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Note

Reaction on D-glucal by an inverting phosphorylase to synthesize derivatives of 2-deoxy-β-D-*arabino*-hexopyranosyl-(1→4)-D-glucose (2^{II}-deoxycellobiose)

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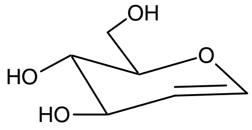
Abstract—Four derivatives of 2^{II}-deoxycellobiose were synthesized from D-glucal and acceptor sugars (D-glucose, D-xylose, D-mannose, and 2-deoxy-D-*arabino*-hexose) using a cellobiose phosphorylase from *Cellvibrio gilvus*. The enzyme was found to be an effective catalyst to synthesize the β-(1→4) linkage of 2-deoxy-D-*arabino*-hexopyranoside. The acceptor specificity for the D-glucal reaction was identical to that for the α-D-glucose 1-phosphate reaction, but the activity of D-glucal was approximately 500 times less than that of α-D-glucose 1-phosphate, using 10 mM substrates.

Keywords: Cellobiose phosphorylase; D-Glucal; Glycal; 2-Deoxyglycoside

Glycosides of 2-deoxy derivatives of carbohydrates are useful for the characterization of various carbohydrate-related enzymes. For example, α-glucosidases belonging to glycoside hydrolase family (GHF) 31 hydrolyze α-glycosides of 2-deoxy-D-*arabino*-hexopyranose (2-deoxy-D-glycopyranose), whereas those of GHF13 do not, suggesting that the 2-OH group participates in the reaction by GHF13 enzymes.[1] In such a manner, β-glycosides of 2-deoxy-D-*arabino*-hexopyranose are useful compounds for investigating β-glucanases. However, glycosylation of a 2-deoxy glycoside with strict specificity in the anomeric configuration is difficult, due to the lack of neighboring groups that enhance selectivity. Because enzymatic syntheses are strictly specific in the anomeric configuration, enzymatic procedures to produce glycosides of 2-deoxy sugars are desired.

Hydrations of glycals by hydrolases have been reported to form the corresponding 2-deoxy sugars.[2–7] In these cases, water molecules are added to glycals to form free 2-deoxy sugars. Synthesis of 2-deoxy glycosides by a hydrolytic enzyme has also been reported.[8] For example, a β-glycosidase from *Sulfolobus solfataricus* was found to catalyze the addition of alcohols to D-glucal (**1**) to form 2-deoxy-β-D-*arabino*-hexopyranosides through a tranglycosylation-like reaction. The addition of alcohols to sugars has also been reported. These reactions have shown excellent stereoselectivity but not strict regio-specificity, with poor yields due to competition with their hydrolysis.



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Phosphorolytic enzymes of carbohydrates show excellent stereo- and regio-specificities in these reactions.[9]

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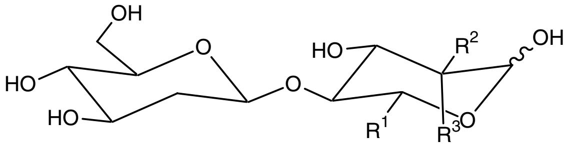
Cellobiose phosphorylase (CBP) is one of the phosphorolytic enzymes that catalyze the reversible phosphorolysis of cellobiose to form glucose and α-D-glucose 1-phosphate, with an inversion of the anomeric configuration.[10] In the reverse reaction, CBP catalyzes the production of heterodisaccharides from various derivatives of glucose.[11–19] CBP was expected to react with D-glucal as a hydrolytic enzyme, because the reaction mechanism of CBP is considered to share the identical reaction mechanism with inverting hydrolytic enzymes, as evidenced by 3D structural analysis of chitobiose phosphorylase that possesses high sequence similarity with CBP.[20] If CBP were to utilize D-glucal as a glycosyl donor, it would be an excellent catalyst for the synthesis of the β-(1→4) linkage of 2-deoxy-D-*arabino*-hexopyranose.

Only glycogen phosphorylase,[21] one of the retaining phosphorolytic enzymes, has been reported to react with D-glucal, producing α-(1→4)-linked 2-deoxy-D-*arabino*-hexopyranosyl oligosaccharides. However, no D-glucal reaction with an inverting phosphorolytic enzyme has been described. In addition, *Cellulomonas uda* CBP was found not to react with D-glucal.[19] We report herein that *Cellvibrio gilvus* CBP selectively utilizes D-glucal as a donor substrate in the production of a β-(1→4) linkage of 2-deoxy-D-*arabino*-hexopyranose.

CBP was used to synthesize several derivatives of 2^{II}-deoxycellobiose (2-deoxy-β-D-*arabino*-hexopyranosyl-(1→4)-D-glucose) using D-glucal as the donor substrate and derivatives of D-glucose (D-glucose, D-xylose, D-mannose, and 2-deoxy-D-*arabino*-hexose) as acceptor substrates. Using this procedure, we successfully synthesized 2-deoxy-β-D-*arabino*-hexopyranosyl-(1→4)-D-glucose in reasonable yields. The disaccharides were purified by gel-filtration chromatography and were subjected to NMR analyses. Assignments of the signals are shown in Tables 1 and 2. All the compounds were determined to be the corresponding 2^{II}-deoxycellobiose derivatives. The 2-deoxy-β-D-*arabino*-hexopyranosyl-(1→4) linkages were identified by HMBC cross peaks, in that inter-ring cross peaks appeared between each C-4^{I} and H-1^{II} and between each H-4^{I} and C-1^{II}. We successfully synthesized 2-deoxy-β-D-*arabino*-hexopyranosyl-(1→4)-D-glucose (**2**), 2-deoxy-β-D-*arabino*-hexopyranosyl-(1→4)-D-xylose (**3**), 2-deoxy-β-D-*arabino*-hexopyranosyl-(1→4)-D-Mannose (**4**), and 2-deoxy-β-D-*arabino*-hexopyranosyl-(1→4)-2-deoxy-D-*arabino*-hexose (**5**). The acceptor specificity of the D-glucal reaction was identical to that of the α-D-glucose 1-phosphate reaction, where D-glucose was the best acceptor.

The activity on D-glucal was approximately 500 times less than that on α-D-glucose 1-phosphate at 10 mM of each donor with 10 mM D-glucose by comparing the initial rate of the production of the corresponding disaccharide using high-performance ion-exchange chromatography on a CARBOPAC PA1 column (4 × 250 mm, Dionex, Sunnyvale, CA, USA), equipped with a pulsed amperometric detector (DX-3, Dionex).[22] Because the amino acid sequences of *C. gilvus* and *C. uda* CBP are 89% identical,[23] *C. uda* CBP may possess the same level of activity with *C. gilvus* CBP, which may be negligible in some cases.



2	$R^1 = CH_2OH$,	$R^2 = OH$,	$R^3 = H$
3	$R^1 = H$,	$R^2 = OH$,	$R^3 = H$
4	$R^1 = CH_2OH$,	$R^2 = H$,	$R^3 = OH$
5	$R^1 = CH_2OH$,	$R^2 = H$,	$R^3 = H$

1. Experimental

1.1. Materials

D-Glucal was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). All other chemicals used in this study were of reagent grade and were commercially available. Recombinant *C. gilvus* CBP was prepared as described.[24] *Escherichia coli* BL21 (DE3) harboring pET28a-cbp was cultivated in a 5-L flask containing 1 L of LB medium with 30 μg/mL of kanamycin at 30 °C with rotary shaking (150 rpm). When the optical density at 600 nm reached 0.6, isopropyl 1-thio-β-D-galactoside was added to a final concentration of 0.1 mM, followed by cultivation at 22 °C for 22 h. The cells were harvested by centrifugation, washed with 50 mM phosphate buffer (pH 7.5), and resuspended in 15 mL of the same buffer. CBP was extracted by sonication using a sonifier (250D, Branson, Danbury, CT, USA). CBP having an additional His_6 sequence at its N-terminus was purified by Ni–NTA column chromatography. One unit of the enzyme was defined as the amount that synthesized 1 μmol/min glucose from 10 mM cellobiose and 10 mM phosphate in 50 mM MOPS buffer (pH 7.0) at 37 °C, as measured by a glucose oxidase method.[25]

1.2. 2-Deoxy-β-D-*arabino*-hexopyranosyl-(1→4)-D-glucose (2)

To 10 mL of 25 mM MOPS buffer (pH 7.0) containing 0.02% BSA, 2.0 mmol D-glucal, and 2.0 mmol D-glucose was added 50 U CBP, and the reaction was performed at 37 °C for 72 h. The reaction mixture was subsequently treated by standard methods to isolate the disaccharide formed: 359 mg (1.1 mmol, 55% yield) of **2**.

Table 1. Assignment of the ^{1}H NMR signals of the products

			Chemical shifts (ppm)									Coupling constants (Hz)											
			H-1	H-2a	H-2e	H-3	H-4	H-5a	H-5e	H-6x	H-6y	$J_{1,2a}$	$J_{1,2e}$	$J_{2,2}$	$J_{2a,3}$	$J_{2e,3}$	$J_{3,4}$	$J_{4,5a}$	$J_{4,5e}$	$J_{5,5}$	$J_{5a,6x}$	$J_{5a,6y}$	$J_{6,6}$
2	α	I	5.26	3.61		3.85	3.70	3.94		3.80	3.86	3.8			9.8		9.1	10.0			5.6	2.3	12.2
		II	4.83	1.62	2.38	3.78	3.35	3.46		3.80	3.97	9.8	2.0	12.2	12.2	5.0	8.9	10.1			5.9	2.0	12.3
	β	I	4.63	3.32		3.64	3.71	3.58		3.77	3.91	8.0			9.8		9.0	9.8			5.0	2.2	12.3
		II	4.82	1.61	2.37	3.78	3.35	3.46		3.80	3.97	9.8	2.1	12.2	12.1	5.0	8.9	10.1			5.9	2.0	12.3
3	α	I	5.17	3.53		3.72	3.82	3.70	3.80			3.7			9.3		9.4	10.4	5.6	11.6			
		II	4.81	1.53	2.24	3.70	3.27	3.38		3.72	3.91	9.1	2.1	12.2	11.8	5.0	9.1	9.6			6.3	2.3	12.3
	β	I	4.56	3.23		3.51	3.84	3.33	4.04			7.9			9.3		9.2	10.6	5.4	11.7			
		II	4.81	1.52	2.23	3.70	3.27	3.38		3.72	3.91	9.8	1.9	12.2	11.6	5.1	9.1	9.7			6.3	2.3	12.3
4	α	I	5.16		3.96	3.91	3.81	3.86		3.75	3.86		1.8			3.3	9.6	10.1			2.5	4.8	10.4
		II	4.76	1.55	2.32	3.72	3.28	3.42		3.73	3.92	9.9	2.0	12.3	12.3	5.0	9.3	9.6			6.4	2.2	12.4
	β	I	4.89		3.97	3.75	3.79	3.44		3.72	3.85		1.1			4.7	10.1	9.8			2.0	5.5	12.3
		II	4.75	1.54	2.32	3.72	3.28	3.42		3.73	3.92	9.9	2.0	12.3	12.3	5.0	9.3	9.6			6.4	2.2	12.4
5	α	I	5.37	1.68	2.15	4.01	3.58			3.78–3.85[a]		3.3	1.1	13.5	11.7	5.2	4.6	9.6			[b]	[b]	[b]
		II	4.76	1.56	2.33	3.72	3.29	3.41		3.71	3.90	9.5	1.7	12.1	12.5	5.5	9.1	9.6			5.5	2.1	12.2
	β	I	4.93	1.49	2.28	3.80	3.51	3.44		3.84	3.72	9.8	2.0	12.3	12.0	5.2	8.9	9.5			4.9	2.2	12.2
		II	4.76	1.55	2.32	3.72	3.29	3.41		3.71	3.90	9.8	1.8	12.1	12.4	5.5	9.1	9.6			5.5	1.6	12.1

α and β indicate the anomeric configuration of the compounds. I and II denote the first and second glycosyl residues from the reducing end, respectively.

[a] Not separated (H-5a, H-6x, and H-6y).

[b] Not determined due to their close chemical shifts.

Table 2. Assignment of the ^{13}C NMR signals of the products

			C-1	C-2	C-3	C-4	C-5	C-6
2	α	I	93.5	73.0	73.0	80.5	71.7	61.8
		II	101.8	39.9	72.0	72.4	77.9	62.5
	β	I	97.5	75.6	76.0	80.3	76.4	61.9
		II	101.9	39.9	72.0	72.4	77.9	62.5
3	α	I	92.4	71.8	70.7	76.6	59.3	
		II	98.7	38.7	70.7	71.4	76.5	61.5
	β	I	98.6	74.4	74.4	76.4	63.4	
		II	98.7	38.7	70.7	71.4	76.5	61.5
4	α	I	94.0	70.6	69.4	77.2	71.3	60.8
		II	100.6	38.6	70.7	71.0	76.6	61.2
	β	I	94.1	70.6	72.1	77.2	76.8	60.8
		II	100.7	38.6	70.7	71.0	76.6	61.2
5	α	I	91.4	36.9	67.1	81.0	70.8	60.6
		II	100.6	38.6	70.7	71.1	76.5	61.1
	β	I	93.7	38.6	69.6	80.7	75.0	60.8
		II	100.6	38.6	70.7	71.1	76.5	61.1

Values are given in ppm. α and β indicate the anomeric configuration of the compounds. I and II denote the first and second glycosyl residues from the reducing end, respectively.

1.3. 2-Deoxy-β-D-*arabino*-hexopyranosyl-(1→4)-D-xylose (3), 2-deoxy-β-D-*arabino*-hexopyranosyl-(1→4)-D-mannose (4), and 2-deoxy-β-D-*arabino*-hexopyranosyl-(1→4)-2-deoxy-D-*arabino*-hexose (5)

To 10 mL of 25 mM MOPS buffer (pH 7.0) containing 0.02% BSA, 2.0 mmol D-glucal, and 2.0 mmol D-xylose, 2.0 mmol D-mannose, or and 2.0 mmol 2-deoxy-D-*arabino*-hexose, respectively, was added 50 U CBP, and the reaction was performed at 37 °C for 170 h. Each reaction mixture was subsequently treated by standard methods to isolate the disaccharide. Products were obtained as follows: 289 mg (0.98 mmol, 49% yield) of **3**, 222 mg (0.68 mmol, 34% yield) of **4**, and 367 mg (1.2 mmol, 59% yield) of **5**.

1.4. General methods

Reactions were monitored by thin-layer chromatography (TLC) on Silica Gel 60 F_{254} (E. Merck, Darmstadt, Germany). Each TLC plate was developed twice with 4:1 acetonitrile–water. To detect the presence of carbohydrates, the TLC plate was dipped into a solution of 5% sulfuric acid in MeOH and heated at 120 °C. After the completion of each reaction, the reaction mixture was desalted by passing it through a mixed-bed ion-exchange resin (Amberlite MB-3; Organo, Tokyo, Japan), and the eluant was concentrated to approximately 1/5 of the initial volume. The concentrated sample was applied to a Toyopearl HW-40S gel-filtration column equilibrated with H_2O (25×900 mm; Tosoh, Tokyo, Japan) and fractionated with H_2O at a rate of 1 mL/min. Separation of the products was confirmed by TLC analysis, as described above.

1.5. Structural analyses

NMR data were obtained on a Bruker DRX 600 spectrometer operating at 600.13 MHz for ^{1}H nuclei and at 150.92 MHz for ^{13}C nuclei. Spectra were run at 298 K in 99.9% D_2O (Wako). As an internal reference standard for the ^{1}H and ^{13}C NMR chemical shifts, *t*-BuOH (1.23 and 74.1 ppm, respectively) 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, and HMBC). Each signal in the ^{1}H 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.

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