Carbohydrate Research, 84 (1980) 365–369
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

Note

Structural analysis of the glycuronan 'protuberic acid' from *Kobayasia nipponica*: configurational and linkage analysis*

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(Received November 23th, 1979; accepted for publication, December 14th, 1979)

The water-soluble glycuronan "protuberic acid" (PA), isolated[1] from *Kobayasia nipponica*, a fungus of the Rhizopogonaceae, contains D-glucuronic acid and L-iduronic acid residues[2]. We now report on further structural studies.

Reduction of PA was performed by the methods of Timell and Mian[3] and of Taylor and Conrad[4]. The molar ratios of the components of the respective products, R-PA-1 and R-PA-2, were not conspicuously different (Tables I and II), but R-PA-1 was more water-soluble.

Methylation analysis (see Experimental and Table II) indicated that the reduced and methylated R-PA-1 and R-PA-2 contained 2,3,4,6-tetra-*O*-methylglucose, 2,3,6-tri-*O*-methylglucose, and 2,3,6-tri-*O*-methylidose. Demethylation of

TABLE I

G.L.C.[a] AND G.L.C.–M.S. DATA[b] FOR ALDITOL ACETATES FROM R-PA

Compounds	T	*Molar ratio*		*Mass of fragment-ion*	
		R-PA-1	*R-PA-2*	$M^+ - 59$	$M^+ - 59 - 14$
2,3,4-Tri-*O*-acetyl-1,6-anhydroidose	0.17	1.0	1.0	229	215
Hexa-*O*-acetylglucitol	1.00	2.0	2.8	375	361
Hexa-*O*-acetyliditol	1.20	0.2	0.2	375	361
Glucose/Idose ratio	—	1.7	2.3	—	—

[a]Obtained at 210° on a glass column (0.3 × 200 cm) of Uniport KS (60/80 mesh) coated with 3% of OV-225; nitrogen flow-rate, 60 ml/min. Retention time (*T*) relative to that of hexa-*O*-acetylglucitol (24.5 min). [b]Obtained at 150° on a glass column of Chromosorb W coated with 1.5% of OV-225; helium, 2 kg/cm².

*Studies on Fungal Polysaccharides, Part XXV. For Part XXIV, see ref. 2.
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TABLE II

G.L.C. DATA[a] FOR METHYLATED ALDITOL ACETATES FROM METHYLATED R-PA

Product	T	*Molar ratio*	
		R-PA-1	*R-PA-2*
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol	1.00	1.0	1.0
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol	2.53	12.0	30.3
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyliditol	2.76	5.2	14.3
Glucose/Idose ratio	—	2.5	2.2

[a]Obtained at 180° on a glass column (0.3 × 300 cm) of Gas Chrom Q (100/120 mesh) coated with 3% of ECNSS-M; nitrogen flow-rate, 60 ml/min. Retention time (*T*) relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol (16.9 min).

1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyliditol (Table II) followed by acetylation gave a product having the g.l.c. behavior of hexa-*O*-acetyliditol. As indicated by the ratio of tri- to tetra-*O*-methylhexose, R-PA-2 was less degraded than R-PA-1 (Table II); hence, R-PA-1 was analysed by periodate oxidation and n.m.r. spectroscopy.

When R-PA-1 was subjected in sequence to periodate oxidation, borohydride reduction, hydrolysis, borohydride reduction, and acetylation, tri-*O*-acetylglycerol, tetra-*O*-acetylerythritol, tetra-*O*-acetylthreitol, and a small proportion of hexa-*O*-acetylglucitol were formed. The glycerol was derived from reducing and non-reducing ends, and the glucitol reflected incomplete oxidation. These results indicate that R-PA has a (1→4)-linked, linear structure consisting of D-glucosyl and L-idosyl residues.

The p.m.r. spectrum of R-PA-1 contained two doublets in the range τ 5.0–5.5 associated with anomeric protons, and these signals were assigned to H-1 of the D-glucopyranosyl and L-idopyranosyl residues. The chemical shift of the signal at τ 5.47 (J 7.3 Hz) was similar to that (τ 5.42, J 7.2 Hz) for H-1 of the β-D-glucopyranosyl residue in cellobiose. The signal at τ 5.15 (J 5.9 Hz) differed from that (τ 4.65, J 3.1 Hz) for H-1 of the α-D-glucopyranosyl residue in maltose, but was similar to that (τ 4.92, J 6.0 Hz) for H-1 in α-D-idose. The ratio of the signals at τ 5.15 and 5.47 was 1:2.5 and reflects the molar ratio of α-L-idopyranosyl and β-D-glucopyranosyl residues. The p.m.r. spectrum of PA was characterised by three downfield signals in the ratios 1.0:1.1:2.3 at τ 4.96 (J 4.0 Hz), 5.25 (J 3.1 Hz), and 5.41 (J 7.2 Hz). In the spectrum of decationised-PA (D-PA), the signal at τ 5.25 was shifted downfield by 0.3 p.p.m. as a result of acidification, which is characteristic of the signal of H-5 of uronic acid residues[5]. Furthermore, the signals at τ 4.96 and 5.25 were attributed to H-1 and H-5 of α-L-iduronic acid residues by comparison with literature data[5–8]. The signal at τ 5.41 (J 7.2 Hz) was similar to that (τ 5.32, J 8.0 Hz) for H-1 of sodium β-D-glucuronate. The ^{13}C-n.m.r. data for R-PA-1 in Table III were consistent with the foregoing structural assignments.

TABLE III

OBSERVED AND CALCULATED ^{13}C-CHEMICAL SHIFT OF MAJOR SIGNALS IN THE SPECTRA OF R-PA-1

Carbon atom	α-D-*Idose* *Obs.*[a]	L-*Idose in R-PA-1*			*Methyl* β-D-*gluco-pyranoside* *Obs.*	D-*Glucose in R-PA-1*		
		Increment[b]	*Calc.*	*Obs.*		*Increment*[b]	*Calc.*	*Obs.*
C-1	94.0	+7	101.0	101.9	104.1	0	104.1	103.3
C-2	71.2	−1	70.2	71.3	74.0	0	74.0	74.0
C-3	72.8	−1	71.8	72.1	76.8	−1	75.8	75.8
C-4	70.3	+9	79.3	79.2	70.6	+9	79.6	79.2
C-5	70.7	−1	69.7	70.8	76.7	−1	75.7	75.0
C-6	59.5	0	59.5	59.9	61.7	0	61.7	61.3

[a]Assigned to the signals of the carbon atoms of α-D-idose by reference to the literature data[19,20]. [b]Approximate change in chemical shift expected relative to the corresponding carbon atom of the model compound: on formation of a glycosidic bond at the anomeric (+7 p.p.m.) or at a secondary C (+9 p.p.m.)[21,22].

EXPERIMENTAL

PA was isolated[1] from the fungus *Kobayasia nipponica*. T.l.c. was performed on silica gel (Merck, 5721) with *A*, ether–benzene (1:1); *B*, acetone–benzene (1:1); and *C*, benzene–ethanol (20:3); and detection with the Molisch[9] reagent. Reduction of PA was performed by the methods of Timell and Mian[3] (to give R-PA-1), and Taylor and Conrad[4] (to give R-PA-2). R-PA-1 was soluble in water, but R-PA-2 was slightly soluble. Hexa-*O*-acetyl-D-iditol was prepared[10] from D-idose (Sigma), and 2,3,4-tri-*O*-acetyl-1,6-anhydro-D-idose from 1,6-anhydro-D-idose[11]. The latter product was purified by t.l.c. (solvent *A*), and identified on the basis of mass-spectral and p.m.r. data[12,13]. 2,3,6-Tri-*O*-methyl-L-idose was synthesised by the method of Morgenlie[14]. G.l.c. was performed on a Shimadzu GC-6A instrument with flame-ionization detectors. G.l.c.–m.s. was performed on a Hitachi K-53 gas chromatograph coupled to a Hitachi RMU-7L double-focussing mass spectrometer. The mass range, chamber voltage, and chamber temperature were 750, 70 eV, and 200° respectively.

R-PA-1 and R-PA-2 were hydrolysed with 0.5M H_2SO_4 at 100° for 1, 2, 4, 6, 8, or 10 h. The reducing power of each hydrolysate was determined by the method of Park and Johnson[15]. The highest value of reducing power was at 4 and 6 h on R-PA-1, and at 8 and 10 h on R-PA-2. Therefore, the times of hydrolysis of R-PA-1 and R-PA-2 were fixed at 5 and 9 h, respectively. Each sample was hydrolysed, and the products were reduced with $NaBH_4$. The reduced products were converted into alditol acetates[10]. Each alditol acetate was examined by t.l.c. (solvent *A*), g.l.c., and g.l.c.–m.s. (Table I).

Methylated sugars and the corresponding alditol acetates of R-PA-1 and R-PA-2 were prepared as described previously[1]. The methylated sugars were analysed by t.l.c.

(solvents *B* and *C*) and as the corresponding alditol acetates by g.l.c. (Table II). Each methylated sugar was isolated by t.l.c. (solvent *B*), converted into the alditol acetate derivative, and subjected to g.l.c. and g.l.c.–m.s. Demethylation of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylliditol was performed by the method of Allen *et al.*[16], and the product was analysed as the alditol acetate by g.l.c.

R-PA-1 (4 mg) was oxidised with 10mM $NaIO_4$ in 40mM acetate buffer (pH 5.2) for 105 h at room temperature in the dark. Oxidant uptake (mol per hexosyl residue), determined by the method of Avigad[17], for R-PA-1 was 0.62 (4 h), 0.68 (9 h), 0.78 (30 h), 0.89 (56 h), 0.93 (93 h), and 0.93 (105 h). After oxidation, the reaction mixture was treated with ethylene glycol, reduced with $NaBH_4$, and then dialysed. The non-dialysable product (yield 90%) was hydrolysed (0.5M H_2SO_4, 100°, 5 h), and the products were converted into alditol acetates and subjected to g.l.c. (3% OV-225, 160°).

P.m.r. spectra in D_2O were obtained with JNM-4H-100 or JEOL-FX-100 spectrometers at 83°, and chemical shifts were recorded as p.p.m. downfield from the signal for internal sodium 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionate. D-PA was prepared as described previously[2]. D-Idose, cellobiose, maltose, and sodium D-glucuronate were used as reference materials for assigning the chemical shifts of anomeric protons. The data of Angyal[18] were used for the assignment of the anomeric protons of D-idopyranose. ^{13}C-N.m.r. spectra in D_2O were recorded at room temperature on a JEOL-FX-100 spectrometer at 25.0 MHz, in the pulsed, Fourier-transform mode with complete proton-decoupling. Chemical shifts are recorded as p.p.m. downfield from the signal for internal methanol. The assignment of chemical shifts for carbon atoms of α-D-idose is based on published data[19,20].

ACKNOWLEDGMENTS

The authors thank Mr. Y. Shida for the mass spectrometry measurements, Miss C. Takagai for the n.m.r. spectrometry measurements, and Mr. K. Kuramoto for technical assistance.

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