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Enzymatic transglycosylation of xylose using a glycosynthase

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Abstract—The application of the hyperactive glycosynthase derived from *Agrobacterium* sp. β-glucosidase (AbgE358G-2F6) to the synthesis of xylo-oligosaccharides by using α-D-xylopyranosyl fluoride as donor represents the first successful use of glycosynthase technology for xylosyl transfer. Transfer to *p*-nitrophenyl β-D-glucopyranoside yields di- and trisaccharide products with β-(1→4) linkages in 63% and 35% yields, respectively. By contrast, transfer to *p*-nitrophenyl β-D-xylopyranoside yielded the β-(1→3) linked disaccharide and β-D-Xyl-(1→4)-β-D-Xyl-(1→3)-β-D-Xyl-*p*NP as major products in 42% and 30% yields, respectively. Transfer of xylose to β-D-Xyl-(1→4)-β-D-Xyl-*p*NP yielded the β-(1→4) linked trisaccharide in 98% yield, thereby indicating that transfers to xylo-disaccharides occur with formation of β-(1→4) bonds. Xylosylation of carbamate-protected deoxyxylonojirimycin produced a mixture of di- and tri-'saccharide' products in modest yields.

Keywords: *Agrobacterium* sp. β-glucosidase; Glycosynthase; Regioselectivity; Xylo-oligosaccharides; Nitrogen-containing inhibitors

1. Introduction

The important role of oligosaccharides and their conjugates in biology has been increasingly recognized in recent years, leading to an upsurge of interest in this field.[1,2] Unfortunately, in contrast to the situation with nucleic acids and proteins, the assembly of oligosaccharides remains a substantial challenge. This difficulty has its roots in the fact that glycosidic bond formation requires exquisite control of both regio- and stereochemistry, the former being made more challenging by the similar reactivities of the hydroxyl groups. In order to circumvent these difficulties, it is generally necessary to employ extensive protecting group chemistry with all its attendant difficulties. The use of solid-supported synthesis approaches can prove helpful, but control of stereochemistry remains a challenge. These problems are particularly acute in attempts to scale up synthesis. Increasing attention is therefore now being paid to the use of enzymes for such syntheses, particularly for products required on a large scale. In this way, complete control over both stereo- and regiochemistry can be attained. Useful in this regard are the naturally occurring glycosyl transferases, especially those from bacterial sources, along with glycosidases run in transglycosylation mode and the recently introduced glycosynthases.[3,4]

Glycosynthases are retaining glycosidases in which the catalytic nucleophile has been mutated. They catalyze the formation of glycosidic bonds using glycosyl fluoride donors with anomeric configuration opposite to that of the original substrate and various glycosides as acceptor sugars[5,6] (Chart 1). The glycosynthase methodology is gaining favor for the synthesis of oligosaccharides because these engineered glycosidases utilize cheaper donor substrates than do glycosyltransferases,[7] and because the yields are typically high due to the inability of the glycosynthase to hydrolyze the transfer products. Although xylosides and xylo-oligosaccharides are important components of plant cell walls, as well as showing up as constituents of certain mammalian

Abbreviations: Abg, *Agrobacterium* sp. β-glucosidase; α-XylF, α-D-xylopyranosyl fluoride; *p*NP, 4-nitrophenyl; *p*NP-Glc, 4-nitrophenyl β-D-glucopyranoside; *p*NP-Xyl, 4-nitrophenyl β-D-xylopyranoside; *p*NP-Xyl2, 4-nitrophenyl β-D-xylopyranosyl (1→4)-β-D-xylopyranoside; XNJ, 1-deoxyxylonojirimycin; Cbz, *N*-benzyloxycarbonyl.

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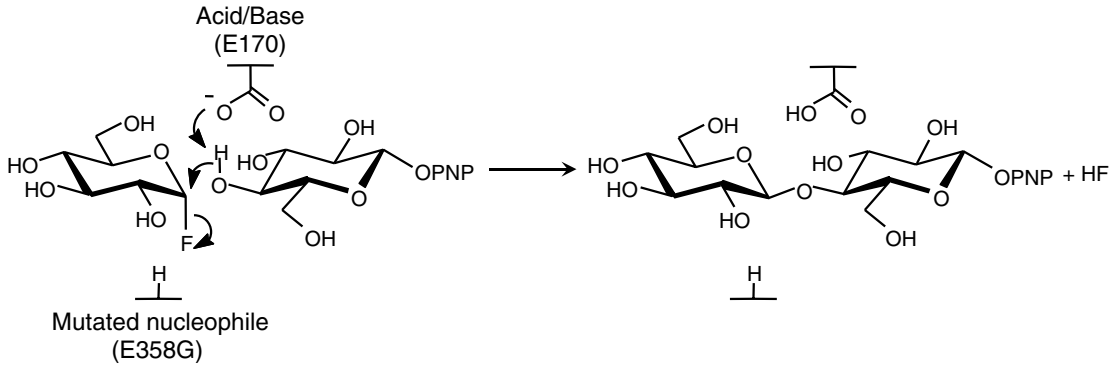


Chart 1. Mechanism of Abg glycosynthase.

glycosaminoglycans, glycosynthases originating from β-xylosidases or xylanases have not yet been reported.[6] Recently, a hyperactive glycosynthase originating from *Agrobacterium* sp. β-glucosidase (Abg) belonging to glycoside hydrolase family 1 has been developed *via* directed evolution.[8] This enzyme not only transfers glucose and galactose but was also shown to be capable of both mannosyl and xylosyl transfer. In this study, we describe the use of this evolved glycosynthase (AbgE358G-2F6) to carry out xylo-oligosaccharide synthesis using α-D-xylopyranosyl fluoride (α-XylF, **1**, Table 1) as a donor and aryl glycosides as acceptors. The structures of the resultant oligosaccharides reveal that the regioselective preference of AbgE358G-2F6 depends on the acceptors employed. This is the first report on the synthesis of xylo-oligosaccharides using glycosynthase methodology.

2. Results and discussion

2.1. Kinetic parameters for transglycosylation by AbgE358G-2F6

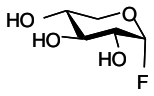
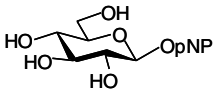
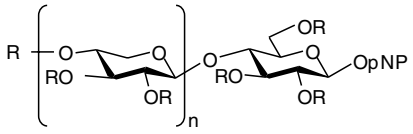
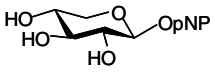
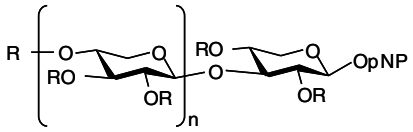
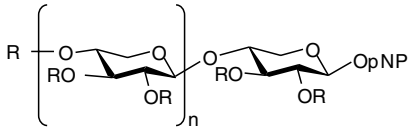
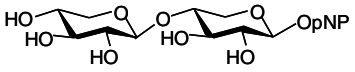
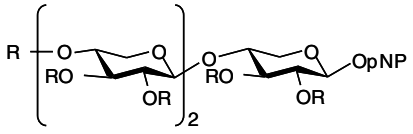
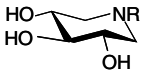
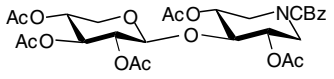
Kinetic parameters for the reaction of α-XylF (**1**) with 4-nitrophenyl β-D-xylopyranoside (*p*NP-Xyl, **3**) catalyzed by AbgE358G-2F6 were determined by the measurement of initial rates using a fluoride selective electrode.[9,10] Since in some cases, Abg glycosynthase shows substrate inhibition at high acceptor concentrations,[8,10] an optimal acceptor concentration was first determined. The measurement of rates at varying concentrations of an acceptor *p*NP-Xyl, at a fixed concentration of donor, α-XylF (50 mM) revealed standard saturation kinetic behavior, allowing the determination of an apparent K_m of 3.7 mM (*p*NP-Xyl) and k_{cat} of 0.1 min^{-1} (Fig. 1). Since no substrate inhibition was observed at high concentrations of the acceptor, the V_{max} value for α-XylF was measured at various concentrations of α-XylF using 20 mM *p*NP-Xyl (five times K_m for *p*NP-Xyl). Under these conditions AbgE358G-2F6 showed standard saturation kinetics with apparent kinetic parameters of $K_m = 28$ mM (α-XylF) and $k_{cat} = 0.1\ min^{-1}$.

2.2. Products of transglycosylation of xylosyl fluoride to *p*-nitrophenyl glycosides

The reaction catalyzed by AbgE358G-2F6 was first explored using α-XylF (**1**) and 4-nitrophenyl β-D-glucopyranoside (*p*NP-Glc, **2**) as a donor and an acceptor, respectively (Table 1). The use of an equimolar ratio of **1** and **2** resulted in incomplete consumption of the acceptor, largely due to the efficient conversion of the disaccharide product initially formed into a trisaccharide. This presumably occurs because the disaccharide is a better acceptor than the monosaccharide. Some spontaneous hydrolysis of α-XylF was also observed. The use of a 2:1 ratio of α-XylF to *p*NP-Glc resulted in the complete consumption of *p*NP-Glc after 48 h incubation. TLC and HPLC analysis revealed two products corresponding to a *p*NP-disaccharide (**4a**, 63%) and a *p*NP-trisaccharide (**5a**, 35%). No tetrasaccharide was detected under these conditions, though presumably, with higher ratios of α-XylF to *p*NP-Glc, this would be possible since Abg glycosynthase has previously been shown to form tetra- and pentasaccharides using α-D-glucopyranosyl fluoride.[11] Compounds **4b** and **5b** were purified using flash column chromatography after acetylation of the enzymatic products with acetic anhydride/pyridine, and subjected to detailed structural investigation by ^1H and ^{13}C NMR. The formation of a β-(1→4) linkage in **4b** was determined by the presence of a correlation of H-4 and C-1′ in the HMBC spectrum. Further, H-4 was shifted upfield to δ 3.84 relative to its shift (δ 5.10) in the per-*O*-acetate of *p*NP-Glc,[12] as would be expected upon replacement of the OAc substituent with a sugar moiety. This result is entirely consistent with previous studies of Abg glycosynthase in which glucosides were used as acceptors.[9]

Structural analysis of trisaccharide **5a**, formed by subsequent addition of a xylose to disaccharide **4a**, yielded a surprise. A precedent with xylo-acceptors in Abg glyco-

Table 1. Reactions catalyzed by AbgE358G-2F6

Donor	Acceptor	Products	Yields (%)
			
1	**2**	**4a** n = 1, R = H **4b** n = 1, R = Ac	63[a]
		5a n = 2, R = H **5b** n = 2, R = Ac	35[a]
			
	3	**6a** n = 1, R = H **6b** n = 1, R = Ac	42[a]
		7a n = 2, R = H **7b** n = 2, R = Ac	30[a]
			
		8a n = 1, R = H **8b** n = 1, R = Ac	4[a]
		9a n = 2, R = H **9b** n = 2, R = Ac	10[a]
			
	8a	**9a** R = H **9b** R = Ac	98[a]
			
	10a R = H	**11**	28[b]
	10b R = Cbz		

[a] The values were obtained by HPLC analysis of reaction mixture.
[b] The values are isolation yields after acetylation and flash column chromatography.

synthase suggests that a β-(1→3) linkage would be formed.[9,10] However, our NMR analysis of **5b** clearly confirmed the presence of a second β-(1→4) linkage. Again a clear correlation was observed between H-4′ and C-1″ in the HMBC spectrum, and H-4′ was again found at higher field (δ 3.80) than either H-3′ (δ 5.06) or H-4′ in disaccharide **4b** (δ 4.90). Previous rationales for the formation of β-(1→3) linkages to xylosides have invoked the binding of the acceptor sugar in an inverted mode, permitted by the greater symmetry of this sugar than that of glucose.[9] Presumably the constraints imposed by binding of a disaccharide in the acceptor (+1 and +2) subsites prohibit this inverted binding mode.

Incubation of α-XylF (**1**) and *p*NP-Xyl (**3**) in a 3:1 molar ratio in the presence of AbgE358G-2F6 resulted in the formation of two major and two minor products as analyzed by TLC using UV detection. Two major products were isolated after acetylation and flash chromatography. The major disaccharide (**6a**) had a β-(1→3) linkage, as expected from previous studies. However, the second glycosidic linkage in trisaccharide **7a**, formed by transfer of the second xylosyl moiety to disaccharide **6a**, was a β-(1→4) bond, exactly as seen earlier in this study for the transfer of xylose to β-D-Xyl-(1→4)-β-D-Glc-*p*NP (**4a**). These assignments were based on comparison with the known spectra and on similar NMR protocols to these described earlier. This result again points to the presence of different binding constraints for disaccharide acceptors with non-reducing end xylosyl moieties than for a monosaccharide xyloside.

The minor products were not isolated from the reaction mixture. However, a minor disaccharide was confirmed as β-D-Xyl-(1→4)-β-D-Xyl-*p*NP (**8a**) by HPLC analysis and comparison with authentic material (Fig. 2). The minor trisaccharide, therefore, was presumably β-D-Xyl-(1→4)-β-D-Xyl-(1→4)-β-D-Xyl-*p*NP (**9a**).

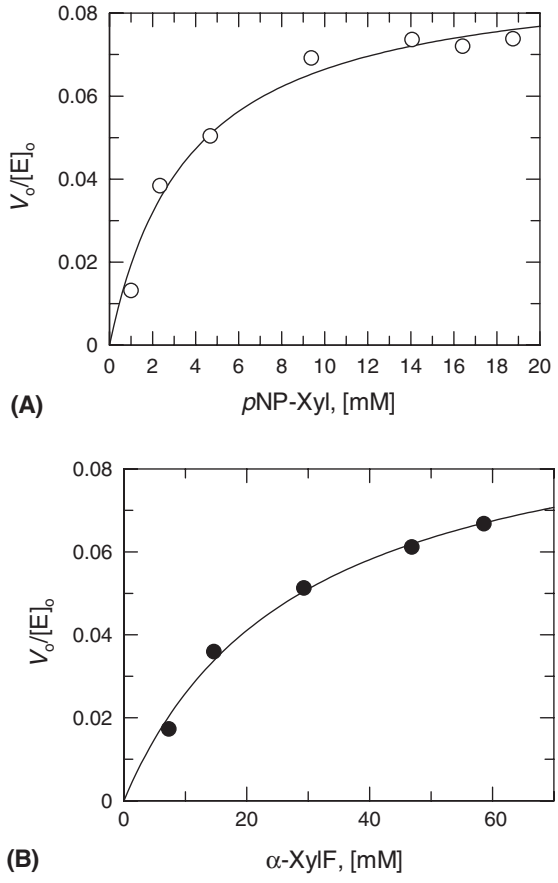


Figure 1. Transglycosylation kinetics. (A) Reaction rates of AbgE358G-2F6 were measured with respect to *p*NP-Xyl donor, with α-XylF fixed at 50 mM (○). (B) Reaction rates of AbgE358G-2F6 were measured with respect to α-XylF donor, with *p*NP-Xyl fixed at 20 mM (●). All assays were performed at 25 °C in 100 mM phosphate buffer (pH 7.0) containing 150 mM NaCl using a fluoride electrode. Error range in this figure is from 5% to 10%.

To confirm this proposal, the reaction of **1** with independently prepared **8a** was carried out. Only one product was detected by TLC, all acceptor sugars being converted into products after 48 h. As expected, the second linkage of the isolated trisaccharide (**9a**) was a β-(1→4) bond.

2.3. Oligomerization of xylose-based nitrogen-containing inhibitors

Various nitrogen-containing carbohydrate derivatives have been described as powerful inhibitors of glycosidase and have found their use not only in the study of glycosidase structures and mechanisms but also as tools for controlling the glycosylation of glycoproteins and even as therapeutics.[13] Most of these studies have been performed on *exo*-glycosidases with 'monosaccharide' aza-sugars. Such monosaccharide-based nitrogen-containing inhibitors, however, have a low affinity for *endo*-glycanases, thus attention has been paid to the synthesis of di- and trisaccharide-derived nitrogen-containing inhibitors in recent years,[14–18] and the compounds so

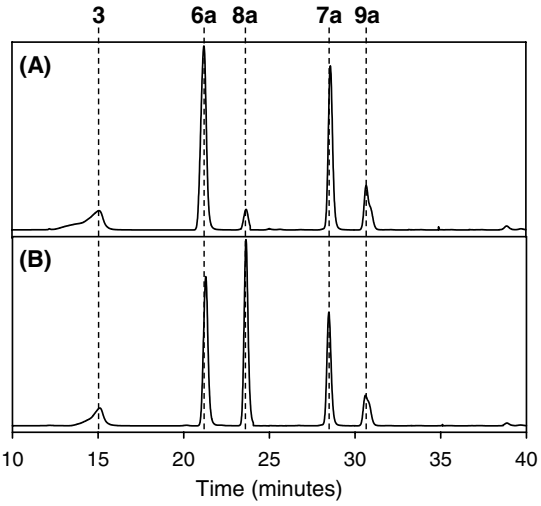


Figure 2. HPLC analysis of the products of the reaction of compounds **1** and **3**. The eluate was analyzed using a UV detector. (A) The reaction mixture, (B) the reaction mixture plus *p*NP-Xyl2 standard. Conditions: Tosoh Amide-80 column, 1 mL/min (CH_3CN–H_2O 100:0 to 65:35 for 45 min).

produced have provided useful insights into mechanisms of cellulases.[19,20] The glycosynthase strategy was successfully applied to the synthesis of cello-oligomer derivatives of isofagomine and tetrahydrooxazine.[15] The synthesis of xylose versions, however, was unsuccessful both because of the low rates of xylosyl transfer with the glycosynthases available at that time as well as the low affinity of the xylose-derived nitrogen-containing inhibitors acting as the acceptors (Hiebert, T.; Withers, S. G. Unpublished data). We thought that it might be possible to xylosylate 1-deoxyxylonojirimycin (XNJ, **10a**) using the more efficient evolved glycosynthase, AbgE358G-2F6, thereby providing easier access to useful tools. Given the substantial difference in structure between XNJ and *p*NP-Xyl, it was not clear what regioselectivity would be observed.

In our previous study, *N*-benzyloxycarbonyl (Cbz) aza-sugars such as Cbz *gluco*-isofagomine and Cbz *gluco*-tetrahydrooxazine worked as acceptors for Abg glycosynthase. The Cbz group suppresses the basicity of nitrogen, thus its inhibitory power, as well as providing an aromatic moiety to assist binding.[15] Unfortunately, Cbz XNJ was a significantly worst acceptor compared to *p*NP-Xyl, resulting in a considerably lower transglycosylation rate. This permitted substantial self-transglycosylation of α-XylF as well. Nonetheless, transfer to the aza-sugar was indeed observed and two products were detected using ESIMS. Acetylation of these products and separation by flash chromatography revealed the formation of the Xyl β-(1→3) linked disaccharide product **11** in 28% yield. The same regioselectivity as was seen for transfer to *p*NP-Xyl suggests that Cbz XNJ, which, like xylose, lacks a hydroxymethyl group, binds to the acceptor subsite in the same mode as *p*NP-Xyl. In the

case of the acetylated trisaccharide isolated in 10% yield, the complexity of the NMR spectra of this compound, arising presumably from the contamination by other sugars as well as from the partial double bond character within the carbamate functionality, made it difficult to generate convincing evidence concerning the formation of the Xyl β-(1→4)-Xyl β-(1→3) linkage that would be expected based on previous observations of this study.

2.4. Conclusions

The improved activity of Abg glycosynthase obtained through directed evolution has resulted in an extension of the range of useful donors to now include α-XylF. Indeed, this is the first report of enzymatic synthesis of xylo-oligosaccharides using the glycosynthase technology. The observed β-(1→3) regioselectivity for xyloside-derived acceptors (**3**, **10b**) will be useful in the synthesis of substrates and inhibitors for the study of β-(1,3)-xylanases (EC 3.2.1.32). Such β-(1,3)-xylanases have been discovered within marine bacteria and have received relatively little attention compared to β-(1,4)-xylanases (EC 3.2.1.8).[21–23] The complete conversion of compound **8a** to β-(1→4)-linked xylotrisaccharide (**9a**) is particularly noteworthy since xylobiose is relatively readily available through xylanase-catalyzed hydrolysis of xylan, whereas xylotriose is obtained only in small quantities. Previous enzymatic syntheses of β-(1→4)-linked arylxylo-oligosaccharides using a wild type β-xylosidase from *Aspergillus niger* were reported to yield mixtures of xylo-oligosaccharides with a degree of polymerization up to 7.[24] From a practical viewpoint, a synthetic pathway producing a single product with a high yield is generally preferable to routes generating a mixture of products, if only because purification steps are enormously simplified.

3. Materials and methods

3.1. Materials

α-XylF (**1**),[25] *p*NP-Xyl2 (**8a**),[26] and Cbz XNJ (**10b**)[16] were synthesized according to known methods. *p*NP-Glc (**2**) and *p*NP-Xyl (**3**) were purchased from Aldrich Chemical Co. All other chemicals and reagents were purchased from Sigma Chemical Co. unless otherwise noted.

3.2. General analytical methods

All ^{1}H and ^{13}C NMR spectra were recorded at 400 MHz using a Bruker AV-400 spectrometer. Mass spectrometry for small molecules was recorded using a PE-Sciex API 300 triple quadrupole mass spectrometer (Sciex, Thornhill, Ont., Canada) equipped with an electrospray ionization ion source. Thin layer chromatography (TLC) was performed on aluminum-backed sheets of Silica Gel $60F_{254}$ (Merck) of thickness 0.2 mm using 12:2:1 EtOAc–MeOH–water. The plates were visualized using UV light (254 nm) and/or by exposure to 10% H_2SO_4 in MeOH followed by charring. All analysis of oligosaccharides were performed on a Waters 600 HPLC system equipped with a Waters 2487 UV detector. The protein was first removed by adding MeCN (final concentration was 75% (v/v)), followed by centrifugation. Approximately 30 μL of the sample was loaded onto a Tosoh Amide-80 (4.6 mm × 25 cm) column. The products were then eluted at 1 mL/min with an MeCN–water (100:0 to 65:35 for 45 min) gradient. Reaction yields were determined by integration of the peaks within the HPLC chromatograms using an analysis program developed by Waters Co. Column chromatography was carried out using Silica Gel 60 (230 ± 400 mesh).

3.3. Purification and kinetic analysis of AbgE358G-2F6

AbgE358G-2F6 was purified by affinity chromatography using Ni-nitrilotriacetic agarose (QIAGEN), as described previously.[8] Protein concentrations were determined by measuring absorbance at 280 nm, using an extinction coefficient of 102,850 $M^{-1}\,cm^{-1}$.[27] An Orion fluoride electrode (model 96-09BN), interfaced with a Fischer Scientific Accumet 925 pH/ion meter, was used to monitor fluoride release during reaction at 25 °C. All enzymatic rates were corrected for spontaneous hydrolysis of α-XylF. The concentration of either a donor (α-XylF, 50 mM) or an acceptor (*p*NP-Xyl, 20 mM) sugar was fixed and that of the counterpart was varied to allow K_m and k_{cat} determinations. GraFit version 4.0[28] was used to calculate kinetic parameters by direct fit of initial rates.

3.4. Transglycosylation reactions and purification of transfer products

The transglycosylation reactions were carried out at 30 or 20 °C in 100 mM sodium phosphate buffer, pH 7.0. Reactions were monitored by TLC. Upon completion, reaction solutions were lyophilized and dissolved in pyridine (3 mL) and Ac_2O (2 mL). The reaction mixture was stirred overnight at rt. This mixture was quenched upon the addition of MeOH (3 mL) and then concentrated to give a residue.

3.4.1. 4-Nitrophenyl (2,3,4-tri-*O*-acetyl-β-D-xylopyranosyl)-(1→4)-*O*-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (4b) and 4-nitrophenyl [(2,3,4-tri-*O*-acetyl-β-D-xylopyranosyl)-(1→4)-*O*-2,3-di-*O*-acetyl-β-D-xylopyranosyl]-(1→4)-*O*-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (5b). A mixture of α-XylF (**1**, 25 mM) and *p*NP-Glc (**2**, 12.5 mM) in phosphate buffer (5 mL of 0.1 M) was treated with AbgE358G-2F6 (10 mg) and the mixture then incubated

(48 h, 30 °C). After acetylation, flash chromatography (1:1 EtOAc–petroleum ether) of the residue gave, firstly, disaccharide **4b**. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.18 (m, 2H, Ar–H), 7.03 (m, 2H, Ar–H), 5.27 (dd, 1H, $J_{2,3}$ 8.3 Hz, $J_{3,4}$ 9.9 Hz, H-3), 5.19 (dd, 1H, H-2), 5.15 (d, 1H, $J_{1,2}$ 7.5 Hz, H-1), 5.12 (dd, 1H, $J_{3',4'}$ 8.6 Hz, H-3′), 4.90 (ddd, 1H, H-4′), 4.86 (dd, 1H, $J_{2',3'}$ 8.8 Hz, H-2′), 4.50 (d, 1H, $J_{1',2'}$ 6.9 Hz, H-1′), 4.46 (dd, 1H, $J_{5,6a}$ 1.7 Hz, $J_{6a,6b}$ 12.0 Hz, H-6a), 4.10 (dd, 1H, $J_{5,6b}$ 4.9 Hz, H-6b), 4.06 (dd, 1H, $J_{4',5'a}$ 5.1 Hz, $J_{5'a,5'b}$ 11.9 Hz, H-5′a), 3.84 (dd, 1H, H-4), 3.32 (dd, 1H, $J_{4',5'b}$ 8.5 Hz, H-5′b), 2.06 (s, 3H, CH_3CO), 2.03 (s + s + s, 9H, CH_3CO), 2.02 (s, 3H, CH_3CO), 2.00 (s, 3H, CH_3CO). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 170.2, 170.0, 169.8, 169.7, 169.4, 169.3 (CH_3CO); 161.2, 143.2, 125.8 (2C), 116.6 (2C) (Ar–C); 101.6, 97.9, 77.1, 73.1, 72.8, 71.4, 71.1 (2C), 68.7, 62.4, 61.9; 20.8, 20.7 (2C), 20.6 (3C) (CH_3CO). ESIMS: Calcd for $C_{29}H_{35}NO_{18} + Na^+$: 708.6. Found: 708.5.

Next to elute was trisaccharide **5b**. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.18 (m, 2H, Ar–H), 7.02 (m, 2H, Ar–H), 5.26 (dd, 1H, $J_{2,3}$ 8.4 Hz, $J_{3,4}$ 7.6 Hz, H-3), 5.18 (dd, 1H, H-2), 5.15 (d, 1H, $J_{1,2}$ 7.4 Hz, H-1), 5.07 (dd, 1H, $J_{2'',3''}$ 7.7 Hz, $J_{3'',4''}$ 7.6 Hz, H-3″), 5.06 (dd, 1H, $J_{2',3'}$ 9.1 Hz, $J_{3',4'}$ 8.5 Hz, H-3′), 4.85 (ddd, 1H, H-4″), 4.79 (dd, 1H, H-2′), 4.76 (dd, 1H, H-2″), 4.54 (d, 1H, $J_{1'',2''}$ 5.7 Hz, H-1″), 4.47 (dd, 1H, $J_{5,6a}$ 1.7 Hz and $J_{6a,6b}$ 12.1 Hz, H-6a), 4.45 (d, 1H, $J_{1',2'}$ 7.2 Hz, H-1′), 4.09 (dd, 1H, $J_{5,6b}$ 1.3 Hz, H-6b), 4.05 (dd, 1H, $J_{4'',5''a}$ 1.6 Hz, $J_{5''a,5''b}$ 12.1 Hz, H-5″a), 3.90 (dd, 1H, $J_{4',5'a}$ 5.2 Hz, H-5′a), 3.84–3.80 (m, 3H, H-4, H-5, H-4′), 3.38 (dd, 1H, $J_{4'',5''b}$ 7.3 Hz, H-5″b), 3.28 (dd, 1H, $J_{4',5'b}$ 9.2 Hz, $J_{5'a,5'b}$ 11.7 Hz, H-5′b), 2.05 (s, 3H, CH_3CO), 2.04 (s, 3H, CH_3CO), 2.034 (s + s, 6H, 2 × CH_3CO), 2.03 (s, 3H, CH_3CO), 2.02 (s, 3H, CH_3CO), 2.01 (s, 3H, CH_3CO), 2.00 (s, 3H, CH_3CO). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 170.2, 169.9, 169.8 (2C), 169.7, 169.5, 169.4, 169.1 (CH_3CO); 161.1, 143.2, 125.8 (2C), 116.6 (2C) (Ar–C); 101.8, 99.2, 97.8, 77.2, 74.2, 73.0, 72.8, 72.5, 71.5, 71.1, 70.1 (2C), 68.1, 63.1, 61.9, 61.3; 20.8, 20.7 (3C), 20.6 (4C) (CH_3CO). ESIMS: Calcd for $C_{38}H_{47}NO_{24} + Na^+$: 924.8. Found: 924.7.

3.4.2. 4-Nitrophenyl (2,3,4-tri-*O*-acetyl-β-D-xylopyranosyl)-(1→3)-*O*-2,4-di-*O*-acetyl-β-D-xylopyranoside (6b) and 4-nitrophenyl [(2,3,4-tri-*O*-acetyl-β-D-xylopyranosyl)-(1→4)-*O*-2,3-di-*O*-acetyl-β-D-xylopyranosyl]-(1→3)-*O*-2,4-di-*O*-acetyl-β-D-xylopyranoside (7b). A mixture of α-XylF (**1**, 60 mM) and *p*NP-Xyl (**3**, 20 mM) in phosphate buffer (5 mL of 0.1 M) was treated with AbgE358G-2F6 (25 mg) and the mixture was then incubated (72 h, 30 °C). After acetylation, flash chromatography (1:2, 2:3, and 1:1 EtOAc–petroleum ether) of this residue gave, firstly, the acetylated *p*NP-Xyl (**3**).

Next to elute was disaccharide **6b**. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.19 (m, 2H, Ar–H), 7.07 (m, 2H, Ar–H), 5.35 (d, 1H, $J_{1,2}$ 4.1 Hz, H-1), 5.12 (dd, 1H, $J_{2',3'} = J_{3',4'}$ 8.0 Hz, H-3′), 5.05 (dd, 1H, $J_{2,3}$ 5.6 Hz, H-2), 4.95–4.80 (m, 3H, H-4, H-2′ and H-4′), 4.71 (d, 1H, $J_{1',2'}$ 6.3 Hz, H-1′), 4.19 (m, 2H, H-5a and H-5′a), 3.95 (dd, 1H, $J_{3,4}$ 5.7 Hz, H-3), 3.59 (dd, 1H, $J_{4,5b}$ 4.8 Hz, $J_{5a,5b}$ 12.8 Hz, H-5b), 3.40 (dd, 1H, $J_{4',5'b}$ 8.0 Hz, $J_{5'a,5'b}$ 12.0 Hz, H-5′b), 2.05 (s + s + s, 9H, CH_3CO), 2.02 (s + s, 6H, CH_3CO). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 170.0, 169.9, 169.8, 169.4, 169.3 (CH_3CO); 160.9, 142.9, 125.8 (2C), 116.5 (2C) (Ar–C); 101.0, 96.6, 74.9, 70.8, 70.2, 69.3, 68.5, 68.3, 61.9, 60.7; 21.0, 20.84, 20.76, 20.7, 20.66 (CH_3CO). ESIMS: Calcd for $C_{26}H_{31}NO_{16} + Na^+$: 636.5. Found: 636.4.

Last to elute was trisaccharide **7b**. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.18 (m, 2H, Ar–H), 7.02 (m, 2H, Ar–H), 5.36 (d, 1H, $J_{1,2}$ 3.7 Hz, H-1), 5.09 (dd, 1H, $J_{2'',3''} = J_{3'',4''}$ 8.7 Hz, H-3″), 5.08 (dd, 1H, $J_{2',3'} = J_{3',4'}$ 7.7 Hz, H-3′), 5.02 (dd, 1H, $J_{2,3}$ 5.2 Hz, H-2), 4.94 (ddd, 1H, H-4), 4.90–4.86 (m, 2H, H-2″ and H-4″), 4.78 (dd, 1H, H-2′), 4.63 (d, 1H, $J_{1'',2''}$ 7.1 Hz, H-1″), 4.55 (d, 1H, $J_{1',2'}$ 5.9 Hz, H-1′), 4.14 (dd, 1H, $J_{4,5a}$ 3.8 Hz, $J_{5a,5b}$ 12.8 Hz, H-5a), 4.08 (dd, 1H, $J_{4'',5''a}$ 4.8 Hz, $J_{5''a,5''b}$ 12.2 Hz, H-5″a), 3.98 (dd, 1H, $J_{4',5'a}$ 4.9 Hz, $J_{5'a,5'b}$ 11.8 Hz, H-5′a), 3.93 (dd, 1H, $J_{3,4}$ 5.3 Hz, H-3), 3.83 (ddd, 1H, H-4′), 3.59 (dd, 1H, $J_{4,5b}$ 4.4 Hz, H-5b), 3.38 (dd, 1H, $J_{4'',5''b}$ 7.6 Hz, H-5″b), 3.34 (dd, 1H, $J_{4',5'b}$ 9.4 Hz, H-5′b), 2.04 (s, 3H, CH_3CO), 2.03 (s, 3H, CH_3CO), 2.027 (s + s, 6H, CH_3CO), 2.02 (s, 3H, CH_3CO), 2.01 (s, 3H, CH_3CO), 2.00 (s, 3H, CH_3CO). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 170.0, 169.88, 169.85, 169.8, 169.7, 169.3, 169.2 (CH_3CO); 160.8, 142.8, 125.8 (2C), 116.5 (2C) (Ar–C); 101.5, 99.5, 96.6, 74.7, 74.4, 72.2, 70.8, 70.3, 70.2, 69.2, 68.23, 68.16, 62.8, 61.5, 60.4; 21.0, 20.9, 20.8, 20.76, 20.7, 20.6 (2C) (CH_3CO). ESIMS: Calcd for $C_{35}H_{43}NO_{22} + Na^+$: 852.7. Found: 852.7.

3.4.3. 4-Nitrophenyl [(2,3,4-tri-*O*-acetyl-β-D-xylopyranosyl)-(1→4)-*O*-2,3-di-*O*-acetyl-β-D-xylopyranosyl]-(1→4)-*O*-2,3-di-*O*-acetyl-β-D-xylopyranoside (9b). A mixture of α-XylF (**1**) (30 mM) and *p*NP-Xyl2 (**8a**) (10 mM) in phosphate buffer (5 mL of 0.1 M, pH 7.0) was treated with AbgE358G-2F6 (25 mg) and the mixture was then incubated (48 h, 30 °C). After acetylation, flash chromatography (1:2, 2:3, and 1:1 EtOAc–petroleum ether) of this residue gave trisaccharide **9b**. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.19 (m, 2H, Ar–H), 7.04 (m, 2H, Ar–H); 5.22 (d, 1H, $J_{1,2}$ 6.0 Hz, H-1), 5.19 (dd, 1H, $J_{2,3}$ 7.8 Hz, $J_{3,4}$ 7.6 Hz, H-3), 5.09 (dd, 1H, H-2), 5.07 (dd, 1H, $J_{2',3'}$ 7.9 Hz, $J_{3',4'}$ 9.4 Hz, H-3′), 5.06 (dd, 1H, $J_{2'',3''}$ 7.0 Hz, $J_{3'',4''}$ 7.6 Hz, H-3″), 4.86 (ddd, 1H, $J_{4'',5''a}$ 4.6 Hz, $J_{4'',5''b}$ 7.6 Hz, H-4″), 4.78 (dd, 1H, H-2″), 4.77 (dd, 1H, H-2′), 4.54 (d, 1H, $J_{1'',2''}$ 5.9 Hz, H-1″), 4.50 (d, 1H, $J_{1',2'}$ 6.9 Hz, H-1′), 4.07 (dd, 1H, $J_{5''a,5''b}$

11.8 Hz, H-5″a), 4.05 (dd, 1H, $J_{4,5a}$ 4.5 Hz, H-5a), 3.95 (dd, 1H, $J_{4'5'a}$ 5.0 Hz, $J_{5'a,5'b}$ 11.9 Hz, H-5′a), 3.86 (ddd, 1H, $J_{4,5b}$ 7.5 Hz, H-4), 3.81 (ddd, 1H, $J_{4'5'b}$ 8.5 Hz, H-4′), 3.51 (dd, 1H, $J_{5a,5b}$ 12.1 Hz, H-5b), 3.38 (dd, 1H, H-5″b), 3.32 (dd, 1H, H-5′b), 2.07 (s, 3H, CH_3CO), 2.05 (s, 3H, CH_3CO), 2.04 (s, 3H, CH_3CO), 2.03 (s, 3H, CH_3CO), 2.026 (s, 3H, CH_3CO), 2.02 (s, 3H, CH_3CO), 2.01 (s, 3H, CH_3CO). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 170.0, 169.9, 169.8, 169.63, 169.58, 169.4, 169.2 (CH$_3$*C*O); 161.1, 143.1, 125.8 (2C), 116.5 (2C) (Ar–C); 100.5, 99.4, 97.8, 74.4, 74.2, 72.0, 71.1, 71.0, 70.4, 70.3, 69.9, 68.2, 62.7, 62.4, 61.5; 20.83, 20.77, 20.76, 20.7 (3C), 20.6 (*C*H$_3$CO). ESIMS: Calcd for $C_{35}H_{43}NO_{22} + Na^+$: 852.7. Found: 852.7.

3.4.4. (2,3,4-Tri-*O*-acetyl-β-D-xylopyranosyl)-(1→3)-*O*-2,4-di-*O*-acetyl-*N*-benzyloxycarbonyl-1,5-dideoxy-1,5-imino-D-xylitol (11). A mixture of α-XylF (**1**) (11.5 mg, 150 μmol) and 1-deoxyxylonojirimycin carbamate **10b** (69.4 mg, 230 μmol) in phosphate buffer (5 mL of 0.1 M, pH 7.0) was treated with AbgE358G-2F6 (25 mg) and the mixture was then incubated (7 days, 20 °C). Additional aliquots of α-XylF (11.5 mg) were supplied after 24, 48, and 96 h. After acetylation, flash chromatography (EtOAc–petroleum ether, gradient from 3:7 to 3:2) of the residue gave, firstly, acetylated compound **10b** (40 mg, 100 μmol 43%).

Next to elute was pseudo-disaccharide **11** (40 mg, 65 μmol 28%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.33 (m, 5H, Ar–H), 5.12 (s, 2H, PhCH_2O), 5.08 (dd, 1H, $J_{2',3'} = J_{3',4'}$ 8.8 Hz, H-3′), 5.02 (dd, 1H, $J_{3,4}$ 9.1 Hz, H-4), 4.90 (ddd, 1H, H-4′), 4.84 (dd, 1H, H-2′), 4.76 (m, 1H, H-2a or H-6a), 4.54 (d, 1H, $J_{1',2'}$ 7.1 Hz, H-1′), 4.30 (m, 1H, H-2a or H-6a), 4.07 (m, 2H, H-2 and H-5′a), 3.64 (m, 1H, H-3), 3.29 (m, 1H, H-5′b), 2.78 (dd, 2H, $J_{2b,3} = J_{6b,5}$ 11.7 Hz, $J_{2a,2b} = J_{6a,6b}$ 13.0 Hz, H-2b and H-6b), 2.05 (s, 3H, CH_3CO), 2.02 (s, 3H, CH_3CO), 2.00 (s, 3H, CH_3CO), 1.99 (s, 3H, CH_3CO), 1.98 (s, 3H, CH_3CO). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 170.1, 169.7 (2C), 169.3 (2C) (CH$_3$*C*O); 154.7 (N*C*O); 136.1, 128.5 (2C), 128.2 (2C), 127.9 (Ar–C); 101.3, 75.2, 74.9, 71.5, 70.6, 68.7, 67.7, 62.2, 46.9, 45.0; 20.8, 20.7 (2C), 20.6, 20.4 (*C*H$_3$CO). ESIMS: Calcd for $C_{28}H_{35}NO_{14} + Na^+$: 632.6. Found: 632.5.

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