Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 339 (2004) 2019–2025
Carbohydrate RESEARCH

Synthesis of pentose-containing disaccharides using a thermostable α-L-arabinofuranosidase

Caroline Rémond,[a] Richard Plantier-Royon,[b] Nathalie Aubry,[a] Emmanuel Maes,[d] Christophe Bliard[c] and Michael J. O'Donohue[a,*]

[a]*Laboratoire de Technologie Enzymatique et Physico-chimie des Agroressources, UMR URCA/INRA FARE, 8, rue Gabriel Voisin, BP 316, F-51688 Reims, France*
[b]*Laboratoire Réactions Sélectives et Applications, UMR URCA/CNRS 6519, Faculté des Sciences, BP 1039, F-51687 Reims, France*
[c]*Laboratoire de Pharmacognosie, UMR URCA/CNRS 6013, Faculté des Sciences, BP 1039, F-51687 Reims, France*
[d]*Unité de Glycobiologie Structurale et Fonctionnelle, UMR USTL/CNRS 8576, Université des Sciences et Technologies de Lille, Bat. C9, F-59655 Villeneuve d'Ascq, France*

Received 29 January 2004; accepted 28 April 2004
Available online 1 July 2004

Abstract—To date, the enzymatically-catalysed synthesis of pentose-containing compounds has been limited to the production of oligo-β-(1 → 3) and oligo-β-(1 → 4)-linked xylopyranosides. To our knowledge, no such syntheses have involved arabinofuranose or, indeed, any other sugars in the furanose configuration. In this report, we describe the use of a thermostable α-L-arabinofuranosidase for the synthesis of *p*-nitrophenyl α-L-arabinofuranosyl-(1 → 2)-α-L-arabinofuranoside, *p*-nitrophenyl β-D-xylopyranosyl-(1 → 2)-β-D-xylopyranoside, *p*-nitrophenyl β-D-xylopyranosyl-(1 → 3)-β-D-xylopyranoside and benzyl α-D-xylopyranosyl-(1 → 2)-α-L-arabinofuranoside. Importantly, this latter compound is synthesised in a highly regiospecific reaction, which leads to the production of a single disaccharide.

Keywords: α-L-Arabinofuranosidase; Transglycosylation; Pentoses; Furanoses; Arabinofuranosides; Xylopyranosides

1. Introduction

Increasingly, enzymes are being adopted for the synthesis of oligosaccharides and glycoconjugates via enzymatic or mixed chemo-enzymatic routes. The use of glycosyltransferases (EC 3.2.4) or glycoside hydrolases (EC 3.2.1) in synthetic strategies is justified by the fact that such enzymes perform glycosylation in one stereoselective step. In the case of glycosyltransferases, which use nucleotide sugars as donors, the glycosylation reaction is also regioselective, whereas glycoside hydrolases (mainly *exo*-acting hydrolases) often display more relaxed regioselectivity. However, unlike glycosyltransferases, an extensive palette of glycoside hydrolases, displaying a wide range of sugar specificities, is available. Many of these enzymes are robust and thermostable and do not require the use of costly sugar donors.

It is now clearly established that glycoside hydrolases, functioning in their hydrolytic mode, cleave the glycosidic linkage via retaining or inverting mechanisms.[1] In either mechanism, two acidic amino acid residues (aspartic or glutamic acid) provide catalytic assistance.[2] Reactions catalysed by inverting enzymes proceed via a single step displacement during which an ephemeral

Abbreviations: AbfD3, α-L-arabinofuranosidase D3; *p*NP, *p*-nitrophenol; *p*NPAra*f*, *p*-nitrophenyl α-L-arabinofuranoside; *p*NPXyl*p*, *p*-nitrophenyl-β-D-xylopyranoside; *p*NPAra*p*, *p*-nitrophenyl α-L-arabinopyranoside; *p*NPXyl*f*, *p*-nitrophenyl β-D-xylofuranoside

* Corresponding author. Tel.: +33-326-355-365; fax: +33-326-355-369; e-mail: michael.odonohue@univ-reims.fr

0008-6215/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2004.04.017

oxocarbenium ion is formed. Importantly, the products of such reactions display an inverted anomeric configuration. In contrast, retaining enzymes catalyse a two-step reaction, which proceeds via the formation of a covalently-linked enzyme–substrate intermediate, which, in the second step (deglycosylation), reacts with water to generate a product, which displays an anomeric configuration identical to that of the substrate saccharide.[2–4]

In addition to hydrolysis, many retaining glycoside hydrolases also possess the ability to transfer donor sugars onto nucleophilic molecules other than water. Likewise, when sugar acceptors are present, they will compete with water in the deglycosylation step and often lead to the formation of oligosaccharides.

A considerable number of studies have described the use of glycoside hydrolases for the synthesis of a wide variety of oligosaccharides and glycoconjugates. Generally, enzymatic-mediated synthesis is performed either by exploiting the ability of glycoside hydrolases to perform reverse hydrolysis (thermodynamic control) or by employing activated substrates, which, by generating a steady-state concentration of the glycosyl-enzyme intermediate, exert a kinetic control over the transglycosylation reaction. Most frequently, the activated substrates are *p*-nitrophenyl glycosides, probably because of their commercial availability. Likewise, the target compounds are mainly hexose-containing oligosaccharides, almost certainly because of the widespread biological importance of such sugar motifs. Consequently, the most studied examples of glycoside hydrolase-mediated synthesis involve glucosidases[5–7] and galactosidases.[8–10]

To date, relatively few studies have explored the use of glycoside hydrolases for the synthesis of pentose-containing oligosaccharides. Furthermore, among the published examples, most concern the use of β-xylosidases or xylanases for the synthesis of xylo-oligosaccharides.[11–14] To our knowledge, very few studies have described the enzyme-mediated synthesis of arabinose-containing compounds and none have reported the use of an α-L-arabinofuranosidase for this purpose.

Arabinosidases (EC 3.2.1.55) belonging to family 51 of the glycoside hydrolase classification system are retaining, *exo*-acting glycoside hydrolases, which generally catalyse the hydrolysis of α-(1 → 5) bonds linking two α-L-arabinofuranosidic moieties or α-(1 → 2) and/or α-(1 → 3) bonds linking a L-arabinofuranose to a D-xylose in polymers such arabinan and arabinoxylan, respectively. Previously, the thermostable family 51 arabinosidase from *Thermobacillus xylanilyticus* (AbfD3)[15] was tested for its ability to catalyse transglycosylation in the presence of *p*-nitrophenyl α-L-arabinofuranoside (*p*NPAra*f*) and various alcohols.[16] In this present study, we have analysed the ability of AbfD3 to catalyse transglycosylation and report the synthesis of several pentose-containing oligosaccharides.

2. Results and discussion

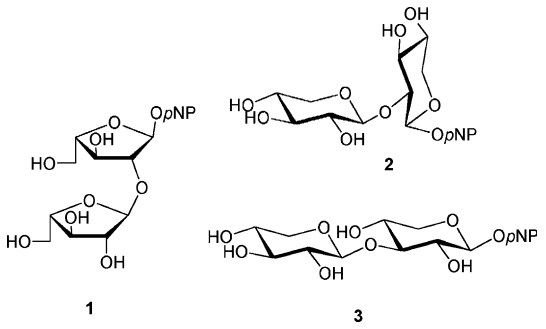
2.1. Hydrolysis of various *p*-nitrophenyl glycosides

The AbfD3-catalysed hydrolysis of various *p*-nitrophenyl glycosides was evaluated by measuring specific activity towards *p*NPAra*f*, *p*-nitrophenyl-β-D-xylopyranoside (*p*NPXyl*p*), *p*-nitrophenyl α-L-arabinopyranoside (*p*NPAra*p*) and *p*-nitrophenyl β-D-xylofuranoside (*p*NPXyl*f*). The activity measured in the presence of *p*NPAra*f* (465 IU/mg) was much higher than that measured in the presence of *p*NPXyl*p* (0.292 IU/mg), *p*NPAra*p* (0.190 IU/mg) or *p*NPXyl*f* (0.066 IU/mg). Attempts to determine the kinetic parameters for the latter reactions were not pursued since K_m values were estimated to be much greater (>15 mM) than the value determined for the hydrolysis of *p*NPAra*f* ($K_m = 0.70$). These results are in agreement with the findings of a recently published study in which the three-dimensional structure of another family 51 arabinosidase was revealed.[17] Analysis of the active site of this enzyme indicated that, while the −1 (donor) subsite is highly specific for binding of L-arabinofuranosides, xylopyranose can also occupy this subsite. Therefore, even if pyranosidic compounds constitute poor substrates for hydrolysis, the possibility that these groups might act as donors in transglycosylation cannot be excluded.

2.2. AbfD3-mediated synthesis of homo-disaccharides

To screen the ability of AbfD3 to catalyse transglycosylation, individual reactions in the presence of each *p*NPglycoside (*p*NPAra*f*, *p*NPXyl*p*, *p*NPAra*p* and *p*NPXyl*f*) were performed and the products were monitored by TLC analysis. After short incubation periods (10 min), autocondensation constituted the main reaction in the case of reactions performed in the presence of *p*NPXyl*p* or *p*NPAra*f*. However, in the case of *p*NPAra*p* and *p*NPXyl*f*, only the products of hydrolysis were detected (*p*NP and the corresponding monosaccharide), indicating that these compounds can only act as donors and not acceptors. In view of the structural data and the results of a recent mutagenesis study,[18] it can be expected that inversed configuration of the C-4 (OH) (compared to L-arabinofuranose) displayed by L-arabinopyranosyl and D-xylofuranosyl residues would have a major impact on −1 (donor) subsite binding.

To pursue our investigation, the products of the reactions involving *p*NPAra*f* and *p*NPXyl*p* were purified and identified by NMR analysis. In the case of *p*NPAra*f*, the major product of transglycosylation (60–70% as determined by relative integration of the anomeric protons H-1 and H-1′) was *p*-nitrophenyl α-L-arabinofuranosyl-(1 → 2)-α-L-arabinofuranoside (**1**). Minor disaccharides, probably *p*NP α-L-arabinofuranosyl-(1 → 3)-α-L-arabinofuranoside and *p*-nitrophen-



Scheme 1.

yl α-L-arabinofuranosyl-(1 → 5)-α-L-arabinofuranoside, were also detected. Using *p*NPXyl*p* as substrate, *p*-nitrophenyl β-D-xylopyranosyl-(1 → 2)-β-D-xylopyranoside (**2**) and *p*-nitrophenyl β-D-xylopyranosyl-(1 → 3)-β-D-xylopyranoside (**3**) were generated in approximately equal amounts. The determination of the corresponding structures for compounds **2** and **3** were performed by careful analysis of the ^{1}H and ^{13}C NMR spectra (Scheme 1). For compound **2**, taking into account the nature of the solvent and the calibration reference, the ^{13}C chemical shift for C-2 was very downfield shifted ($\delta = 82.2$ ppm) when compared to an analogous free carbon ($\Delta\delta = +7$ ppm),[19] but was identical to C-2 of the β-D-xylose unit at the reducing end of xylopyranosyl-(1 → 2)-β-D-xylopyranoside.[20] This indicates that in compound **2**, O-2 is substituted. Finally, the chemical shifts of the neighbouring carbons, C-1 and C-3, were weakly upfield shifted ($\Delta\delta = -1$ ppm), thus unambiguously confirming O-2 substitution. It is noteworthy that our results clearly indicate that the spectral attributions for the H-4 and H-5 of the aglycon-linked Xyl residue, that appeared in a previous report by Armand et al.[12] are erroneous. These authors performed a 1D TOCSY experiment in selective excitation mode, unaware that both H3 and H4 closely resonate at about 3.67 ppm. In fact these protons resonated together at approximately 3.8 ppm, which is confirmed by their corresponding carbons C-3 and C-4 (chemical shifts of 75.9 and 69.8 ppm, respectively), whereas C-5/H-5b resonated at 66.4/3.44 ppm, in agreement with published data. Therefore, it can be concluded that the transglycosylation reactions described by Armand et al.[12] led to the production of oligo-β-(1 → 2) and oligo-β-(1 → 3)-linked xylopyranosides, and not oligo-β-(1 → 4)-linked xylopyranosides. For compound **3**, the ^{13}C chemical signal for C-3 was very downfield shifted ($\delta = 84.5$ ppm), in accordance with available data for C-3 substituted xylopyranose,[19–21] whereas C-2 and C-4 exhibited chemical shifts corresponding to unsubstituted analogous carbons. These results show that AbfD3 displays poor regioselectivity, although in the presence of *p*NPXyl*p* the formation of a β-(1 → 4)-linked disaccharide was not detected. These results are unexceptional since, unlike polysaccharide hydrolases, most *exo*-acting glycoside hydrolases display relaxed regioselectivity. Moreover, numerous investigations have shown that reaction conditions, the nature and the anomeric configuration of the aglycon moiety of the acceptor[8,22,23] and the exact source of the enzymes influence regioselectivity.[23–25] Importantly, it is also known that frequently the regioselectivity of the transglycosylation reaction can be directly correlated to the hydrolytic specificity of glycoside hydrolases.[6] In the case of AbfD3, it has been previously shown that this enzyme can hydrolyse both the α-(1 → 2) and α-(1 → 3) bonds in arabinoxylans and, to a lesser extent, the α-(1 → 5) bond in arabinan.[15]

Time course analysis of the formation of compounds **1**, **2** and **3** (Figs. 1 and 2) revealed that, like the previously reported transglycosylation reactions,[16] transglycosylation was rapid since maximum product yields were obtained after 30 s (**1**) and after 10 min (**2** and **3**) in the presence of 0.11 or 22 IU of AbfD3, respectively.

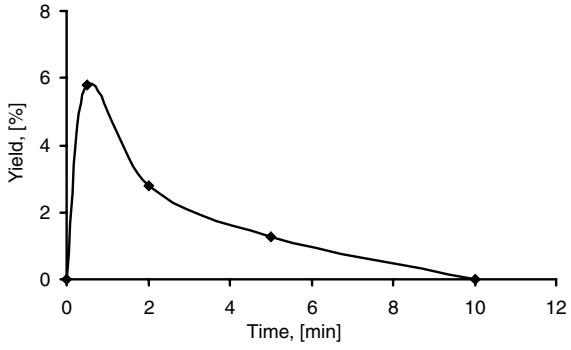


Figure 1. Kinetics of the formation of compound **1** obtained through AbfD3-catalysed transarabinosylation of *p*NPAra*f*. Yields are calculated as percentage of the initial quantity of *p*NPAra*f*.

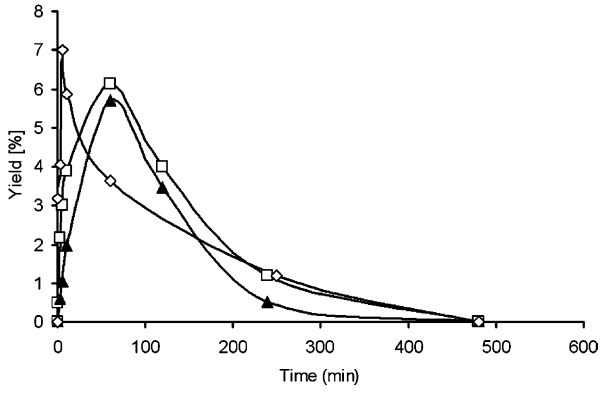


Figure 2. Kinetics of the formation of β-(1 → 2)-linked compound **2** (▲) and β-(1 → 3)-linked compound **3** (□) obtained through AbfD3-catalysed transxylosylation of *p*NPXyl*p* and the β-(1 → 2)-linked compound **4** (◇) obtained through AbfD3-catalysed transarabinosylation of $PhCH_2$-Xyl*p* with *p*NPAra*f*. Yields are calculated either as percentage of the initial quantity of *p*NPXyl*p* (compounds **2** and **3**) or as percentage of the initial quantity of *p*NPAra*f* (compound **4**).

However, maximum yields were low (5.8%, 6.1% and 5.7%, respectively, for products **1**, **2** and **3**). After reaching maximum yield, products were the subject of secondary hydrolysis and progressively disappeared. In the case of *p*-nitrophenyl arabinobioside (**1**), no product remained after 10 min of incubation, whereas the *p*-nitrophenyl xylobiosides (**2** and **3**) were progressively degraded over a 6-h period. These results clearly reflect the superior hydrolytic efficiency (k_{cat}/K_m) of AbfD3 towards **1** and provide an explanation for the presence of *p*-nitrophenyl xylotrioside among the products. The lower efficiency (k_{cat}/K_m) of AbfD3-mediated hydrolysis of *p*-nitrophenyl xylobiosides would allow products **2** and **3** to act as acceptors for xylosylation. With regard to the poor yields, several parameters (substrate concentration, enzyme concentration and incubation temperature) were varied, but no improvement was measured. Surprisingly, the combined yield (compounds **2** and **3**) (11.8%) of the reaction involving *p*NPXyl*p* was higher than that involving *p*NPAra*f* (between 8.3% and 8.7%). Since the −1 (donor) subsite is highly selective for *p*NPAra*f*, this observation may indicate that, inversely, the +1 (acceptor) subsite of the enzyme is better adapted for occupation by a xylopyranoside than an arabinofuranoside, or that water-mediated deglycosylation of the arabinofuranosyl-enzyme intermediate is more efficient than water-mediated deglycosylation of the xylopyranosyl-enzyme intermediate.

2.3. AbfD3-mediated synthesis of arabinofuranosyl-xylopyranoside

To test the ability of AbfD3 to catalyse the transxylosylation of an arabinofuranoside, *p*NPAra*f* and benzyl α-D-xylopyranoside ($PhCH_2$-Xyl*p*) were provided as substrates. Presumably, because $PhCH_2$-Xyl*p* possesses a relatively stable glycosidic bond that also exhibits α-anomeric configuration, it is not hydrolysed by AbfD3 (data not shown). Therefore, it was assumed that the more reactive *p*NPAra*f* would act as the donor and $PhCH_2$-Xyl*p* as the acceptor. Analysis of the reaction products partially confirmed this assumption and revealed that one of the disaccharides produced was a benzyl α-L-arabinofuranosyl-(1 → 2)-α-D-xylopyranoside (**4**) (Scheme 2). However, NMR analysis revealed that arabinobiosides, resulting from the autocondensation of *p*NPAra*f*, were also produced, indicating that *p*NPAra*f* competes with $PhCH_2$-Xyl*p* for acceptor subsite occupation. Appealingly though, the transxylosylation of *p*NPAra*f* appeared to be regioselective. Analysis of the ^{13}C NMR data revealed a downfield shift of the C-2 of the D-xylopyranoside moiety from 73.5 (for $PhCH_2$-Xyl*p*) to 78.8 ppm. Moreover, a ^{13}C–^{1}H (HMBC) spectrum showed an intense three-bond coupling between C-1′ (109.2 ppm) and H-2, whereas no correlation was observed between C-1′ and H-3 or H-4. A time course experiment of the production of **4** (Fig. 2) once again revealed a poor maximal yield of 7%, similar to that of the arabinobiosides, which was achieved after 10 min. However, the mixed disaccharide was more stable than the arabinobiosides since it was progressively hydrolysed over an 8-h period. These results indicate that xylopyranosides are not necessarily better acceptors and support the hypothesis that the different yields of disaccharides observed in this study are related to the aptitude of the glycosyl-enzyme for water-mediated deglycosylation. Interestingly, the information gleaned from the recently published structural study agrees with this hypothesis and suggests that xylopyranose lacks the necessary distortion for efficient catalysis.[17]

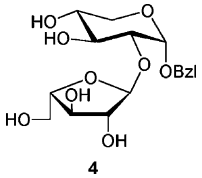
With regard to the observed regioselectivity of the reaction, improved regioselectivity using other glycoside hydrolases has been achieved using acceptors with bulky hydrophobic aglycon groups and α-anomeric configuration.[22,23] Since the $PhCH_2$-Xyl*p* we used also exhibits α-anomeric configuration, this may have been a determining factor.

2.4. Concluding remarks

We have demonstrated that AbfD3 possesses the ability to synthesise oligosaccharides in kinetically controlled transglycosylation reactions. The products of these reactions, in particular compound **4**, could be useful as analytic tools for the study of plant cell walls, which often contain arabinoxylans, polymers consisting of main a chain composed of (1 → 4)-linked β-D-xylopyranosyl residues substituted at O-2 and/or O-3 by α-L-arabinofuranosyl groups.[26] Such synthetic oligosaccharide motifs could be used as reference compounds for the analysis of hemicellulase action and for raising antibodies to well-defined motifs for immunochemical-based analyses of plant cell walls.

Although the reaction yields presented here are rather poor, at least one reaction displayed tight regioselective control. Improvements to reaction yields can be expected if water activity can be reduced. Therefore, future work will concentrate on this area in order to identify suitable co-solvents.

Importantly, the relaxed stereoselectivity of the +1 (acceptor) subsite of AbfD3 observed in this study may indicate that other sugar acceptors could be employed. In particular, it may be interesting to investigate the use



4

Scheme 2.

of D-galactopyranosides and D-galactofuranosides as acceptors since L-arabinopyranosyl-D-galactopyranoside motifs are present in plant cell walls and D-arabino-D-galactofuranoside motifs are present in the cell walls of many pathogenic bacteria, including *Mycobacterium tuberculosis*, the causative agent of tuberculosis.

Finally, AbfD3 may constitute an interesting enzymatic scaffold for protein engineering work. Indeed, in a recent study we demonstrated that the modification of a single amino acid in the (−1) donor subsite is sufficient to radically alter stereoselectivity and/or to preferentially diminish hydrolytic activity.[18] In the latter case, although transglycosylation is also slower, maximal yields are unaffected and the apparent product stability is increased due to the low level of secondary hydrolysis. Therefore, it is tempting to speculate that in the future it will be possible to create AbfD3 variants adapted for the synthetic catalysis of a wide variety of oligosaccharides.

3. Experimental

3.1. AbfD3 production and activity measurement

Recombinant AbfD3 expressed from the plasmid-borne *AbfD3* gene was produced and purified from *Escherichia coli* cells as previously described.[15] The hydrolytic activity of AbfD3 towards *p*NP glycosides was determined by continuous measurement of *p*NP release. Reactions were performed in buffered conditions (50 mM sodium acetate buffer, pH 5.8) in the presence of *p*NP glycoside (5 mM) and 0.1 mL of enzyme soln. The total reaction volume was 1 mL. Reactions were incubated at 60 °C and *p*NP release was monitored spectrophotometrically at 401 nm. One unit of activity was defined as the amount of enzyme releasing 1 μmol of *p*NP per min.

3.2. Glycosides

*p*NPXyl*p* and *p*NPAra*p* were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). *p*NPAra*f* and *p*NPXyl*f* were synthesised on a multi-gram scale according to methods previously described for other monosaccharides.[16]

3.2.1. *p*-Nitrophenyl α-L-arabinofuranoside. White needles (39% overall yield from L-arabinose in a five-step synthesis): mp 157–159 °C, lit.[27] 155–156 °C; $[\alpha]_D^{22}$ −211 (*c* 1.00, MeOH), lit.[27] $[\alpha]_D^{20}$ −205 (*c* 0.50, water); ^{1}H NMR (CD_3OD): δ 8.18 (d, 2H, $J_{3',2'}$ 9.2 Hz, H-3′, H-5′), 7.20 (d, 2H, $J_{2',3'}$ 9.2 Hz, H-2′, H-6′), 5.67 (d, 1H, $J_{1,2}$ 1.5 Hz, H-1), 4.31 (d, 1H, $J_{2,3}$ 3.4, $J_{2,1}$ 1.5 Hz, H-2), 3.99–4.12 (m, 2H, H-3, H-4), 3.79 (dd, 1H, $J_{5a,5b}$ 12.0, $J_{5a,4}$ 2.9 Hz, H-5a), 3.72 (dd, 1H, $J_{5b,5a}$ 12.0, $J_{5b,4}$ 4.4 Hz, H-5b); ^{13}C NMR (CD_3OD): δ 163.4 (C-1′), 143.4 (C-4′), 126.5 (C-3′, C-5′), 117.6 (C-2′, C-6′), 107.7 (C-1), 86.9 (C-4), 83.5 (C-2), 78.1 (C-3), 62.6 (C-5); Anal. Calcd for $C_{11}H_{13}O_7N$: C, 48.71; H, 4.83; N, 5.16. Found: C, 48.81; H, 4.91; N, 4.93.

3.2.2. *p*-Nitrophenyl β-D-xylofuranoside. White needles (32% overall yield from D-xylose in a five-step synthesis): mp 129–130 °C, $[\alpha]_D^{22}$ −181 (*c* 1.00, MeOH), ^{1}H NMR (CD_3OD): δ 8.19 (d, 2H, $J_{3',2'}$ 9.2 Hz, H-3′, H-5′), 7.20 (d, 2H, $J_{2',3'}$ 9.2 Hz, H-2′, H-6′), 5.64 (s, 1H, H-1), 4.41 (dt, 1H, $J_{4,5a}$ 9.2, $J_{4,5b} = J_{4,3}$ 4.8 Hz, H-4), 4.33 (d, 1H, $J_{2,3}$ 1.6 Hz, H-2), 4.22 (dd, 1H, $J_{3,4}$ 4.8, $J_{3,2}$ 1.6 Hz, H-3), 3.84 (dd, 1H, $J_{5a,5b}$ 11.7, $J_{5a,4}$ 4.7 Hz, H-5a), 3.72 (dd, 1H, $J_{5b,5a}$ 11.7, $J_{5b,4}$ 6.8 Hz, H-5b); ^{13}C NMR (CD_3OD): δ 163.4 (C-1′), 143.4 (C-4′), 126.6 (C-3′, C-5′), 117.6 (C-2′, C-6′), 107.7 (C-1), 85.7 (C-4), 82.7 (C-2), 77.1 (C-3), 62.4 (C-5); Anal. Calcd for $C_{11}H_{13}O_7N$: C, 48.71; H, 4.83; N, 5.16. Found: C, 48.35; H, 4.68; N, 4.85.

3.2.3. Benzyl α-D-xylopyranoside. Conversion of D-xylose to $PhCH_2$-Xyl*p* was achieved using a slight modification of the procedure described by Ballou for benzyl α-D-arabinopyranoside.[28] Briefly, 50 g of D-xylose were mixed with 8 mol/equiv of purified benzyl alcohol. The stirred mixture was cooled in an ice bath and dry HCl was bubbled through until saturation. The mixture was allowed to reach room temperature overnight under constant stirring. Diethylether was added to the resulting clear soln and the soln was cooled to 0 °C to allow crystallisation. The crystals were separated by filtration, washed with ether and dried under diminished pressure to yield 24 g (30%) of product. A further crystallisation step afforded an other 5 g (36%) of crystals: mp 128°C, lit.[29] (127–128.5 °C); ^{1}H NMR ($CD_3OD + D_2O$): δ 7.42–7.20 (m, 5H, Ph), 4.83 (d, 1H, $J_{1,2}$ 3.8 Hz, H-1), 4.70 (d, 1H, J_{gem} 11.8 Hz, H-benzyl), 4.50 (d, 1H, J_{gem} 11.8 Hz, H-benzyl), 3.65 (t, 1H, $J_{3,2} = J_{3,4}$ 9.5 Hz, H-3), 3.63–3.47 (m, 3H, H-4, H-5a, H-5b), 3.42 (dd, 1H, $J_{2,3}$ 9.5, $J_{2,1}$ 3.8 Hz, H-2); ^{13}C NMR ($CD_3OD + D_2O$): δ 138.6, 129.0, 128.6 (Ph), 99.4 (C-1), 75.1 (C-2), 73.4 (C-3), 71.4 (C-4), 70.2 (CH_2 Ph), 63.1 (C-5).

3.3. Analytical methods

Transglycosylation reactions were monitored by TLC using Kieselgel 60 F_{254} aluminium-backed sheets (E. Merck) and 7:2:2 EtOAc–HOAc–water as the mobile phase. Products were detected by charring (at 100 °C) with 0.2% v/v orcinol in H_2SO_4 (20% v/v). Prior to TLC analysis, heating at 100 °C for 10 min stopped enzyme-catalysed reactions.

The kinetics of transglycosylation reactions were determined by high performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) on a Dionex system (Dionex Corpo-

ration, CA, USA) equipped with a CarboPac PA-1 column (Dionex, 250×4.5 mm) and a suitable guard column. Separation was performed at a flow rate of 1 mL/min with a gradient of 1 M sodium acetate in 0.1 M NaOH.[30] Prior to product quantification by HPAEC, enzyme-catalysed reactions were stopped by addition of an equal volume of universal buffer at pH 11 (6.008 g citric acid, 3.893 g KH_2PO_4, 1.769 g H_3BO_3, 5.266 g sodium barbital in 1 L adjusted at pH 11 with NaOH).

The structural analysis of transglycosylation products was performed using ^{1}H and ^{13}C NMR spectroscopy. Spectra were recorded at 500 and 125 MHz, respectively, (Bruker DRX spectrometer) in D_2O at 293 K for compounds **1** and **4** and 400.3 and 100.7 MHz (Bruker ASX-400NB spectrometer) in D_2O at 300 K for compounds **2** and **3**. Chemical shifts were expressed in ppm downfield from the signal of the methyl group of internal acetone (^{1}H NMR $\delta = 2.22$ ppm, ^{13}C NMR $\delta = 31.5$ ppm).

Electrospray-ionisation mass spectra of the disaccharides (ESIMS) were recorded on samples dissolved in water using a Q-TOF mass spectrometer (Micromass, UK). Cone voltage was 30 V and the source temperature was 80 °C. Measurements were performed in either positive ($[M+Na]^+$ ion detection) or negative mode ($[M-H]^-$ ion detection).

3.4. Procedures for AbfD3-catalysed transglycosylation and purification of disaccharides

For the production of compound **1**, 0.11 IU, or for compounds **2** and **3**, 22 IU, of AbfD3 were added to a 5 mM soln of *p*NPAra*f* (compound **1**) or *p*NPXyl*p* (compounds **2** and **3**) in 0.28 mL of 50 mM sodium acetate buffer, pH 5.8. Reactions were incubated at 60 °C for an appropriate time. To produce compound **4**, 0.11 IU of AbfD3 was added to 0.46 mL of a buffered soln (50 mM sodium acetate, pH 5.8) containing *p*NPAra*f* (3 mM) and $PhCH_2$-Xyl*p* (2 mM). Reactions were incubated at 60 °C.

Preparative scale production of disaccharides was performed either by preparative TLC using the conditions described above (for compounds **1**, **2** and **3**) or by flash chromatography on silica gel columns (E. Merck) with 20:1 EtOAc–MeOH as the mobile phase (compound **4**).

3.4.1. *p*-Nitrophenyl α-L-arabinofuranosyl-(1 → 2)-α-L-arabinofuranoside (1). ^{1}H NMR (D_2O): δ 8.11 (d, 2H, J 9.1 Hz, H-3″, H-5″), 7.09 (d, 2H, J 9.1 Hz, H-2″, H-6″), 5.82 (d, 1H, $J_{1,2}$ 1.5 Hz, H-1), 5.08 (d, 1H, $J_{1',2'}$ 1.5 Hz, H-1′), 4.30 (dd, 1H, $J_{2,3}$ 4.0, $J_{2,1}$ 1.5 Hz, H-2), 4.11 (dd, 1H, $J_{3,4}$ 6.2, $J_{3,2}$ 4.0 Hz, H-3), 4.03 (ddd, 1H, $J_{4,3}$ 6.2, $J_{4,5a}$ 5.1,$J_{4,5b}$ 3.3 Hz, H-4), 4.00 (dd, 1H, $J_{2',3'}$ 3.3, $J_{2',1'}$ 1.5 Hz, H-2′), 3.88 (ddd, 1H, $J_{4',5'a} = J_{4',3'}$ 6.2, $J_{4',5'b}$ 3.3 Hz, H-4′), 3.79 (dd, 1H, $J_{3',4'}$ 6.2, $J_{3',2'}$ 3.3 Hz, H-3′), 3.69 (dd, 1H, $J_{5b,5a}$ 12.8, $J_{5b,4}$ 3.3 Hz, H-5b), 3.59 (dd, 1H, $J_{5b,5a}$ 12.8, $J_{5a,4}$ 3.3 Hz, H-5a), 3.55 (dd, 1H, $J_{5'b,5'a}$ 12.8, $J_{5'b,4'}$ 3.3 Hz, H-5′b), 3.30 (dd, 1H, $J_{5'a,5'b}$ 12.8, $J_{5'a,4'}$ 6.2 Hz, H-5′a); ^{13}C NMR (D_2O): δ 161.2 (C-1″), 142.1 (C-4″), 125.9 (C-3″, C-5″), 116.5 (C-2″, C-6″), 107.4 (C-1′), 104.3 (C-1), 87.2 (C-2), 84.0 (C-4′), 83.9 (C-2′), 81.2 (C-4), 76.4 (C-3′), 74.3 (C-3), 60.8 (C-5′), 60.3 (C-5). ESIMS: m/z calcd for $[C_{16}H_{21}N0_{11}]Na^+$: 426.34. Found: 426.03.

3.4.2. *p*-Nitrophenyl β-D-xylopyranosyl-(1 → 2)-β-D-xylopyranoside (2). ^{1}H NMR (D_2O): δ 8.28 (d, 2H, J 9.1 Hz, H-3″, H-5″), 7.25 (d, 2H, J 9.1 Hz, H-2″, H-6″), 5.39 (d, 1H, $J_{1,2}$ 7.2 Hz, H-1), 4.74 (d, 1H, $J_{1',2'}$ 7.7 Hz, H-1′), 4.06 (dd, 1H, $J_{5eq,5ax}$ 11.7 $J_{5eq,4}$ 4.9 Hz, H-5_{eq}), 3.83 (dd, 1H, $J_{2,3}$ 9.8, $J_{2,1}$ 7.2 Hz, H-2), 3.82–3.74 (m, 3H, H-3, H-4, H-$5'_{eq}$), 3.61–3.53 (m, 2H, H-4′, H-5_{ax}), 3.47 (dd, 1H, $J_{3',4'}$ 9.4, $J_{3',2'}$ 8.8 Hz, H-3′), 3.30 (dd, 1H, $J_{2',3'}$ 8.8, $J_{2',1'}$ 7.7 Hz, H-2′), 3.29 (dd, $J_{5'ax,5'eq}$ 11.4, $J_{5'ax,4'}$ 10.4 Hz, H-$5'_{ax}$); ^{13}C NMR (D_2O) δ 161.2 (C-1″), 142.1 (C-4″), 125.9 (C-3″, C-5″), 116.5 (C-2″, C-6″), 104.7 (C-1′), 99.7 (C-1), 82.2 (C-2), 76.5 (C-3′), 75.9 (C-3), 74.6 (C-2′), 70.3 (C-4′), 69.8 (C-4), 66.5 (C-5′), 66.2 (C-5). ESIMS: m/z calcd for $[C_{16}H_{21}N0_{11}]Na^+$: 426.34. Found: 426.12.

3.4.3. *p*-Nitrophenyl β-D-xylopyranosyl-(1 → 3)-β-D-xylopyranoside (3). ^{1}H NMR (D_2O): δ 8.28 (d, 2H, J 9.1 Hz, H-3″, H-5″), 7.25 (d, 2H, J 9.1 Hz, H-2″,H-6″), 5.26 (d, 1H, $J_{1,2}$ 7.3 Hz, H-1), 4.72 (d, 1H, $J_{1',2}$ 7.6 Hz, H-1′), 4.10 (dd, 1H, $J_{5eq,5ax}$ 11.5, $J_{5eq,4}$ 4.2 Hz, H-5_{eq}), 4.01 (dd, 1H, $J_{5'eq,5'ax}$ 11.7 $J_{5'eq,4'}$ 5.4 Hz, H-$5'_{eq}$), 3.85–3.78 (m, 3H, H-2, H-3, H-4), 3.67 (ddd, 1H, $J_{4',5'ax}$ 10.8, $J_{4',3'}$ 9.4, $J_{4',5'eq}$ 4.2 Hz, H-4′), 3.59 (dd, 1H, $J_{5ax,5eq}$ 11.5, $J_{5ax,4}$ 9.8 Hz, H-5_{ax}), 3.48 (dd, 1H, $J_{3',2'}$ 9.8 $J_{3',4'}$ 9.1 Hz, H-3′), 3.37 (dd, 1H, $J_{2',3'}$ 9.8, $J_{2',1'}$ 7.6 Hz, H-2′), 3.33 (dd, $J_{5'ax,5'eq}$ 11.7, $J_{5'ax,4'}$ 10.8 Hz, H-$5'_{ax}$); ^{13}C NMR (D_2O) δ 161.2 (C-1″), 142.1 (C-4″), 125.9 (C-3″, C-5″), 116.5 (C-2″, C-6″), 104.9 (C-1′), 101.1 (C-1), 84.5 (C-3), 76.8 (C-3′), 74.8 (C-2′), 73.5 (C-2), 70.4 (C-4′), 68.8 (C-4), 66.5 (C-5′), 66.5 (C-5). ESIMS: m/z calcd for $[C_{16}H_{20}N0_{11}]^-$: 402.35. Found: 402.77.

3.4.4. Benzyl α-D-xylopyranosyl-(1 → 2)-α-L-arabinofuranoside (4). ^{1}H NMR (D_2O): δ 7.42–7.33 (m, 5H, H-Ar), 5.04 (d, 1H, $J_{1,2}$ 3.6 Hz, H-1), 5.02 (d, 1H, $J_{1',2'}$ 1.7 Hz, H-1′), 4.70 (d, 1H, J_{gem} 11.7 Hz, H-benzyl), 4.54 (d, 1H, J_{gem} 11.7 Hz, H′-benzyl), 4.06 (dd, 1H, $J_{2',3'}$ 3.9, $J_{2',1'}$ 1.7 Hz, H-2′), 3.84 (dd, 1H, $J_{3',4'}$ 6.9, $J_{3',2'}$ 3.9 Hz, H-3′), 3.71–3.63 (m, 3H, H-3, H-4, H-5′a), 3.62–3.59 (m, 1H, H-4′), 3.55–3.51 (m, 3H, H-5′b, H-5a, H-5b), 3.48 (dd, 1H, $J_{2,3}$ 9.5, $J_{2,1}$ 3.6 Hz, H-2); ^{13}C NMR (D_2O): δ 136.0 (C-1″), 129.1 (C-3″, C-5″), 128.6 (C-2″, C-6″), 128.4 (C-4″), 109.2 (C-1′), 96.3 (C-1), 82.9 (C-4′), 81.3 (C-2′), 78.8 (C-2), 75.9 (C-3′), 71.9 (C-3), 69.2 (C-4), 68.9 (CH_2-benzyl), 60.8 (C-5′), 60.2 (C-5). ESIMS: m/z calcd for $[C_{17}H_{23}0_9]^-$: 371.38. Found: 371.11.

Acknowledgements

The present work was partially financed through the GLYCOVAL project (EUROPOL'AGRO—Conseil Général de la Marne). The authors express their gratitude to H. Baillia for technical help with the NMR work and D. Harakat for the ESIMS measurements.

References

1. Koshland, D. *Biol. Rev.* **1953**, *28*, 416–436.
2. Sinnott, M. L. *Chem. Rev.* **1990**, *90*, 1171–1202.
3. McCarter, J. D.; Withers, S. G. *Curr. Opin. Struct. Biol.* **1994**, *4*, 885–892.
4. Withers, S. G. *Carbohydr. Polym.* **2001**, *44*, 325–337.
5. Viladot, J. L.; Moreau, V.; Planas, A.; Driguez, H. *J. Chem. Soc., Perkin Trans. 1* **1997**, 2383–2387.
6. Mala, S.; Dvorakova, H.; Hrabal, R.; Kralova, B. *Carbohydr. Res.* **1999**, *322*, 209–218.
7. Prade, H.; Mackenzie, L. F.; Withers, S. G. *Carbohydr. Res.* **1998**, *305*, 371–381.
8. Lopez, R.; Fernandez-Mayoralas, A. *Tetrahedron Lett.* **1992**, *33*, 5449–5452.
9. Binder, W. H.; Kählig, H.; Schmid, W. *Tetrahedron* **1994**, *50*, 10407–10418.
10. Huber, R.; Gaunt, M.; Hurlburt, K. *Arch. Biochem. Biophys.* **1984**, *234*, 151–160.
11. Deleyn, F.; Claeyssens, M.; De Bruyne, C. K.; De Bruyn, A. *Can. J. Biochem. Cell. Biol.* **1985**, *63*, 204–211.
12. Armand, S.; Vieille, C.; Gey, C.; Heyraud, A.; Zeikus, G.; Henrissat, B. *Eur. J. Biochem.* **1996**, *236*, 706–713.
13. Eneyskaya, E. V.; Brumer, H.; Backinowsky, L. V.; Ivanen, D. R.; Kulminskaya, A. A.; Shabalin, K. A.; Neustroev, K. N. *Carbohydr. Res.* **2003**, *338*, 313–325.
14. Matsumara, S.; Sakiyama, K.; Toshima, K. *Biotechnol. Lett.* **1999**, *21*, 17–22.
15. Debeche, T.; Cummings, N.; Connerton, I.; Debeire, P.; O'Donohue, M. J. *Appl. Environ. Microbiol.* **2000**, *66*, 1734–1736.
16. Rémond, C.; Ferchichi, M.; Aubry, N.; Plantier-Royon, R.; Portella, C.; O'Donohue, M. J. *Tetrahedron Lett.* **2002**, *43*, 9653–9655.
17. Hövel, K.; Shallom, D.; Niefind, K.; Belakhov, V.; Shoham, G.; Baasov, T.; Shoham, Y.; Schomburg, D. *EMBO J.* **2003**, *22*, 4922–4932.
18. Ferchichi, M.; Rémond, C.; Simo, R.; O'Donohue, M. J. *FEBS Lett.* **2003**, *553*, 381–386.
19. Bock, K.; Pedersen, C. *Adv. Carbohydr. Chem. Biochem.* **1983**, *41*, 27–66.
20. Bock, K.; Pedersen, C.; Pedersen, H. *Adv. Carbohydr. Chem. Biochem.* **1984**, *42*, 193–225.
21. Gloaguen, V.; Ruiz, G.; Morvan, H.; Mouradi-Givernaud, A.; Maes, E.; Krausz, P.; Strecker, G. *Carbohydr. Res.* **2004**, *339*, 97–103.
22. Nilsson, K. G. I. *Carbohydr. Res.* **1987**, *167*, 95–103.
23. Zeng, X.; Yoshino, R.; Murata, T.; Ajisaka, K.; Usui, T. *Carbohydr. Res.* **2000**, *325*, 120–131.
24. Yoon, J. H.; Ajisaka, K. *Carbohydr. Res.* **1996**, *292*, 153–163.
25. Weignerova, L.; Spizek, J.; Najmanova, L.; Kren, V. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 1897–1899.
26. Joseleau, J.; Comtat, J.; Ruel, K. Chemical structure of xylans and their interaction in plant cell walls. In *Xylans and Xylanases*; Visser, J., Beldman, G., Someren, K.-v., Voragen, A., Eds.; Elsevier: Amsterdam, 1992; Vol. 7, pp 1–15.
27. Fielding, A. H.; Hough, L. *Carbohydr. Res.* **1965**, *1*, 327–329.
28. Ballou, C. E. *J. Am. Chem. Soc.* **1957**, *79*, 165–166.
29. Ballou, C. E.; Roseman, S.; Link, K. P. *J. Am. Chem. Soc.* **1951**, *73*, 1140–1144.
30. Beldman, G.; Searle-van Leeuwen, M. J. F.; De Ruiter, G. A.; Siliha, H. A.; Voragen, A. G. J. *Carbohydr. Polym.* **1993**, *20*, 159–168.