ELSEVIER

Carbohydrate Research 275 (1995) 67–72

CARBOHYDRATE RESEARCH

Synthesis and structural analysis of disaccharides of 4-*O*-β-D-glucopyranosyl-D-glucosamine and 4-*O*-D-β-glucopyranosyl-2-deoxy-D-glucose

M.A. Tariq, K. Hayashi *, K. Tokuyasu, T. Nagata

National Food Research Institute, Tsukuba, Ibaraki 305, Japan

Received 16 January 1995; accepted 27 March 1995

Abstract

The disaccharides 4-*O*-β-D-glucopyranosyl-D-glucosamine (Glc–GlcN) and 4-*O*-β-D-glucopyranosyl-2-deoxy-D-glucose (Glc–2-deoxy-Glc) were synthesized from equimolar amounts of D-glucosamine and α-D-glucose-1-phosphate (G-1-P), and 2-deoxy-D-glucose and α-D-glucose-1-phosphate (G-1-P) respectively, in the presence of cellobiose phosphorylase from *Cellvibrio gilvus*. The yields were 55% and 50% based on the initial amounts of D-glucosamine and 2-deoxy-D-glucose, respectively. The structure of disaccharides were confirmed by NMR analysis.

Keywords: 4-*O*-β-D-glucopyranosyl-D-glucosamine; 4-*O*-β-D-glucopyranosyl-2-deoxy-D-glucose; Cellobiose phosphorylase; *Cellvibrio gilvus*; NMR

1. Introduction

The cellobiose phosphorylase (EC 2.4.1.20) of *Cellvibrio gilvus* is an endocellular enzyme responsible for the reversible phosphorolysis of cellobiose [1]. Several other microorganisms, including *Clostridium thermocellum* [2], *Ruminococcus flavefaciens* [3] and *Cellulomonas* sp. [4] also produce cellobiose phosphorylase. The physiological role of cellobiose phosphorylase is to convert cellobiose into α-D-glucose-1-phosphate (G-1-P) which is more efficiently utilized as a carbon source than D-glucose in some of these microorganisms [5].

Cellobiose phosphorylase from *Clostridium thermocellum* [6] has been partially purified and was found to utilize several monosaccharides as a glycosyl acceptor to form various disaccharides. Using the enzyme of *Clostridium thermocellum*, Alexander [7]

* Corresponding author.

SSDI 0008-6215(95)00143-3

synthesized six disaccharides from G-1-P and D-glucose, 2-deoxy-D-glucose, D-mannose, D-glucosamine, D-xylose, D-arabinose. The structures of these disaccharides were 4-*O*-β-D-glucopyranosyl-D-glucose, 4-*O*-β-D-glucopyranosyl-2-deoxy-D-glucose, 4-*O*-β-D-glucopyranosyl-D-mannose, 4-*O*-β-D-glucopyranosyl-D-glucosamine, 4-*O*-β-D-glucopyranosyl-D-xylose and 4-*O*-β-D-glucopyranosyl-D-arabinose, based on their enzymatic susceptibility and products of periodate oxidation followed by Smith degradation. Sasaki et al. [8] reported the purification of cellobiose phosphorylase from *C. gilvus* and discussed relevant catalytic properties of purified enzyme. The synthetic reactions of cellobiose phosphorylase [9] and the reaction mechanism of cellobiose phosphorylase [10] from *C. gilvus* cells have also been reported. Kitaoka et al. [5] have recently demonstrated the synthesis of 4-*O*-β-D-glucopyranosyl-D-xylose using cellobiose phosphorylase from *C. gilvus* and confirmed its structure by NMR and mass spectroscopy. Interest in oligosaccharides such as cellobiose and xylobiose has been stimulated because of their potential as a constituents for food materials [5]. Nakamura et al. [11] examined the water activity of cello-oligosaccharides containing cellobiose and proposed that these oligosaccharides might serve as soluble dietary fibre. Petrakova et al. [12] investigated the binding of carbohydrate ligands to monoclonal antigalactan and antidextran antibodies using mono- and oligosaccharides and their deoxy and deoxyfluoro analogues. Changes in binding resulting from the replacement of a hydroxyl group by fluorine or hydrogen in saccharides suggest a role of hydrogen binding by proton donation or acceptance. Because commercial applications of mono- and oligosaccharides are immerging slowly, the synthesis of disaccharides described in this paper provides a unique option for synthesis together with the adoption of NMR for confirmation analysis. A simple and efficient method to synthesize the disaccharides of 4-*O*-β-D-glucopyranosyl-D-glucosamine and 4-*O*-β-D-glucopyranosyl-2-deoxy-D-glucose by using cellobiose phosphorylase from *C. gilvus* are also described.

2. Experimental

Chemical.—Cellobiose and α-D-glucose-1-phosphate (G-1-P) dipotassium salt were purchased from Sigma, St. Louis, MO, USA. All other chemicals were of reagent grade.

Preparation of Cellvibrio gilvus *cells.*—*C. gilvus* cells were grown (as well as ethanol treatment of the cells) by the same procedure reported by Kitaoka et al. [5]. The ethanol treatment of cells was effective for selective reduction of phosphoglucomutase activity. Phosphoglucomutase activity in 1 g of dried cell has been reduced from 9 U to 0.1 U but only a slight effect on the cellobiose phosphorylase activity from 48 U to 30 U was observed.

Enzyme assays.—The activity of cellobiose phosphorylase was assayed using the method of Michal [13], by measuring the amount of G-1-P formed in the presence of cellobiose. Phosphoglucomutase activity was assayed by measuring the amount of G-6-P converted from G-1-P by the G-6-P dehydrogenase system [14]. One unit of activity was defined as the amount of the enzyme that produced 1 μmol of product, G-1-P for cellobiose phosphorylase or G-6-P for phosphoglucomutase, per minute.

Analytical methods.—Analyses were carried out using HPLC on a Dionex LCM-3

equipped with a Pulsed Amperometric Detector (Dionex) and a Shimadzu SIL-9A auto-injector. The detector output was displayed by a SIC Chromatocorder 12. The prepacked column was of DIONEX CarboPac^{TM} PA1 (250 × 4 mm). The mobile phase was 100 mM NaOH. The operation temperature was 25°C, the flow rate 1.0 mL/min and the injection volume 10 μL. The ^{13}C NMR spectrum was recorded on a Jeol GSX 270W spectrometer using a 10 mm internal diameter tube. The disaccharide (200 mg) was dissolved in 3 mL of D_2O. The internal reference was 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The chemical shift of DSS versus TMS was −1.38 ppm.

Synthesis of Glc–GlcN and Glc–2-deoxy-Glc.—The acetone–ethanol treatment cells (2.5 g dry weight, 75 U cellobiose phosphorylase and 0.25 U of phosphoglucomutase) were suspended in 200 mL TRIS–HCl buffer (100 mM, pH 7.0) containing G-1-P (100 mM), glucosamine or 2-deoxy-glucose (100 mM) and $MgCl_2 \cdot 6H_2O$ (5 mM). The suspension was incubated at 37°C for 24 h with continuous shaking. After 24 h incubation, the reaction mixture was centrifuged to recover the disaccharide.

Purification of Glc–GlcN.—The Glc–GlcN was purified after synthesis by passing through a column packed with Amberlite CG-120 type 1 resin (300 mL). A linear gradient [H_2O (2 L): 2 N HCl (2 L)] was used to obtain the purified disaccharide. The purified disaccharide sample was analysed by HPLC.

Purification of Glc–2-deoxy-Glc.—The Glc–2-deoxy-Glc was first deionized by treating with Amberlite MB3 and then purified by passing through a column packed with charcoal (300 mL). The column was first washed with 2 L distilled water and then a linear gradient [H_2O (2 L): 30% ethanol (2 L)] to obtain the purified disaccharide. The purified disaccharide sample was analysed by HPLC and the structure confirmed with ^{13}C NMR spectroscopy.

3. Results and discussion

Nuclear magnetic resonance spectroscopy.—^{13}C NMR spectroscopy is a powerful tool for the elucidation of carbohydrate structures [15,16]. The usual method of assignment of oligosaccharides involves comparison of the spectrum of those of the constituent monosaccharides [17,18] or closely related disaccharides [19,20]. The ^{13}C NMR spectra of standard glucose, glucosamine, 2-deoxy-glucose, cellobiose, maltose and chitobiose were used to assign the ^{13}C NMR spectra of Glc–GlcN and Glc–2-deoxy-Glc. The ^{13}C NMR spectra of Glc–GlcN and Glc–2-deoxy-Glc disaccharides displayed clearly resolved signals (Figs 1 and 2), and the assignment of signals to the putative structure are presented in Tables 1 and 2 respectively.

The spectrum of Glc–GlcN shows three distinct signals in the anomeric region. The signal at 103.7 ppm is due to the C-1 of β-D-glucopyranosyl (non-reducing end group) residue, confirmed by comparing with the cellobiose spectrum. The other two signals at 90.0 and 93.6 ppm are due to α- and β-glucosamine residues (reducing end group), respectively, at the anomeric position. The other signals were assigned by comparison of chemical shifts with the monomeric units (D-glucose and D-glucosamine) and cellobiose, chitobiose and maltose, and are given in Table 1. The chemical shift changes created by the replacement of 2-OH by 2-NH_3^+ in cellobiose are similar in magnitude to the

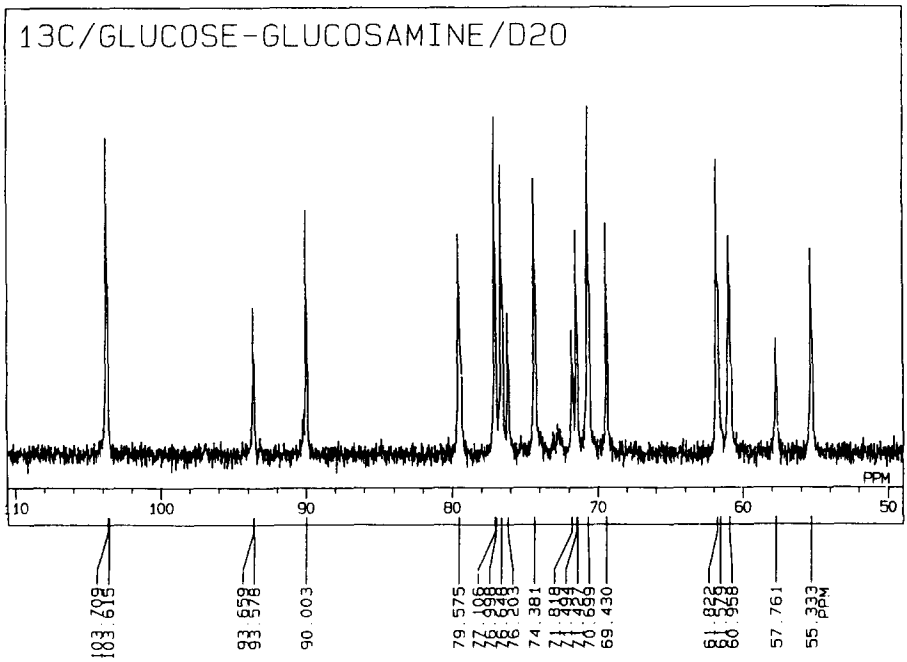

Fig. 1. ^{13}C NMR spectrum of 4-*O*-β-D-glucopyranosyl-D-glucosamine.

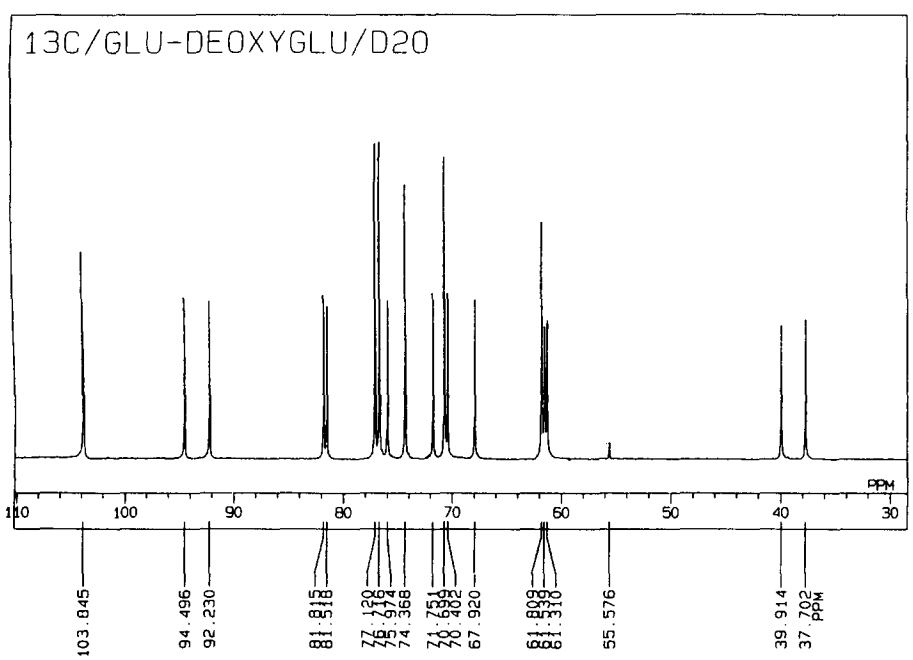

Fig. 2. ^{13}C NMR spectrum of 4-*O*-β-D-glucopyranosyl-2-deoxy-D-glucose (Glc–2-deoxy-Glc).

Table 1
^{13}C NMR data of 4-*O*-β-D-glucopyranosyl-D-glucosamine (Glc–GlcN) [a]

Monosaccharide		Signal locations (ppm)					
		C-1	C-2	C-3	C-4	C-5	C-6
Reducing end glucosamine	α	90.0	55.3	69.4	79.6	71.5	60.9
	β	93.6	57.7	71.8	79.6	76.2	60.9
Non-reducing end glucose		103.6	74.4	76.6	70.7	77.0	61.8

[a] In D_2O at 22°C; internal standard DSS; all the chemical shifts are relative to TMS.

Table 2
^{13}C NMR data of 4-*O*-β-D-glucopyranosyl-2-deoxy-D-glucose (Glc–2-deoxy-Glc) [a]

Monosaccharide		Signal locations (ppm)					
		C-1	C-2	C-3	C-4	C-5	C-6
Reducing end 2-deoxy glucose	α	92.2	37.7	67.9	81.5	71.7	61.3
	β	94.5	39.9	70.4	81.8	76.0	61.5
Non-reducing end glucose		103.8	74.4	76.7	70.7	77.1	61.8

[a] In D_2O at 22°C; internal standard DSS; all chemical shifts relative to TMS.

changes found in the monomer level, with exception of the C-1 (NR) and C-4,5,6 (R), resonances of Glc–GlcN. These observations may result from slight changes in steric constraints imposed on the glycosidic linkage as a result of replacement of the 2-OH group by the protonated amino group ($-NH_3^+$).

The spectrum of Glc–2-deoxy-Glc also shows three distinct signals in the anomeric region. The signal at 103.8 ppm is due to the C-1 of β-D-glucopyranosyl (non-reducing end group) residue, confirmed by comparison with the cellobiose spectrum. The other two signals at 92.2 and 94.5 ppm are due to the α- and β- of 2-deoxy-glucose residues (reducing end group) respectively, and occur at the same position as the 2-deoxy-glucose monosaccharide itself. The other signals were readily assigned by comparison of chemical shifts with those previously assigned in monomeric units (D-glucose and 2-deoxy-glucose) and cellobiose, chitobiose and maltose and shown in Table 2.

After confirmation of the synthesis and the structure of disaccharides Glc–GlcN and Glc–2-deoxy-Glc by HPLC and NMR, experiments were designed to achieve the optimum yield of these disaccharides. A range of pH from 5.0 to 8.7 was used and the optimum yield (55% for Glc–GlcN) was obtained from reaction at pH 7.0 for 8–10 h at 37°C. The synthesis of Glc–2-deoxy-Glc was highest (50%) at pH 7.0 (24 h at 37°C).

Acknowledgements

The STA fellowship provided by JISTEC/JRDC, Japan, to Dr M.A. Tariq, is gratefully acknowledged. Ms C. Aoyagi provided excellent technical assistance. We are thankful to Dr M. Kitaoka and Dr H. Taniguchi for useful discussion. Thanks are also

due to Professor D.J. Nevins, University of California, for critically reading the manuscript.

References

[1] F.H. Hulcher and K.W. King, *J. Bacteriol.*, 76 (1958) 571–577.
[2] C.J. Sih and R.H. McBee, *Proc. Mont. Acad. Sci.*, 15 (1955) 21–22.
[3] W.A. Ayers, *J. Bacteriol.*, 76 (1958) 515–517.
[4] K.L. Schimz, B. Broll, and B. John, *Arch. Microbiol.*, 135 (1983) 241–249.
[5] M. Kitaoka, H. Taniguchi, and T. Sasaki, *Appl. Microbiol. Biotechnol.*, 34 (1990) 178–182.
[6] J.K. Alexander, *J. Biol. Chem.*, 243 (1968) 2899–2904.
[7] J.K. Alexander, *Arch. Biochem. Biophys.*, 123 (1968) 240–246.
[8] T. Sasaki, T. Tanaka, S. Nakagawa, and K. Kainuma, *Biochem. J.*, 209 (1983) 803–807.
[9] M. Kitaoka, T. Sasaki, and H. Taniguchi, *J. Biochem.*, 112 (1992) 40–44.
[10] M. Kitaoka, T. Sasaki, and H. Taniguchi, *Biosci. Biotechnol. Biochem.*, 56 (1992) 652–655.
[11] T. Nakamura, A. Okiyama, and T.G. Harada, Japan laid open patent 87-273921 (1987).
[12] E. Petrakova, P. Kovac, and C.P.J. Glaudemans, *Carbohydr. Res.*, 233 (1992) 101–112.
[13] G. Michal, in H.U. Bergmeyer (Ed.), *Methods of Enzymatic Analysis*, Vol. 4, 2nd ed., Academic Press, New York, 1983, pp 185–191.
[14] G. Michal, in H.U. Bergmeyer (Ed.), *Methods of Enzymatic Analysis*, Vol. 4, 2nd ed., Academic Press, New York, 1983, pp 191–198.
[15] A.S. Shashkov and O.S. Chizhov, *Bioorg. Khim.*, 2 (1976) 437–442.
[16] H.J. Jennings and I.C.P. Smith, *Methods Enzymol.*, 50C (1978) 39–43.
[17] D.E. Dorman and J.D. Roberts, *J. Am. Chem. Soc.*, 93 (1971) 4463–4472.
[18] T. Usui, N. Yamaoka, J. Matsuda, H. Sugiyama, S. Seto, and K. Tuzimura, *J. Chem. Soc., Perkin Trans. I*, (1973) 2425–2432.
[19] H. Friebolin, N. Frank, G. Keilich, and E. Seifert, *Makromol. Chem.*, 177 (1976) 845–858.
[20] Y. Inoue and R. Chujo, *Carbohydr. Res.*, 60 (1978) 367–370.