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Enzymatic synthesis of a library of β-(1→4) hetero- D-glucose and D-xylose-based oligosaccharides employing cellodextrin phosphorylase

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Abstract

Enzymatic synthesis was attempted of six trisaccharides and 14 tetrasaccharides comprising β-(1→4)-linked D-glucose and D-xylose residues, using cellodextrin phosphorylase (CDP, EC 2.4.1.49) as the enzyme catalyst, with α-D-glucose 1-phosphate (**1**) or α-D-xylose 1-phosphate (**2**) as the donor substrates, and cellobiose (**3**), xylobiose (**4**), βGlc-(1→4)-Xyl (**5**), or βXyl-(1→4)-Glc (**6**) as the acceptor substrates. All enzymatic reactions were performed at pH 7.0 and the products purified by gel-filtration chromatography. We successfully synthesized all six hetero-trisaccharides and 10 of the 14 possible hetero-tetrasaccharides. It was not found possible to synthesize the four tetrasaccharides with a Xyl→Glc sequence at their non-reducing ends employing this method. The stereochemistries of the isolated products were assessed by analysis of their 2D NMR spectra (DQF-COSY, TOCSY, HSQC, HMBC), confirming that all of the glycosidic bonds in the products were β-(1→4) linkages.

Keywords: β-(1→4)-Linked hetero-oligosaccharide; Cellodextrin phosphorylase; Xylanase; Cellulase; Xylan; Cellulose

1. Introduction

The β-(1→4)-linked glycan chains found in cellulose, xylan, chitin, and chitosan differ only in their constituent monosaccharides (namely D-glucose, D-xylose, *N*-acetyl-D-glucosamine, and D-glucosamine, respectively), and are hydrolyzed by the enzymes cellulase, xylanase, chitinase, and chitosanase, respectively. Xylanase, the β-(1→4)-glycan-hydrolyzing enzyme specific for xylan, is employed in various industrial applications, as in the bleaching of pulp in paper manufacture, and as an additive in food and animal forages.[1] However, for the most effective use of this enzyme, a more thorough elucidation of its reaction properties is still required.

The enzymatic properties of xylanases were initially studied using its natural substrate, xylan. Xylan, however, is heterogeneous: it contains variable side-chains of L-arabinose and D-glucuronic acid. In addition, xylan has been shown to inhibit the enzymatic activity of xylanases.[2,3] These findings suggest that xylan is probably not the best substrate for analyzing the enzymatic properties of xylanases, and that novel substrates having well-defined, homogeneous structures, would be of more use in such investigations.

Since Family-10 xylanases have been reported that can hydrolyze both xylooligosaccharides and such related derivatives as aryl cellobiosides,[4] the question arises as to how the β-(1→4)-linked glycanases recognize their specific substrates. We therefore sought to synthesize β-(1→4)-linked hetero-oligosaccharides consisting of D-glucose and D-xylose residues in order to facilitate investigations of the substrate-specificity of xylanases.

Oligosaccharides may be synthesized either chemically or enzymatically. The major advantage that enzymatic syntheses have over chemical syntheses is that the glycosidic bonds generated enzymatically are formed with a high degree of stereoselectivity, without the need for protecting groups. Some enzymes also display very strict regiospecificity in the synthesis of specific oligosaccharides, and phosphorolytic enzymes[5] are good examples of such enzymes. One phosphorolytic enzyme, cellodextrin phosphorylase (CDP, EC 2.4.1.49), cata-

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lyzes the reversible phosphorolysis of glucose residues into α-D-glucose 1-phosphate (G-1-P, **1**) in cello-oligosaccharides containing three or more monosaccharide residues. In the reverse reaction, CDP catalyzes the synthesis of β-(1→4) glycosidic bonds using G-1-P as the donor substrate, and dimeric or longer cello-oligosaccharides, and other β-D-glucosides as the acceptor substrates.[6,7] Kawaguchi and co-workers successfully synthesized cellulase inhibitors with CDP using G-1-P as a glucosyl donor and 4-*O*-β-D-glucopyranosyl-1-deoxynojirimycin as the acceptor.[8] Moreau and co-workers reported syntheses of inhibitors against 1,3;1,4-β-D-glucanases using CDP as the enzymatic catalyst.[9] If CDP is able to recognize β-D-xylosides as acceptors and α-D-xylose 1-phosphate (X-1-P, **2**) as the donor substrate for the transfer of xylose, then we rationalized that this enzyme could also be used to synthesize specific β-(1→4)-linked hetero-oligosaccharides. Using four acceptor substrates [cellobiose (G-G, **3**), βGlc-(1→4)-Xyl (G-X, **4**), βXyl-(1→4)-Glc (X-G, **5**), and xylobiose (X-X, **6**)], and two donor substrates (X-1-P and G-1-P) we demonstrate herein that CDP can be employed in the synthesis of all six possible G-X trisaccharides and 10 of the 14 possible G-X tetrasaccharides.

(Abbreviations used: G, D-glucose; X, D-xylose. All synthesized glycosidic bonds are β-(1→4) bonds).

2. Results and discussion

We attempted to synthesize all six possible hetero-trisaccharides (Table 1, **8**–**13**) as well as the 14 possible hetero-tetrasaccharides (Table 1, **16**–**29**) consisting of β-(1→4)-linked D-glucose and D-xylose residues. The syntheses of these hetero-oligosaccharides are depicted in Fig. 1. CDP was able to utilize all four disaccharides as acceptors and both G-1-P and X-1-P as donors, although its activity with X-1-P as a donor was approximately 1/25 that of G-1-P.

The syntheses of the tetrasaccharides having two identical monosaccharide residues at the non-reducing end (for example, G-G-X-G) were achieved in the same reaction system as the synthesis of the corresponding trisaccharide (in this case, G-X-G). When the two non-reducing end residues were different, however, the trisaccharide was isolated by gel-filtration chromatography and the tetrasaccharide was subsequently synthesized by using the isolated trisaccharide and the other donor substrate.

Employing this method, we were successful in synthesizing all six G-X trisaccharides (Table 1, **8**–**13**) and 10 of the 14 possible G-X tetrasaccharides (Table 1, **17**–**19**, **21**–**23**, **25**–**27**, **29**). A typical gel-filtration chromatogram of the purification is depicted in Fig. 2. Deionization of the reaction mixture prior to the chromatography step was necessary because any remaining G-1-P and X-1-P interfered with the separation process. All of the products gave a single spot on thin layer chromatography (TLC) after purification by gel-filtration (Fig. 3). The chemical shifts observed in the ^{1}H NMR spectra of the synthesized trisaccharides are shown in Table 2, and

Table 1
Target oligosaccharides

Starting disaccharides	Trisaccharides	Tetrasaccharides	
G-G	G-G-G	G-G-G-G	15
3	7	***X-G-G-G***	***16***
	X-G-G	**G-X-G-G**	**17**
	8	**X-X-G-G**	**18**
G-X	**G-G-X**	**G-G-G-X**	**19**
4	**9**	***X-G-G-X***	***20***
	X-G-X	**G-X-G-X**	**21**
	10	**X-X-G-X**	**22**
X-G	**G-X-G**	**G-G-X-G**	**23**
5	**11**	***X-G-X-G***	***24***
	X-X-G	**G-X-X-G**	**25**
	12	**X-X-X-G**	**26**
X-X	**G-X-X**	**G-G-X-X**	**27**
6	**13**	***X-G-X-X***	***28***
	X-X-X	**G-X-X-X**	**29**
	14	X-X-X-X	30

Compounds in bold represent the target saccharides. The four compounds in italics could not be synthesized successfully.

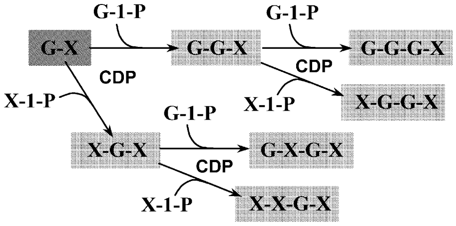


Fig. 1. Synthesis of hetero-oligosaccharides consisting of D-glucose and D-xylose.

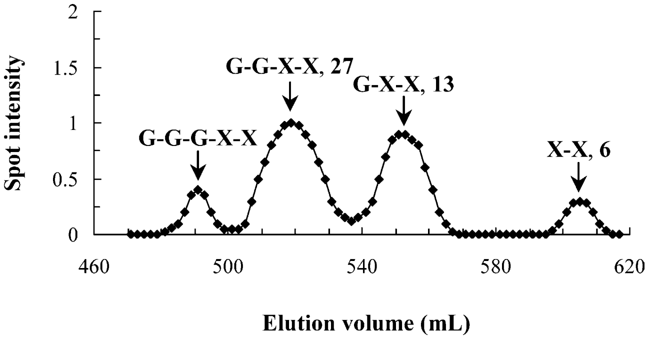
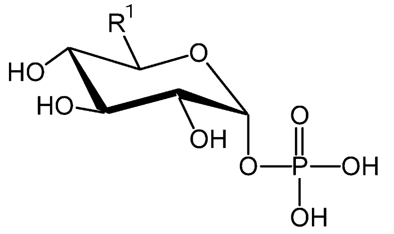
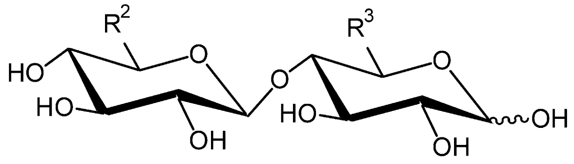


Fig. 2. A typical elution profile of the gel-filtration chromatography performed on the product mixture. This chromatogram shows the purification of the products obtained from the reaction of X-X and G-1-P.

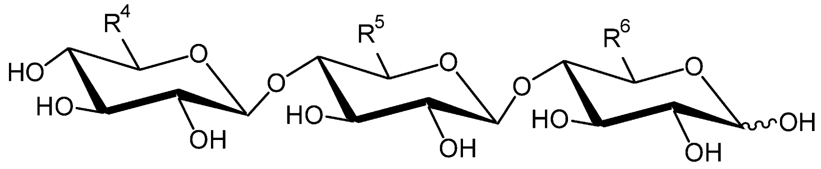
the shifts observed in the ^{13}C NMR spectra, as well as those of the inter-ring cross peaks observed in the HMBC spectra, are listed in Table 3. The inter-ring cross peaks observed in the HMBC spectra of the tetrasaccharides products are listed in Table 4. These NMR data confirmed that all of the glycosidic bonds



1 $R^1 = CH_2OH$
2 $R^1 = H$



3 $R^2 = CH_2OH$, $R^3 = CH_2OH$
4 $R^2 = CH_2OH$, $R^3 = H$
5 $R^2 = H$, $R^3 = CH_2OH$
6 $R^2 = H$, $R^3 = H$



7 $R^4 = CH_2OH$, $R^5 = CH_2OH$, $R^6 = CH_2OH$
8 $R^4 = H$, $R^5 = CH_2OH$, $R^6 = CH_2OH$
9 $R^4 = CH_2OH$, $R^5 = CH_2OH$, $R^6 = H$
10 $R^4 = H$, $R^5 = CH_2OH$, $R^6 = H$
11 $R^4 = CH_2OH$, $R^5 = H$, $R^6 = CH_2OH$
12 $R^4 = H$, $R^5 = H$, $R^6 = CH_2OH$
13 $R^4 = CH_2OH$, $R^5 = H$, $R^6 = H$
14 $R^4 = H$, $R^5 = H$, $R^6 = H$

Table 2
Chemical shifts in ^{1}H NMR spectrum of each trisaccharide

			H-1		$J_{1,2}$(Hz)	H-2		$J_{2,3}$(Hz)	H-3		$J_{3,4}$(Hz)	H-4		$J_{4,5^a}$(Hz)	H-5^a		$J_{4,5^e}$(Hz)	H-5^e		$J_{5,5}$(Hz)	H-6'		$J_{5,6'}$(Hz)	H-6''		$J_{5,6''}$(Hz)	$J_{6,6}$(Hz)
7	I*	α	5.21	d	3.77	3.56	dd	9.85	3.81	dd	10.91	3.63	dd	8.40	3.92-3,95	m					3.87	dd	2.57	3.84	dd	4.55	—
		β	4.65	d	7.98	3.27	dd	9.03	3.61	t	8.69	3.63-3.66	m	—	3.58	ddd					3.94	dd	2.11	3.79	dd	4.81	12.30
	II	α	4.52	d	7.96	3.35	dd	9.04	3.63	dd	10.17	3.67	t	10.07	3.59-3.62	m					3.97	dd	2.01	3.81	dd	5.03	12.31
		β				3.34	dd																				
	III		4.49	d	7.93	3.30	dd	9.38	3.49	t	9.21	3.40	t	9.45	3.45-3.49	m					3.90	dd	2.16	3.72	dd	5.86	12.38
8	I	α	5.21	d	3.79	3.56	dd	9.86	3.86	dd	10.72	3.62	dd	8.57	3.92-3.94	m					3.87	dd	2.50	3.84	dd	4.34	—
		β	4.64	d	7.98	3.27	dd	10.29	3.59-3.62	m	—	3.64	m	—	3.60	m					3.93-3.95	m	—	3.79	dd	5.01	12.26
	II	α	4.511	d	7.95	3.333	dd	8.19	3.57-3.65	m	—	3.57-3.65	m	—	3.57-3.65	m					3.95-3.97	m	—	3.81	dd	5.26	12.98
		β	4.509	d	7.95	3.325	dd	8.89																			
	III		4.40	d	7.85	3.28	dd	9.37	3.43	dd	9.27	3.62	ddd	10.28	3.29	dd	5.34	3.97	dd	11.59							
9	I	α	5.18	d	3.71	3.54	dd	9.18	3.76	t	8.53	3.78-3.82	m	—	3.74	m	—	3.81	m	—							
		β	4.58	d	7.89	3.24	dd	9.40	3.56	t	9.27	3.80-3.84	m	10.76	3.38	dd	5.36	4.05	dd	11.76							
	II	α	4.541	d	7.95	3.30-3.33	m	—	3.61	t	8.83	3.64	t	8.67	3.57-3.60	m					3.97	dd	2.10	3.80-3.82	m	—	12.05
		β	4.545	d	7.97	3.304	dd	9.08																			
	III		4.49	d	7.94	3.298	dd	9.37	3.49	t	9.24	3.40	t	9.45	3.46-3.49	ddd					3.90	dd	2.21	3.72	dd	5.84	12.36
10	I	α	5.17	d	3.70	3.54	dd	9.26	3.76	t	9.34	3.71-3.82	m	—	3.71-3.82	m	—	3.71-3.82	m	—							
		β	4.58	d	7.89	3.24	dd	9.40	3.58	t	9.34	3.82	ddd	10.43	3.37	dd	5.36	4.05	dd	11.72							
	II	α	4.530	d	7.95	3.30	dd	9.18	3.55-3.63	m	—	3.55-3.63	m	—	3.55-3.59	m					3.69-3.98	m	—	3.80	dd	4.65	12.49
		β	4.534	d	7.95	3.29	dd	9.24																			
	III		4.40	d	7.86	3.28	dd	9.39	3.43	t	9.27	3.62	ddd	10.56	3.29	dd	5.49	3.97	dd	11.69							
11	I	α	5.20	d	3.77	3.54	dd	9.85	3.77	t	10.16	3.60	dd	8.11	3.91-3.94	m					3.80-3.86	m	—	3.80-3.86	m	—	—
		β	4.64	d	7.97	3.25	dd	9.19	3.57	t	9.46	3.59-3.63	m	—	3.55-3.58	m					3.94	dd	1.95	3.79	dd	4.91	12.27
	II	α	4.43	d	7.84	3.325	dd	9.46	3.58	t	9.22	3.81-3.86	m	10.27	3.37	dd	5.36	4.11	dd	11.80							
		β				3.318	dd	9.43																			
	III		4.523 4.521	d	7.95/7.93	3.26	dd	9.28	3.47	t	9.13	3.38	dd	10.32	3.43-3.47	m					3.90	dd	2.17	3.71	dd	6.13	12.36
12	I	α	5.20	d	3.78	3.54	dd	9.90	3.77	dd	8.90	3.59	dd	9.80	3.91-3.94	m					3.87	dd	2.45	3.85	dd	4.38	12.24
		β	4.64	d	7.99	3.25	dd	8.90	3.57	t	8.77	3.54-3.61	m	—	3.54-3.61	m					3.92-3.94	m	—	3.79	dd	4.67	11.94
	II	α	4.427	d	7.83	3.318	dd	9.43	3.557	t	9.17	3.76-3.80	m	10.49	3.366	dd	5.33	4.10	dd	11.83							
		β	4.425	d	7.83	3.311	dd	9.43	3.554	t	9.25				3.362	dd											
	III		4.447 4.445	d	7.83	3.24	dd	9.37	3.41	t	9.26	3.61	ddd	10.42	3.29	dd	5.48	3.96	dd	11.69							
13	I	α	5.17	d	3.65	3.53	dd	9.11	3.72-3.75	m	—	3.77-3.83	m	—	3.77-3.83	m	—	3.77-3.83	m	—							
		β	4.57	d	7.90	3.24	dd	9.32	3.53	t	9.19	3.77	ddd	10.69	3.36	dd	5.35	4.04	dd	11.73							
	II	α	4.470	d	7.79	3.289	dd	9.35	3.564	t	9.20	3.83	ddd	10.57	3.37	dd	5.36	4.10	dd	11.83							
		β	4.467	d	7.78	3.282	dd	9.35	3.562	t																	
	III		4.52	d	7.94	3.26	dd	9.38	3.47	t	9.21	3.38	dd	9.72	3.45	ddd					3.90	dd	2.23	3.71	dd	6.13	12.38
14	I	α	5.17	d	3.67	3.53	dd	9.20	3.74	t	9.14	3.79	m	—	3.72-3.81	m	—	3.72-3.81	m	—							
		β	4.57	d	7.89	3.23	dd	9.39	3.53	t	9.23	3.74-3.79	m	10.40	3.36	dd	5.31	4.04	dd	11.69							
	II	α	4.460	d	8.56	3.283	dd	9.88	3.54	t	9.20	3.75-3.80	m	10.40	3.36	dd	5.33	4.09	dd	11.86							
		β	4.464	d	7.78	3.275	dd																				
	III		4.45	d	8.04	3.24	dd	9.36	3.41	t	9.23	3.61	ddd	10.49	3.29	dd	5.48	3.96	dd	11.59							

* I, II, and III denote the first, second, and third glycosyl residue from the reducing-end, respectively.

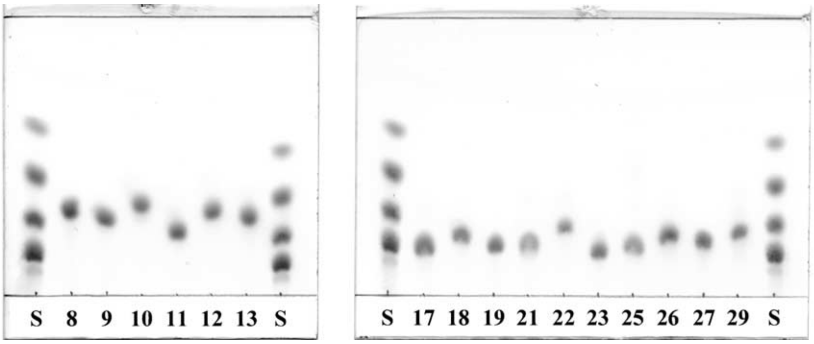
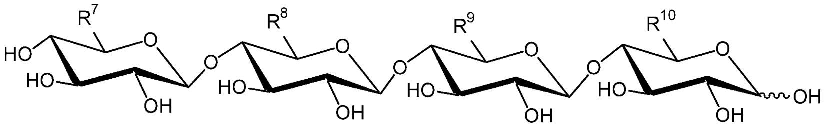


Fig. 3. TLC analysis of the products after purification: Lane numbers represent compound numbers. **8**–**13**, trisaccharides; **17**–**29**, tetrasaccharides; S, standards (glucose, cellobiose, cellotriose and cellotetraose).

formed were β-(1 → 4) linkages.

The syntheses of the four tetrasaccharides with an X-G sequence at their non-reducing end (namely X-G-G-G, **16**; X-G-G-X, **20**; X-G-X-G, **24**; and X-G-X-X, **28**) were not successful. These syntheses were attempted using X-1-P as the donor and the respective trisaccharides with G residues at their non-reducing ends. Unexpectedly, we found that the tetrasaccharides that were produced in these reactions had a G-G sequence at their non-reducing ends, and that the yields of the desired tetrasaccharides (with X-G at their non-reducing ends) were too low to permit isolation by gel-filtration. The formation of the unexpected tetrasaccharides may be attributed to phosphorolysis of the glucosyl residue at the non-reducing end of the trisaccharide to form G-1-P, followed by transglucosylation of the G-1-P onto another molecule of the trisaccharide. This would result in a disproportionation reaction, which would yield the disaccharide and the unexpected tetrasaccharide (Fig. 4). Theoretically, phosphorolysis should not occur in the absence of phosphate ion, however, once the transglycosylation of X-1-P occurs, even to a limited extent, the resultant phosphate ion would act as a catalyst for the disproportionation reaction. Since G-1-P is a much



15 $R^7 = CH_2OH$, $R^8 = CH_2OH$, $R^9 = CH_2OH$, $R^{10} = CH_2OH$
16 $R^7 = H$, $R^8 = CH_2OH$, $R^9 = CH_2OH$, $R^{10} = CH_2OH$
17 $R^7 = CH_2OH$, $R^8 = H$, $R^9 = CH_2OH$, $R^{10} = CH_2OH$
18 $R^7 = H$, $R^8 = H$, $R^9 = CH_2OH$, $R^{10} = CH_2OH$
19 $R^7 = CH_2OH$, $R^8 = CH_2OH$, $R^9 = CH_2OH$, $R^{10} = H$
20 $R^7 = H$, $R^8 = CH_2OH$, $R^9 = CH_2OH$, $R^{10} = H$
21 $R^7 = CH_2OH$, $R^8 = H$, $R^9 = CH_2OH$, $R^{10} = H$
22 $R^7 = H$, $R^8 = H$, $R^9 = CH_2OH$, $R^{10} = H$
23 $R^7 = CH_2OH$, $R^8 = CH_2OH$, $R^9 = H$, $R^{10} = CH_2OH$
24 $R^7 = H$, $R^8 = CH_2OH$, $R^9 = H$, $R^{10} = CH_2OH$
25 $R^7 = CH_2OH$, $R^8 = H$, $R^9 = H$, $R^{10} = CH_2OH$
26 $R^7 = H$, $R^8 = H$, $R^9 = H$, $R^{10} = CH_2OH$
27 $R^7 = CH_2OH$, $R^8 = CH_2OH$, $R^9 = H$, $R^{10} = H$
28 $R^7 = H$, $R^8 = CH_2OH$, $R^9 = H$, $R^{10} = H$
29 $R^7 = CH_2OH$, $R^8 = H$, $R^9 = H$, $R^{10} = H$
30 $R^7 = H$, $R^8 = H$, $R^9 = H$, $R^{10} = H$

Table 3
Chemical shifts in ^{13}C NMR spectrum and inter-ring cross peaks in HMBC spectrum of each trisaccharide

		Chemical shifts in ^{13}C NMR spectrum												Inter-ring cross peaks in HMBC spectrum			
		C-1		C-2		C-3		C-4		C-5		C-6		C-1 → H-4		H-1 → C-4	
		α	β	α	β	α	β	α	β	α	β	α	β	δ (ppm)		δ (ppm)	
7	I *	93.52	97.46	72.92	75.59	73.00	75.94	80.14	80.17	71.82	76.51	61.70	61.58				
	II	104.04		74.64		75.75		80.10		76.51		61.58		3.63–3.66	$(4^{I}\beta)$	80.17	$(4^{I}\beta)$
	III	104.26		74.84		77.17		71.15		77.68		62.26		3.67	(4^{II})	80.10	(4^{II})
8	I	92.18	96.12	71.58	74.25	71.67	74.61	78.99	78.84	70.48	75.16	60.23	60.23				
	II	102.70		73.48		74.23		78.40		75.23		60.36		3.64	$(4^{I}\beta)$	78.84	$(4^{I}\beta)$
	III	103.64		73.44		75.97		69.54		65.53						78.38	(4^{II})
9	I	92.38	96.86	71.75	74.35	71.41	74.35	76.94	76.77	59.10	63.34						
	II	101.27		73.03		74.50		78.96		75.17		60.37		3.80–3.84	$(4^{I}\beta)$	76.77	$(4^{I}\beta)$
	III	102.96		73.52		75.86		69.82		76.35		60.93		3.64	(4^{II})	78.96	(4^{II})
10	I	92.38	96.86	71.41	74.32	71.75	74.35	76.94	76.77	59.13	63.34						
	II	101.28		73.16		75.22		78.60		74.35		60.33		3.82	$(4^{I}\beta)$	76.77	$(4^{I}\beta)$
	III	103.68		73.49		75.99		69.55		65.54						78.60	(4^{II})
11	I	92.22	96.13	71.74	74.47	71.50	74.40	78.68	78.53	70.58	75.22	60.23	60.36				
	II	103.45		73.45		74.17		76.78		63.33				3.59–3.63	$(4^{I}\beta)$	78.53	$(4^{I}\beta)$
	III	101.50		73.24		75.92		69.95		76.34		61.08				76.78	(4^{II})
12	I	92.22	96.13	71.74	74.41	71.50	74.48	78.67	78.53	70.58	75.22	60.23	60.36				
	II	103.46		73.44		74.06		76.74		63.33						78.53	$(4^{I}\beta)$
	III	102.20		73.13		75.96		69.55		65.58				3.76–3.80	(4^{II})	76.74	(4^{II})
13	I	92.39	96.87	71.74	74.36	71.31	74.27	76.94	76.79	59.19	63.35						
	II	102.03		73.07		74.14		76.77		63.35				3.77	$(4^{I}\beta)$	76.79	$(4^{I}\beta)$
	III	101.51		73.25		75.93		69.95		76.34		61.08		3.83	(4^{II})	76.77	(4^{II})
14	I	93.71	98.19	73.06	75.68	72.63	75.35	78.24	78.07	60.51	64.66						
	II	103.35		74.38		75.33		78.07		64.66				3.74–3.79	$(4^{I}\beta)$	78.07	$(4^{I}\beta)$
	III	103.53		74.45		77.29		70.87		66.90				3.75–3.80	(4^{II})	78.07	(4^{II})

* I, II, and III denote the first, second, and third glycosyl residue from the reducing-end, respectively.

Table 4
HMBC inter-ring cross peaks for each tetra-saccharide

		C-1 → H-4			H-1 → C-4		
		δ (ppm)			δ (ppm)		
17	II [a]	102.9	3.63	(4β)	4.51	79.0	(4β)
	III	103.5	3.63	(4^{II})	4.43	78.3	(4^{II})
	IV	101.5	3.84	(4^{III})	4.52	76.9	(4^{III})
18	II	(102.2	3.77) [b]		4.51	79.0	(4β)
	III	(102.7	3.62)		4.42	78.4	(4^{II})
	IV	(103.5	3.65)		4.45	76.9	(4^{III})
19	II	101.3	3.82	(4β)	4.54	76.8	(4β)
	III	102.7			4.51	78.8	(4^{II})
	IV	103.0	3.66	(4^{III})	4.49	78.7	(4^{III})
21	II	101.3	3.83	(4β)	4.53	76.8	(4β)
	III	103.5	3.60	(4^{II})	4.42	78.6	(4^{II})
	IV	101.6	3.83	(4^{III})	4.52	76.8	(4^{III})
22	II	(101.3	3.81)		4.53	76.9	(4β)
	III	(102.2	3.78)		(4.44	76.9)	
	IV				(4.42	78.6)	
23	II	103.5	3.59	(4β)	4.43		
	III	101.3	3.85	(4^{II})	4.55	76.7	(4^{II})
	IV	103.0			4.49	78.9	(4^{III})
25	II	103.6	3.60	(4β)	4.43	78.6	(4β)
	III	(102.0	3.79)		4.47	76.7	(4^{II})
	IV	(101.4	3.83)		4.52	76.7	(4^{III})
26	II	(102.0	3.79)		4.43	78.5	(4β)
	III	(102.2	3.77)		4.47	76.7	(4^{II})
	IV	(103.5	3.59)		4.45	76.7	(4^{III})
27	II	102.0			4.47	76.8	(4β)
	III	101.3	3.82	(4^{II})	4.55	76.7	(4^{II})
	IV	103.0	3.62	(4^{III})	4.49	78.9	(4^{III})
29	II	102.0	3.77	(4β)	4.46	76.7	(4β)
	III	102.0	3.78	(4^{II})	4.47	76.7	(4^{II})
	IV	101.5	3.83	(4^{III})	4.52	76.8	(4^{III})

[a] II, III and IV denote the second, third and fourth glycosyl residue from the reducing-end, respectively.
[b] Ring number is not identified.

better substrate for CDP than X-1-P, the disproportionation reaction would be favored over the X-1-P reaction, resulting in low yields of the intended tetrasaccharides.

Attempts were made to overcome these problems, for example by the addition of calcium ions to the reaction mixture to trap the phosphate ion produced prior to phosphorolysis occurring, and by changing the ratio of acceptor to donor substrate from 1:1 to 1:10. However, these attempts all proved unsuccessful and the major product continued to be the undesired tetrasaccharide. Our inability to synthesize these four target tetrasaccharides demonstrates the difficulty in removing trace amounts of phosphate from the reaction mixture. In order to ensure the complete absence of phosphate ion

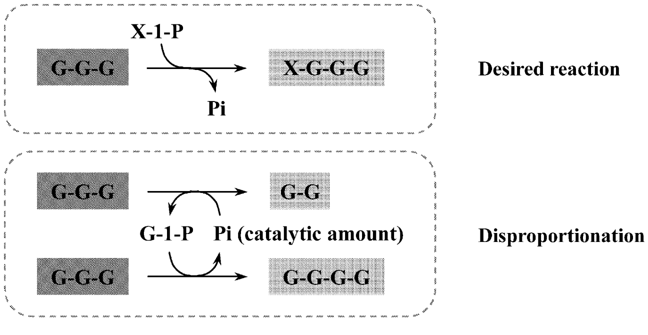


Fig. 4. The transglycosylation reaction of xylose onto cellotriose and its competing disproportionation reaction.

from the reaction system, we attempted to employ α-D-xylosyl fluoride (Xyl-α-F) as a donor substrate. Unfortunately, although α-D-glucosyl fluoride (Glc-α-F) was able to serve as a donor substrate for CDP, this enzyme was not able to use Xyl-α-F as a donor.

In conclusion, we succeeded in synthesizing all six X-G trisaccharides and 10 of the 14 possible X-G tetrasaccharides using CDP as the enzyme catalyst. These hetero-oligosaccharides will be useful in the analysis of substrate specificities of the various β-(1 → 4) glycanases, and in particular those of the xylanases.

3. Experimental

3.1. Materials

α-D-Glucose 1-phosphate disodium salt (G-1-P 2Na), purchased from Wako Pure Chemical Industries (Osaka, Japan), and cellobiose, purchased from Sigma Chemical (St. Louis, USA), were used without further purification. α-D-Xylose 1-phosphate di(monocyclohexylammonium) salt (X-1-P $2C_6H_{13}N$) was purchased from Sigma and prior to use the cyclohexylammonium ions were removed using Amberlite IRC-50 cation exchange resin (Organo, Tokyo, Japan), followed by lyophilization to give the disodium salt. Xylobiose was purchased from Wako (xylooligosaccharides) and was used after purification by gel-filtration chromatography. βGlc-(1 → 4)-Xyl (G-X, **4**)[10] and βXyl-(1 → 4)-Glc (X-G, **5**)[11] were synthesized as reported previously. Cellodextrin phosphorylase from *Clostridium thermocellum* YM4 was prepared from the *Escherichia coli* strain containing a plasmid encoding the cdp gene.[12] The enzyme was purified to give a single protein band by SDS-PAGE, and the specific activity of the purified enzyme was 5.5 IU/mg-protein.

3.2. General methods

Purification of the reaction products was achieved as follows: after the completion of each reaction, the mixture was diluted threefold and desalted by passing it through a mixed-bed ion-exchange resins, [Amberlite MB-3 (Organo)], and the eluate was concentrated to approximately 1/3 of the initial volume. The concentrated sample was applied to a Toyopearl HW-40S gel-filtration column equilibrated with H_2O (25 × 900 mm × 2; Tosoh, Tokyo, Japan), and fractionated with H_2O at a rate of 1 mL/min. Separation of the products was confirmed by TLC analysis on silica gel 60 F_{254} (Merck KGaA, Darmstadt, Germany) and each TLC plate was developed twice with 4:1 MeCN–water. To detect the presence of carbohydrates, the TLC plate was dipped into a solution of 5% H_2SO_4 in MeOH and heated at 120 °C. Optical rotations were measured at 25 °C on a P-1020 polarimeter (JASCO, Tokyo, Japan).

3.3. Structural analyses

NMR data were obtained on a Bruker DRX 600 spectrometer operating at 600.13 MHz for ^1H nuclei and at 150.92 MHz for ^{13}C nuclei, and on a Bruker Avance 800 spectrometer operating at 800.30 MHz for ^1H nuclei and at 201.25 MHz for ^{13}C nuclei. Spectra were run at 298 K in 99.9% D_2O (Wako). As an internal reference standard for the ^1H NMR chemical shifts, *t*-BuOH (1.23 ppm) was added to each sample. The structures of the isolated transglycosylation products were confirmed by analysis of the two-dimensional NMR spectra (DQF-COSY, TOCSY, HSQC, HMBC) employing the following sequence: each signal in the ^1H NMR spectrum was initially assigned from an analysis of the DQF-COSY and TOCSY spectra to determine the chemical shifts and the coupling constants for each proton. The stereochemistries of the individual linkages within each oligosaccharide were determined from the coupling constants of the corresponding anomeric protons. Signals in the ^{13}C NMR spectra were then assigned from an analysis of the corresponding HSQC spectrum. Finally, the linkage positions were determined by identifying the inter-ring cross peaks from the HMBC spectrum, which gives cross peaks with covalently bound H and C.

3.4. Enzyme assays

The enzymatic activity of CDP was measured by quantifying the amount of phosphate ion produced from 10 mM G-1-P and 10 mM cellobiose.[13] The reaction mixture (500 μL) was diluted with 2 mL of 0.2 M acetate buffer (pH 4.0), then 0.25 mL 1% ascorbic acid and 0.25 mL 1% ammonium molybdate were added. After incubation for 30 min at 37 °C, the absorbance at 700 nm was measured to quantify the concentration of phosphate ion. One unit of enzyme is defined as the amount producing 1 μmol of phosphate ion per minute at pH 7.0 and 37 °C.

3.5. β-D-Xylopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-D-glucopyranose (8) and β-D-xylopyranosyl-(1 → 4)-β-D-xylopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-D-glucopyranose (18)

Compounds **2** (32.1 mg, or 0.075 mmol), **3** (12.8 mg, or 0.0375 mmol) and CDP (15 U) were dissolved in 1.5 mL 50 mM MOPS buffer (pH 7.0) and incubated for 96 h at 50 °C (final concentrations: **2**, 50; **3**, 25 mM; and CDP, 10 U/mL). The yield of βXyl-(1 → 4)-βGlc(1 → 4)-Glc (**8**) was 3.5 mg (19.7%), $[\alpha]_D$ +14° (*c* 0.50, H_2O), and the yield of βXyl-(1→4)-βXyl-(1→4)-βGlc-(1→4)-Glc (**18**) was 3.0 mg (13.2%), [α]$_D$ −12° (*c* 0.28, H_2O).

3.6. β-D-Glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-D-xylopyranose (9) and β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-D-xylopyranose (19)

Compounds **1** (152.0 mg, or 0.5 mmol), **4** (154.1 mg, or 0.5 mmol) and CDP (50 U) were dissolved in 10 mL of 50 mM MOPS buffer (pH 7.0) and incubated for 48 h at 37 °C (final concentrations: **1** and **4**, 50 mM; CDP, 5 U/mL). The yield of βGlc-(1→4)-βGlc-(1→4)-Xyl (**9**) was 20.1 mg (8.5%), [α]$_D$ −7° (*c* 0.49, H_2O), and the yield of βGlc-(1→4)-βGlc-(1→4)-βGlc-(1→4)-Xyl (**19**) was 7.9 mg (5.0%), [α]$_D$ −5° (*c* 0.49, H_2O).

3.7. β-D-Xylopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-D-xylopyranose (10) and β-D-xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-D-xylopyranose (22)

Compounds **2** (32.1 mg, or 0.075 mmol), **4** (11.7 mg, or 0.0375 mmol) and CDP (15 U) were dissolved in 1.5 mL of 50 mM MOPS buffer (pH 7.0) and incubated for 48 h at 50 °C (final concentrations: **2** and **4**, 50 mM; CDP, 5 U/mL). The yield of βXyl-(1→4)-βGlc-(1→4)-Xyl (**10**) was 6.3 mg (37.8%), [α]$_D$ −13° (*c* 0.51, H_2O), and the yield of βXyl-(1→4)-βXyl-(1→4)-βGlc(1→4)-Xyl (**22**) was 4.5 mg (20.8%), [α]$_D$ −30° (*c* 0.43, H_2O).

3.8. β-D-Glucopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-D-glucopyranose (11) and β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-D-glucopyranose (23)

Compounds **1** (76.0 mg, or 0.25 mmol), **5** (78.1 mg, or 0.25 mmol) and CDP (12.5 U) were dissolved in 5 mL of 50 mM MOPS buffer (pH 7.0) and incubated for 72 h at 50 °C (final concentrations: **1** and **5**, 50 mM; CDP, 2.5 U/mL). The yield of βGlc-(1→4)-βXyl-(1→4)-Glc (**11**) was 26.1 mg (22.0%), [α]$_D$ −5° (*c* 0.50, H_2O), and the yield of βGlc-(1→4)-βGlc-(1→4)-βXyl-(1→4)-Glc (**23**) was 9.5 mg (11.9%), [α]$_D$ −4° (*c* 0.50, H_2O).

3.9. β-D-Xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-D-glucopyranose (12) and β-D-xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-D-glucopyranose (26)

Compounds **2** (32.1 mg, or 0.075 mmol), **5** (11.7 mg, or 0.0375 mmol) and CDP (15 U) were dissolved in 1.5 mL of 50 mM MOPS buffer (pH 7.0) and incubated for 168 h at 50 °C (final concentrations: **2**, 50 mM; **5**, 25 mM; CDP, 10 U/mL). The yield of βXyl-(1→4)-βXyl-(1→4)-Glc (**12**) was 7.3 mg (43.8%), [α]$_D$ −16° (*c* 0.50, H_2O), and the yield of βXyl-(1→4)-βXyl-(1→4)-βXyl-(1→4)-Glc (**26**) was 1.9 mg (8.8%), [α]$_D$ −23° (*c* 0.28, H_2O).

3.10. β-D-Glucopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-D-xylopyranose (13) and β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-D-xylopyranose (27)

Compounds **1** (152.0 mg, or 0.5 mmol), **6** (141.1 mg, or 0.5 mmol) and CDP (50 U) were dissolved in 10 mL of 50 mM MOPS buffer (pH 7.0) and incubated for 24 h at 37 °C (final concentrations: **1** and **6**, 50 mM; CDP, 5 U/mL). The yield of βGlc-(1→4)-βXyl-(1→4)-Xyl (**13**) was 28.4 mg (12.8%), [α]$_D$ −34° (*c* 0.51, H_2O), and the yield of βGlc-(1→4)-βGlc-(1→4)-βXyl-(1→4)-Xyl (**27**) was 7.3 mg (4.8%), [α]$_D$ −32° (*c* 0.50, H_2O).

3.11. β-D-Glucopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-D-glucopyranose (17)

Compounds **1** (7.6 mg, or 0.025 mmol), **8** (11.9 mg, or 0.025 mmol) and CDP (2.5 U) were dissolved in 0.5 mL of 50 mM MOPS buffer (pH 7.0) and incubated for 96 h at 50 °C (final concentrations: **1** and **8**, 50 mM; CDP, 5 U/mL). The yield of βGlc-(1→4)-βXyl-(1→4)-βGlc-(1→4)-Glc (**17**) was 2.6 mg (16.3%), [α]$_D$ −11° (*c* 0.30, H_2O).

3.12. β-D-Glucopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-D-xylopyranose (21)

Compounds **1** (7.6 mg, or 0.025 mmol), **10** (11.1 mg, or 0.025 mmol) and CDP (2.5 U) were dissolved in 0.5 mL of 50 mM MOPS buffer (pH 7.0) and incubated for 96 h at 50 °C (final concentrations: **1** and **10**, 50 mM; CDP, 5 U/mL). The yield of βGlc-(1→4)-βXyl-(1→4)-βGlc-(1→4)-Xyl (**21**) was 1.1 mg (7.3%), [α]$_D$ −27° (*c* 0.092, H_2O).

3.13. β-D-Glucopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-D-glucopyranose (25)

Compounds **1** (7.6 mg, or 0.025 mmol), **12** (11.1 mg, or 0.025 mmol) and CDP (2.5 U) were dissolved in 0.5 mL 50 mM MOPS buffer (pH 7.0) and incubated for 96 h at 50 °C (final concentrations: **1** and **12**, 50 mM; CDP, 5 U/mL). The yield of βGlc-(1→4)-βXyl-(1→4)-βXyl-(1→4)-Glc (**25**) was 2.0 mg (13.2%), [α]$_D$ −30° (*c* 0.17, H_2O).

3.14. β-D-Glucopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-D-xylopyranose (29)

Compounds **1** (152.0 mg, or 0.5 mmol), **14** (207.2 mg, or 0.5 mmol) and CDP (50 U) were dissolved in 10 mL of 50 mM MOPS buffer (pH 7.0) and incubated for 24 h at

37 °C (final concentrations: **1** and **14**, 50 mM; CDP, 5 U/mL). The yield of βGlc-(1→4)-βXyl-(1→4)-βXyl-(1→4)-Xyl (**29**) was 58.2 mg (20.2%), $[\alpha]_D$ −56° (*c* 0.50, H_2O).

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