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An original chemoenzymatic route for the synthesis of β-D-galactofuranosides using an α-L-arabinofuranosidase

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Abstract—Galactofuranose is a widespread component of cell wall polysaccharides in bacteria, protozoa and fungi, but is totally absent in mammals. Importantly, galactofuranose is a key constituent of major cell envelope polysaccharides in pathogenic mycobacteria. In this respect, galactofuranose-based glycoconjugates are interesting target molecules for drug design. *O*-Glycosidases and notably β-D-galactofuranosidases could be useful tools for the chemoenzymatic synthesis of galactofuranosides, but to date no studies of this type have been reported. Here we report the use of a GH 51 α-L-arabinofuranosidase for the synthesis of β-D-galactofuranosides. We have demonstrated that this enzyme can catalyse both the autocondensation of *p*-nitrophenyl-β-D-galactofuranoside and the trans-galactofuranosylation of benzyl α-D-xylopyranoside, forming *p*-nitrophenyl β-D-galactofuranosyl-(1→2)-β-D-galactofuranoside and benzyl β-D-galactofuranosyl-(1→2)-α-D-xylopyranoside, respectively. Both reactions were very regiospecific and the reaction involving benzyl α-D-xylopyranoside afforded very high yields (74.8%) of the major product. To our knowledge, this demonstration of chemoenzymatic synthesis of galactofuranosides constitutes the very first use of an *O*-glycosidase for the synthesis of galactofuranosides.

Keywords: α-L-Arabinofuranosidase; Transglycosylation; *O*-Glycosidase; GH 51; Galactofuranose; Mycobacteria

1. Introduction

In nature, arabinose is frequently present in the furanose form. Notably, α-L-arabinofuranose is abundant in plant cell walls,[1] whereas α-D-arabinofuranose is characteristic of the cell wall glycoconjugates of several prokaryotes. While being absent in plants and mammals, galactose in the furanose form is also widespread in nature. β-D-Galactofuranose is an abundant constituent of cell wall glycoconjugates of bacteria,[2] protozoa[3] and fungi.[4] Importantly, β-D-galactofuranose and/or α-D-arabinofuranose are cell wall constituents in several pathogenic species. These include *Mycobacterium tuberculosis* and *leprae*, the causative agents of tuberculosis and leprosy, respectively, and *Trypanosama cruzi*, the protozoan responsible for Chaga's disease.

Over the last decade or so, the alarming progression of multidrug-resistant mycobacterial strains (MDRTB) around the world has been the subject of major public concern.[5] MDRTB strains are defined as strains that are resistant to at least isoniazid and rifampicin, two first-line drugs currently used for the treatment of tuberculosis.[6] The former, isoniazid, and another first-line drug ethambutol affect the biosynthesis of the arabinogalactan–peptidoglycan complex (MAPc),[7–9] a major component of the mycobacterial cell envelope. MAPc is composed of a galactan polymer, which is

Abbreviations: AbfD3, α-L-arabinofuranosidase D3; *p*NP, *p*-nitrophenol; *p*NPGal*f*, *p*-nitrophenyl β-D-galactofuranoside; *p*NPAra*f*, *p*-nitrophenyl α-L-arabinofuranoside; *p*NPXyl*p*, *p*-nitrophenyl-β-D-xylopyranoside; *p*NP β-D-Glc*f*, *p*-nitrophenyl-β-D-glucofuranoside; *p*NP β-D-Xyl*f*, *p*-nitrophenyl-β-D-xylofuranoside; *p*NP β-D-Gal*p*, *p*-nitrophenyl-β-D-galactopyranoside; $PhCH_2$ α-D-Xyl*p*, benzyl α-D-xylopyranoside; $PhCH_2$ β-L-Ara*p*, benzyl β-L-arabinopyranoside.

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made of D-galactofuranose monomers, which are interlinked via (1→5) and (1→6) glycosidic bonds, and an arabinan polymer that is mainly composed of (1→5)-linked D-arabinofuranose residues. The arabinan part of MAPc is itself linked to mycolic acids. A second major cell envelope polysaccharide is lipoarabinomannan (LAM) that also contains arabinose and galactose in the furanose form. This polymer, localised in the cell membrane, extends out towards and contacts MAPc.[10]

Because of the absence of galactofuranose in humans, galactofuranose-based conjugates are both highly immunogenic and resistant to endogenous human glycosidases. Therefore, MAPc and LAM-associated galactans constitute attractive targets for new drug development. To this end, two strategies can be envisaged: (i) the synthesis of galactofuranose analogues that could act as inhibitors of the biosynthetic enzymes UDP-galactopyranose mutase[11] and galactofuranosyl transferases[12] or (ii) the synthesis of galactofuranosides that could be used for the elaboration of vaccines. With regard to the inhibition of mycobacterial cell wall biosynthesis, the production of β-D-galactofuranosyl-(1→4)-α-L-rhamnopyranosyl octyl analogues has been described.[13] Although these compounds proved to be effective acceptors for mycobacterial glycosyl transferases, in vitro inhibition tests revealed that they only moderately inhibited the growth of two mycobacterial strains.[13] So far, no reports of new galactofuranose-based vaccines have been reported. However, it is known that cell-surface bacterial polysaccharides often elicit immune responses in humans. Therefore, it is expected that certain mycobacterial cell wall polysaccharides, or motifs thereof, could constitute effective vaccines. Indeed, to combat other pathogenic bacteria such as *Streptococcus pneumoniae* type 23F[14] or *Haemophilus influenzae* type b,[15] the synthesis and use of oligosaccharide motifs for vaccine development has already proved successful.

To obtain relevant oligosaccharides for drug design it is necessary to identify an appropriate strategy. A straightforward approach consists of the extraction via chemical and/or enzymatic means and purification of bacterial polysaccharides. A second widely used approach is the chemical synthesis of specific oligosaccharides. Although several reports have described organic syntheses of galactofuranose-based compounds, generally speaking, the chemical synthesis of oligosaccharides remains a challenge.[13,16–21] This is because appropriate, reactive and anomerically-pure monomers must first be prepared and then linked together in a stereo- and regiospecific fashion via glycosidic bonds. Increasingly, to simplify some or all of the synthetic steps for the production of oligosaccharides, enzymatic or chemoenzymatic strategies are being adopted.[22,23] Generally, by default, *O*-glycosidases and glycosyl transferases catalyse the modification of glycosidic bonds in a stereospecific manner and, in addition, are often regiospecific. Therefore, the use of such enzymes for synthesis can eliminate lengthy protection/deprotection procedures and drastically reduce both the time required and the use of organic solvents and other undesirable chemicals.[23,24]

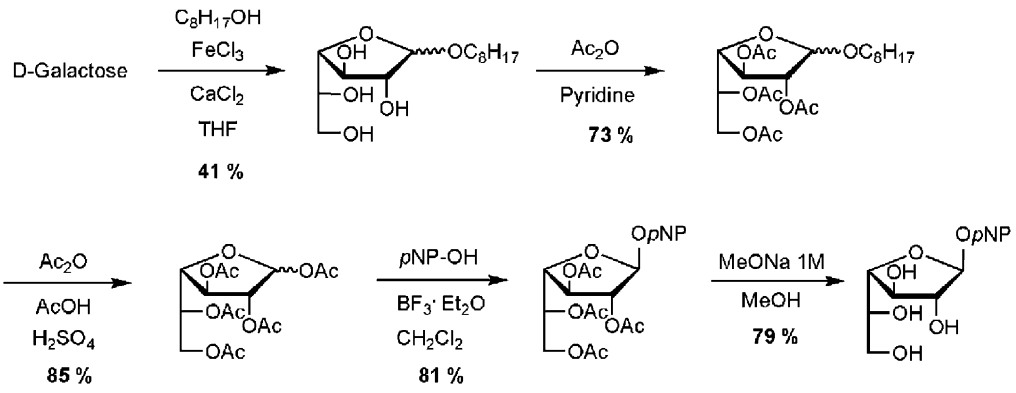
Glycosyl transferases (EC 3.2.4) catalyse regioselective glycosylation and afford high-transfer yields.[25,26] However, the high costs of the nucleotide–sugar substrates and, until recently, the low availability of these enzymes are often perceived as limiting factors. In addition to hydrolysis, many so-called 'retaining' *O*-glycosidases (EC 3.2.1) also possess the ability to catalyse the formation of oligosaccharides. Generally, glycosidase-mediated synthesis is performed either by exploiting the ability of these enzymes 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 and the enzymes used are either glucosidases or galactosidases. Intriguingly, to date, although β-D-galactofuranosidase (EC 3.2.1.146) activities have been identified,[27,28] no genes have been isolated and expressed and no transgalactofuranosylation activities have been associated with these enzymes.

Until now, most studies describing the enzymatic production of oligosaccharides have been concerned with the synthesis of oligopyranosides. However, recently two reports, one from our group[29] and the other from Sakamoto et al.,[30] have demonstrated that both an arabinofuranosidase and an arabinanase can perform transglycosylation in the presence of an appropriate donor sugar. With regards to our study, we showed that the α-L-arabinofuranosidase from *Thermobacillus xylanilyticus* (AbfD3) is able to catalyse the autocondensation of α-L-arabinofuranose or β-D-xylopyranose, as well as the synthesis of benzyl α-L-arabinofuranosyl-(1→2)-α-D-xylopyranoside. Here, taking advantage of the similar spatial orientation of the C-2, C-3 and C-4–OH groups in α-L-arabinofuranose and β-D-galactofuranose, we have explored the ability of AbfD3 to perform transgalactofuranosylation using *p*-nitrophenyl β-D-galactofuranoside (*p*NPGal*f*) as the donor sugar.

2. Results and discussion

2.1. Synthesis of *p*-nitrophenyl β-D-galactofuranoside

Despite several attempts, we were unable to perform the synthesis of *p*NPGal*f* according to the previously described protocol.[31] Therefore, the intermediate 1,2,3,5,6-tetra-*O*-acetyl-D-galactofuranose was prepared according to a more recent procedure as described in



Scheme 1. Synthesis of the *p*-nitrophenyl β-D-galactofuranoside donor.

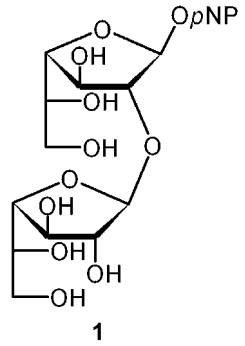
Scheme 1.[32,33] Introduction of the *p*-nitrophenyl group onto the anomeric position was achieved with reasonable yield via a classical method using *p*-nitrophenol and $BF_3{\cdot}Et_2O$ in dichloromethane. Deprotection of the acetyl groups was carried out using sodium methanolate 1 M solution in methanol as described.[31] The overall yield for the synthesis from D-galactose was 16%.

2.2. Hydrolytic activity of AbfD3 on *p*-nitrophenyl β-D-galactofuranoside

In order to investigate the specificity of AbfD3 towards *p*-nitrophenyl β-D-galactofuranoside (*p*NPGal*f*), this compound was used as a substrate for hydrolysis. Specific activity measured in the presence of *p*NPGal*f* (0.64 IU/mg) was much lower than that measured for *p*-nitrophenyl α-L-arabinofuranoside (*p*NPAra*f*, 465 IU/mg), but was similar to that measured for *p*-nitrophenyl β-D-xylopyranoside (*p*NPXyl*p*, 0.292 IU/mg). Attempts to determine the kinetic parameters for AbfD3-catalysed hydrolysis of *p*NPGal*f* were not pursued because K_M values were estimated to be extremely high (>50 mM) and hence incompatible with the solubility and availability of the substrate. This indicates that the presence of the extra hydroxymethyl group (C-6) is extremely detrimental for substrate binding in the −1 (donor) subsite. In a previous study, despite being a poor substrate for AbfD3-catalysed hydrolysis, *p*NPXyl*p* was shown to act as both donor and acceptor in AbfD3-mediated transglycosylation reactions.[29] Therefore, the low but measurable activity on *p*NPGal*f* was taken to be an encouraging indication of the ability of this sugar to act as a donor for transglycosylation.

2.3. Synthesis of galactofuranobioside with AbfD3

For initial tests, *p*NPGal*f* was incubated with AbfD3 and the reactions products were examined by TLC. One major and two minor products were detected. The major product was purified for further analysis by NMR. Analysis of the R_f values of the minor products led us to speculate that these were trisaccharides, but further verification was not performed due to the low availability of these compounds. Analysis of the ^{13}C NMR data for the major product revealed a downfield shift of the C-2 of the D-galactofuranoside moiety from 81.7 (for *p*NP-Gal*f*) to 88.5 ppm. Moreover, the ^{13}C–^{1}H (HMBC) spectrum showed an intense three-bond coupling between C-1′ (108.4 ppm) and H-2, clearly indicating that this compound was *p*-nitrophenyl β-D-galactofuranosyl-(1→2)-β-D-galactofuranoside (**1**) (Scheme 2). Importantly, no correlations were observed between C-1′ and H-3, H-5 or H-6, confirming the presence, within the major product fraction, of only one (1→2)-linked regioisomer. Therefore the AbfD3-catalysed autocondensation of *p*NPGal*f* was quite regioselective. Interestingly, we have previously shown that the autocondensation of *p*NPAra*f* or *p*NPXyl*p* occurs with only moderate regioselectivity (60–70% of *p*-nitrophenyl α-L-arabinofuranosyl-(1→2)-α-L-arabinofuranoside and 50% each of *p*-nitrophenyl β-D-xylopyranosyl-(1→2)-β-D-xylopyranoside and *p*-nitrophenyl β-D-xylopyranosyl-(1→3)-β-D-xylopyranoside),[29] although a (1→2)-linked compound was always present among the reaction products.



Scheme 2.

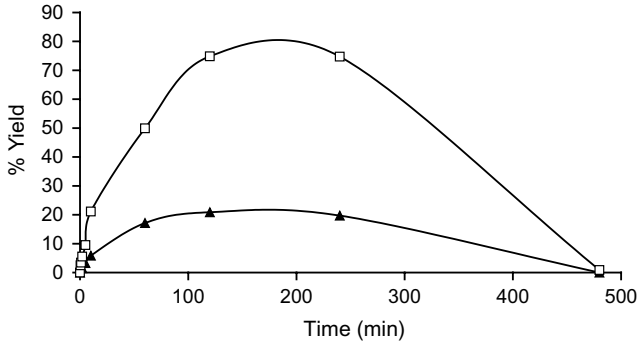


Figure 1. Kinetics of the formation of *p*-nitrophenyl β-D-galactofuranosyl-(1→2)-β-D-galactofuranoside (▲) and benzyl α-D-xylopyranosyl-(1→2)-β-D-galactofuranoside (□) obtained through AbfD3-catalysed transglycosylation reactions using *p*NPGal*f* as the donor sugar. Yields are calculated as a percentage of the initial quantity of *p*NPGal*f*.

Time-course analysis revealed that AbfD3-catalysed autocondensation of *p*NPGal*f* was relatively slow, since maximum disaccharide yield was obtained after 2 h (Fig. 1). In comparison, in the presence of *p*NPAra*f* and *p*NPXyl*p*, maximum yields (8.3% for *p*-nitrophenyl α-L-arabinofuranosyl-(1→2)-α-L-arabinofuranoside and 11.8% for *p*-nitrophenyl β-D-xylopyranosyl-(1→2 or 1→3)-β-D-xylopyranosides) were achieved after 30 s and 10 min, respectively (Table 1).[29] After reaching maximum yield (20.8%), secondary hydrolysis was responsible for the progressive disappearance of (**1**). No disaccharide was detectable after 8 h. Overall, these results reflect the poor binding (high K_M) of galactofuranose in the −1 (donor) subsite of AbfD3 and reveal that the catalytic efficiency (k_{cat}/K_M) of AbfD3-mediated hydrolysis of (**1**) is lower than that of the homodisaccharides *p*-nitrophenyl β-D-xylopyranosyl-(1→2 or 1→3)-β-D-xylopyranosides and *p*-nitrophenyl α-L-arabinofuranosyl-(1→2)-α-L-arabinofuranoside. Despite reaching 20.8% yield during transglycosylation, the final yield of compound (**1**) after purification was only 6%.

Table 1. Maximal yields for the AbfD3-catalysed synthesis of disaccharides in presence of various acceptor and donor molecules at 60 °C

Donor	Acceptor	Maximal yields	Linkages formed	References
*p*NPGal*f*	*p*NPGal*f*	20.8	β-(1→2)	This study
*p*NPGal*f*	$PhCH_2$ α-D-Xyl*p*	74.8	β-(1→2)	This study
*p*NPAra*f*	$PhCH_2$ α-D-Xyl*p*	7	α-(1→2)	Rémond et al.[29]
*p*NPAra*f*	*p*NPAra*f*	8.3	α-(1→2) α-(1→3) α-(1→5)	Rémond et al.[29]
*p*NPXyl*p*	*p*NPXyl*p*	11.8	β-(1→2) β-(1→3)	Rémond et al.[29]

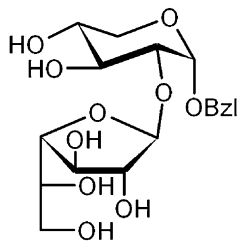
Yields are calculated as a percentage of the initial quantity of donor.

2.4. Transgalactofuranosylation of various acceptors using *p*NPGal*f*

As well as *p*NPAra*f* and *p*NPXyl*p*, several other monosaccharide derivatives (*p*-nitrophenyl α-D-xylopyranoside, *p*NP α-D-Xyl*p*; *p*-nitrophenyl β-D-xylofuranoside, *p*NP β-D-Xyl*f*; *p*-nitrophenyl β-D-glucofuranoside, *p*NP β-D-Glc*f*; *p*-nitrophenyl α-L-arabinopyranoside, *p*NP α-L-Ara*p*; benzyl α-D-xylopyranoside, $PhCH_2$ α-D-Xyl*p*; benzyl β-L-arabinopyranoside, $PhCH_2$ β-L-Ara*p*; *p*-nitrophenyl β-D-galactopyranoside, *p*NP β-D-Gal*p*) were tested for their ability to act as acceptors for AbfD3-catalysed transgalactofuranosylation. Control reactions, in which *p*NPGal*f* was omitted, were performed in order to monitor autocondensation reactions. The products of all the reactions were examined by TLC analysis. Most of the monosaccharide derivatives tested were not subject to autocondensation and could therefore only act as acceptors for transgalactofuranosylation. In the majority of cases, one reaction product was detected, although in reactions involving *p*NP β-D-Xyl*f* or *p*NP α-D-Xyl*p*, two and three products, respectively, were formed. Overall, despite the fact that poor yields precluded further structural analysis of all but one of the products, it is reasonable to suppose that they were all galactofuranose-containing disaccharides. These results indicate that the specificity in the acceptor (+1) subsite of AbfD3 is rather large. In the light of structural data available for another GH 51 arabinofuranosidase from *Geobacillus stearothermophilus* (GS-Abf)[34] such permissiveness is expected. The crystal structure of this enzyme complexed with Abf Ara*f*-α(1→3)-Xyl*p* revealed that the xylose residue at the +1 subsite formed only one hydrogen bond, compared to the multiple hydrogen bonding at the −1 subsite. Unlike all of the other monosaccharide derivatives tested, $PhCH_2$ α-D-Xyl*p* led to the appearance of one very intense spot on TLC. Being sufficiently abundant, this product was therefore purified for analysis (see below). Like (**1**), the purification of this product was characterised by a disappointingly low yield (15%). In the case of *p*NPAra*f* and *p*NPXyl*p*, autocondensation was the dominant reaction and so the major products formed were arabinobiosides and xylobiosides, respectively.

2.5. Structural characterisation of a galactofuranosyl-xylopyranoside

Analysis of the ^{13}C NMR data for the product of the reaction involving *p*NPGal*f* and $PhCH_2$ α-D-Xyl*p* revealed a downfield shift of C-2 of the D-xylopyranoside moiety from 73.5 (for $PhCH_2$-Xyl*p*) to 78.4 ppm. Moreover, a ^{13}C–^{1}H (HMBC) spectrum showed an intense three-bond coupling between C-1′ (109.0 ppm) and H-2, while revealing no correlation between C-1′ and H-3 or H-4. Overall, these data show that the



2

Scheme 3.

AbfD3-catalysed transgalactofuranosylation of $PhCH_2$ α-D-Xyl*p* occurs in a highly regioselective manner and leads to the production of benzyl β-D-galactofuranosyl-(1→2)-α-D-xylopyranoside (**2**) (Scheme 3). A time-course analysis of this reaction revealed that, like for compound **1**, maximum yield of **2** was reached after 2 h. However, in this case transglycosylation yield was much higher (74.8%) (Fig. 1). Significantly, this represents the highest yield obtained so far for any AbfD3-catalysed transglycosylation reaction, being 10-fold higher than that obtained for the transarabinosylation of $PhCH_2$ α-D-Xyl*p* (7%) (Table 1).[29] This indicates that in the presence of the covalently linked AbfD3-Gal*f* intermediate, $PhCH_2$ α-D-Xyl*p* is a much better acceptor than water. One might speculate that this situation arises because of a specific configuration of the donor sugar that favours $PhCH_2$ α-D-Xyl*p*-mediated deglycosylation, coupled to a favourable interaction of $PhCH_2$ α-D-Xyl*p* with AbfD3 in the +1 subsite. Moreover, the reaction was highly regioselective because, like in the previously described transarabinosylation of $PhCH_2$ α-D-Xyl*p*,[29] only one (1→2)-linked product was generated. Previously, others have suggested that there is a causal relationship between the bond specificity of a *O*-glycosidase working in hydrolytic mode and regiospecificity during transglycosylation.[35] Likewise, taken together, our findings infer that in hydrolytic mode AbfD3 should preferentially cleave (1→2) linkages. In our previous study, based on the findings of others,[36–38] we suggested that the high regioselectivity of reactions involving $PhCH_2$-Xylp might be correlated to the α configuration of the anomeric carbon in this sugar derivative. These present data add further weight to this argument, but do not provide confirmation because the equivalent β anomer was not tested. Furthermore, the fact that more products are generated when $PhCH_2$α–D-Xylp is replaced by *p*NP α-D-Xyl*p* (three in the former and one in the latter), implies that the aglycon group might also influence regiospecificity. After achieving maximum yield, **2** was subject to secondary hydrolysis and, like **1**, was totally degraded after 8 h.

2.6. Concluding remarks

Herein, we report the first use of an *O*-glycosidase for the synthesis of galactofuranose-based disaccharides. The chemoenzymatic method uses an α-L-arabinofuranosidase and the experimental rationale is based upon the fact that β-D-Gal*f* is a C-5 hydroxymethylated analogue of α-L-Ara*f*.

Galactofuranosyl-containing oligosaccharides are potentially useful compounds for the development of new vaccines or chemotherapeutic strategies for the prevention or treatment of tuberculosis and other serious pathologies. In mycobacterial cell walls, galactofuranosyl units are linked to each other via β-(1→5) or β-(1→6) bonds and to rhamnose through β-(1→4) bonds. In some fungal cell walls D-Gal*f* is interlinked via β-(1→2) and β-(1→3) bonds[39] and elsewhere in nature it can be linked to other sugar moieties such as Man or Glc via β-(1→3) bonds.[40–43] Although very regiospecific, the transglycosylation reactions in this study disappointingly led to the formation of β-(1→2) linkages. Nevertheless, it might be interesting to test whether either of the two β-(1→2)-linked disaccharides could inhibit enzymes involved in galactofuran biosynthesis in *Mycobacteria*. In this respect, the aryl groups on **1** and **2** (*p*NP and benzyl groups, respectively), might be useful. In the case of *n*-octyl-5-(α-D-arabinofuranosyl)-β-D-galactofuranoside derivatives, it was demonstrated that the hydrophobic group blocking the reducing end of disaccharides presented better biological activity against mycobacterial arabinosyl transferases than their less hydrophobic analogues.[44]

As a preliminary study, the results obtained for AbfD3 are extremely encouraging and open several exciting prospects for further work. Concerning the regioselectivity, potentially more useful β-(1→5) or β-(1→6) linkages between Gal*f* residues might be obtained in a number of ways: (i) through enzyme engineering of AbfD3; (ii) via a thioligase approach[45] or (iii) by using other arabinofuranosidases displaying other hydrolytic specificities. Exploration of each of these approaches is currently underway.

Finally, taken together our present and previous[29,46] data indicate that AbfD3 is a useful and quite versatile tool for chemoenzymatic syntheses involving furanosides. Therefore, it might be interesting to extend the rationale of this study towards D-fucofuranose, which is 6-deoxygalactofuranose.

3. Experimental

3.1. Enzyme and substrates

Recombinant arabinofuranosidase (AbfD3) was produced in *Escherichia coli* and purified as previously described.[47] The hydrolytic activity of AbfD3 was quantified after incubation of the enzyme (0.1 mL corresponding to 0.1 IU) with different *p*NP glycosides (0.9 mL, 5 mM in 50 mM sodium acetate, pH 5.8) at

60 °C. Continuous release of *p*NP was measured at 401 nm. One unit of activity corresponds to the amount of enzyme releasing 1 μmol of *p*NP per minute.

Benzyl α-D-xylopyranoside and benzyl β-L-arabinopyranoside were obtained by a previously described method.[48] *p*NP β-D-Xyl*p*, *p*NP α-D-Xyl*p*, *p*NP α-L-Ara*p* and *p*NP β-D-Gal*p* were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). *p*NP α-L-Ara*f*,[29,46] *p*NP β-D-Xyl*f*[29] and *p*NP β-D-Glu*f* were synthesised on a multigram scale according to the method previously described for the monosaccharides. *p*NP β-D-Gal*f* was obtained according to the synthetic approach in Scheme 1.

3.1.1. *p*-Nitrophenyl β-D-glucofuranoside. White needles (35% overall yield from 1,2-*O*-isopropylidene-α-D-glucofuranose, in a four-step synthesis): mp 118–119 °C (EtOAc); $[\alpha]_D^{22}$ −204 (*c* 1.00, methanol); ^{1}H NMR (CD_3OD): δ 8.21 (d, 2-H, $J_{3',2'}$ 9.3 Hz, H-3′, H-5′), 7.18 (d, 2-H, $J_{2',3'}$ 9.3 Hz, H-2′, H-6′), 5.63 (s, 1-H, H-1), 4.34 (s, 1-H, H-2), 4.31–4.27 (m, 2-H, H-3, H-4), 3.91 (ddd, 1-H, $J_{5,4}$ 8.5, $J_{5,6b}$ 5.9, $J_{5,6a}$ 3.0 Hz, H-5), 3.70 (dd, 1-H, $J_{6a,6b}$ 11.5, $J_{6a,5}$ 3.0 Hz, H-6a), 3.54 (dd, 1-H, $J_{6b,6a}$ 11.5, $J_{6b,5}$ 5.9 Hz, H-6b); ^{13}C NMR (CD_3OD): δ 163.3 (C-1′), 143.4 (C-4′), 126.6 (C-3′, C-5′), 117.4 (C-2′, C-6′), 107.8 (C-1), 84.4 (C-4), 82.5 (C-2), 76.9 (C-3), 71.4 (C-5), 65.1 (C-6). Anal. Calcd for $C_{12}H_{15}O_8N$: C, 47.84; H, 5.02; N, 4.65. Found: C, 47.97; H, 4.99; N, 4.61.

3.1.2. *p*-Nitrophenyl β-D-galactofuranoside. White needles (16% overall yield from D-galactose, in a five-step synthesis): mp 153–154 °C (EtOAc), lit.[31] 152–154 °C; $[\alpha]_D^{22}$ −205 (*c* 1.00, MeOH), lit.[31] $[\alpha]_D$ −203 (*c* 1.0, MeOH); ^{1}H NMR (CD_3OD): δ 8.18 (d, 2-H, $J_{3',2'}$ 9.2 Hz, H-3′, H-5′), 7.19 (d, 2-H, $J_{2',3'}$ 9.2 Hz, H-2′, H-6′), 5.64 (d, 1-H, $J_{1,2}$ 2.0 Hz, H-1), 4.28 (dd, 1-H, $J_{2,3}$ 4.2, $J_{2,1}$ 2.0 Hz, H-2), 4.18 (dd, 1-H, $J_{3,4}$ 6.5, $J_{3,2}$ 4.2 Hz, H-3), 4.08 (dd, 1-H, $J_{4,3}$ 6.5, $J_{4,5}$ 3.0 Hz, H-4), 3.75 (dt, 1-H, $J_{5,6a} = J_{5,6b}$ 6.4, $J_{5,4}$ 3.0 Hz, H-5), 3.61 (d, 2-H, $J_{6,5}$ 6.4 Hz, H-6a, H-6b); ^{13}C NMR (CD_3OD): δ 163.5 (C-1′), 143.5 (C-4′), 126.6 (C-3′, C-5′), 117.6 (C-2′, C-6′), 107.7 (C-1), 85.5 (C-4), 83.4 (C-2), 78.1 (C-3), 72.0 (C-5), 64.2 (C-6). Anal. Calcd for $C_{12}H_{15}O_8N$: C, 47.84; H, 5.02; N, 4.65. Found: C, 48.11; H, 4.89; N, 4.54.

3.2. Transglycosylation reactions

For analytic purposes, small amounts of compounds **1** and **2** were obtained by incubating 11 IU of AbfD3 with 5 mM *p*NPGal*f* alone (compound **1**), or in combination with an equimolar amount of $PhCH_2$ α-D-Xyl*p* (compound **2**), in a final reaction volume of 0.3 mL. Reactions involving *p*NPGal*f* and other sugar acceptors were prepared in a similar way by replacing $PhCH_2$ α-D-Xyl*p*. All reactions were performed in 50 mM sodium acetate buffer, pH 5.8 and at 60 °C. Incubation periods were variable and reactions were stopped by enzyme denaturation at 100 °C for 10 min. The reaction products were examined by TLC using Kieselgel 60 F_{254} aluminium-backed sheets (E. Merck) and 7:2:2 EtOAc–AcOH–water as the mobile phase. Detection of products was achieved at 100 °C using 0.2% v/v orcinol in H_2SO_4 (20% v/v). Larger amounts of compounds **1** and **2** were prepared in an identical manner by scaling up reactions by a factor of 40 (12 mL final volume). In this case, the compounds were purified by flash chromatography on silica gel columns (E. Merck) using 7:1 EtOAc–MeOH as the mobile phase.

For time-course analyses, reactions were stopped after different incubation periods by the addition of an equal volume of veronal buffer, 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). Afterwards, product yield was monitored and quantified by high-performance anion-exchange chromatography (HPAEC).

3.2.1. *p*-Nitrophenyl β-D-galactofuranosyl-(1→2)-β-D-galactofuranoside (1). ^{1}H NMR (D_2O): δ 8.16 (d, 2-H, *J* 9.2 Hz, H-3″, H-5″), 7.11 (d, 2-H, *J* 9.2 Hz, H-2″, H-6″), 5.78 (d, 1-H, $J_{1,2}$ 1.9 Hz, H-1), 5.13 (d, 1-H, $J_{1',2'}$ 1.7 Hz, H-1′), 4.31 (dd, 1-H, $J_{2,3}$ 4.4, $J_{2,1}$ 1.9 Hz, H-2), 4.24 (dd, 1-H, $J_{3,4}$ 7.2, $J_{3,2}$ 4.4 Hz, H-3), 4.06 (dd, 1-H, $J_{2',3'}$ 3.6, $J_{2',1'}$ 1.7 Hz, H-2′), 4.00 (ddd, 1-H, $J_{4,3}$ 7.2, $J_{4,5}$ 3.6 Hz, H-4), 3.97 (dd, 1-H, $J_{3',4'}$ 6.2, $J_{3',2'}$ 3.6 Hz, H-3′), 3.83 (dd, 1-H, $J_{4',3'}$ 6.6, $J_{4',5'}$ 3.4 Hz, H-4′), 3.76 (ddd, 1-H, $J_{5,6b}$ 7.4, $J_{5,6a}$ 5.0, $J_{5,4}$ 3.6 Hz, H-5), 3.66 (ddd, 1-H, $J_{5',6'b}$ 7.7, $J_{5',6'a}$ 4.6, $J_{5',4'}$ 3.4 Hz, H-5′), 3.56 (dd, 1-H, $J_{6a,6b}$ 11.8, $J_{6a,5}$ 5.0 Hz, H-6a), 3.53 (dd, 1-H, $J_{6b,6a}$ 11.8, $J_{6b,5}$ 7.4 Hz, H-5a), 3.29 (dd, 1-H, $J_{6'a,6'b}$ 11.6, $J_{6'a,5'}$ 4.6 Hz, H-6′a), 3.29 (dd, 1-H, $J_{6'b,6'a}$ 11.6, $J_{6'b,5'}$ 7.7 Hz, H-6′b); ^{13}C NMR (D_2O) δ 161.6 (C-1″), 142.6 (C-4″), 126.7 (C-3″, C-5″), 117.3 (C-2″, C-6″), 108.4 (C-1′), 105.1 (C-1), 88.5 (C-2), 83.5 (C-4′), 83.2 (C-4), 82.0 (C-2′), 77.2 (C-3′), 75.4 (C-3), 70.9 (C-5′), 70.5 (C-5), 63.3 (C-6′), 63.2 (C-6). ESI HRMS: *m*/*z* calcd for $[C_{18}H_{25}NO_{13}Na]^+$: 486.1224. Found: 486.1213.

3.2.2. Benzyl β-D-galactofuranosyl-α-D-xylanopyranoside (2). ^{1}H NMR (D_2O): δ 7.45–7.36 (m, 5-H, H-aromatic), 5.03 (d, 1-H, $J_{1',2'}$ 1.9 Hz, H-1′), 4.94 (d, 1-H, $J_{1,2}$ 3.7 Hz, H-1), 4.73 (d, 1-H, J_{gem} 11.9 Hz, H-benzyl), 4.56 (d, 1-H, J_{gem} 11.9 Hz, H′-benzyl), 4.09 (dd, 1-H, $J_{2',3'}$ 4.3, $J_{2',1'}$ 1.9 Hz, H-2′), 3.99 (dd, 1-H, $J_{3',4'}$ 7.1, $J_{3',2'}$ 4.3 Hz, H-3′), 3.73–3.63 (m, 4-H, H-3, H-4, H-5′, H-5a), 3.62 (dd, 1-H, $J_{4',3'}$ 7.1, $J_{4',5'}$ 3.3 Hz, H-4′), 3.53 (t, 1-H, $J_{5b,5a} = J_{5b,4}$ 10.4 Hz, H-5b), 3.50 (dd, 1-H, $J_{2,3}$ 9.6, $J_{2,1}$ 3.7 Hz, H-2), 3.43 (dd, 1-H, $J_{6'a,6'b}$ 11.6, $J_{6'a,5'}$ 8.0 Hz, H-6′a), 3.24 (dd, 1-H, $J_{6'b,6'a}$ 11.6,

$J_{6'b,5'}$ 3.9 Hz, H-6′b); ^{13}C NMR (D_2O): δ 136.0 (C-1″), 128.9 (C-3″, C-5″), 128.6 (C-2″, C-6″), 128.3 (C-4″), 109.0 (C-1′), 96.3 (C-1), 82.0 (C-4′), 81.1 (C-2′), 78.4 (C-2), 76.2 (C-3′), 71.9 (C-3), 69.8 (C-5′), 69.1 (C-4), 68.7 (CH_2 benzyl), 62.7 (C-5), 60.8 (C-6′). ESI HRMS: *m/z* calcd for $[C_{18}H_{26}O_{10}Na]^+$: 425.1424. Found: 424.1422.

3.3. Analytical methods

To monitor the production of **1** and **2**, HPAEC equipped with tandem with amperometric detection (PAD) (Dionex Corporation, CA, USA) was performed. Products of transglycosylation were separated on a chromatographic system consisting of a CarboPac PA1 precolumn and a CarboPac PA1 analytical column (Dionex, USA) to which a gradient of 0.3 M sodium acetate in 0.1 M NaOH was applied at a flow rate of 1 mL/min.

^{1}H and ^{13}C NMR analyses for the disaccharides were performed in D_2O at 20 °C on a Bruker DRX spectrometer at 500 and 125 MHz, respectively. Chemical shifts were referenced to the signal of the methyl group of internal acetone (^{1}H NMR δ = 2.22 ppm, ^{13}C NMR δ = 31.5 ppm).

Mass analysis of disaccharides was performed using electrospray-ionisation technology and Q-TOF mass analysis (ES-Q-TOF, Micromass, UK). ESI-Q-TOF data were acquired in positive mode ($[M+Na]^+$ ion detection). The cone voltage was set to 30 V and the source temperature was 80 °C.

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