Carbohydrate Research, 98 (1981) 65–74
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

APPLICATION OF ^{1}H- AND ^{13}C-N.M.R. SPECTROSCOPY FOR STRUCTURAL STUDIES OF THE GLYCURONAN "PROTUBERIC ACID"*

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(Received March 23rd, 1981; accepted for publication, May 27th, 1981)

ABSTRACT

The structures of four oligosaccharides, isolated from a partial acid-hydrolysate of the glycuronan "protuberic acid" (PA), have been examined by chemical analysis, and by ^{1}H- and ^{13}C-n.m.r. spectroscopy. They were identified as *O*-(β-D-glucopyranosyluronic acid)-(1→4)-D-glucuronic acid, *O*-(α-L-idopyranosyluronic acid)-(1→4)-D-glucuronic acid, *O*-(β-D-glucopyranosyluronic acid)-(1→4)-*O*-(α-L-idopyranosyluronic acid)-(1→4)-D-glucuronic acid, and *O*-(β-D-glucopyranosyluronic acid)-(1→4)-*O*-(β-D-glucopyranosyluronic acid)-(1→4)-*O*-(α-L-idopyranosyluronic acid)-(1→4)-D-glucuronic acid. The acid-resistant portion of PA had properties similar to those of the original PA. These results suggested that the linear structural-sequence of PA is derived from the trisaccharide repeating-unit [→4)-β-D-GlcA-(1→4)-α-L-IdoA-(1→4)-β-D-GlcA-(1→].

INTRODUCTION

A water-soluble glycuronan, "protuberic acid" (PA), has been isolated from the fungus *Kobayasia nipponica* and is composed of one part of (1→4)-linked α-L-iduronic acid and two parts of (1→4)-linked β-D-glucuronic acid[2–4].

Generally, the determination of structures of glycuronans is difficult. For example, the preparation of oligosaccharides from alginate is difficult because of its acid-resistance[5]. It is known that alginate has (1→4)-linked residues of α-L-guluronic acid and β-D-mannuronic acid in a block polymer[5]. By the use of ^{13}C-n.m.r. spectroscopy, the structures of heparin[6,7], hyaluronic acid[8], and chondroitin sulfate A[8,9], B[9], and C[8,9] were shown to have repeating units, in contrast to the block-polymer structure of alginate[10,11].

We now report on the preparation of oligosaccharides by partial hydrolysis of PA with acid, and on the application of ^{13}C-n.m.r. spectroscopy to the oligosaccharides and PA.

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*Studies on Fungal Polysaccharides, Part XXIX. For Part XXVIII. See ref. 1.
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0008-6215/81/0000–0000/$ 02.75,

EXPERIMENTAL

General. — PA was obtained from the fruiting bodies of *K. nipponica*, as previously described[2]. The carbazole/orcinol (C/O) ratio was determined by the method of Hoffman *et al.*[12]. Uronic acid was determined by the orcinol method[13], and the reducing power by the method of Park and Johnson[14] (as glucuronic acid, in both cases). Specific rotations were measured for solutions in 5.0-cm semimicro tubes at 20° with a JASCO DIP-Digital polarimeter. T.l.c. of mono- and oligosaccharides (2 developments) was performed on cellulose-coated plastic sheet (Merck, 5577), and preparative p.c. of the Oligo 2 fraction (5 developments) on Toyo Roshi No. 50 paper; the solvent system was ethyl acetate–acetic acid–water (3:3:1) in both cases, and detection was performed with alkaline silver nitrate[15]. ^{1}H-N.m.r. spectra were recorded at 70° for solutions in D_2O (internal TSP) with a JEOL-FX-100 spectrometer in the pulsed Fourier-transform (F.t.) mode. ^{13}C-N.m.r. spectra were recorded at room temperature for solutions in D_2O with a JEOL-FX-100 spectrometer at 25.0 MHz in the pulsed F.t. mode with complete proton-decoupling. The chemical shifts are expressed as p.p.m. downfield from that of tetramethylsilane using internal MeOH (49.8 p.p.m.). Proton-decoupled F.t. spectra were measured using a repetition time of 2.0 s, a pulse width of 7 μs (45°), 8k real-data points, a sweep width of 5000 Hz, and, typically, 10,000–100,000 scans. Dermatan sulfate and chondrosine (Seikagaku Kogyo Co.; see Table II, legend), decationised with a column of Dowex 50 (H^+) resin, were used as reference materials for assigning the chemical shifts in ^{13}C-n.m.r. spectra. D-PA was prepared from PA by decationisation on Dowex 50 (H^+) resin. ^{13}C-N.m.r. spectra of PA (sodium salt) were recorded at 70° for solutions in D_2O with complete, off-resonance-, and non-decoupling. For off-resonance spectra, the repetition time was 2 s, and the pulse width 7 μs (45°). For non-decoupled spectra, the repetition time was 4 s, and the pulse width 7 μs (45°).

Preparation of oligosaccharides from PA. — PA (800.9 mg) was hydrolysed with 0.01M H_2SO_4 for 5.5 h at 100°. The hydrolysate was dialysed against water for 62 h, and divided into non-diffusible and diffusible fractions. The non-diffusible fraction was adjusted to pH 6.5 with 0.05M NaOH in a cold-water bath, concentrated, and lyophilised, to give PA-1. After measurement of its ^{1}H-n.m.r. spectrum, PA-1 was decationised with Dowex 50 (H^+) resin, to give D-PA-1, which was analysed by C/O ratio, specific rotation, and ^{13}C-n.m.r. spectroscopy. The diffusible fraction was neutralised with $BaCO_3$, filtered, concentrated, and then lyophilised, to give PA-2. An aqueous solution of PA-2 was fractionated on a column (90 × 5 cm) of DEAE-Sephadex A-50 (Cl^- form), by the method of Liu and Luh[16], by stepwise elution with aqueous NaCl as follows; 0.0 (100 ml), 0.03, 0.05, 0.10, 0.125, 0.15, 0.175, 0.20, 0.225, 0.25, and 2.0M NaCl (300 ml of each). The separated, oligosaccharide solutions were desalted twice on a column (97 × 1.9 cm) of Sephadex G-25, and then decationised with Dowex 50 (H^+) resin at 4° and lyophilised. The decationised oligosaccharides were analysed by C/O ratio, orcinol/reducing power (O/R) ratio, specific rotation, t.l.c., and ^{13}C-n.m.r. spectroscopy. The oligosaccharides were adjusted to

pH 6.5 with 0.05M NaOH in a cold-water bath and then analysed by ^{1}H-n.m.r. spectroscopy. The Oligo 2 fraction, which was eluted with 0.05M NaCl, was separated into major (Oligo 2-A) and minor (Oligo 2-B) fractions by preparative p.c. Analysis of the decationised and sodium salt forms of Oligo 2-A and Oligo 2-B was carried out as described earlier.

Preparation of oligosaccharides from PA-1. — PA-1 (102.5 mg) was hydrolysed with 0.01M H_2SO_4 for 5.5 h at 100°. The hydrolysate was separated into non-diffusible (PA-3) and diffusible (PA-4) fractions as described above. PA-4 was fractionated on a column (47 × 1.9 cm) of DEAE-Sephadex A-50 (Cl^- form) by stepwise elution with one third of the elution volume used for PA-2. The separated oligosaccharides were desalted with Sephadex G-25, and then decationised with Dowex 50 (H^+) resin at 4° and lyophilised. The decationised oligosaccharides were analysed by C/O ratio and t.l.c. ^{1}H-N.m.r. spectroscopic measurements were made as described earlier. PA-3 and decationised PA-3 (D-PA-3) were analysed as for PA-1 and D-PA-1.

Estimation of molecular size of PA-1 and PA-3. — The molecular sizes of PA-1 and PA-3 were estimated on a column (120 × 1.2 cm) of Sephadex G-100, equilibrated and eluted with 0.2M NaCl, using the following standards: Dextran T-70 (mol. wt. 70,000), T-40 (40,000), and T-10 (10,000). Fractions (2.5 ml) were collected at a flow rate of 10–15 ml/h.

RESULTS

Preparation of oligosaccharides. — Yields of PA-1 and PA-2 from PA were 39.0 and 42.9%, those of PA-3 and PA-4 from PA-1 were 20.1 and 53.9%, and those of oligosaccharides (d.p. 1–7) prepared from PA-2 and PA-4 were 48.5 and 61.6%, respectively. The elution patterns of PA-2 and PA-4 were similar (Fig. 1). Analytical

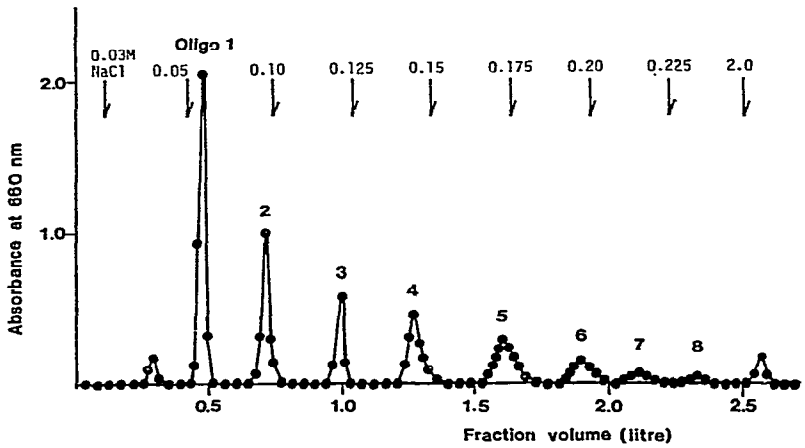

Fig. 1. Ion-exchange chromatography of PA-2 on a DEAE-Sephadex column.

TABLE I

ANALYTICAL DATA FOR MONO-, OLIGO-, AND POLY-SACCHARIDES

Oligosaccharides from PA-2	*1*	*2-A*	*2-B*	*3*	*4*	*5*	*6*	*7*	*PA-1*	*PA-3*	*PA*
Yield (%)[a]	21.6	16.0	4.0	21.8	16.6	13.8	4.3	1.3	39.0	20.1	—
$[\alpha]_D$ (degrees)	—	−10.5	−27.8	−59.9	−86.0	—	—	—	−85.7	−83.8	−83.6
C/O ratio	0.86	1.05	0.61	0.82	0.86	0.91	0.86	0.85	0.81	0.80	0.75
O/R ratio	1.19	1.50	1.90	2.87	3.70	5.16	5.65	7.11	—	—	—
R_{GlcA}	1.30, 1.00	0.36	0.46	0.13	0.03	0.005	0	0	—	—	—

Oligosaccharides from PA-4	*1*	*2-A*	*2-B*	*3*	*4*	*5*	*6*	*7*
Yield (%)[a]	12.0	15.0	3.8	20.8	10.0	10.8	6.5	6.5
C/O ratio	0.87	1.02	0.66	0.83	0.88	0.93	0.85	0.89
R_{GlcA}	1.31, 1.00	0.37	0.46	0.13	0.03	0.005	0	0

[a]Percentage of total oligosaccharides obtained.

TABLE II

^{13}C-N.M.R. DATA

Compound	*Chemical shifts (p.p.m.)*								*Assignment*
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C=O*	*CH_3*	
GlcA	96.9	74.5	75.2	72.1	76.1	174.0			β-GlcA
	93.1	71.9	72.3	71.2	73.2	173.0			α-GlcA
Chondrosine[a]	104.4, 104.2	73.5	75.3	72.0	75.9	173.2			β-GlcA
	93.8	53.9	80.5	67.7	75.9	61.9			β-GalN
	90.2	50.8	78.3	68.4	71.1	61.7			α-GalN
Dermatan sulfate[b]	103.2	70.7	70.4	78.9	70.5	172.5			α-IdoA
	104.9	52.8	82.3	76.9	75.4	62.1	175.7	23.3	β-GalNAc
Oligo 2-A	103.1	73.5	75.1	72.1	75.9	173.4			β-GlcA
	97.1	74.4	74.8	80.8	75.9	173.4			β-GlcA
	93.0	71.9	71.1	80.7	72.1	173.0			α-GlcA
Oligo 2-B	102.3	70.3	71.2	70.1	71.2	172.9			α-IdoA
	96.9	74.3	74.8	79.7	74.8	173.9			β-GlcA
	93.0	71.9	70.9	79.5	72.1	172.9			α-GlcA
Oligo 3	104.4	73.5	75.3	72.2	75.8	173.3			β-GlcA
	102.2	70.3	69.5	78.9	69.9	172.8			α-IdoA
	96.8	74.4	74.9	79.8	74.9	173.3			β-GlcA
	93.0	71.8	70.9	79.7	72.2	172.8			α-GlcA
Oligo 4	103.0	73.5	75.4	72.1	75.9	173.2			β-GlcA
	104.6	73.5	74.5	80.7	75.8	173.2			β-GlcA
	102.3	70.3	69.5	78.9	69.9	172.9			α-IdoA
	96.7	74.4	74.8	79.7	74.8	173.2			β-GlcA
	93.0	71.9	71.0	79.6	72.2	172.7			α-GlcA
D-PA-3	103.0	73.3	74.4	79.5	75.1	173.3			β-GlcA
	104.6	73.5	73.9	80.6	75.8	173.3			β-GlcA
	102.3	70.2	69.4	78.8	70.0	172.7			α-GlcA
D-PA-1	103.1	73.1	74.7	79.7	74.9	174.2			β-GlcA
	104.4	73.6	74.1	81.0	75.7	174.2			β-GlcA
	102.2	70.7	70.0	78.9	70.0	173.5			α-IdoA
D-PA	103.1	73.4	74.7	79.5	75.2	174.0			β-GlcA
	104.4	73.6	74.0	81.0	75.5	174.0			β-GlcA
	102.3	70.4	69.7	79.0	69.9	173.5			α-IdoA
PA (sodium salt)	103.6	74.2	75.4	80.6	76.6	176.1			β-GlcA
	104.4	74.5	75.4	82.2	77.3	176.1			β-GlcA
	102.6	71.6	70.4	80.6	71.0	175.8			α-IdoA

[a] β-D-GlcA-(1→3)-D-GalN. [b] [→4)-α-L-IdoA-(1→3)-β-D-GalNAc-(1→]$_n$.

data for the oligosaccharides are shown in Tables I–III. As shown in Table I, oligosaccharides **1**–**5** constituted ~95% of PA-2 and 75% of PA-4, respectively.

Characterisation of Oligo 1 and Oligo 2. — The O/R ratio indicated the Oligo 1 fractions to be monosaccharides. These were identified as L-iduronic acid and D-glucuronic acid, in the molar ratio of ~1:2 from the results of t.l.c., and from the C/O ratio.

TABLE III

^{1}H-N.M.R. DATA

Compound	*Chemical shift (p.p.m.)* (J, *Hz*)	*Integral*	*Assignment*
PA	5.04 (4.0)	1.0	α-IdoA
	4.75 (3.0)	1.1	H-5 of α-IdoA
	4.59 (7.2)	2.3	β-GlcA
PA-1	5.07 (4.0)	1.0	α-IdoA
	4.76 (3.0)	0.9	H-5 of α-IdoA
	4.61 (7.2)	1.8	β-GlcA
PA-3	5.01 (4.0)	1.0	α-IdoA
	4.73 (3.0)	1.0	H-5 of α-IdoA
	4.57 (7.2)	2.3	β-GlcA
Oligo 2-A	5.23 (2.7)	0.4	α-GlcA
	4.65 (8.0)	0.6	β-GlcA
	4.53 (7.2)	1.0	β-GlcA
Oligo 2-B	5.25 (3.0)	0.4	α-GlcA
	4.87 (4.0)	1.0	α-IdoA
	4.68 (3.0)	1.0	H-5 of α-IdoA
	4.65 (8.0)	0.6	β-GlcA
Oligo 3	5.21 (3.0)	0.3	α-GlcA
	5.02 (4.4)	1.0	α-IdoA
	4.70 (3.1)	1.1	H-5 of α-IdoA
	4.55 (7.3)	1.8	β-GlcA
Oligo 4	5.21 (3.0)	0.3	α-GlcA
	5.01 (3.8)	1.0	α-IdoA
	4.72 (3.1)	1.0	H-5 of α-IdoA
	4.58 (7.3)	0.6	β-GlcA
	4.55 (6.8)	1.8	β-GlcA
Oligo 5	5.21 (3.0)	0.3	α-GlcA
	5.01 (4.2)	1.0	α-IdoA
	4.69 (3.5)	1.1	H-5 of α-IdoA
	4.57 (7.1)	0.7	β-GlcA
	4.54 (6.7)	2.6	β-GlcA

Oligo 2 fractions obtained from PA-2 and PA-4 contained major (Oligo 2-A) and minor (Oligo 2-B) components (t.l.c.; R_{GlcA} 0.36 and 0.46) obtained in yields of 80 and 20%, respectively. From the analytical data (Tables I–III), the structures of Oligo 2-A and Oligo 2-B were determined as *O*-(β-D-glucopyranosyluronic acid)-(1→4)-D-glucuronic acid and *O*-(α-L-idopyranosyluronic acid)-(1→4)-D-glucuronic acid, respectively.

Characterisation of Oligo 3, 4, and 5. — The analytical data (Tables I–III) suggested that the ratios of glucuronic acid to iduronic acid in Oligos 3 and 4 were 2:1 and 3:1, respectively. In the ^{13}C-n.m.r. spectrum of Oligo 3, the C-1 signal at 102.2 p.p.m., which agreed with that of Oligo 2-B, must be assigned to the iduronic acid portion of idopyranosyluronic acid–glucuronic acid (IG). However, the C-1 signal at 104.4 p.p.m. did not agree with that of the glucopyranosyluronic acid residue in

Oligo 2-A. Also, the C-4 signals at 79.7 and 79.8 p.p.m. in α- and β-D-glucuronic acid agreed with those of Oligo 2-B but not with those of Oligo 2-A. The signals at 93.0 and 96.8 p.p.m. were assigned to C-1 in α- and β-D-glucuronic acid at the reducing end, and the signal at 72.2 p.p.m. was assigned to C-4 in β-D-glucuronic acid at the non-reducing end. Therefore, the structure of Oligo 3 is β-D-GlcA-(1→4)-α-L-IdoA-(1→4)-D-GlcA. From the results for Oligo 2-A, 2-B, and 3, the signals at 102.2, 103.1, and 104.4 p.p.m. were assigned to C-1 of IG (iduronic acid with a neighbouring glucuronic acid), GG (glucuronic acid with a neighbouring glucuronic acid), and GI (glucuronic acid with a neighbouring iduronic acid).

In the ^{13}C-n.m.r. spectrum of Oligo 4, the signals at 93.0 and 96.7 p.p.m. were assigned to C-1 in α- and β-D-glucuronic acid of the reducing end, and the signal at 72.1 p.p.m. was assigned to C-4 in β-D-glucuronic acid of the non-reducing end. The signals at 79.6 and 79.7 p.p.m. were assigned to C-4 in α- and β-D-glucuronic acid at the reducing end which was substituted by iduronic acid at C-4. The signals at 104.6 and 103.0 p.p.m. were assigned to C-1 of β-D-glucuronic acid in GI and GG residues, respectively (Table II). On the basis of these results, the structure of Oligo 4 is β-D-GlcA-(1→4)-β-D-GlcA-(1→4)-α-L-IdoA-(1→4)-D-GlcA.

The structure of Oligo 5 could not be determined because of the complexity of its ^{13}C-n.m.r. spectrum, but the chemical shifts of Oligo 5 were similar to those of Oligo 4. Also, Oligo 5 did not migrate significantly in t.l.c., and the glucuronic acid/iduronic acid ratio was 3–4 (Tables I and III).

Characterisation of PA-1 and PA-3. — The molecular sizes of PA-1 and PA-3, estimated by gel filtration on Sephadex G-100, were 3500 and 2800, respectively (Fig. 2). The data for C/O ratio, specific rotation, and ^{1}H- and ^{13}C-n.m.r. spectra agreed with those of PA (Tables I–III). Thus, the structures of PA-1 and PA-3 were essentially the same as that of PA.

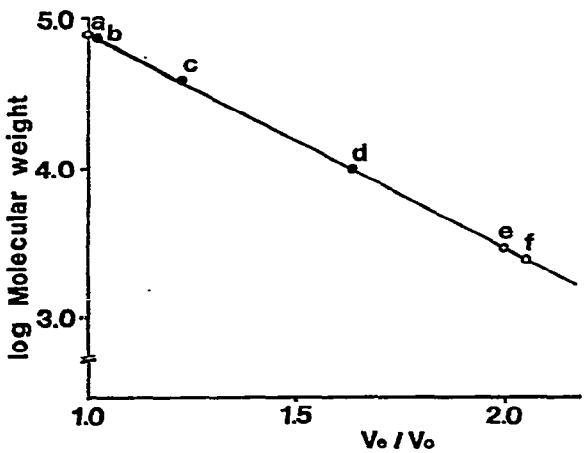

Fig. 2. Molecular size determination of PA-1 and PA-3 on a calibrated column of Sephadex G-100. (a) PA, (b) Dextran T-70, (c) T-40, (d) T-10, (e) PA-1, and (f) PA-3. Dextran T-2000 (mol. wt. 2,000,000) and D-glucose were used for the estimation of Void- and Ved-volumes.

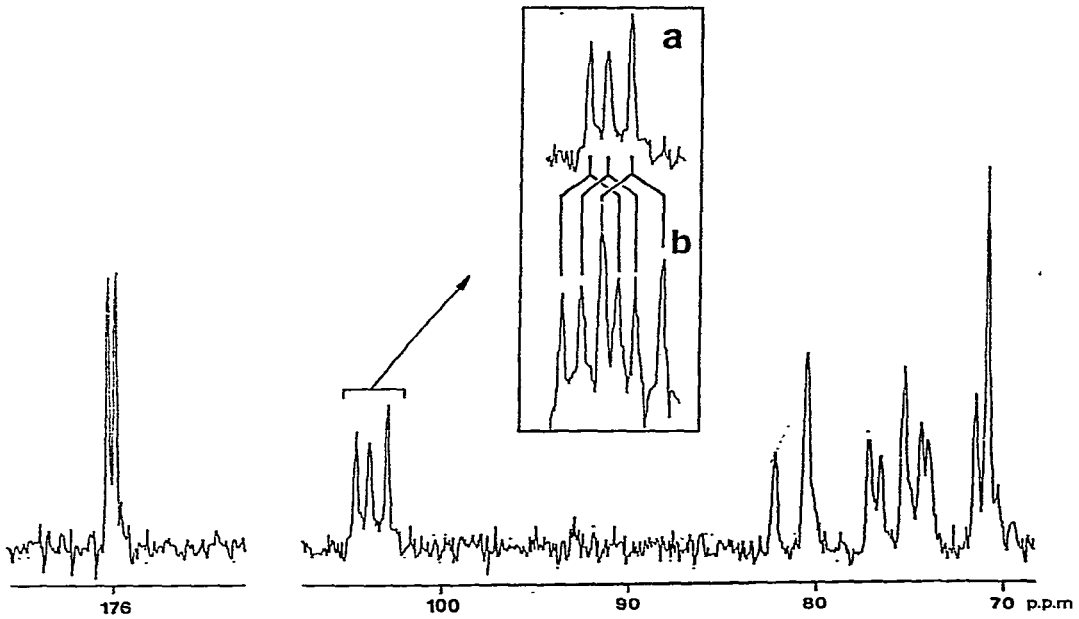

Fig. 3. ^{13}C-N.m.r. spectrum of PA. Inset: (a) complete proton decoupling, (b) off-resonance decoupling.

^{13}C-N.m.r. spectra of PA. — Three signals for anomeric carbon atoms (C-1) were revealed by complete decoupling and six by off-resonance and non-decoupling (Fig. 3), and the C–H coupling constants at 104.4, 103.6, and 102.6 p.p.m. were 161.8, 161.7 and 171.9 Hz, respectively. The signals at 104.4, 103.6, and 102.6 p.p.m. were assigned to β-D-glucuronic acid, β-D-glucuronic acid, and α-L-iduronic acid, respectively (Table II).

DISCUSSION

We have reported[2–4] that the glycuronan "protuberic acid" (PA) contains (1→4)-linked α-L-iduronic acid and β-D-glucuronic acid residues in the molar ratio 1:2. We have now investigated whether these residues are assembled in a blockwise or a repeating fashion.

As described previously[2,3], PA was hydrolysed to monosaccharides by 0.5M H_2SO_4 for 3 h at 100°, and the prolonged heating resulted in the disappearance of the L-iduronic acid. The acid-lability of L-iduronic acid was noted by Conrad[17]. In this study, the diffusible oligosaccharide fractions were obtained from PA by hydrolysis with 10mM H_2SO_4 for 5.5 h at 100°, in a 42.9% yield. Therefore, the preparation of the oligosaccharide units required a mild, desalting procedure. As shown in Fig. 1, the fraction eluted with 2.0M NaCl contained oligosaccharides having d.p. >9, in low yield (<1%). Low yields of the oligosaccharides having d.p. 1–7 (48.5%

from PA-2 and 61.6% from PA-4) may be due to non-specific adsorption on the columns used. For PA-2, the desalting was repeated twice.

By use of ^{13}C-n.m.r. spectroscopy, the structures of glycosaminoglycans[6-9] and alginate[10,11] were determined to be of the repeating and block type, respectively. In the ^{13}C-n.m.r. spectrum[10,11] of alginate, four signals for anomeric carbon atoms (C-1) were detected after complete proton-decoupling, associated with GU–GU (guluronic acid with a neighbouring guluronic acid), GU–MU (guluronic acid with a neighbouring mannuronic acid), MU–MU (mannuronic acid with a neighbouring mannuronic acid), and MU–GU (mannuronic acid with a neighbouring guluronic acid) units. Three signals for anomeric carbon atoms at 104.4, 103.6, and 102.6 p.p.m. were seen for PA (Fig. 3). These signals were assigned to GI (104.4), GG (103.6), and IG (102.6 p.p.m.) units, from the analysis of ^{13}C-n.m.r. spectra of oligosaccharides and by off-resonance and non-decoupling techniques applied to PA. Any oligosaccharide composed only of iduronic acid residues was not isolated and also a signal attributable to I–I units was not observed. From these facts, it can be concluded that I–I units are not present in PA. Possible structures for PA are:

(A) ---GIGIGGIGGGGIGIGIGIGGGIGGGGGIGGIGGGGIGIGIG---,

and

(B) ---GIGGIGGIGGIGGIGGIGGIGGIGGIGGIGGIGGIGGIGGIG---

Furthermore, Oligo 2-A, 3, and 4 were obtained in relatively high yield (~15%) from PA-2 and PA-4. The largest oligosaccharide composed only of glucuronic acid was Oligo 2-A. The acid-resistant portions (PA-1 and PA-3) from PA were similar to PA in their glucuronic acid/iduronic acid ratio, and chemical shifts (^{13}C-n.m.r. spectra), but not in molecular size (Tables II and III, and Fig. 2). From these results, we conclude that the structure of PA is mainly the (B) form.

Enzymic and biosynthetic studies of PA will be reported elsewhere.

ACKNOWLEDGMENTS

The authors thank Mr. Y. Shida and Mrs. C. Sakuma for the n.m.r. measurements.

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