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Glycosynthase-Mediated Assembly of Xylanase Substrates and Inhibitors

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An *exo*-β-xylosidase mutant with glycosynthase activity was created to aid in the synthesis of xylanase substrates and inhibitors. Simple monosaccharides were easily elaborated into di-, tri- and tetrasaccharides by using this enzyme. Some products proved to be surprisingly potent inhibitors of xylanases from glycoside hydrolase families 10 and 11.

Introduction

The present interest in biofuels and renewable chemical feedstocks has inspired a resurgence of interest in plant polysaccharide-degrading enzymes. While enzymes that degrade cellulose remain the primary focus of attention, those that act on other cell-wall polysaccharides, such as hemicellulose and pectin, are also of great interest. Indeed, enzymes acting upon these heterogeneous polysaccharides have long been exploited in various industrial processes.[1,2]

Xylan is a common component of hemicellulose, and an abundant plant cell wall polysaccharide. It is a polymer of D-xylose linked in a β-(1→4) fashion and is often modified with acetate esters and branching glycosides of L-arabinose and D-glucuronic acid.[2] Some algae also synthesise xylans, although these linear biopolymers typically contain a mixture of β-(1→3) and β-(1→4) glycosidic bonds, or might even be assembled entirely with β-(1→3) linkages.[3,4]

Xylanases, glycoside hydrolases (GHs) responsible for the hydrolysis of xylans into smaller oligosaccharides, are ubiquitous among bacteria and fungi that subsist on plant matter.[5] The most common, and best studied of these enzymes are the *endo*-β-(1→4)-xylanases (E.C. 3.2.1.8). In accordance with the sequence-based classification system defined by the Carbohydrate Active enZyme (CAZy) database (http://www.cazy.org)[6] these enzymes hail from GH families 5, 8, 10, 11 and 43, and facilitate hydrolysis with either inversion (GH families 8 and 43)[7,8] or retention (GH families 5, 10 and 11)[9,10] of the substrate's anomeric configuration. The less common *endo*-β-(1→3)-xylanases (E.C. 3.2.1.32) are all of GH family 26, and so presumably hydrolyse their algal xylan substrates with retention of anomeric configuration.[11]

The mechanisms by which xylanases, particularly those of GH families 10 and 11, accomplish catalysis are well studied.[5,12] Synthetic substrates, competitive and mechanism-based inhibitors, as well as activity-based probes have played crucial roles in such studies.[13–16] These compounds will no doubt feature prominently in future efforts to obtain a better understanding of how the various components of cellulolytic enzyme cocktails secreted by many organisms work in concert to deconstruct the heterogeneous polysaccharide matrix that is the plant cell wall.

Synthesising xylanase substrates and inhibitors can be a laborious task, since these di- or trisaccharides are usually chemically assembled from monosaccharide synthons.[13,14] A similar issue concerning the preparation of oligosaccharide cellulase inhibitors was resolved some time ago with the introduction of the glycosynthase paradigm,[17–19] which suggested that an analogous approach might simplify the synthesis of xylanase substrates and inhibitors.

Glycosynthases are hydrolytically incompetent GH mutants that can catalyse efficient glycoside bond formation using activated glycosyl donors. For a conventional retaining glycosidase, this is usually accomplished by mutating the catalytic nucleophile residue to a small, non-nucleophilic residue, such as glycine or alanine.[19] This mutation ablates the enzyme's hydrolytic activity, but also creates sufficient room for a glycosyl fluoride with an anomeric configuration opposite to that of the natural substrate to bind. This complex mimics the glycosyl–enzyme intermediate of the wild-type enzyme and, with the aid of base catalysis from the remaining catalytic residue, might glycosylate an acceptor alcohol occupying the aglycone binding site (Scheme 1).

Thus, a β-xylosidase-derived glycosynthase should catalyse the addition of D-xylosyl units onto a host of *xylo*-configured acceptors, making it ideal for the preparation of xylanase substrates and inhibitors. Early attempts to generate glycosynthases from GH family 39 xylosidases were unsuccessful. The weak xylosidase activity of a GH family 1 glucosidase was exploited to produce a glycosynthase capable of forming xylosidic bonds, although it had rather poor activity and provided the undesired β-(1→3) regioisomers.[20] Efficient glycosynthase mutants of an *exo*-β-xylosidase were recently reported by Shoham

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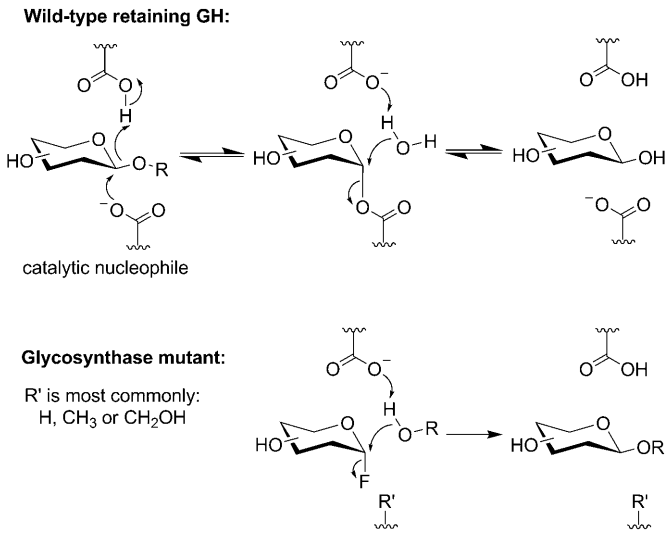


Scheme 1. Retaining glycoside hydrolase and glycosynthase mechanisms.

and co-workers.[21] This particular xylosidase belonged to GH family 52—a relatively new GH family.[22]

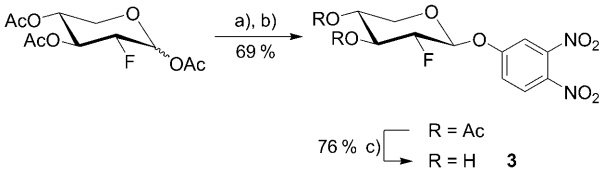
Results and Discussion

Glycosynthase mutants of *Bacillus halodurans* C-125 xylosidase

Bacillus halodurans C-125 possesses open reading frames (ORFs) encoding putative retaining β-xylosidases from both GH family 39 and 52—the latter being a prime candidate for conversion into a glycosynthase. This gene was cloned, recombinantly expressed in *E. coli* and, as anticipated, produced protein with β-xylosidase activity. Site directed mutagenesis was performed on this *B. halodurans* xylosidase (Bhx) to substitute the putative catalytic nucleophile (E334, predicted based on sequence homology) with a glycine, alanine or serine residue to provide three mutant enzymes. None of the *Bhx* mutants catalysed the hydrolysis of 4-nitrophenyl β-D-xylopyranoside; this demonstrates that E334 was indeed crucial for catalysis and supports the assertion that it was the catalytic nucleophile. The *Bhx* mutants were then evaluated as glycosynthases by incubating each one with α-D-xylopyranosyl fluoride in buffer (this compound functions as both donor and acceptor), and the reactions were analysed by thin layer chromatography and mass spectrometry to see if xylobiosyl fluoride was produced.[21] All mutants demonstrated the requisite activity, but it was the E334G mutant that proved to be the most competent. To assess the synthetic utility of the E334G Bhx glycosynthase, a number of D-*xylo*-configured glycosyl acceptors were prepared—acceptors that, once glycosylated, could serve as xylanase substrates or inhibitors.

Synthesis of acceptors

Simple acceptors, like 4-nitrophenyl β-D-xylopyranoside (**1**) and phenyl 1-thio-β-D-xylopyranoside (**2**) were obtained by using literature procedures.[23,24] Glycosynthase-mediated glycosylation of xyloside **1** would provide xylanase substrates, while a similar elaboration of thioglycoside **2** would presumably give a substrate-like competitive inhibitor. The 2-deoxy-2-fluoro-xyloside **3** was prepared by using a modified literature procedure (Scheme 2).[13] Enzymatic conversion of compound **3** into xylan-like oligosaccharides would provide a simple route to mechanism-based inactivators of retaining xylanases.



Scheme 2. Conditions: a) HBr/AcOH, CH_2Cl_2, b) 3,4-DNP, 2,6-collidine, Ag_2CO_3, MeCN, $CaSO_4$, c) HCl, MeOH.

To generate glycosylated iminosugars using a glycosynthase, the corresponding *N*-benzyloxycarbonyl (Cbz) derivative of the iminosugar is commonly used as a glycosyl acceptor.[25] Converting the iminosugar to the carbamate ablates the iminosugar's basicity, thereby, reducing its affinity for the donor-binding subsite of the enzyme and minimising substrate inhibition. Additionally, the aromatic substituent increases the iminosugar's affinity for the acceptor binding subsite. The benzyloxycarbonyl group also aids in product purification by ensuring that the desired products carry no charge, have a hydrophobic "handle" and a chromophore for product detection. Of course, after glycosylation, this protecting group is easily removed by hydrogenolysis.

Thus, iminosugar derivatives **4** and **5** were prepared from diacetone glucose by way of aldehyde **6** (Scheme 3).[26] Hydrolysis of the acetonide from this intermediate, followed by reductive amination and N-acylation provided the desired carbamate **4**. A Henry reaction of the aldehyde **6** with nitromethane, followed by dehydration and reduction gave the nitro compound **7**.[27] Removal of the acetonide group and treatment of the product with periodate yielded the unstable aldehyde **8**. This product was immediately subjected to hydrogenation, followed by N-acylation, presumably with concomitant transesterification, to provide the carbamate **5** in good yield.

Glycosynthase reactions

Each of the putative acceptors **1**–**5** was dissolved, along with donor substrate (α-D-xylopyranosyl fluoride), in phosphate buffer at pH 7 and incubated with the E334G Bhx mutant at room temperature for one day. Although the products from these glycosynthase reactions could have been isolated without further derivatisation, each was per-O-acetylated to simplify product isolation and characterisation. To this end, each glycosynthase reaction was first passed through a short column packed with reverse-phase C-18 silica to separate the desired acceptor-derived products (all possessing a hydrophobic

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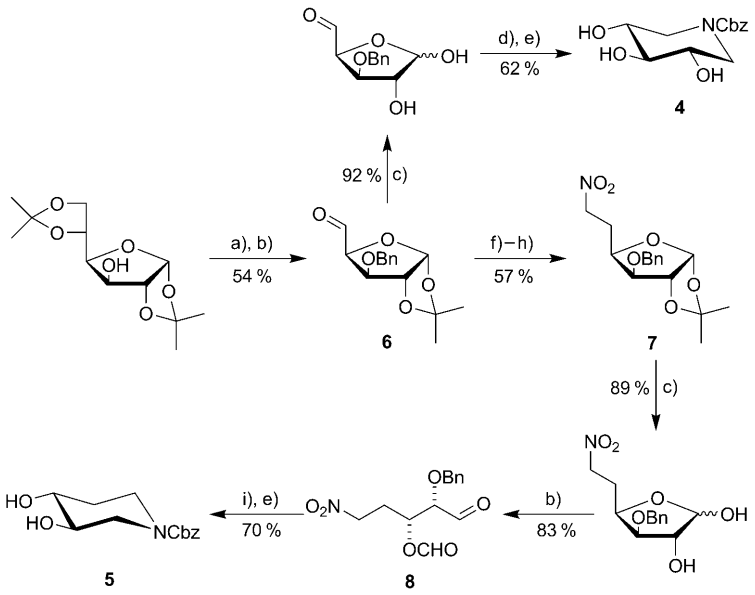


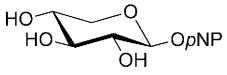
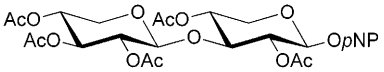
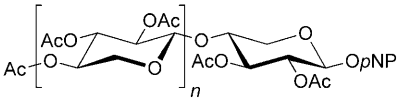
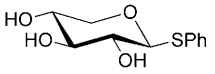
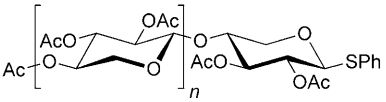
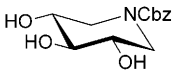
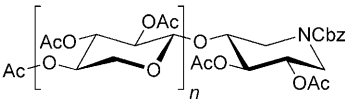
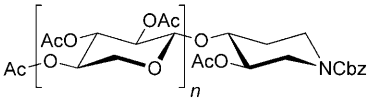
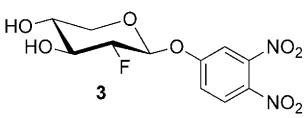
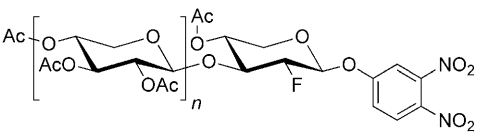
Scheme 3. Conditions: a) i: NaH, BnBr, DMF, ii: AcOH/H_2O (9:1); b) $NaIO_4$, MeOH, H_2O; c) 6 N HCl, THF; d) $BnNH_2$, H_2 (3 atm), Pd/C, MeOH, 1 N HCl; e) CbzCl, K_2CO_3, H_2O/MeOH (1:1); f) $MeNO_2$, EtOH, 1 N NaOH; g) MsCl, Et_3N, CH_2Cl_2; h) $NaBH_4$, EtOH; i) H_2 (3 atm), Pd/C, MeOH, 1 N HCl.

group) from the glycosyl fluorides, their hydrolysis products, and buffer salts. This quick, preliminary purification step made the subsequent acetylation reaction and separation of oligosaccharide products much simpler. Each mixture of semipurified products was dissolved in pyridine and acetylated by the action of acetic anhydride and a catalytic amount of 4-dimethylaminopyridine. Subsequent purification by flash chromatography on silica gel afforded each individual oligosaccharide. The results for each glycosynthase reaction are provided in Table 1.

The proficiency of this particular glycosynthase is demonstrated by the high total yield of glycosylated products for each acceptor tested, ranging from 64 to 86%. For the xyloside **1** (Table 1, entry 1), the β-(1→4) linked disaccharide **9** and trisaccharide **10** were obtained in good yields, as expected. However, a small amount of the β-(1→3) linked disaccharide **11** was also isolated. In the case of the thioglycoside **2** (entry 2), only the β-(1→4) linked disaccharide **12** and trisaccharide **13** were obtained. The iminosugar-derived carbamate **4** (entry 3) gave the (1→4) xylan-like di-, tri- and tetrasaccharides **14**, **15** and **16**, respectively. Likewise, the protected iminosugar **5** (entry 4) was elaborated to the desired disaccharide **17** and trisaccharide **18**.

In the case of the 2-deoxy-2-fluoro-xyloside **3** (entry 5) both disaccharide **19** and trisaccharide **20** were obtained, here the first glycosylation reaction proceeded in a β-(1→3) manner and the second in a β-(1→4) sense (see NMR COSY experiments in the Supporting Information). The reasons for this lapse in fidelity are unclear.

Table 1. BhxE334G-catalysed glycosylation of various acceptors.

	Acceptor	Product	*n*	Compound number	Yield [%] isolated	Yield [%] glycosylated products
1	 **1**		–	**11**	5	64
			0	–	19	
			1	**9**	36	
			2	**10**	23	
2	 **2**		0	–	14	
			1	**12**	32	67
			2	**13**	35	
3	 **4**		0	–	7	
			1	**14**	13	80
			2	**15**	53	
			3	**16**	14	
4	 **5**		0	–	11	
			1	**17**	24	71
			2	**18**	47	
5	 **3**		0	–	2	
			1	**19**	63	86
			2	**20**	23	

Conditions: A solution of 0.2 mmol acceptor and 0.5 mmol α-D-xylopyranosyl fluoride in sodium phosphate buffer (pH 7) was treated with 480 μg BhxE334G glycosynthase and incubated at room temperature for 24 h. Products were isolated after per-*O*-acetylation by treatment with excess Ac_2O and catalytic DMAP in pyridine.

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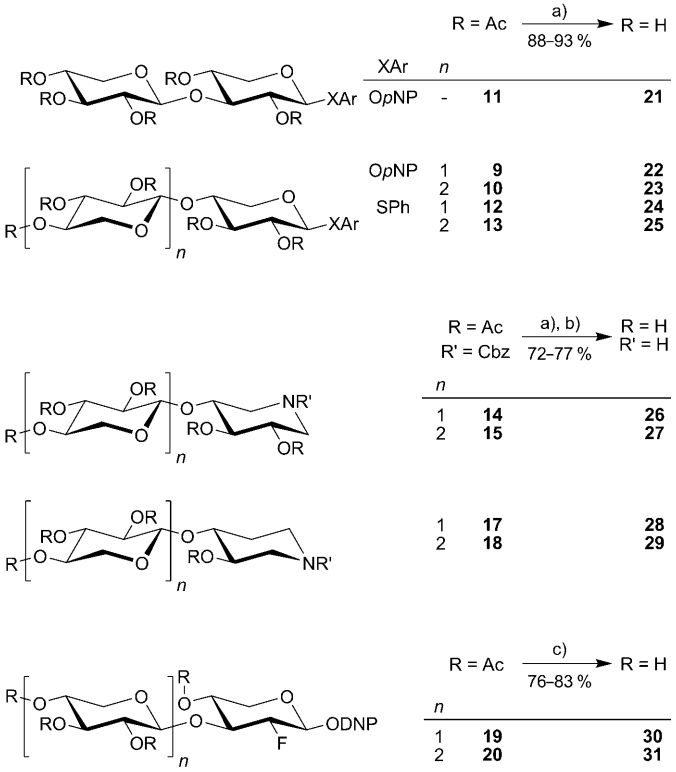
The products of these glycosynthase reactions were subsequently deprotected by using conventional procedures (Scheme 4). The 4-nitrophenyl glycosides **9**–**11** and thioglycosides **12**–**13** were deprotected by a Zemplén transesterification to give the corresponding polyols **21**–**25**. Similar treatment of the carbamates **14**–**18**, with a subsequent hydrogenation provided the iminosugars **26**–**29** (Scheme 4). Acid catalysed transesterification, effected by methanolic hydrogen chloride, was sufficient for the deprotection of the 2-deoxy-2-fluoro-xylosides **19**–**20** to provide the corresponding polyols **30**–**31**.



Scheme 4. Deprotection of oligosaccharide products. Conditions: a) i: NaOMe, MeOH, ii: Amberlite IR-120 (H^+); b) i: H_2 (1 atm), Pd/C, MeOH, ii: 1 N HCl; c) HCl, MeOH.

Inhibition of xylanases

The disaccharide iminosugars **26** and **28** have been synthesised previously—both are relatively good inhibitors of *Cellulomonas fimi* xylanase (Cex), a GH family 10 enzyme, but rather poor inhibitors of the GH family 11 xylanase from *Bacillus circulans* (Bcx; Table 2).[14] The structures of both enzymes have been solved by using X-ray diffraction methods, and in neither is there any obvious −3 subsite in the substrate-binding cleft.[28,29] Thus, one might expect that the novel trisaccharides **27** and **29** would be little better as inhibitors than their disaccharide counterparts **26** and **28**, respectively. Intriguingly, the inhibition constants for trisaccharides **27** and **29** against both enzymes (determined by using the 4-nitrophenyl glycoside substrate **22** prepared with the Bhx glycosynthase) were found to be over an order of magnitude smaller than those reported for the corresponding disaccharides **26** and **28**, respectively (Table 2). Both trisaccharides are relatively good inhibitors of the GH family 11 enzyme Bcx—in fact they are the most potent competitive inhibitors of this xylanase reported to date. Similarly, both **27** and **29** are excellent inhibitors of Cex, although **29**, with a K_i of just 18 nM, is clearly the better of the two. The large difference in the inhibitory potency of iminosugars like **26**–**29** against the two different xylanases has previously been rationalised by the fact that these enzymes facilitate hydrolysis using different conformational itineraries, and so have evolved to stabilise quite different transition states.[14,30] It would appear then, that **26**–**29** more closely resemble the transition state of Cex than of Bcx.

Table 2. Competitive inhibition constants.

Compound	**26**	**27**	**28**	**29**
K_i for Bcx [μM]	1500[14]	88 ± 6	1100[14]	63 ± 4
K_i for Cex [μM]	5.8[14]	0.16 ± 0.03	0.13[14]	0.018 ± 0.002

The di- and trisaccharide 2-fluoro-xylosides **30** and **31** were incapable of inactivating Bcx or Cex. However, these molecules might prove to be mechanism-based inhibitors of the GH family 26 xylanases.[31,32] Likewise, iminosugars **28** and **29** might also serve as inhibitor candidates for this less-common class of xylanases.

Conclusions

In support of previous studies,[21] this body of work has illustrated the synthetic utility and limitations of an *exo*-β-xylosidase-derived glycosynthase. This enzyme can efficiently catalyse the glycosylation of xylose-like acceptors; for the most part forming β-(1→4)-linked products in good to excellent yield. Kinetic studies demonstrated that trisaccharide iminosugars are better inhibitors than the corresponding disaccharides for two xylanases of different GH families. The reasons for enhanced binding of the trisaccharides **27** and **29** relative to the disaccharides **26** and **28** are unclear; this matter is the subject of ongoing structural studies. The β-(1→3)-linked oligosaccharides synthesised by an apparent lapse in glycosynthase fidelity might yet prove to be useful as substrates and inhibitors of the (1→3)-xylanases from GH family 26.

Experimental Section

General materials and methods: All chemicals were obtained from Sigma–Aldrich and were of reagent grade. Thin layer chromatography (TLC) was performed on Merck silica gel 60 F_{254} plates. Flash chromatography was performed with Merck silica gel 60 (230–400 mesh). ^1H and ^{13}C NMR (referenced by using the solvent peak, or an internal MeOH standard in the case of ^{13}C for D_2O) spectra were recorded on a 400 MHz Bruker instrument. High-resolution mass spectra of all compounds were obtained in the mass spectrometry laboratory of the Chemistry Department at UBC. Bcx and Cex were obtained as described previously.[33,34] Inhibition constants were determined at 37 °C by using a NaH_2PO_4/Na_2HPO_4 buffer (50 mM, pH 7.5) containing NaCl (150 mM), and 4-nitrophen-

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yl β-xylobioside (**22**) as a substrate. Reactions were initiated by the addition of xylanase, and the increase in absorption at 400 nm over time, in a continuous assay, gave the initial reaction rate. This increase was linear for all measurements (3 min). Michaelis parameters (V_{max} and K_M) were extracted from these data by best fit to the Michaelis–Menten equation by using the program GraFit 5.0.13. Full K_i determinations were performed by measurement of rates at a series of five substrate concentrations for a number of inhibitor concentrations (typically seven concentrations bracketing the K_i value ultimately determined such that $0.2\,K_i < [I] < 5\,K_i$). K_i values were then calculated from these data by nonlinear regression analysis by using the GraFit program.

Cloning, mutagenesis and expression of *Bhx*

Cloning: The DNA sequence encoding *Bhx*, BAB05833.1 was amplified by PCR (Bio-Rad, MyCycler) from genomic DNA of *B. halodurans* C-125, ATCC BAA-125D, by using PWO DNA polymerase (Roche) with primers 5′-GCATCG GCTAGC ATGTTT ACACCT AAAAAT ATATTT TTTAAC GCACAT CATTCA CC-3′ and 5′-GCAGTC TGCGGC CGCTTA TTATGT TATTTC CTCTAG CCAGAG AAT-3′ including restriction sites for NheI and NotI, respectively. The PCR product was double digested with NheI and NotI (Fermentas) and cloned into the pET-28a(+) vector (Novagen) resulting in an N-terminal His-tag. The pET-28a(+)-*Bhx* construct was transformed into BL21(DE3) cells (Novagen) by electroporation.

Mutagenesis: Site-directed mutagenesis of the wild-type *Bhx* gene was carried out following the QuickChange protocol (Stratagene) by using Pfu DNA polymerase (Stratagene), with pET-28a(+)-*Bhx* wild type as template. Mutagenic primers were: 5′-CCATTA TGGGTC GTGAAT G**C**GGGC GAATAC AGAATG-3′ and 5′-CATCAT TCTGTA TTCGCC C**G**CATT CACGAC CCATAA TG-3′ for the E334A mutant; 5′-CCATTA TGGGTC GTGAAT G**G**GGGC GAATAC AGAATG-3′ and 5′-CATCAT TCTGTA TTCGCC C**C**CATT CACGAC CCATAA TG-3′ for the E334G mutant (mutated nucleotides in bold type). The E334S mutant was created by using a four primer protocol with: 5′-CGACCA TTATGG GTCGTG AAT**TC**G GGCGAA TACAGA ATG-3′ and 5′-GTATTC ATCATT CTGTAT TCGCCC **GA**ATTC ACGACC CATAAT G-3′ as mutagenic primers as well as the two primers used for cloning of the wild-type gene. The mutants were transformed into BL21(DE3) cells (Novagen) as for the wild type. Correct mutagenesis was validated by DNA sequencing.

Protein expression: Wild-type and mutant *Bhx* clones in BL21(DE3) cells were cultured in TYP medium at 37 °C. When cultures reached an OD_{600} of approximately 1.0, isopropyl β-D-thiogalactopyranoside (IPTG) was added (final concentration 0.1 mM) and the cultures were incubated for a further 16 h. Cells were harvested by centrifugation (4500 *g*, 30 min) and suspended in buffer A (60 mL per liter of culture; buffer A: 20 mM potassium phosphate, pH 7.4, 0.5 M NaCl, 5 mM imidazole). Cell extracts were prepared by French press. The soluble extracts were loaded onto HisTrap FF columns (GE Healthcare) on a FPLC system (GE Healthcare), washed with 15 column volumes of buffer A, and then eluted with a gradient of buffers A and B (buffer B: 20 mM potassium phosphate, pH 7.4, 0.5 M NaCl, 0.5 M imidazole). The yield of wild-type and mutant Bhx protein was around 70 mg L^{-1}.

Glycosynthase reaction protocol: Glycosynthase (50 μL, 9.6 mg mL^{-1} BhxE334G) was added to a solution of the acceptor (0.20 mmol) and α-D-xylopyranosyl fluoride[35] (76 mg, 0.50 mmol) in NaH_2PO_4/Na_2HPO_4 buffer (15 mL, 350 mM, pH 7) and the solution was left at room temperature (24 h). Methanol (2 mL) was added, then the solution was concentrated to a third of its volume and filtered (Millex GV 0.22 μm filter unit). The filtrate was applied to a Waters tC18 SepPak cartridge (2 g) and eluted under gravity with a H_2O/MeOH gradient (1:0–1:4). The fractions containing glycosylated acceptor were combined, concentrated and co-evaporated with toluene (2×10 mL). Acetic anhydride (0.38 mL, 4.0 mmol) was added to a solution of the residue and DMAP (5 mg) in C_5H_5N (10 mL) and left at room temperature (16 h). Methanol (1 mL) was added to the solution at 0 °C and the mixture warmed to room temperature. The mixture was concentrated to dryness, the residue was dissolved in EtOAc (50 mL) and washed with HCl (1 N, 50 mL), water (50 mL) and sat. $NaHCO_3$ (50 mL). The organic solution was dried over $MgSO_4$ and filtered. Concentration of the filtrate and flash chromatography (EtOAc/hexanes, 1:2 to 2:1) enabled individual isolation of the per-O-acetylated acceptor and its glycosylation products.

3,4-Dinitrophenyl 2-deoxy-2-fluoro-β-D-xylopyranoside (3): Tri-*O*-acetyl-2-deoxy-2-fluoro-D-xylopyranose[15] (1.1 g, 3.8 mmol) was converted into 3,4-di-*O*-acetyl-2-deoxy-2-fluoro-α-D-xylopyranosyl bromide according to the procedure of Ziser et al.[13] Silver carbonate (2.1 g, 7.6 mmol) and anhydrous $CaSO_4$ (2.0 g) were added to a solution of anhydrous 3,4-dinitrophenol (1.0 g, 5.7 mmol) and 2,6-lutidine (0.89 mL, 7.6 mmol) in anhydrous MeCN (40 mL) and the mixture was stirred under Ar in the dark (room temperature, 10 min). A solution of the crude 3,4-di-*O*-acetyl-2-deoxy-2-fluoro-α-D-xylopyranosyl bromide in anhydrous MeCN (10 mL) was added drop-wise and the mixture was stirred (room temperature, 30 min). The mixture was filtered, the residue was washed with EtOAc, and the combined filtrate was concentrated. The residue was subjected to flash chromatography (EtOAc/hexanes; 1:7). A suspension of this product (1.1 g, 2.6 mmol) in dry MeOH (30 mL) was treated with AcCl (0.30 mL) at 0 °C, and the solution was stirred (room temperature, 24 h). The solution was concentrated to dryness and the residue was recrystallised (MeOH/Et_2O) to give the diol **3** as pale yellow needles (630 mg, 52% yield). ^{1}H NMR (400 MHz, CD_3OD): $\delta = 3.51$ (dd, $J = 9.6$, 11.6 Hz, 1H; H-5), 3.63 (ddd, $J = 5.2$, 9.6, 9.6 Hz, 1H; H-4), 3.72 (ddd, $J = 8.8$, 9.6, 15.2 Hz, 1H; H-3), 3.97 (dd, $J = 5.2$, 11.6 Hz, 1H; H-5), 4.32 (ddd, $J = 8.4$, 8.8, 51.2 Hz, 1H; H-2), 5.44 (dd, $J = 4.0$, 8.4 Hz, 1H; H-1), 7.44 (dd, $J = 2.4$, 8.8 Hz, 1H; ArH), 7.62 (d, $J = 2.4$ Hz, 1H; ArH), 8.13 (d, $J = 8.8$ Hz, 1H; ArH); ^{13}C NMR (100.6 MHz, CD_3OD): $\delta = 67.1$ (C-5), 70.5 (d, $J = 7.0$ Hz, C-4), 75.8 (d, $J = 18.1$ Hz, C-3), 93.0 (d, $J = 186.2$ Hz, C-2), 99.7 (d, $J = 24.2$ Hz, C-1), 114.1, 121.0, 128.8, 137.6, 146.6, 162.0 (Ar). HRMS (ES): *m/z* 341.0390; $[M+Na]^+$ requires 341.0397.

***N*-Benzyloxycarbonyl-1,5-dideoxy-1,5-imino-D-xylitol (4):** Hydrochloric acid (6 N, 5 mL) was added to a solution of the aldehyde **6**[26] (1.5 g, 5.5 mmol) in THF (10 mL) and the mixture was stirred (room temperature, 16 h). The solution was concentrated to dryness. A mixture of the residue, Pd/C (10%, 100 mg), benzylamine (0.72 mL, 6.6 mmol) and hydrochloric acid (6 N, 1 mL) in MeOH (20 mL) was stirred under H_2 (3 atm, 48 h). The mixture was filtered and concentrated. Potassium carbonate (3.0 g, 22 mmol) and CbzCl (1.2 mL, 8.2 mmol) were added to a solution of the residue in MeOH/H_2O (1:1, 20 mL) and the mixture was stirred (16 h). The mixture was concentrated, and the residue was repeatedly washed with Me_2CO (5×10 mL). The Me_2CO washings were combined and concentrated to dryness. The residue was subjected to flash chromatography (EtOAc/hexanes; 19:1–1:0) to return the triol **5** as colourless needles (832 mg, 57% yield). ^{1}H NMR (400 MHz, D_2O): $\delta =$ 2.65–2.88 (m, 2H; H-1,5), 3.29–3.54 (m, 3H; H-2,3,4), 4.02–4.31 (m, 2H; H-1,5), 5.12–5.19 (m, 2H; CH_2Ph), 7.35–7.55 (m, 5H; Ph); ^{13}C NMR (100.6 MHz, D_2O): $\delta = 47.6$ (C-1,5), 68.1 (CH_2Ph), 69.2 (C-2,4), 77.9 (C-3), 127.9, 128.6, 128.9, 136.3 (Ph), 156.7 (NCO_2). HRMS (ES): *m/z* 290.1006; $[M+Na]^+$ requires 290.1004.

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Benzyl (3*R*,4*R*)-3,4-dihydroxy-piperidine-1-carboxylate (5): A mixture of Pd/C (10%, 100 mg), the aldehyde **8**[27] (0.77 g, 2.7 mmol) and hydrochloric acid (1 N, 0.5 mL) in MeOH (20 mL) was stirred under H_2 (3 atm, 48 h). The mixture was filtered and concentrated. Potassium carbonate (0.75 g, 5.4 mmol) and CbzCl (0.59 mL, 4.1 mmol) were added to a solution of the residue in MeOH/H_2O (1:1, 10 mL) and the mixture was stirred (16 h). The mixture was concentrated, and the residue was repeatedly washed with Me_2CO (5×10 mL). The Me_2CO washings were combined and concentrated to dryness. The residue was subjected to flash chromatography (EtOAc/hexanes; 17:3–9:1) to return the diol **5** as a colourless foam (482 mg, 70% yield). ^1H and ^{13}C NMR spectra obtained for this material were in agreement with the data previously reported.[14]

Glycosylation of 4-nitrophenyl β-D-xylopyranoside (1): The xyloside **1**[23] was subjected to the glycosynthase reaction protocol described above. The following products were obtained after flash chromatography and are listed in order of elution (yields are presented in Table 1).

*4-Nitrophenyl 2,3,4-tri-*O*-acetyl-*β*-D-xyloside:* ^1H and ^{13}C NMR spectra obtained for this compound were in agreement with the data previously reported.[36]

*4-Nitrophenyl tri-*O*-acetyl-*β*-D-xylopyranosyl-(1→4)-2,3-di-*O*-acetyl-*β*-D-xyloside (**9**):* ^1H and ^{13}C NMR spectra obtained for this compound were in agreement with the data previously reported.[37]

*4-Nitrophenyl tri-*O*-acetyl-*β*-D-xylopyranosyl-(1→3)-2,4-di-*O*-acetyl-*β*-D-xyloside (**11**):* ^1H and ^{13}C NMR spectra obtained for this compound were in agreement with those data previously reported.[20]

*4-Nitrophenyl tri-*O*-acetyl-*β*-D-xylopyranosyl-(1→4)-2,3-di-*O*-acetyl-*β*-D-xylosyl-(1→4)-2,3-di-*O*-acetyl-*β*-D-xyloside (**10**):* ^{13}C NMR spectra obtained for this compound were in agreement with the data previously reported.[38]

Glycosylation of phenyl 1-thio-β-D-xylopyranoside (2): The xyloside **2**[24] was subjected to the glycosynthase reaction protocol described above. The following products were obtained after flash chromatography and are listed in order of elution (yields are presented in Table 1).

*Phenyl 2,3,4-tri-*O*-acetyl-1-thio-*β*-D-xyloside:* ^1H and ^{13}C NMR spectra obtained for this compound were in agreement with the data previously reported.[39]

*Phenyl tri-*O*-acetyl-*β*-D-xylopyranosyl-(1→4)-2,3-di-*O*-acetyl-1-thio-*β*-D-xyloside (**12**):* ^1H NMR (400 MHz, $CDCl_3$): $\delta = 2.01$, 2.03, 2.04, 2.07 (4 s, 15 H; $COCH_3$), 3.30–3.43 (m, 2 H; H-5,5′), 3.81 (ddd, $J = 5.2$, 9.2, 9.2 Hz, 1 H; H-4), 4.02–4.16 (m, 2 H; H-5,5′), 4.54 (d, $J = 6.0$ Hz, 1 H; H-1′), 4.71 (d, $J = 9.2$ Hz, 1 H; H-1), 4.77 (dd, $J = 6.0$, 7.6 Hz, 1 H; H-2′), 4.82–4.92 (m, 2 H; H-2,4′), 5.03–5.18 (m, 2 H; H-3,3′), 7.27–7.51 (m, 5 H; Ph); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 20.78$, 20.81, 20.89, 20.93, 21.0 ($COCH_3$), 61.7 (C-5′), 66.5 (C-5), 68.5 (C-4′), 70.3 (C-2), 70.5, 70.6 (C-2′,3′), 73.5 (C-3), 74.7 (C-4), 86.6 (C-1), 99.7 (C-1′), 128.4, 129.2, 132.3, 132.9 (Ph), 169.3, 169.7, 169.9, 170.0, 170.1 ($COCH_3$). HRMS (ES): *m/z* 607.1451; $[M+Na]^+$ requires 607.1461.

*Phenyl tri-*O*-acetyl-*β*-D-xylopyranosyl-(1→4)-2,3-di-*O*-acetyl-*β*-D-xylosyl-(1→4)-2,3-di-*O*-acetyl-1-thio-*β*-D-xyloside (**13**):* ^1H NMR (400 MHz, $CDCl_3$): $\delta = 2.01$, 2.02, 2.03, 2.04, 2.05, 2.07 (6 s, 21 H; $COCH_3$), 3.26–3.43 (m, 3 H; H-5,5′,5″), 3.72–3.84 (m, 2 H; H-4,4′), 3.93 (dd, $J = 4.8$, 12.0 Hz, 1 H; H-5′), 4.03–4.15 (m, 2 H; H-5,5″), 4.47 (d, $J = 6.8$ Hz, 1 H; H-1′), 4.55 (d, $J = 5.6$ Hz, 1 H; H-1″), 4.69–4.76 (m, 2 H; H-1,2′), 4.79 (dd, $J = 5.6$, 6.0 Hz, 1 H; H-2″), 4.83–4.92 (m, 2 H; H-2,4″), 5.01–5.16 (m, 3 H; H-3,3′,3″), 7.28–7.50 (m, 5 H; Ph); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 20.80$, 20.82, 20.91, 20.93, 20.96, 21.00 ($COCH_3$), 61.7 (C-5″), 62.7 (C-5′), 66.4 (C-5), 68.5 (C-4″), 70.2 (C-2), 70.5, 70.6 (C-2″,3″), 71.2 (C-2′), 72.1 (C-3′), 73.4 (C-3), 74.3 (C-4′), 75.2 (C-4), 86.6 (C-1), 99.6 (C-1″), 100.6 (C-1′), 128.4, 129.2, 132.4, 132.9 (Ph), 169.3, 169.5, 169.7, 169.9, 169.98, 170.01, 170.1 ($COCH_3$). HRMS (ES): *m/z* 823.2108; $[M+Na]^+$ requires 823.2095.

Glycosylation of *N*-benzyloxycarbonyl-1,5-dideoxy-1,5-imino-D-xylitol (4): The carbamate **4** was subjected to the glycosynthase reaction protocol described above. The following products were obtained after flash chromatography and are listed in order of elution (yields are presented in Table 1).

*2,3,4-Tri-*O*-acetyl-*N*-benzyloxycarbonyl-1,5-dideoxy-1,5-imino-D-xylitol:* ^1H NMR (400 MHz, $CDCl_3$ at 318 K): $\delta = 2.00$, 2.04 (2 s, 9 H; $COCH_3$), 3.22 (dd, $J = 8.4$, 13.6 Hz, 2 H; H-1,5), 4.08 (dd, $J = 4.4$, 13.6 Hz, 2 H; H-1,5), 4.85 (ddd, $J = 4.4$, 7.6, 8.4 Hz, 2 H; H-2,4), 5.09 (t, $J = 7.6$ Hz, 1 H; H-3), 5.16 (br s, 2 H; CH_2Ph), 7.28–7.37 (m, 5 H; Ph); ^{13}C NMR (100.6 MHz, $CDCl_3$ at 318 K): $\delta = 20.76$, 20.79 ($COCH_3$), 45.0 (C-1,5), 67.9 (CH_2Ph), 68.4 (C-2,4), 72.2 (C-3), 128.2, 128.4, 128.7, 136.5 (Ph), 155.3 (NCO_2), 169.7, 169.8 ($COCH_3$). HRMS (ES): *m/z* 416.1325; $[M+Na]^+$ requires 416.1321.

*Benzyl (3S,4S,5R)-3,4-diacetoxy-5-(tri-*O*-acetyl-β-D-xylopyranosyloxy)-piperidine-1-carboxylate (**14**):* ^1H NMR (400 MHz, $CDCl_3$ at 318 K): $\delta = 2.01$, 2.02, 2.04 (3 s, 15 H; $COCH_3$), 2.87–3.06 (m, 2 H; H-2,6), 3.37 (dd, $J = 8.0$, 11.6 Hz, 1 H; H-5′), 3.71 (ddd, $J = 4.8$, 8.8, 8.8 Hz, 1 H; H-5), 4.02–4.25 (m, 3 H; H-2,6,5′), 4.59 (d, $J = 6.0$ Hz, 1 H; H-1′), 4.75–4.95 (m, 3 H; H-3,2′,4′), 5.01–5.22 (m, 4 H; CH_2Ph, H-4,3′), 7.29–7.41 (m, 5 H; Ph); ^{13}C NMR (100.6 MHz, $CDCl_3$ at 318 K): $\delta = 20.7$, 20.8, 21.0 ($COCH_3$), 45.2 (C-2), 45.3 (C-6), 62.0 (C-5′), 68.0 (CH_2Ph), 68.8 (C-3,4′), 70.8 (C-2′), 71.1 (C-3′), 73.6 (C-4), 74.4 (C-5), 99.6 (C-1′), 128.2, 128.5, 128.8, 136.4 (Ph), 155.1 (NCO_2), 169.3, 169.8, 169.9, 170.0 ($COCH_3$). HRMS (ES): *m/z* 632.1964; $[M+Na]^+$ requires 632.1955.

*Benzyl (3S,4S,5R)-3,4-diacetoxy-5-[tri-*O*-acetyl-β-D-xylopyranosyl-(1→4)-di-*O*-acetyl-β-D-xylosyloxy]-piperidine-1-carboxylate (**15**):* ^1H NMR (400 MHz, $CDCl_3$ at 318 K): $\delta = 2.00$, 2.018, 2.021, 2.03, 2.04, 2.05 (6 s, 21 H; $COCH_3$), 2.89–3.09 (m, 2 H; H-2,6), 3.30 (dd, $J = 8.4$, 11.6 Hz, 1 H; H-5′), 3.39 (dd, $J = 7.6$, 12.0 Hz, 1 H; H-5″), 3.68 (ddd, $J = 4.4$, 8.8, 8.8 Hz, 1 H; H-5), 3.80 (ddd, $J = 4.8$, 8.4, 8.4 Hz, 1 H; H-4′), 3.94 (dd, $J = 4.8$, 11.6 Hz, 1 H; H-5′), 4.02–4.20 (m, 3 H; H-2,6,5″), 4.51 (d, $J = 6.4$ Hz, 1 H; H-1′), 4.56 (d, $J = 5.6$ Hz, 1 H; H-1″), 4.72–4.84 (m, 3 H; H-3,2′,2″), 4.88 (ddd, $J = 4.4$, 7.6, 7.6 Hz, 1 H; H-4″), 4.99–5.20 (m, 5 H; CH_2Ph, H-4,3′,3″), 7.28–7.40 (m, 5 H; Ph); ^{13}C NMR (100.6 MHz, $CDCl_3$ at 318 K): $\delta = 20.7$, 20.78, 20.84, 20.9, 21.0 ($COCH_3$), 45.1 (C-2), 45.3 (C-6), 61.8 (C-5″), 62.8 (C-5′), 68.0 (CH_2Ph), 68.6 (C-3,4″), 70.66, 70.74 (C-2″,3″), 71.3 (C-2′), 72.3 (C-3′), 73.5 (C-4), 74.5 (C-4′), 74.8 (C-5), 99.6 (C-1″), 100.3 (C-1′), 128.2, 128.5, 128.8, 136.3 (Ph), 155.1 (NCO_2), 169.2, 169.5, 169.7, 169.91, 169.93, 170.0, 170.1 ($COCH_3$). HRMS (ES): *m/z* 848.2582; $[M+Na]^+$ requires 848.2589.

*Benzyl (3S,4S,5R)-3,4-diacetoxy-5-[tri-*O*-acetyl-β-D-xylopyranosyl-(1→4)-di-*O*-acetyl-β-D-xylosyl-(1→4)-di-*O*-acetyl-β-D-xylosyloxy]-piperidine-1-carboxylate (**16**):* ^1H NMR (400 MHz, $CDCl_3$ at 318 K): $\delta =$ 1.99, 2.00, 2.01, 2.02, 2.026, 2.029, 2.04 (7 s, 27 H; $COCH_3$), 2.88–3.07 (m, 2 H; H-2,6), 3.22–3.42 (m, 3 H; H-5′,5″,5‴), 3.67 (ddd, $J = 4.4$, 8.8, 8.8 Hz, 1 H; H-5), 3.71–3.84 (m, 2 H; H-4′,4″), 3.86–3.98 (m, 2 H; H-5′,5″), 3.99–4.19 (m, 3 H; H-2,6,5‴), 4.47 (d, $J = 6.8$ Hz, 1 H; H-1″), 4.50 (d, $J = 6.8$ Hz, 1 H; H-1′), 4.55 (d, $J = 6.0$ Hz, 1 H; H-1‴), 4.68–4.82 (m, 4 H; H-3,2′,2″,2‴), 4.87 (ddd, $J = 4.8$, 7.6, 7.6 Hz, 1 H; H-4‴), 4.97–5.19 (m, 6 H; CH_2Ph,H-4,3′,3″,3‴), 7.29–7.39 (m, 5 H; Ph); ^{13}C NMR (100.6 MHz, $CDCl_3$ at 318 K): $\delta = 20.71$, 20.73, 20.80, 20.84, 20.9, 21.0 ($COCH_3$), 45.1 (C-2), 45.2 (C-6), 61.8 (C-5‴), 62.6, 62.7 (C-5′,5″), 68.0 (CH_2Ph), 68.6 (C-3,4‴), 70.7, 70.8 (C-2‴,3‴), 71.1, 71.2 (C-2′,2″), 72.1, 72.2 (C-3′,3″), 73.4 (C-4), 74.4, 74.9 (C-4′,4″), 74.7 (C-5),

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99.7 (C-1‴), 100.1, 100.3 (C-1′,1″), 128.2, 128.4, 128.8, 136.3 (Ph), 155.1 (NCO_2), 169.2, 169.4, 169.5, 169.7, 169.8, 169.87, 169.90 ($COCH_3$). HRMS (ES): *m/z* 1064.3199; $[M+Na]^+$ requires 1064.3223.

Glycosylation of benzyl (3*R*,4*R*)-3,4-dihydroxy-piperidine-1-carboxylate (5): The carbamate **5** was subjected to the glycosynthase reaction protocol described above. The following products were obtained after flash chromatography and are listed in order of elution (yields are presented in Table 1).

Benzyl (3R,4R)-3,4-diacetoxy-piperidine-1-carboxylate: ^1H and ^{13}C NMR spectra obtained for this compound were in agreement with the data previously reported.[40]

*Benzyl (3R,4R)-4-acetoxy-3-(tri-O-acetyl-β-D-xylopyranosyloxy)-piperidine-1-carboxylate (**17**):* ^1H NMR (400 MHz, $CDCl_3$ at 318 K): $\delta =$ 1.51–1.63 (br m, 1H; H-5), 1.88–2.12 (br m, 13H; $COCH_3$, H-5), 3.20–3.79 (br m, 5H; H-2,2,3,6,5′), 3.81–3.90 (br m, 1H; H-6), 4.04–4.18 (br m, 1H; H-5′), 4.58–4.69 (br m, 1H; H-1′), 4.78–5.01 (br m, 3H; H-4,2′,4′), 5.05–5.23 (br m, 3H; CH_2Ph, H-3′), 7.24–7.43 (br m, 5H; Ph). The ^{13}C NMR (100.6 MHz, $CDCl_3$ at 318 K) was so broad as to be uninterpretable. HRMS (ES): *m/z* 574.1913; $[M+Na]^+$ requires 574.1900.

*Benzyl (3R,4R)-4-acetoxy-3-[tri-O-acetyl-β-D-xylopyranosyl-(1→4)-di-O-acetyl-β-D-xylosyloxy]-piperidine-1-carboxylate (**18**):* ^1H NMR (400 MHz, $CDCl_3$ at 318 K): $\delta =$ 1.48–1.64 (br m, 1H; H-5), 1.90–2.10 (br m, 19H; $COCH_3$, H-5), 3.10–3.73 (br m, 7H; H-2,2,3,6,6,5′,5″), 3.73–3.87 (br m, 1H; H-4′), 3.88–4.02 (br m, 1H; H-5′), 4.02–4.16 (br m, 1H; H-5″), 4.49–4.62 (br m, 2H; H-1′,1″), 4.72–4.96 (br m, 4H; H-4,2′,2″,4″), 5.00–5.20 (br m, 4H; CH_2Ph, H-3′,3″), 7.28–7.40 (br m, 5H; Ph). The ^{13}C NMR (100.6 MHz, $CDCl_3$ at 318 K) was so broad as to be uninterpretable. HRMS (ES): *m/z* 790.2547; $[M+Na]^+$ requires 790.2534.

Glycosylation of 3,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-xylopyranoside (3): The xyloside **3** was subjected to the glycosynthase reaction protocol described above. The following products were obtained after flash chromatography and are listed in order of elution (yields are presented in Table 1).

3,4-Dinitrophenyl 3,4-di-O-acetyl-2-deoxy-2-fluoro-β-D-xyloside: ^1H NMR (400 MHz, $CDCl_3$): $\delta =$ 2.11, 2.17 (2s, 6H; $COCH_3$), 3.68 (dd, $J=$6.8, 12.4 Hz, 1H; H-5), 4.18 (dd, $J=$4.0, 12.4 Hz, 1H; H-5), 4.64 (ddd, $J=$5.2, 6.8, 47.6 Hz, 1H; H-2), 4.98 (ddd, $J=$4.0, 6.8, 6.8 Hz, 1H; H-4), 5.37 (ddd, $J=$6.8, 6.8, 12.8 Hz, 1H; H-3), 5.46 (dd, $J=$5.2, 7.2 Hz, 1H; H-1), 7.33 (dd, $J=$2.4, 8.8 Hz, 1H; ArH), 7.44 (d, $J=$ 2.4 Hz, 1H; ArH), 8.04 (d, $J=$8.8 Hz, 1H; ArH); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta =$ 20.89, 20.91 ($COCH_3$), 62.1 (C-5), 67.6 (d, $J=$4.0 Hz, C-4), 69.4 (d, $J=$23.1 Hz, C-3), 87.0 (d, $J=$188.2 Hz, C-2), 97.8 (d, $J=$ 29.2 Hz, C-1), 113.2, 119.9, 127.6, 136.9, 145.3, 159.8 (Ar), 169.6, 170.1 ($COCH_3$). HRMS (ES): *m/z* 425.0615; $[M+Na]^+$ requires 425.0608.

*3,4-Dinitrophenyl 4-O-acetyl-2-deoxy-2-fluoro-3-(tri-O-acetyl-β-D-xylopyranosyl)-β-D-xyloside (**19**):* ^1H NMR (400 MHz, $CDCl_3$): $\delta =$ 2.02, 2.03, 2.05, 2.09 (4s, 12H; $COCH_3$), 3.41 (dd, $J=$8.4, 12.0 Hz, 1H; H-5′), 3.58 (dd, $J=$6.4, 12.4 Hz, 1H; H-5), 4.02–4.17 (m, 3H; H-3,5,5′), 4.54 (ddd, $J=$4.8, 4.9, 47.6 Hz, 1H; H-2), 4.72 (d, $J=$6.8 Hz, 1H; H-1′), 4.87–5.02 (m, 3H; H-4,2′,4′), 5.16 (dd, $J=$8.4, 8.4 Hz, 1H; H-3′), 5.42 (dd, $J=$4.9, 8.0 Hz, 1H; H-1), 7.33 (dd, $J=$2.6, 9.2 Hz, 1H; ArH), 7.46 (d, $J=$2.6 Hz, 1H; ArH), 8.00 (d, $J=$9.2 Hz, 1H; ArH); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta =$ 20.77, 20.80, 20.82, 20.9 ($COCH_3$), 61.7 (C-5), 62.2 (C-5′), 68.1 (d, $J=$5.0 Hz, C-4), 68.8 (C-4′), 70.7 (C-2′), 71.0 (C-3′), 76.5 (d, $J=$21.1 Hz, C-3), 88.4 (d, $J=$185.2 Hz, C-2), 97.3 (d, $J=$ 30.2 Hz, C-1), 101.8 (C-1′), 113.0, 119.9, 127.5, 136.6, 145.2, 159.8 (Ar), 169.5, 169.9, 170.0, 170.1 ($COCH_3$). HRMS (ES): *m/z* 641.1238; $[M+Na]^+$ requires 641.1242.

*3,4-Dinitrophenyl 4-O-acetyl-2-deoxy-2-fluoro-3-[tri-O-acetyl-β-D-xylopyranosyl-(1→4)-di-O-acetyl-β-D-xylosyl]-β-D-xyloside (**20**):* ^1H NMR (400 MHz, $CDCl_3$): $\delta =$ 2.04, 2.05, 2.07, 2.12 (4s, 18H; $COCH_3$), 3.32–3.45 (m, 2H; H-5′,5″), 3.61 (dd, $J=$5.6, 12.4 Hz, 1H; H-5), 3.86 (ddd, $J=$5.2, 8.8, 8.8 Hz, 1H; H-4′), 3.97–4.16 (m, 4H; H-3,5,5′,5″), 4.44–4.62 (m, 2H; H-2,1″), 4.65 (d, $J=$7.2 Hz, 1H; H-1′), 4.80 (dd, $J=$6.0, 7.6 Hz, 1H; H-2″), 4.86–5.04 (m, 3H; H-4,2′,4″), 5.05–5.19 (m, 2H; H-3′,3″), 5.47 (dd, $J=$4.4, 8.8 Hz, 1H; H-1), 7.34 (dd, $J=$2.4, 9.2 Hz, 1H; ArH), 7.49 (d, $J=$2.4 Hz, 1H; ArH), 8.23 (d, $J=$9.2 Hz, 1H; ArH); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta =$ 20.84, 20.85, 20.87, 20.95, 20.99, 21.03 ($COCH_3$), 61.4 (C-5), 61.7 (C-5″), 63.2 (C-5′), 68.1 (d, $J=$4.0 Hz, C-4), 68.4 (C-4″), 70.49, 70.51 (C-2″,3″), 71.3 (C-2′), 72.2 (C-3′), 74.6 (C-4′), 76.3 (d, $J=$23.1 Hz, C-3), 87.8 (d, $J=$184.2 Hz, C-2), 97.0 (d, $J=$31.2 Hz, C-1), 99.6 (C-1″), 102.5 (C-1′), 113.1, 119.8, 127.5, 136.7, 145.3, 159.8 (Ar), 169.4, 169.8, 169.98, 170.01, 170.04, 170.1 ($COCH_3$). HRMS (ES): *m/z* 857.1869; $[M+Na]^+$ requires 857.1876.

Deprotection of 9–13: The 4-nitrophenyl glycosides **9**–**11** and phenyl thioglycosides **12**–**13**, were subjected to a base-catalysed transesterification. Sodium methoxide in MeOH (0.1 M) was added drop-wise to a stirred solution (or suspension) of the per-*O*-acetate in anhydrous MeOH (0.5 mM) until the solution remained basic. After completion of the reaction (as determined by TLC), the solution was neutralised with cation-exchange resin (Amberlite IR-120, H^+ form), filtered and concentrated. The residue was recrystallised or subjected to chromatography, as described below or in accordance with literature procedures. ^1H and ^{13}C NMR spectra obtained for products **21**–**23** were in agreement with the data previously reported.[37,41,42]

*Phenyl β-D-xylopyranosyl-(1→4)-1-thio-β-D-xyloside (**24**):* This compound was eluted under gravity on a Waters tC18 SepPak cartridge (2 g) with a H_2O/MeOH gradient (1:0–1:4) and was lyophilised to give a colourless foam (21 mg, 88% yield). ^1H NMR (400 MHz, $CDCl_3$): $\delta =$ 3.21–3.45 (m, 5H; H-2,5,2′,3′,5′), 3.53–3.75 (m, 3H; H-3,4,4′), 3.94 (dd, $J=$5.6, 11.6 Hz, 1H; H-5′), 4.07 (dd, $J=$5.2, 11.6 Hz, 1H; H-5), 4.37 (d, $J=$8.0 Hz, 1H; H-1′), 4.70 (d, $J=$10.0 Hz, 1H; H-1), 7.28–7.56 (m, 5H; Ph); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta =$ 65.3 (C-5′), 66.6 (C-5), 69.3 (C-4′), 71.7 (C-2), 72.8 (C-2′), 75.3 (C-3), 75.7 (C-3′), 76.1 (C-4), 88.0 (C-1), 101.8 (C-1′), 128.4, 129.5, 131.6, 132.3 (Ph). HRMS (ES): *m/z* 397.0937; $[M+Na]^+$ requires 397.0933.

*Phenyl β-D-xylopyranosyl-(1→4)-β-D-xylosyl-(1→4)-1-thio-β-D-xyloside (**25**):* This compound was eluted under gravity on a Waters tC18 SepPak® cartridge (2 g) with a H_2O/MeOH gradient (1:0–1:4) and lyophilised to give a colourless foam (32 mg, 91% yield). ^1H NMR (400 MHz, $CDCl_3$): $\delta =$ 3.22–3.47 (m, 7H; H-2,5,2′,5′,2″3″,5″), 3.51–3.83 (m, 5H; H-3,4,3′,4′,4″), 3.97 (dd, $J=$5.6, 11.6 Hz, 1H; H-5″), 4.06–4.17 (m, 2H; H-5,5′), 4.42–4.49 (m, 2H; H-1′,1″), 4.77 (d, $J=$9.9 Hz, 1H; H-1), 7.38–7.60 (m, 5H; Ph); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta =$ 63.1, 65.3 (C-5′,5″), 66.6 (C-5), 69.3 (C-4″), 71.8 (C-2), 72.8, 72.9 (C-2′,2″), 73.8, 75.3, 75.7 (C-3,3′,3″), 76.1 (C-4), 76.5 (C-4′), 88.0 (C-1), 101.7, 101.9 (C-1′,1″), 128.5, 129.5, 131.5, 132.2 (Ph). HRMS (ES): *m/z* 529.1352; $[M+Na]^+$ requires 529.1356.

Deprotection of 14–15 and 17–18: The carbamates **14**–**15** and **17**–**18** were subjected to a base-catalysed transesterification, followed by hydrogenolysis. Sodium methoxide in MeOH (0.1 M) was added drop-wise to a stirred solution of the per-*O*-acetate in anhydrous MeOH (0.5 mM) until the solution remained basic. After completion of the reaction (as determined by TLC) the mixture was neutralised with cation-exchange resin (Amberlite IR-120, H^+ form), filtered and concentrated. A mixture of Pd/C (10%, 15 mg) and the

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residue in anhydrous MeOH (5 mL) was stirred under H_2 (1 atm, 4 h). The mixture was filtered, concentrated, and the residue was purified by ion-exchange chromatography (BioRad AG 50W×2, H^+ form, eluted with H_2O then 1 M NH_3). Fractions containing product were combined and lyophilised. ^1H and ^{13}C NMR spectra obtained for products **26** and **28** were in agreement with the data previously reported.[14]

*(3R,4R,5S)-4,5-Dihydroxy-3-[β-D-xylopyranosyl-(1→4)-β-D-xylosyloxy]-piperidine (**27**):* The product was obtained as a colourless foam (32 mg, 77% yield). ^1H NMR (400 MHz, D_2O): $\delta = 2.40–2.55$ (m, 2H; H-2,6), 3.06–3.69 (m, 12H; H-2,3,5,6,2′,3′,4′,5′,2″,3″,4″,5″), 3.73–3.84 (m, 1H; H-4), 3.93–4.15 (m, 2H; H-5′,5″), 4.42–4.53 (m, 2H; H-1′,1″); ^{13}C NMR (100.6 MHz, D_2O): $\delta = 47.6$, 49.9 (C-2,6), 64.0, 66.3 (C-5′,5″), 70.3, 71.7, 73.8, 73.9, 74.8, 76.7, 76.9, 77.5, 79.0 (C-3,4,5,2′,3′,4′,2″,3″,4″), 102.4, 102.9 (C-1′,1″). HRMS (ES): *m/z* 398.1672; $[M+H]^+$ requires 398.1662.

*(3R,4R)-4-Hydroxy-3-[β-D-xylopyranosyl-(1→4)-β-D-xylosyloxy]-piperidine (**29**):* Product was obtained as a colourless foam (26 mg, 72% yield). ^1H NMR (400 MHz, D_2O): $\delta = 1.39–1.59$ (m, 1H; H-5), 1.94–2.07 (m, 1H; H-5), 2.50–2.70 (m, 2H; H-2,6), 2.92–3.81 (m, 12H; H-2,3,4,6,2′,3′,4′,5′,2″,3″,4″,5″), 3.89–4.11 (m, 2H; H-5′,5″), 4.39–4.56 (m, 2H; H-1′,1″); ^{13}C NMR (100.6 MHz, D_2O): $\delta = 31.7$ (C-5), 43.0, 47.0 (C-2,6), 64.1, 66.3 (C-5′,5″), 70.3, 70.4, 73.87, 73.90, 74.8, 76.7, 76.9, 77.5, 79.4 (C-3,4,5,2′,3′,4′,2″,3″,4″), 102.6, 103.0 (C-1′,1″). HRMS (ES): *m/z* 382.1720; $[M+H]^+$ requires 382.1713.

Deprotection of 19–20*:* The dinitrophenyl glycosides 1**9**–**20** were all subjected to an acid-catalysed transesterification. Acetyl chloride (0.10 mL) was added drop-wise to a stirred suspension of the per-*O*-acetate in anhydrous MeOH (3 mL) at 0 °C. The mixture was warmed to room temperature and left for 24 h. The solution was concentrated and the residue recrystallized or subjected to flash chromatography, as described below.

*3,4-Dinitrophenyl β-D-xylopyranosyl-(1→3)-2-deoxy-2-fluoro-β-D-xylopyranoside (**30**):* This was recrystallised from MeOH/Et_2O to give pale yellow needles (47 mg, 83% yield). ^1H NMR (400 MHz, CD_3OD): $\delta = 3.20–3.39$ (m, 3H; H-2′,4′,5′), 3.48–3.61 (m, 2H; H-5,3′), 3.69–3.78 (m, 1H; H-4), 3.89–4.07 (m, 3H; H-3,5,5′), 4.52 (d, J = 7.6 Hz, 1H; H-1′), 4.61 (ddd, J = 6.8, 8.0, 50.0 Hz, 1H; H-2), 5.51 (dd, J = 4.8, 6.8 Hz, 1H; H-1), 7.46 (dd, J = 2.4, 8.8 Hz, 1H; ArH), 7.46 (d, J = 2.4 Hz, 1H; ArH), 8.15 (d, J = 8.8 Hz, 1H; ArH); ^{13}C NMR (100.6 MHz, CD_3OD): $\delta = 66.6$ (C-5), 67.1 (C-5′), 68.9 (d, J = 7.0 Hz, C-4), 71.2 (C-3′), 75.0 (C-4′), 77.7 (C-2′), 82.3 (d, J = 17.1 Hz, C-3), 92.3 (d, J = 186.2 Hz, C-2), 99.5 (d, J = 19.1 Hz, C-1), 105.0 (C-1′), 114.2, 121.0, 128.8, 137.6, 146.6, 161.9 (Ar). HRMS (ES): *m/z* 473.0824; $[M+Na]^+$ requires 473.0820.

*3,4-Dinitrophenyl β-D-xylopyranosyl-(1→4)-β-D-xylosyl-(1→3)-2-deoxy-2-fluoro-β-D-xylopyranoside (**31**):* Flash chromatography (EtOAc/MeOH; 9:1–8:2) gave a pale yellow foam (20 mg, 76% yield). ^1H NMR (400 MHz, D_2O): $\delta = 3.23–4.22$ (m, 14H; H-3,4,5,5,2′,3′,4′,5′,5′,2″,3″,4″,5″,5″), 4.41–4.74 (m, 3H; H-2,1′,2′), 5.20–5.38 (m, 1H; H-1′), 7.10–7.42 (m, 2H; ArH), 7.79–7.92 (m, 1H; ArH); ^{13}C NMR (100.6 MHz, D_2O): $\delta = 64.1$, 66.1, 66.4, 68.0, 70.3, 73.8, 74.1, 74.8, 76.7, 77.5 (C-4,5,2′,3′,4′,5′,2″,3″,4″,5″), 81.7 (d, J = 1.5 Hz, C-3), 91.9 (d, J = 186.2 Hz, C-2), 98.8 (d, J = 2.3 Hz, C-1), 103.9, 102.9 (C-1′,1″), 114.0, 120.6, 128.8, 136.5, 145.2, 160.9 (Ar). HRMS (ES): *m/z* 605.1230; $[M+Na]^+$ requires 605.1242.

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