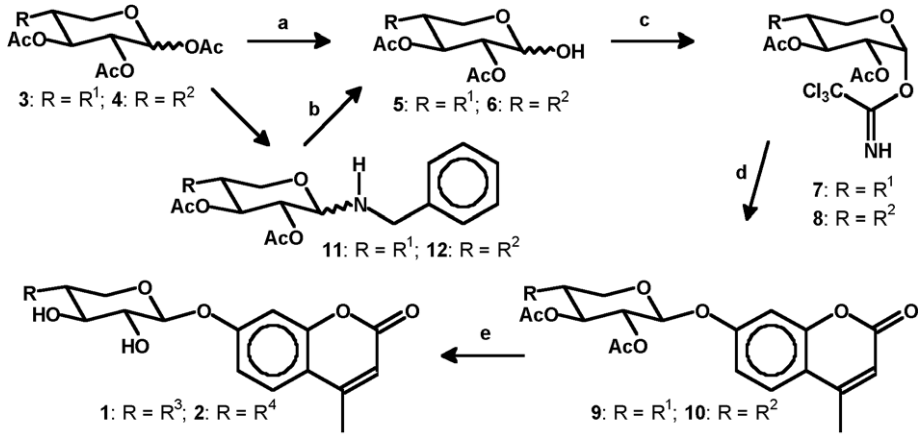
were isolated by chromatography on silica gel. The syntheses of Umb-Xyl_2 (**1**) and Umb-Xyl_3 (**2**) from per-*O*-acetylxylobiose (**3**) and per-*O*-acetylxylotriose (**4**) were accomplished in four steps, involving selective anomeric deprotection to **5** and **6** by treatment with benzylamine, conversion to the α-trichloroacetimidates[18] **7** and **8** and subsequent coupling with 4-methylumbelliferone (7-hydroxy-4-methylcoumarin) to give **9** and **10**, and finally Zemplén deacetylation (Scheme 1). The overall yields of compounds **1** and **2** were reproducible, at around 35% from the per-*O*-acetylated sugars. The structure of the products was confirmed by ^1H and ^{13}C NMR spectroscopy (Table 1). Earlier published ^1H NMR data of only the carbohydrate regions of Umb-Xyl_2[12,17] and Umb-Xyl_3[17] were helpful for proton assignments.

The anomeric deprotection of hexa-*O*-acetyl-xylobiose (**3**) and octa-*O*-acetyl-xylotriose (**4**) with benzylamine to give **5** and **6** is accompanied by formation of *N*-benzyl-penta-*O*-acetyl-xylobiosylamine (**11**) and *N*-benzyl-penta-*O*-acetyl-xylotriosylamine (**12**), respectively, in yields up to 30%. Both benzyl derivatives **11** and **12** can be hydrolyzed to give compounds **5** and **6** and returned to the respective reaction pathways to increase the overall yields of the final products.

They were obtained as yellowish hygroscopic powders, which proved to be stable during storage under dry conditions at −20 °C. Care should also be taken that the final products are not contaminated with the highly fluorescent aglycon, 4-methylumbelliferone.

The present route is straightforward and more suitable for a large-scale synthesis of Umb-Xyl_2 and Umb-Xyl_3 than previously published procedures.[11,12,17] The compounds are obtained in five steps, starting from the unprotected oligosaccharides and avoiding the preparation of unstable acetobromoxylo-oligosaccharides.

Both Umb-Xyl_2 and Umb-Xyl_3 served as substrates of EXs of families 10 and 11. Umb-Xyl_2 was found particularly useful for fluorometric determination of activity of microbial EXs during investigation of their interaction with plant proteinaceous inhibitors.[19] The same work emphasized the significance of low-molecular mass EX substrates in such studies due to interference of the inhibitors with polymeric xylan used as EX substrate.[19] Umb-Xyl_2 was also successfully used in a rapid semi-quantitative test of EX inhibitors present in proteins extracted from germinated maize.[20] Our findings that Umb-Xyl_2 is hydrolyzed to give 4-methylumbelliferone by members of EX families 10 and 11 is in contrast to



Scheme 1. Synthesis of fluorogenic xylo-oligosaccharides. Reagents and conditions: (a) benzylamine, CH_2Cl_2, 88%/87%; (b) Et_2O, HCl 1 N; (c) Cl_3C–CN, DBU, CH_2Cl_2, 79%/80%; (d) 4-methylumbelliferone, BF_3OEt_2, CH_2Cl_2, 4 Å molecular sieves, 57%/56%; (e) NaOMe, MeOH, 87%/97%.

Table 1. Chemical shifts (ppm) for D-xylopyranosyl residues of Umb-Xyl_3

Residue	H-1	C-1	H-2	C-2	H-3	C-3	H-4	C-4	H-5a	H-5b	C-5
Xylp^{I}	5.18	101.1	3.69	73.70	3.78	74.41	3.94	77.20	4.24	3.65	64.04
Xylp^{II}	4.58	102.8	3.35	73.76	3.63	74.79	3.88	77.44	4.19	3.46	64.00
Xylp^{III}	4.53	102.9	3.35	73.76	3.49	76.66	3.69	70.13	4.04	3.38	66.21

Xylp^{I}, xylosyl residue at the reducing end; Xylp^{II}, xylosyl residue in the middle; Xylp^{III}, xylosyl residue at the non-reducing end.

the report of Kaneko et al.[12] that EXs of families 11 do not liberate the aromatic aglycon from the xylobioside. We did not have in our hands an EX of family 11 that would not be detectable with Umb-Xyl_2. A more detailed study is required to find out whether the two substrates could be used for a simple differentiation of family 10 and 11 EXs. Both substrates were earlier shown not to be hydrolyzed by the family 8 xylanase from *Pseudoalteromonas haloplanktis*.[21] The same enzyme did not hydrolyze 6,8-difluoro-4-methylumbelliferyl xylobioside either.[16] In this work we found out that Umb-Xyl_2 and Umb-Xyl_3 are not hydrolyzed by the appendage-dependent EX of family 5 from *E. chrysanthemi*.

Both Umb-Xyl_2 and Umb-Xyl_3 are outstanding substrates for rapid localization of EXs in electrophoretic gels. An example of their use for this purpose was presented in our earlier article, describing the first synthesis of the substrates.[11] The fluorogenic substrates have a great advantage in comparison with RBB-xylan[22] or the Congo Red stain.[23] Indeed, whereas the detection of EXs with RBB-xylan or Congo Red, including the destaining and staining step, respectively, takes several hours, the localization of EXs by Umb-Xyl_2 and Umb-Xyl_3 is a matter of minutes. The disadvantage of fluorogenic substrates is sometimes the rapid diffusion of the released 4-methylumbelliferone in the gels, and the need to use Umb-Xyl as the control substrate for β-xylosidase, which may liberate the fluorescent aglycon from the glycosides after a two or three step hydrolysis. Umb-Cel may be recommended as the control substrate for non-specific *endo*-β-(1→4)-glucanases hydrolyzing xylans.[24]

Localization of EXs in situ can be done easily using agar gels replicas containing the fluorogenic substrates. A successful detection of EXs on sections of fruiting bodies of unripe tomatoes is shown in Figure 1. The sections were brought into contact with the gels containing Umb-Xyl_2 and Umb-Xyl_3, Umb-Xyl and Umb-Cel. Fluorescence was observed already after 5 min, but only with the gels containing Umb-Xyl_2 or Umb-Xyl_3. No evidence for the presence of EXs was obtained with sections of red (ripe) tomatoes, which indicates that EXs observed on seeds of green unripe tomatoes have disappeared completely in the process of ripening. Examination of EXs on cuts of green tomato seeds showed that the enzyme is confined to the seed coat and does not occur in the seed interior, including the endosperm. The identity of the enzyme detected with Umb-Xyl_2 and Umb-Xyl_3 as an EX was confirmed in an experiment in which isolated unripe tomato seed coats were incubated with a 1.5% solution of 4-*O*-methylglucuronoxylan in 0.05 M sodium acetate buffer at pH 5.4. The EX bound to the outer surface of the seed coat hydrolyzed the polysaccharide to Xyl, Xyl_2, aldotetraouronic acid and traces of Xyl_3 (Fig. 2). TLC in an alkaline solvent system[25] confirmed the acidic nature of the third product. Such product profile is characteristic for the action of family 10 EXs.[25] All so far identified plant EXs belong to glycoside hydrolase family 10. When different parts

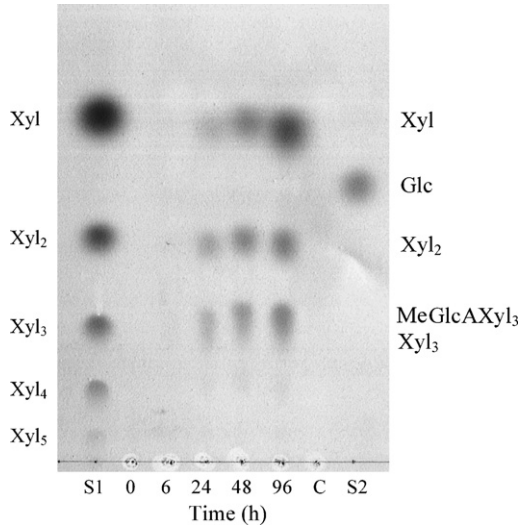


Figure 2. Hydrolysis of beechwood 4-*O*-methylglucuronoxylan by the EX(s) localized on unripe tomato seed coats (isolated from green tomato seeds) followed by TLC on cellulose (E. Merck) in 3:2:2 EtOAc–AcOH–water. Reducing sugars were detected with the aniline–hydrogen phthalate reagent. S1, standards Xyl–Xyl_5; S2, glucose; C, enzyme control; $MeGlcAXyl_3$, aldotetraouronic acid.

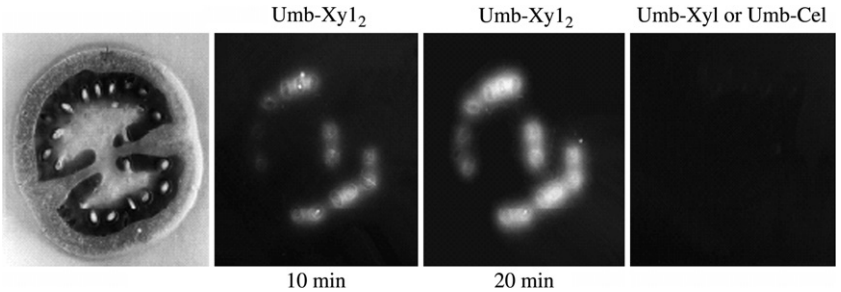


Figure 1. Detection of *endo*-β-(1→4)-xylanase on a section of unripe (green) tomato (left panel) using Umb-Xyl_2 (**1**) as a substrate after 10 and 20 min contact with the substrate-containing agar gel. The detection was followed under UV light. Umb-Xyl was used as a control for β-xylosidase and Umb-Cel as a control for *endo*-β-(1→4)-glucanases.

of other plants were tested for the presence of EX using Umb-Xyl_2 and Umb-Xyl_3, the enzyme activity was revealed only on seeds of the fruiting bodies of two other plant species, potato and eggplant, which belong together with tomato to the *Solanaceae* family.

1. Experimental

1.1. General methods

^{1}H NMR spectra were recorded in $CDCl_3$ or D_2O with a Bruker WP360 or WP500, or a Bruker DPX Avance 300 spectrometer (Madison, WI); ^{13}C NMR spectra were recorded with the latter instrument. Chemical shifts are expressed in parts per million (ppm) with reference to Me_4Si. IR spectra were recorded with a Beckman Accu-lab 4 (Fullerton, CA) spectrometer. MS data were recorded with a Hewlett–Packard 5988-A (EIMS, ionization potential of 70 eV), or with an Agilent 1100 series MSD-VL as electrospray in positive mode from 1:1 0.5 mM aq ammonium acetate–CH_3CN (ESIMS). Melting points were determined with a Reichert Microscope 269156 (Vienna, Austria) and are uncorrected. Specific rotations were measured at 20 °C after 20 h in distilled water with a Perkin–Elmer 241 polarimeter. TLC was performed with Silica gel $60F_{254}$ precoated glass plates (E. Merck, Darmstadt, Germany) with detection under UV light (254 nm) and subsequent dipping of the plates in a 10% soln of H_2SO_4 in 2-propanol and carbonization on a hot plate. Silica gel 100 (0.063–0.200 mm, E. Merck), dried at 150–200 °C under diminished pressure, was used for column chromatographic separations. All reactions were carried out with magnetic stirring and at room temperature unless otherwise indicated.

1.2. Chemicals

The syrupy mixture of D-xylose and xylo-oligosaccharides (22.2% D-xylose, 57.2% Xyl_2 and 17.3% Xyl_3) was from Suntory Ltd. (Japan), obtained by courtesy of Professor T. Yasui, (University of Tsukuba, Japan). Umb-Xyl and Umb-Cel were from Sigma (St. Louis, MO, USA). Benzylamine, trichloroacetonitrile, 4-methylumbelliferone, reagents and solvents were commercial; CH_2Cl_2 was freshly distilled from P_2O_5 prior to use.

1.3. Preparation of fully acetylated xylo-oligosaccharides

The syrupy mixture of D-xylose, Xyl_2 and Xyl_3 was lyophilized and then dried to constant weight in a desiccator over P_2O_5. The dry mixture (10 g) was dissolved in pyridine (200 mL) and Ac_2O (200 mL) at 0 °C. The acetylation was complete after 20 h. The mixture was poured into ice-cold water and stirred for 2–3 h. $CHCl_3$ (500 mL) was added and the solution was washed in a separatory funnel with a saturated soln of $NaHCO_3$ (300 mL). The $CHCl_3$ layer was washed once with water, dried over Na_2SO_4, filtered, and concentrated under diminished pressure. Purification by column chromatography on Silica gel 100 (120×2.5 cm; 3:4 *n*-hexane–EtOAc or 3:2 toluene–EtOAc) yielded fractions containing hexa-*O*-acetyl-β-xylobiose (**3**) and octa-*O*-acetyl-β-xylotriose (**4**) (R_f resp. 0.63 and 0.44 in 2:3 toluene–EtOAc), which were separately pooled, evaporated and used as starting compounds for further syntheses.

1.4. 2,3,2′,3′,4′-Penta-*O*-acetyl-xylobiose (5)

To a soln of hexa-*O*-acetyl xylobiose (**3**, 1.64 g, 2 mmol) in CH_2Cl_2 (5 mL), benzylamine (1 equiv, 218 μL) was added. A second equiv of benzylamine was added after 2 h, and an extra 0.5 equiv after another 2 h (total 2.5 equiv). The reaction was completed after 12 h. Washing with a 1 N HCl soln (2×10 mL) and with a saturated $NaHCO_3$ soln (10 mL), drying over anhyd $MgSO_4$ and purification by column chromatography (eluent: 1:1 toluene–EtOAc) yielded **5** (863 mg, 1.75 mmol, 88%). It should be noted that substantial amounts (up to 30%) of *N*-benzyl-penta-*O*-acetyl xylobiosylamine (**11**) may be formed. After separation by column chromatography, this can be subsequently hydrolyzed to **5** by stirring in the two-phase system Et_2O–1 N HCl, the reaction being complete after 6 h and worked up as above. *Penta-O-acetyl xylobiose* (**5**): white crystals; mp 171 °C; R_f 0.40 (1:1 toluene–EtOAc); IR (KBr): 1750, 1430, 1370, 1240, 1220, 1130, 1050 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$, α-anomer): δ 5.44 (dd, 1H, $J_{2,3}$ 8.7, $J_{3,4}$ 9.6 Hz, H-3^{I}), 5.33 (dd, 1H, $J_{1,2}$ 2.8, $J_{1,OH}$ 3 Hz, H-1^{I}), 5.08 (dd, 1H, $J_{2,3}$ 7.5, $J_{3,4}$ 7.5 Hz, H-3^{II}), 4.87 (ddd, 1H, $J_{4,5a}$ 4.6, $J_{4,5b}$ 7.5 Hz, H-4^{II}), 4.79 (dd, 1H, H-2^{I}), 4.79 (dd, 1H, $J_{1,2}$ 5.9 Hz, H-2^{II}), 4.57 (dd, 1H, H-1^{II}), 4.10 (dd, 1H, $J_{5a,5b}$ 11.9 Hz, H-5a^{II}), 3.85 (ddd, 1H, $J_{4,5a}$ 4.8, $J_{4,5b}$ 10 Hz, H-4^{I}), 3.80 (dd, 1H, $J_{5a,5b}$ 10.4 Hz, H-5b^{I}), 3.70 (dd, 1H, H-5a^{I}), 3.40 (dd, 1H, H-5b^{II}), 2.79 (d, 1H, O*H*), 2.078 (s, 3H, C*H*$_3$CO), 2.061 (s, 3H, C*H*$_3$CO), 2.054 (s + s, 6H, C*H*$_3$CO), 2.051 (s, 3H, C*H*$_3$CO) ppm; ESIMS: *m*/*z* 146 (50%), 188 (4%), 245 (4%), 313 (3%), 391 (8%), 510 $[M+NH_4]^+$ (100%), 515 $[M+Na]^+$ (50%). *N-Benzyl-penta-O-acetyl xylobiosylamine* (**11**): colourless oil; R_f 0.51 (1:1 toluene–EtOAc); IR (KBr): 1750, 1500–1420, 1370, 1220, 1040, 940 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.33–7.25 (m, 5H, Ph), 5.12 (dd, 1H, $J_{2,3}$ 9.5, $J_{3,4}$ 9.5 Hz, H-3^{I}), 5.07 (dd, 1H, $J_{2,3}$ 7.5, $J_{3,4}$ 7.5 Hz, H-3^{II}), 4.86 (ddd, 1H, $J_{4,5a}$ 4.3, $J_{4,5b}$ 7.6 Hz, H-4^{II}), 4.77 (dd, 1H, $J_{1,2}$ 9 Hz, H-2^{I}), 4.75 (dd, 1H, $J_{1,2}$ 5.6 Hz, H-2^{II}), 4.65 (d, 1H, H-1^{II}), 4.09 (dd, 1H, $J_{5a,5b}$ 12 Hz, H-5a^{II}), 4.01 (d, 1H, J_{gem} 13.8 Hz, C*H*$_2$Ph), 3.97 (d, 1H, H-1^{I}), 3.94 (dd, 1H, $J_{4,5a}$ 5.5, $J_{5a,5b}$ 12 Hz, H-5a^{I}), 3.84 (d, 1H, C*H*$_2$Ph),

3.80 (ddd, 1H, $J_{4,5b}$ 10 Hz, H-4^{I}), 3.39 (dd, 1H, H-5b^{II}), 3.21 (dd, 1H, H-5b^{I}), 2.056 (s + s + s, 9H, CH_3CO), 2.033 (s, 3H, CH_3CO), 2.030 (s, 3H, CH_3CO) ppm.

1.5. 2,3,2′,3′,4′-Penta-*O*-acetyl-α-D-xylobiosyl trichloroacetimidate (7)

To a soln of **5** (685 mg, 1.39 mmol) and trichloroacetonitrile (5 equiv, 700 μL) in CH_2Cl_2 (20 mL), 1,8-diazabicyclo[5,4,0]undec-7-ene (0.2 equiv, 41 μL) was added and stirring was continued for 30 min. Concentration under diminished pressure and purification by column chromatography (dried Silica gel 100; eluent: 7:3 toluene–EtOAc) yielded **7** (701 mg, 1.101 mmol, 79%) as a white powder; mp 159 °C; R_f 0.56 (1:1 toluene–EtOAc); IR (KBr): 1760, 1680, 1440, 1370, 1340, 1230, 1040 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.60 (s, 1H, CNHCCl$_3$), 6.40 (d, 1H, $J_{1,2}$ 3.7 Hz, H-1^{I}), 5.51 (dd, 1H, $J_{2,3}$ 9.6, $J_{3,4}$ 9.6 Hz, H-3^{I}), 5.07 (dd, 1H, $J_{2,3}$ 8.0, $J_{3,4}$ 7.8 Hz, H-3^{II}), 4.98 (dd, 1H, H-2^{I}), 4.80 (ddd, 1H, $J_{4,5a}$ 4.8, $J_{4,5b}$ 7.8 Hz, H-4^{II}), 4.77 (dd, 1H, $J_{1,2}$ 6.5 Hz, H-2^{II}), 4.53 (d, 1H, H-1^{II}), 3.96 (dd, 1H, $J_{4,5a}$ 5.0, $J_{5a,5b}$ 12 Hz, H-5a^{I}), 3.88 (ddd, 1H, $J_{4,5b}$ 10 Hz, H-4^{I}), 4.05 (dd, 1H, $J_{5a,5b}$ 12 Hz, H-5a^{II}), 3.77 (dd, 1H, H-5b^{I}), 3.38 (dd, 1H, H-5b^{II}), 2.060 (s, 3H, CH_3CO), 2.053 (s, 3H, CH_3CO), 2.032 (s, 3H, CH_3CO), 2.030 (s, 3H, CH_3CO), 2.005 (s, 3H, CH_3CO) ppm. This compound is moderately stable and should be used within days.

1.6. Preparation of 4-methylumbelliferyl 2,3,2′,3′,4′-penta-*O*-acetyl-β-xylobioside (9)

A mixture of **7** (780 mg, 1.23 mmol) and 4-methylumbelliferone (1.5 equiv, 338 mg, 1.84 mmol) in CH_2Cl_2 (40 mL) in the presence of 4 Å molecular sieves (0.5 g) was stirred for 2 h and then brought to −15 °C (cooling bath benzylalcohol–solid CO_2). A soln of $BF_3{\cdot}OEt_2$ (15 μL) in CH_2Cl_2 (1 mL) was added dropwise during 5 min. The reaction was completed after 1 h at −15 °C. Solid $NaHCO_3$ (100 mg) was added and the mixture was brought to room temperature. Washing with water (20 mL), with a saturated $NaHCO_3$ soln (5 × 20 mL), drying over anhyd $MgSO_4$ and purification by column chromatography (eluent: 7:3 toluene–EtOAc) yielded **9** (456 mg, 701 μmol, 57%) as white crystals; mp 99 °C, lit.[12] mp 108.5–110 °C (from MeOH); R_f 0.38 (1:1 toluene–EtOAc); IR (KBr): 1760–1750, 1620, 1430, 1380, 1230, 1140, 1060 cm^{-1}; ^{1}H NMR (360 MHz, $CDCl_3$): δ 7.53 (d, 1H, $J_{5,6}$ 8.7 Hz, H-5$_{MUF}$), 6.96 (d, 1H, $J_{6,8}$ 2.5 Hz, H-8$_{MUF}$), 6.92 (dd, 1H, H-6$_{MUF}$), 6.20 (s, 1H, H-3$_{MUF}$), 5.22 (dd, 1H, $J_{2,3}$ 8.0, $J_{3,4}$ 8.0 Hz, H-3^{II}), 5.16 (s, 1H, $J_{1,2}$ 6.3 Hz, H-1^{I}), 5.16 (dd, 1H, $J_{2,3}$ 10.0 Hz, H-2^{I}), 5.12 (dd, 1H, $J_{3,4}$ 10.5 Hz, H-3^{I}), 4.91 (ddd, 1H, $J_{4,5a}$ 4.8, $J_{4,5b}$ 8.0 Hz, H-4^{II}), 4.84 (dd, 1H, $J_{1,2}$ 6.2 Hz, H-2^{II}), 4.59 (d, 1H, H-1^{II}), 4.12 (dd, 1H, $J_{5a,5b}$ 12 Hz, H-5a^{II}), 4.09 (dd, 1H, $J_{4,5a}$ 4.9, $J_{5a,5b}$ 12 Hz, H-5a^{I}), 3.92 (ddd, 1H, $J_{4,5b}$ 8.5 Hz, H-4^{I}), 3.51 (dd, 1H, H-5b^{I}), 3.40 (dd, 1H, H-5b^{II}), 2.40 (s, 3H, CH_{3MUF}), 2.091 (s, 3H, CH_3CO), 2.081 (s, 3H, CH_3CO), 2.073 (s, 3H, CH_3CO), 2.063 (s, 3H, CH_3CO), 2.044 (s, 3H, CH_3CO) ppm; EIMS: *m*/*z* 97 (100%), 139 (55%), 157 (55%), 199 (20%), 259 (14%) [XylOAc$_3^+$], 273 (2%), 475 (2%) [Xyl$_2$OAc$_5^+$].

1.7. Preparation of 4-methylumbelliferyl β-xylobioside (1)

To a mixture of **9** (3.5 g, 5.385 mmol) in MeOH (20 mL), a chip of sodium (0.1 equiv) was added. The reaction was completed after 1 h. Silica gel 100 (200 mg) was added and stirring was continued for 5 min. Filtration and concentration under diminished pressure yielded **1** (2.063 g, 87%) as a slightly yellowish powder; mp >200 (dec); $[\alpha]_D^{20}$ −82.5 (*c* 1.0, water), lit.[12] $[\alpha]_D$ −81.6 (*c* 1.0, water); R_f 0.53 (1:1 toluene–MeOH); ^{1}H NMR (500 MHz, D_2O): δ 7.47 (dd, 1H, $J_{5,6}$ 3.8, $J_{6,8}$ 8.8 Hz, H-6$_{MUF}$), 6.93 (d, 1H, H-5$_{MUF}$), 6.83 (d, 1H, H-8$_{MUF}$), 6.04 (s, 1H, H-3$_{MUF}$), 5.05 (d, 1H, $J_{1,2}$ 7.5 Hz, H-1^{I}), 4.45 (d, 1H, $J_{1,2}$ 7.8 Hz, H-1^{II}), 4.14 (dd, 1H, $J_{4,5a}$ 4.9, $J_{5a,5b}$ 11.2 Hz, H-5a^{I}), 3.96 (dd, 1H, $J_{4,5a}$ 5.4, $J_{5a,5b}$ 11.6 Hz, H-5a^{II}), 3.83 (ddd, 1H, $J_{3,4}$ 9, $J_{4,5b}$ 9 Hz, H-4^{I}), 3.68 (dd, 1H, $J_{2,3}$ 8 Hz, H-3^{I}), 3.60 (ddd, 1H, $J_{3,4}$ 8, $J_{4,5b}$ 9 Hz, H-4^{II}), 3.59 (dd, 1H, H-5b^{I}), 3.57 (dd, 1H, H-5b^{II}), 3.42 (dd, 1H, $J_{2,3}$ 8 Hz, H-3^{II}), 3.29 (dd, 1H, H-2^{I}), 3.26 (dd, 1H, H-2^{II}), 2.27 (s, 3H, CH_{3MUF}) ppm. The ^{1}H resonances at the carbohydrate regions matched previously reported data, and the ^{13}C NMR spectrum was identical.[12,17] This compound is hygroscopic and should be kept in a desiccator. Anal. Calcd for $C_{20}H_{24}O_{11}{\cdot}H_2O$: C, 52.40; H, 5.72. Found: C, 52.60; H, 5.65.

1.8. 2,3,2′,3′,2″,3″,4″-Hepta-*O*-acetyl-xylotriose (6)

To a soln of **4** (2.05 g, 2.73 mmol) in CH_2Cl_2 (10 mL), benzylamine (1 equiv, 313 μL) was added. A second equiv of benzylamine was added after 2 h, and an extra 0.5 equiv after another 2 h (total 2.5 equiv). The reaction was completed after 22 h. Washing with 1 N HCl soln (2 × 30 mL) and with a saturated $NaHCO_3$ soln (30 mL), drying over anhyd $MgSO_4$ and purification by column chromatography (2:3 toluene–EtOAc) yielded amorphous **6** (1.685 g, 2.38 mmol, 87%); R_f 0.36 (3:7 toluene–EtOAc); IR (KBr): 1760, 1440, 1380, 1260, 1220, 1050 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$, major α-anomer): δ 5.41 (dd, 1H, $J_{2,3}$ 9, $J_{3,4}$ 9 Hz, H-3^{I}), 5.31 (dd, 1H, $J_{1,2}$ 2.3, $J_{1,OH}$ 4 Hz, H-1^{I}), 5.08 (dd, 1H, $J_{2,3}$ 7.7, $J_{3,4}$ 7.6 Hz, H-3^{III}), 5.05 (dd, 1H, $J_{2,3}$ 8.0, $J_{3,4}$ 8.0 Hz, H-3^{II}), 4.87 (ddd, 1H, $J_{4,5a}$ 4.7, $J_{4,5b}$ 7.6 Hz, H-4^{III}), 4.79 (dd, 1H, $J_{1,2}$ 5.9 Hz, H-2^{III}), 4.75 (dd, 1H, $J_{1,2}$ 6.7 Hz, H-2^{II}), 4.74 (dd, 1H, H-2^{I}), 4.55 (d, 1H, H-1^{III}), 4.58 (d, 1H, H-1^{II}), 4.08 (dd, 1H, $J_{5a,5b}$

12 Hz, H-5a^{III}), 3.94 (ddd, 1H, $J_{4,5a}$ 5, $J_{4,5b}$ 10 Hz, H-4^{I}), 3.80 (dd, 1H, $J_{5a,5b}$ 11 Hz, H-5a^{I}), 3.79 (ddd, 1H, $J_{4,5a}$ 5.1, $J_{4,5b}$ 9 Hz, H-4^{II}), 3.67 (dd, 1H, $J_{5a,5b}$ 11 Hz, H-5a^{II}), 3.39 (dd, 1H, H-5b^{III}), 3.32 (dd, 1H, H-5b^{I}), 3.32 (dd, 1H, H-5b^{II}), 3.10 (d, 1H, O*H*), 2.071 (s, 3H, C*H*$_3$CO), 2.053 (s, 3H, C*H*$_3$CO), 2.046 (s, 3H, C*H*$_3$CO), 2.038 (s, 3H, C*H*$_3$CO), 2.028 (s, 3H, C*H*$_3$CO), 2.026 (s, 3H, C*H*$_3$CO), 2.023 (s, 3H, C*H*$_3$CO) ppm; separately resolved resonances of minor β-anomer: 5.14 (dd, 1H, $J_{2,3}$ 8, $J_{3,4}$ 9 Hz, H-3^{I}), 4.63 (dd, 1H, $J_{1,2}$ 8 Hz, H-2^{I}), 4.57 (d, 1H, $J_{1,2}$ 6.7 Hz, H-1^{II}), 3.60 (d, 1H, $J_{1,OH}$ 8.5 Hz, H-O*H*) ppm; ratio of α/β: 1.8/1.0; ESIMS: *m*/*z* 315 (10%), 726 ([M+NH$_4$]$^+$, 100%); 731 ([M+Na]$^+$, 36%). It should be noted that substantial amounts (up to 30%) of *N*-benzyl-hepta-*O*-acetyl xylotriosylamine (**12**), colourless oil, R_f 0.84 (3:7 toluene–EtOAc), may be formed. After separation by column chromatography, this can be subsequently hydrolyzed to **6** by stirring in the two-phase system Et$_2$O–1 N HCl, the reaction being complete after 6 h and worked up as above.

1.9. 2,3,2′,3′,2″,3″,4″-Hepta-*O*-acetyl-α-D-xylotriosyl trichloroacetimidate (8)

To a soln of **6** (576 mg, 0.813 mmol) and trichloroacetonitrile (5 equiv, 410 μL) in CH_2Cl_2 (15 mL), 1,8-diazabicyclo[5,4,0]undec-7-ene (0.2 equiv, 25 μL) was added and stirring was continued for 30 min. Concentration under diminished pressure and purification by column chromatography (dried Silica gel 100; eluent: 1:1 toluene–EtOAc) yielded **8** (551 mg, 0.646 mmol, 80%) as a white powder, mp 92 °C; R_f 0.66 (3:7 toluene–EtOAc); IR (KBr): 1760, 1690, 1450, 1430, 1380, 1250, 1220, 1050 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.63 (s, 1H, CN*H*CCl$_3$), 6.42 (d, 1H, $J_{1,2}$ 3.7 Hz, H-1^{I}), 5.47 (dd, 1H, $J_{2,3}$ 9.6, $J_{3,4}$ 9.6 Hz, H-3^{I}), 5.08 (dd, 1H, $J_{2,3}$ 7.6, $J_{3,4}$ 7.6 Hz, H-3^{III}), 5.06 (dd, 1H, $J_{2,3}$ 8.5, $J_{3,4}$ 8.3 Hz, H-3^{II}), 4.99 (dd, 1H, H-2^{I}), 4.87 (ddd, 1H, $J_{4,5a}$ 4.7, $J_{4,5b}$ 7.6 Hz, H-4^{III}), 4.79 (dd, 1H, $J_{1,2}$ 5.8 Hz, H-2^{III}), 4.74 (dd, 1H, $J_{1,2}$ 6.7 Hz, H-2^{II}), 4.56 (d, 1H, H-1^{III}), 4.50 (d, 1H, H-1^{II}), 4.09 (dd, 1H, $J_{5a,5b}$ 12 Hz, H-5a^{III}), 3.95 (dd, 1H, $J_{4,5a}$ 5.0, $J_{5a,5b}$ 12 Hz, H-5a^{I}), 3.87 (ddd, 1H, $J_{4,5b}$ 10 Hz, H-4^{I}), 3.85 (dd, 1H, $J_{4,5a}$ 5.0, $J_{5a,5b}$ 12 Hz, H-5a^{II}), 3.82 (ddd, 1H, $J_{4,5b}$ 8.9 Hz, H-4^{II}), 3.75 (dd, 1H, H-5b^{I}), 3.39 (ddd, 1H, H-5b^{III}), 3.34 (dd, 1H, H-5b^{II}), 2.059 (s, 3H, C*H*$_3$CO), 2.053 (s + s, 6H, C*H*$_3$CO), 2.031 (s, 3H, C*H*$_3$CO), 2.029 (s, 3H, C*H*$_3$CO), 2.012 (s, 3H, C*H*$_3$CO), 2.004 (s, 3H, C*H*$_3$CO) ppm.

1.10. 4-Methylumbelliferyl 2,3,2′,3′,2″,3″,4″-hepta-*O*-acetyl-β-xylotrioside (10)

A mixture of **8** (730 mg, 0.865 mmol) and 4-methylumbelliferone (1.5 equiv, 226 mg) in CH_2Cl_2 (20 mL) in the presence of 4 Å molecular sieves (0.5 g) was stirred for 2 h and then brought to −15 °C (cooling bath benzyl-alcohol–solid CO_2). A soln of $BF_3{\cdot}OEt_2$ (0.1 equiv, 10 μL) in CH_2Cl_2 (1 mL) was added dropwise during 5 min. The reaction was completed after 1 h at −15 °C. Solid $NaHCO_3$ (200 mg) was added and the mixture was brought to room temperature. Washing with water (20 mL), with a saturated $NaHCO_3$ soln (5 × 20 mL), drying over anhyd $MgSO_4$ and purification by column chromatography (55:45 toluene–EtOAc) yielded **10** (415 mg, 0.479 mmol, 56%) as white crystals; mp 121 °C; R_f 0.39 (2:3 toluene–EtOAc); IR (KBr): 1760–1740, 1620, 1510–1500, 1430, 1370, 1250–1240, 1150, 1120, 1060–1030, 870 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.51 (d, 1H, $J_{5,6}$ 8.7 Hz, H-5$_{MUF}$), 6.94 (d, 1H, $J_{6,8}$ 2.4 Hz, H-8$_{MUF}$), 6.91 (dd, 1H, H-6$_{MUF}$), 6.18 (q, 1H, $J_{3,CH3}$ 1 Hz, H-3$_{MUF}$), 5.20 (dd, 1H, $J_{2,3}$ 8.1, $J_{3,4}$ 8.1 Hz, H-3^{I}), 5.17 (d, 1H, $J_{1,2}$ 6.5 Hz, H-1^{I}), 5.11 (dd, 1H, H-2^{I}), 5.09 (dd, 1H, $J_{2,3}$ 7.7, $J_{3,4}$ 7.6 Hz, H-3^{III}), 5.08 (dd, 1H, $J_{2,3}$ 8.4, $J_{3,4}$ 8.7 Hz, H-3^{II}), 4.87 (ddd, 1H, $J_{4,5a}$ 4.7, $J_{4,5b}$ 7.6 Hz, H-4^{III}), 4.79 (dd, 1H, $J_{1,2}$ 5.8 Hz, H-2^{III}), 4.78 (dd, 1H, $J_{1,2}$ 6.5 Hz, H-2^{II}), 4.56 (d, 1H, H-1^{III}), 4.51 (d, 1H, H-1^{II}), 4.09 (dd, 1H, $J_{4,5a}$ 4.8, $J_{5a,5b}$ 12.1 Hz, H-5a^{I}), 4.06 (dd, 1H, $J_{5a,5b}$ 12.1 Hz, H-5a^{III}), 3.96 (dd, 1H, $J_{4,5a}$ 5.1, $J_{5a,5b}$ 11.9 Hz, H-5a^{II}), 3.88 (ddd, 1H, $J_{4,5b}$ 8.4 Hz, H-4^{I}), 3.82 (ddd, 1H, $J_{4,5b}$ 9.0 Hz, H-4^{II}), 3.50 (dd, 1H, H-5b^{I}), 3.39 (dd, 1H, H-5b^{III}), 3.33 (dd, 1H, H-5b^{II}), 2.40 (d, 3H, C*H*$_{3MUF}$), 2.081 (s, 3H, C*H*$_3$CO), 2.066 (s, 3H, C*H*$_3$CO), 2.058 (s + s, 6H, C*H*$_3$CO), 2.052 (s, 3H, C*H*$_3$CO), 2.036 (s, 3H, C*H*$_3$CO), 2.027 (s, 3H, C*H*$_3$CO) ppm. ESIMS: *m*/*z* 392 (22%), 883 ([M+O+H]$^+$ or [M+16+1]$^+$, 100%), 889 ([M+Na]$^+$, 82%); MW 866.

1.11. Preparation of 4-methylumbelliferyl β-xylotrioside (2)

Compound **10** (415 mg, 479 μmol) was taken in a soln of sodium methanolate in MeOH (0.1 equiv, 11 mL of a 4.35 mM soln). The reaction was completed after 2 h. Silica gel 100 (100 mg) was added and stirring was continued for 5 min. Filtration and concentration under diminished pressure yielded **2** (267 mg, 97%) as a slightly yellowish powder; mp >200 °C (dec); R_f 0.40 (1:1 toluene–MeOH); $[\alpha]_D^{20}$ −97.5 (*c* 0.60, water); ^{1}H NMR (300 MHz, D_2O): δ 7.62 (dd, 1H, $J_{5,6}$ 3.8, $J_{6,8}$ 8.8 Hz, H-6$_{MUF}$), 7.02 (d, 1H, H-5$_{MUF}$), 6.97 (d, 1H, H-8$_{MUF}$), 6.16 (s, 1H, H-3$_{MUF}$), 5.18 (d, 1H, $J_{1,2}$ 7.6 Hz, H-1^{I}), 4.58 (d, 1H, $J_{1,2}$ 7.8 Hz, H-1^{II}), 4.53 (d, 1H, $J_{1,2}$ 7.8 Hz, H-1^{III}), 4.24 (dd, 1H, $J_{4,5a}$ 5.0, $J_{5a,5b}$ 11.5 Hz, H-5a^{I}), 4.19 (dd, 1H, $J_{4,5a}$ 5.4, $J_{5a,5b}$ 11.7 Hz, H-5a^{II}), 4.04 (dd, 1H, $J_{4,5a}$ 5.4, $J_{5a,5b}$ 11.5 Hz, H-5a^{III}), 3.94 (ddd, 1H, $J_{3,4}$ 9.4, $J_{4,5b}$ 9.4 Hz, H-4^{I}), 3.88 (ddd, 1H, $J_{3,4}$ 9.4, $J_{4,5b}$ 9.4 Hz, H-4^{II}), 3.78 (dd, 1H, $J_{2,3}$ 10.2 Hz, H-3^{I}), 3.69 (ddd, 1H, $J_{3,4}$ 9.3, $J_{4,5b}$ 9.4 Hz, H-4^{III}), 3.69 (dd, 1H, H-2^{I}), 3.65 (dd, 1H, H-5a^{I}), 3.63 (dd, 1H, $J_{2,3}$ 9.0 Hz, H-3^{II}), 3.49 (dd, 1H, $J_{2,3}$ 9.3 Hz,

H-3^{III}), 3.46 (dd, 1H, H-5b^{II}), 3.38 (dd, 1H, H-5b^{III}), 3.35 (dd, 1H, H-2^{II}), 3.35 (dd, 1H, H-2^{III}), 2.35 (s, 3H, CH_{3MUF}) ppm. ^{1}H and ^{13}C NMR chemical shifts are compiled in Table 1. The ^{1}H resonances of the carbohydrate regions matched previously reported data, and the ^{13}C NMR spectrum was identical.[17] This compound is hygroscopic and should be kept in a desiccator. Anal. Calcd for $C_{25}H_{32}O_{15}\cdot H_2O$: C, 50.84; H, 5.80. Found: C, 51.04; H, 5.64.

1.12. Enzymes

The following EXs were used in the present work: family 10 EXs were from *Cryptococcus albidus*[26] and from *Streptomyces lividans* (XlnA),[27] family 11 EXs were from *Schizophyllum commune*,[28] *Thermomyces lanuginosus*[29] and from *S. lividans* (Xln C).[30] Family 5 EX from *Erwinia chrysanthemi*[31] was provided by J.F. Preston (University of Florida, Gainesville, FL, USA). The activity of *endo*-β-(1→4)-xylanase was determined on 4-*O*-methylglucuronoxylan.[26] One unit of activity is defined as the amount of enzyme, which liberates 1 μmol of D-xylose equiv in 1 min.

1.13. Detection of *endo*-β-(1→4)-xylanase on plant sections

Sections of various plants or fruits were applied on four different flat gels, containing the following fluorogenic substrates: Umb-Xyl, Umb-Xyl$_2$, Umb-Xyl$_3$, as well as Umb-Cel (15 mg of each substrates in 30 mL of 2% agar gel prepared in 0.05 M sodium acetate buffer, pH 5.4). After 10 min of incubation at 30 °C in damp atmosphere, sections were removed and gels were examined under UV light (365 nm). Fluorescence (emission at 445 nm) was observed on the locations of enzymes where liberation of 4-methylumbelliferone from the above substrates had occurred. Detection of EX was considered to be positive only in the case when fluorescence bands observed with Umb-Xyl$_2$ and Umb-Xyl$_3$ did not correspond to those obtained with Umb-Xyl and Umb-Cel. The lack of fluorescence occurrence at the same place with Umb-Xyl served as a proof for the absence of β-xylosidase, which could generate 4-methylumbelliferone from Umb-Xyl$_2$ and Umb-Xyl$_3$ in a two- and/or three-step hydrolysis. The lack of fluorescence at the same place with Umb-Cel served as an evidence for the absence of non-specific *endo*-β-(1→4)-glucanases.[24]

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