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Partial structure of an anti-ulcer pectic polysaccharide from the roots of *Bupleurum falcatum* L.*,†

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ABSTRACT

Methylation analysis of a pectic polysaccharide (Bupleuran 2IIc) with anti-ulcer activity, isolated from the roots of *Bupleurum falcatum* L., revealed (1→4)-linked α-GalA together with small proportions of 2,4- and 3,4-linked GalA, and variously linked neutral sugars. Digestion of Bupleuran 2IIc with endo-α-(1→4)-polygalacturonase gave mainly galacturono-oligosaccharides (PG-4) and small proportions of enzyme-resistant regions (PG-1–3). PG-1 contained the sequence →4)-GalA-(1→2)-Rha-(1→4)-GalA-(1→, and partial acid hydrolysis gave GalA-(1→4)-Rha, GlcA-(1→4)-Rha, and several di- and oligo-saccharides consisting variously of Xyl, Glc, Gal, and Man. PG-2 and PG-3 each contained Rha, Fuc, Ara, Xyl, Man, Gal, Glc, GalA, GlcA, 2-Me-Fuc, 2-Me-Xyl, apiose (Api), aceric acid (AceA), and 3-deoxy-D-*manno*-2-octulosonic acid (Kdo). PG-4 contained (1→4)-linked α-galacturono-di- to -penta-saccharides and GalA. The galacturono-tetra- and -penta-saccharides had one and three methyl-esterified GalA units, respectively, and some of the galacturono-oligosaccharides contained 2,4- or 2,3-linked GalA.

INTRODUCTION

The roots of *Bupleurum falcatum* L. (Japanese name = Saiko) have been used in Chinese and Japanese herbal medicine for the treatment of chronic hepatitis, nephrosis syndrome, and autoimmune diseases. We have isolated the neutral[2] and acidic[3] (BR-2IIb) anti-complementary polysaccharides from the hot-water extract of the root of *B. falcatum*, and characterised[2,3] them as a 3,5-linked α-arabinan, an amylose-type glucan, an α-arabinoglucan, and a pectic polysaccharide. The polysaccharide fraction of *B. falcatum* showed[1] potent inhibitory activity against HCl–ethanol induced ulcerogenesis, and Bupleuran 2IIc was purified therefrom as the most potent anti-ulcer polysaccharide.

We now report on the structure of Bupleuran 2IIc.

* Studies on Anti-ulcer Pectic Polysaccharides from *Bupleurum falcatum*, Part II. For Part I, see ref 1.
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EXPERIMENTAL

Materials. — The roots of *B. falcatum* L. were purchased from Uchida Wakanyaku Co. Ltd. Sepharose CL-6B and Sephadex G-50 were obtained from Pharmacia, Bio-gel P-2 (200–400 mesh) and P-10 (200–400 mesh) from Bio-Rad, and Sep-Pak C_{18} cartridges from Waters Associates. Pectinase from *Aspergillus niger* was purchased from Sigma and endo-α-(1→4)-polygalacturonanase [poly(1,4-α-D-galacturonide) glycanohydrolase; EC 3.2.1.15] was purified using the procedure of Thibault and Mercier[4]. BR-2 was purified from BR-1 by hot-water extraction and precipitations with ethanol and Cetavlon as described[3].

General. — Total carbohydrate, uronic acid, and protein contents were assayed by the phenol–sulfuric acid[5], *m*-hydroxybiphenyl[6], and Lowry methods[7], respectively, using arabinose, galacturonic acid, and bovine serum albumin as the respective standards. Pentose was assayed by the phloroglucinol–acetic acid method[8], and methyl ester and acetyl groups by the methods of Wood *et al.*[9] and McComb *et al.*[10], respectively, using methanol and D-glucose penta-acetate as the respective standards. Optical rotations were determined at 23° with a JASCO DIP digital polarimeter. Polysaccharides were hydrolysed with 2M trifluoroacetic acid for 1.5 h at 121°, and t.l.c. of hydrolysates was performed on cellulose (Merck) with ethyl acetate–pyridine–acetic acid–water (5:5:1:3). Reducing sugars were detected with alkaline silver nitrate[11], and uronic acid with *p*-anisidine hydrochloride[12]. Neutral sugars and uronic acids in hydrolysates were converted[13] into the corresponding alditol acetates and analysed by g.l.c. at 125→190° (3°/min), using a Simadzu GC-6A gas chromatograph equipped with an SP-2380 capillary column (0.2-μm film, 0.25 mm i.d. × 30 m, SUPELCO). The molar ratios were calculated from the peak areas, and the molecular factors were estimated from the molecular weights of the corresponding alditol acetates. The molar ratios of uronic acid and neutral sugars were calculated from the contents of uronic acid. H.p.l.c. was performed on a Waters Model ALC/GPC 244 equipped with columns (0.76 × 50 cm each) of Asahi-pak GS-510 + GS-320 (Asahi Chemical Industry Co. Ltd.) and developed with 0.2M sodium chloride. Molecular weights of polysaccharides were estimated from the calibration curve of the elution volume of standard pullulans (P-400, 200, 100, 50, 20, and 5, Showa Denko Co. Ltd.). Electrophoresis (cellulose acetate membrane, Fuji Film Co. Ltd.) was performed in 0.08M pyridine–0.04M acetate buffer (pH 5.4) at 70 V for 30 min with detection[14] using Toluidine Blue.

*Identification of 2-Me-Fuc, 2-Me-Xyl, apiose (Api), aceric acid (AceA), 3-deoxy-*D-lyxo-*2-heptulosaric acid (Dha), and 3-deoxy-*D-manno-*2-octulosonic acid (Kdo).* — 2-Me-Fuc, 2-Me-Xyl, Api, and AceA were converted[13] into alditol acetates, and Dha and Kdo into deuterated alditol acetates by the procedure of Stevenson *et al.*[15], then analysed by g.l.c. and g.l.c.–m.s. using an SP-2380 capillary column with split-less injection. G.l.c. and g.l.c.–m.s. were performed on Hewlett–Packard model 5840 and 5890A gas chromatographs, respectively, with temperature programs of 60° for 1 min, 60→210° at 18°/min, 210° for 10 min, 210→250° at 8°/min, and 250° for 10 min (for 2-Me-Fuc, 2-Me-Xyl, Api, and AceA); and 80° for 2 min, 80→240° at 30°/min, and 240°

for 35 min (for Dha and Kdo). E.i.-m.s. was carried out using a Hewlett–Packard 5970B mass spectrometer, and c.i. (isobutane)-m.s. was performed using a JEOL DX-300 mass spectrometer.

Purification of Bupleuran 2IIc[1,3]. — The acidic polysaccharide fraction BR-2[3] was applied on a column (3.8 × 40 cm) of DEAE-Sepharose CL-6B (HCO_3^- form). The column was washed with water and then stepwise with 0.1, 0.3, 0.5, 1, and 2M ammonium hydrogencarbonate, to give the acidic polysaccharides. The major fraction, eluted with 0.5M ammonium hydrogencarbonate, was dialysed and lyophilised to give Bupleuran 2IIc (24.4% from BR-2).

Enzymic digestion of Bupleuran 2IIc. — Bupleuran 2IIc (120 mg) was digested with endo-α-(1→4)-polygalacturonase[4] (0.1 U) for 4 days at 37° in 50mM acetate buffer (pH 4.2, 30 mL). After neutralisation, the products were eluted from a column of Sephadex G-50, to give fractions PG-1–4 in the ratios 1.0:0.3:0.4:10.0.

Partial acid hydrolysis of PG-1. — PG-1 (2 mg) was hydrolysed with 0.1M trifluoroacetic acid for 1 h at 100°, the hydrolysate was applied to a column (1.5 × 5 cm) of DEAE-Sephadex A-25 ($HCOO^-$ form), and the neutral (N) and acidic fractions (A) were obtained by elution with water then 5M formic acid.

Purification of the galacturono-oligosaccharides from PG-4. — (*a*) *Gel filtration.* PG-4 was eluted from a column (2.2 × 120 cm) of Bio-gel P-2 with 50mM acetate buffer (pH 5.2), to give P2-1–4 in the ratios 1.0:2.8:4.5:2.3.

(*b*) *Anion-exchange chromatography.* P2-2–4 from (*a*) were each fractionated on a column (1.5 × 30 cm) of QAE-Sephadex A-25, using a linear gradient of 10→600mM ammonium hydrogencarbonate (600 mL), to give P2-2-1–4 (from P2-2), P2-3-1 and -2 (from P2-3), and P2-4-1 (from P2-4).

H.p.l.c. of the galacturono-oligosaccharides. — A solution of the oligosaccharides obtained above in 20 μL of the 2-aminopyridine reagent [prepared from 2-aminopyridine (1 g), 6M HCl (0.8 mL), and water (1.6 mL)] was incubated at 65° for 20 h to give[16] the 2-aminopyridine-labelled oligosaccharides. Each reaction mixture (1 μL) was injected into a Simadzu LC 6A instrument equipped with a column (7.5 × 10 cm) of Asahi-pak ES-502N (Asahi Chemical Industry), and the labelled oligosaccharides were eluted using a linear gradient (1.0 mL/min) from ~77→500mM acetate buffer (pH 5.2) [*i.e.*, 10% of 500mM acetate buffer (pH 5.2) and 90% of 30mM acetate buffer (pH 5.2)] in 90 min. The 2-aminopyridine-labelled oligosaccharides were detected by u.v. absorbance at 290 nm and fluorescence (excitation at 320 nm and emission at 400 nm). Standard galacturono-oligosaccharides were prepared by partial acid hydrolysis[17] of the polygalacturonic acid from orange (Sigma).

Methylation analysis. — Polysaccharides were methylated once (Hakomori)[18] in order to prevent[19] β-elimination, but methylsulphinylmethanide was added two or three times until the conversion of polysaccharide into a polyalkoxide was complete, as checked[20] by using triphenylmethane. The methylated polysaccharides were recovered on a Sep-pak C_{18} cartridge by the procedure of Waeghe *et al.*[21] except that samples were eluted with ethanol. Uronic acids of methylated samples were reduced[21,22] with sodium borodeuteride in tetrahydrofuran–ethanol (7:3) for 18 h at room temperature followed

by incubation for 1 h at 80°, and samples were recovered by desalting with AG50W-X8 (H^+) resin. Oligosaccharides, obtained by partial acid hydrolysis, were reduced with sodium borodeuteride, and then methylated. The methylated acidic oligosaccharide-alditols were reduced[21,22] and remethylated. The methylated oligosaccharide-alditols were fractionated on a column (1.0 × 25 cm) of Sephadex LH-20 (chloroform–methanol, 1:1) to give products of high (HMW) and low molecular weight (LMW) (detection[23] with the α-naphthol–sulphuric acid reagent). Each methylated sample was hydrolysed with 2M trifluoroacetic acid for 1 h at 121°, and the products were converted into alditol acetates, and analysed by g.l.c. and g.l.c.–m.s. using an SP-2380 capillary column. The carrier gas was He (0.9 mL/min in g.l.c. and 0.5 mL/min in g.l.c.–m.s.), and the temperature programs were 60° for 1 min, 60→180° at 30°/min, 180→250° at 1.5°/min, and 250° for 5 min (in g.l.c.); and 60° for 1 min, 60→150° at 30°/min, and 150→250° at 1.5°/min (in g.l.c.–m.s.). Methylated alditol acetates were identified by their fragment ions and relative retention times in g.l.c., and their molar ratios were estimated from the peak areas and response factors[24].

Base-catalysed β-elimination[19] *of methylated PG-1.* — A solution of dry, methylated PG-1 in methyl sulphoxide (1 mL) was stirred with 2M methylsulphinylmethanide for 24 h at room temperature, then treated with an excess of ethyl iodide. The mixture was kept overnight at room temperature, the ethyl iodide was evaporated, and the products were recovered using a Sep-pak C_{18} cartridge and fractionated[25] on a column (1.0 × 25 cm) of Sephadex LH-20 equilibrated with chloroform–methanol (1:1), to give a Molisch-reagent[23]-positive material in the void volume.

The material was hydrolysed and the products were converted into the methylated alditol acetates, which were analysed by g.l.c. and g.l.c.–m.s. on an SP-2380 capillary column.

G.l.c.–m.s. of methylated oligosaccharide-alditols. — Solutions of methylated oligosaccharide-alditols in acetone were injected into an HP-1 capillary column (0.1-μm film, 0.25 mm × 30 m, Hewlett–Packard). The g.l.c. program was 60° for 3 min, 60→190° at 30°/min, and 190→340° at 3.5°/min. C.i.-m.s.[26,27] and e.i.-m.s. fragment ions [A, J, and alditol (ald)][28] were used to determine the structures of the methylated oligosaccharide-alditols.

Negative ion f.a.b.-m.s. of the galacturono-oligosaccharides. — A JEOL DX-303 mass spectrometer interfaced with a DA-5000 computer was used. A solution of each sample in water was mixed with glycerol as matrix, and loaded on a silver plate.

N.m.r. spectrometry. — ^{1}H-N.m.r. (400 MHz) and ^{13}C-n.m.r. (100 MHz) spectra of Bupleuran 2IIc were recorded for solutions in D_2O with a Varian XL-400 spectrometer, and ^{1}H-n.m.r. (300 MHz) spectra of the galacturono-oligosaccharides were recorded with a Varian XL-VXR 300 spectrometer at room temperature.

RESULTS

Properties of Bupleuran 2IIc. — Bupleuran 2IIc gave a single spot on electrophoresis on a cellulose acetate membrane (staining with Toluidine Blue), was eluted from

Toyopearl HW-65 as a single peak, and gave a single peak in h.p.l.c. on Asahi-pak GS-510 + GS-320. It had $[\alpha]_D$ +200° (*c* 1, water), contained uronic acid (93%, 8.1% methyl-esterified), hexose (2.9%), protein (1.1%), and acetyl (1.9%), had a molecular weight of 63,000 (h.p.l.c.), and consisted of GalA:Ara:Xyl:Rha:Man:Gal:Glc in the ratios 61.5:1.1:1.1:0.2:0.3:1.0:0.4 together with traces of Fuc and GlcA. The ^{1}H-n.m.r. spectrum contained a signal at 5.10 p.p.m. in the region for anomeric protons, and the ^{13}C-n.m.r. spectrum contained signals at 177.53 (COOH of GalA) and 102.15 p.p.m. (C-1 of α-GalA). Methylation analysis revealed large proportions of 4- and 2,4-linked GalA in addition to terminal GalA*f* and 3,4-linked GalA, and mainly terminal Ara*f*, 2-linked Rha, and terminal Gal as the neutral sugars (Table I).

Enzymic digestion of Bupleuran 2IIc. — Bupleuran 2IIc was digested with endo-α-(1→4)-polygalacturonase. When the products were eluted from Sephadex G-50 (Fig. 1), a small proportion (PG-1) was eluted in the void volume and there were two intermedi-

TABLE I

Methylation analysis of Bupleuran 2IIc

Residue	*Position of OMe groups*	*Position of deuterium*	*Linkages*	*Mol. %*	
				Neutral sugars	*Carboxyl-reduced*
Ara	2,3,5		Terminal (furanosyl)	10.1	
	2,3,4		Terminal (pyranosyl)	3.3	
	2,3		4 or 5	5.1	
	2		3,4 or 3,5	4.1	
Xyl	2,3		4 or 5	4.4	
Rha	2,3,4		Terminal	4.6	
	3,4		2	13.2	
	2,4		3	2.2	
	2		3,4	2.6	
	3		2,4	6.5	
	4		2,3	1.7	
Fuc	2,3,4		Terminal	3.0	
Glc	2,3,6		4	6.2	
Gal	2,3,4,6		Terminal	10.8	
	2,3,6		4	9.2	
	2,4,6		3	5.4	
	2,6		3,4	1.7	
	2,3		4,6	4.6	
	2,4		3,6	1.3	
GalA	2,3,5	6,6-d_2	Terminal (furanosyl)		3.2
	2,3	6,6-d_2	4		64.9
	2	6,6-d_2	3,4		6.5
	3	6,6-d_2	2,4		25.3

ate fractions (PG-2 and -3) and a large proportion of the lowest-molecular-weight fraction (PG-4). PG-1 was eluted as a single peak from Sepharose CL-6B, and contained mainly Rha and Gal together with small proportions of Fuc, Ara, Xyl, Man, Glc, GalA, and GlcA (Table II). PG-4 contained a large proportion of GalA, a small proportion of GlcA, and traces of Rha, Ara, Man, Glc, and Gal. PG-2 and PG-3 each contained mainly Rha, Ara, and Gal, in addition to Fuc, Xyl, Man, Glc, GalA, and GlcA. G.l.c. of alditol acetates from PG-2 and -3 each gave, amongst others (Fig. 2A), peaks **1**–**3** which, when analysed by g.l.c.–c.i.- and –e.i.-m.s., gave ions at *m/z* 349, 335, and 362, respectively, due to $(M + H)^+$, and *m/z* 289, 275, and 302, respectively, due to $(M + H)^+ - HOAc$ in c.i.-m.s. (Table III). Comparison of fragment ions in e.i.-m.s. and retention times with data for standards indicated that peaks **1** and **2** contained 2-*O*-methylfucitol tetra-acetate and 2-*O*-methylxylitol triacetate, respectively (Table III). E.i.-m.s. suggested that peak **3** contained apiitol penta-acetate (Table III). These unusual sugars have been found[29] in rhamnogalacturonan II (RGII)[30] as plant cell-wall polysaccharides. Since RGII also contains[29] AceA, Dha, and Kdo, PG-2 and -3 were reanalysed for these unusual sugars. Peak **4** (Fig. 2B) was shown to contain a derivative of AceA by c.i.- and e.i.-m.s. (Table III). G.l.c.–c.i.-m.s. (Table III) also showed that the deuterium-labelled alditol acetate derived[15] from PG-2 and -3 gave ions at *m/z* 524 $[(M + H)^+]$ and 464 $[(M + H)^+ - HOAc]$, and indicated it to be the derivative of Kdo from comparisons of fragment ions in e.i.-m.s. and retention time with those of a standard. However, Dha was not detected. Methylation analysis (Table IV) indicated

TABLE II

Component sugars of products (PG-1–4) from Bupleuran 2IIc

Glycose [a]	*Molar ratio*			
	PG-1	*PG-2*	*PG-3*	*PG-4*
Rha	1.9	2.7	3.1	0.6
Fuc	0.1	0.5	0.9	
Ara	0.4	1.1	2.8	1.2
Xyl	0.1	0.1	0.2	
Man	0.2	0.1	0.08	1.3
Gal	1.0	1.0	1.0	1.0
Glc	0.4	0.4	0.2	0.2
2-Me-Fuc	n.d.[b]	0.3	0.3	
2-Me-Xyl	n.d.	0.1	0.4	
Api	n.d.	0.2	0.4	
GalA	4.0	4.3	5.9	14.4
GlcA	1.0	1.0	1.0	1.0
AceA	n.d.	0.2	0.08	n.d.
Dha	n.d.	n.d.	n.d.	n.d.
Kdo	n.d.	trace	trace	n.d.

[a] Neutral sugar, uronic acid (GalA, GlcA, and AceA), and ketose (Dha and Kdo) were analysed separately, and these molar ratios could not be compared. [b] Not detected.

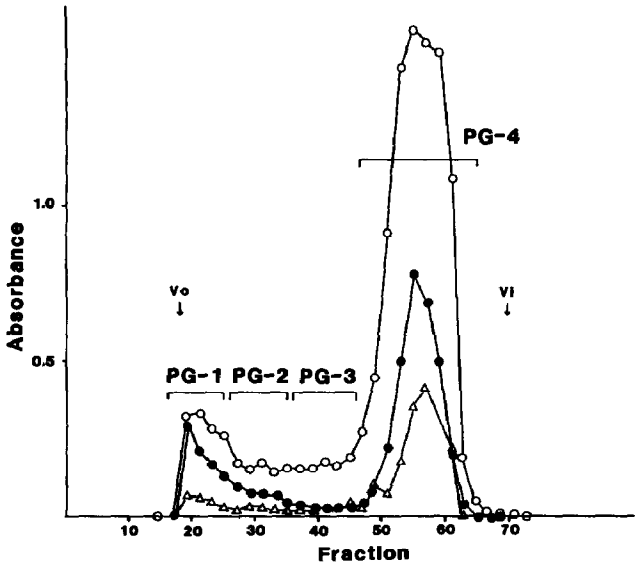


Fig. 1. Gel filtration on Sephadex G-50 of products from Bupleuran 2IIc by the digestion of endo-α-(1→4)-polygalacturonase: ●, carbohydrate (490 nm); ○, uronic acid (520 nm); △, pentose (552 nm); Vo, void volume; Vi, inner volume.

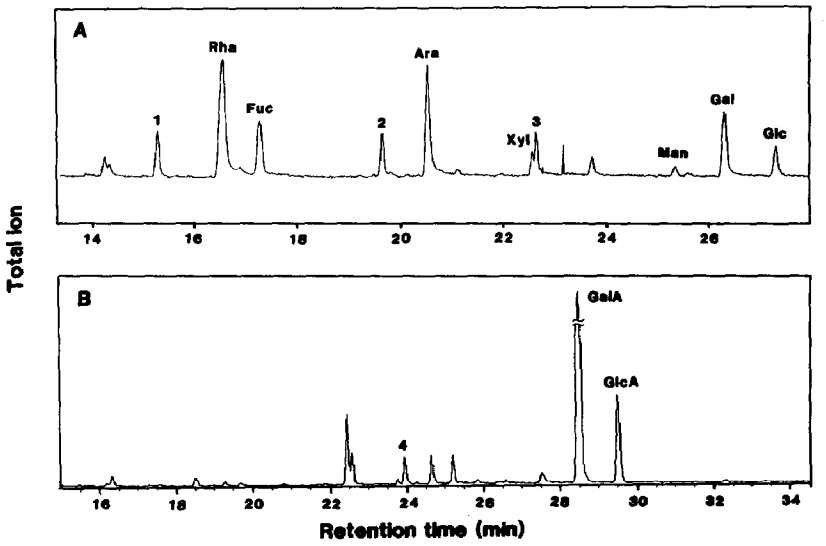


Fig. 2. Gas chromatogram patterns of alditol acetates derived from *A*, neutral sugars; and *B*, acidic sugars of PG-2 and -3: **1**, 2-Me-Fuc; **2**, 2-Me-Xyl; **3**, Api; **4**, AceA.

TABLE III

Diagnostic ions in c.i- and e.i.-m.s. of the alditol acetates from PG-2 and PG-3

Peak	*Glycose*	*C.i.-m.s. (relative abundance)*		*E.i.-m.s. (relative abundance)*							
		$(M+H)^+$	$(M+H)^+ - CH_3COOH$								
1	2-Me-Fuc	349 (56.8)	289 (100)	43 (100)	58 (7.0)	87 (8.8)	99 (9.9)	117 (61.2)	129 (8.4)	141 (1.7)	159 (2.9)
				173 (5.7)	201 (2.3)	215 (0.5)					
2	2-Me-Xyl	335 (40.0)	275 (100)	43 (100)	58 (5.2)	85 (9.9)	99 (7.4)	103 (2.2)	115 (2.6)	117 (57.9)	127 (9.2)
				145 (0.8)	201 (3.0)	261 (3.5)					
3	Api	362 (19.2)	302 (100)	43 (100)	98 (8.5)	103 (8.9)	115 (4.1)	116 (2.9)	127 (4.8)	128 (4.8)	140 (6.0)
				145 (11.7)	175 (2.9)	187 (13.8)	217 (0.5)	247 (1.5)			
4	AceA	377 (5.6)	317 (100)	43 (100)	103 (6.7)	112 (9.8)	128 (11.5)	145 (8.4)	154 (4.0)	170 (6.3)	187 (2.3)
				189 (2.5)	201 (5.2)	231 (3.0)					
5	Kdo	524 (12.0)	464 (100)	43 (100)	72 (14.8)	103 (5.1)	115 (12.5)	128 (12.6)	132 (15.2)	145 (5.8)	157 (6.6)
				170 (8.2)	187 (3.6)	204 (4.0)	217 (0.7)	234 (10.2)	276 (0.5)	289 (1.4)	

that PG-1 consisted mainly of 2-linked Rha and 4-linked GalA together with variously linked neutral sugars. PG-2 and -3 each contained mainly 4-linked GalA and neutral sugars linked as in PG-1 (Table V). PG-4 consisted mainly of terminal, 4-linked, and 3,4-linked GalA, together with a relatively small proportion of 2,4-linked GalA.

Methylation analysis of PG-1 after base-catalysed β-elimination. — Base-catalysed β-elimination of methylated PG-1 exposed[19] hydroxyl groups which were ethylated. Methylation analysis then showed that 2-linked Rha, terminal Rha, terminal Ara*p*, and 6-linked Gal had been lost ($\geq 50\%$) (Table IV). These results indicated that these sugars were originally linked to position 4 of GalA. Partially ethylated alditol acetates were not detected.

Partial acid hydrolysis of PG-1. — Partial acid hydrolysis of PG-1 gave one neutral (N) and one acidic carbohydrate fraction (A) (data not shown) that were each reduced with sodium borodeuteride, methylated, carboxyl-reduced with sodium borodeuteride, and re-methylated. The resulting methylated oligosaccharide-alditols were fractionated on Sephadex LH-20, to give high- (HMW-N and HMW-A) and low-molecular-weight fractions (LMW-N and LMW-A).

TABLE IV

Methylation analysis of PG-1 before and after base-catalysed β-elimination

Residue	*Position of OMe groups*	*Position of deuterium*	*Linkages*	*Carboxyl-reduced*	*Mol. %*	
					Before elimination	*After elimination*
Ara	2,3,5		Terminal (furanosyl)	1.9	2.8	5.8
	2,3,4		Terminal (pyranosyl)	1.8	2.6	0.7
	2,3		4 or 5	3.9	4.5	15.9
	2		3,4 or 3,5	1.1	1.5	4.6
Rha	2,3,4		Terminal	0.7	1.3	0.6
	3,4		2	15.5	16.9	5.9
	4		2,3	1.4	0.6	n.d.
	2		3,4	7.6	7.8	7.2
Gal	2,3,4,6		Terminal	9.1	10.5	11.1
	2,3,6		4	5.0	6.6	7.7
	2,4,6		3	6.3	8.3	14.5
	2,3,4		6	n.d.	4.5	1.7
	2,3		4,6	n.d.	8.1	4.7
	2,4		3,6	4.9	7.0	6.1
Glc	2,3,6		4	7.8	14.9	9.6
	2,3,4		6	4.8	3.9	4.0
GalA	2,3,4	6,6-d_2	Terminal	3.3		
	2,3	6,6-d_2	4	19.0		
	3	6,6-d_2	2,4	2.3		
	2	6,6-d_2	3,4	4.1		

TABLE V

Methylation analysis of PG-2, PG-3, and PG-4

Residue	*Position of OMe groups*	*Position of deuterium*	*Deduced glycosidic linkages*	*Mol. %*		
				PG-2	*PG-3*	*PG-4*
Ara	2,3,5		Terminal (furanosyl)	2.9	8.0	
	2,3,4		Terminal (pyranosyl)	2.0	2.5	
	2,3		4 or 5	4.5	7.1	
	2		3,4 or 3,5	1.9	2.6	
	5		2,3,4	5.6	3.8	
Xyl	2,3		4 or 5	3.7	4.6	
	2		3,4 or 3,5	2.7	0.9	
Rha	2,3,4		Terminal	3.0	2.8	
	3,4		2	8.2	2.9	
	2,4		3	3.7	2.0	
	2		3,4	3.3	3.3	
	4		2,3	1.6	1.1	
	3		2,4	3.6	1.3	
Fuc	2,3,4		Terminal	2.7	2.4	
Gal	2,3,4,6		Terminal	6.0	4.4	
	3,4,6		2	1.9		
	2,3,6		4	1.4		
	3,6		2,4	5.0	2.7	
	2		3,4,6	4.9	5.2	
GalA[a]	2,3,4	6,6-d_2	Terminal	9.0	8.8	20.4
	3,4	6,6-d_2	2		3.2	
	2,3	6,6-d_2	4	13.1	25.3	61.4
	3	6,6-d_2	2,4	5.0	5.0	8.2
	2	6,6-d_2	3,4			10.0

[a] Samples were methylated, carboxyl-reduced with sodium borodeuteride, and then converted into alditol acetates.

Analysis of acidic oligosaccharides from PG-1. — Methylation analysis showed that LMW-A mainly contained terminal GalA and a small proportion of terminal GlcA (Table VI), and that HMW-A mainly contained 4-, 5-, and 3,4- or 3,5-linked Ara, 4-linked Glc, terminal and 4-linked GalA, and terminal GlcA. G.l.c.–c.i.-m.s. indicated that LMW-A mainly contained two components (**1A** and **2A**), each derived from a hexuronosyl→6-deoxyhexitol-*1-d* (Table VII). G.l.c.–e.i.-m.s. of **1A** and **2A** gave fragment ions due to bA_1 (*m/z* 221), bA_2 (*m/z* 189), aJ_1 (*m/z* 266), and aJ_2 (*m/z* 206) (Table VIII), and for the *ald* series at *m/z* 340 and 308, *m/z* 309, 277, and 134, and *m/z* 384 and 352, indicative of HexA-(1→2)-Rha-ol-*1-d* and HexA-(1→4)-Rha-ol-*1-d*. Component **1A** was eluted faster than component **2A** on an HP-1 capillary column and, since Glc was eluted faster than Gal on this capillary column, it is suggested that the HexA units in **1A** and **2A** were GlcA and GalA, respectively (Table VIII).

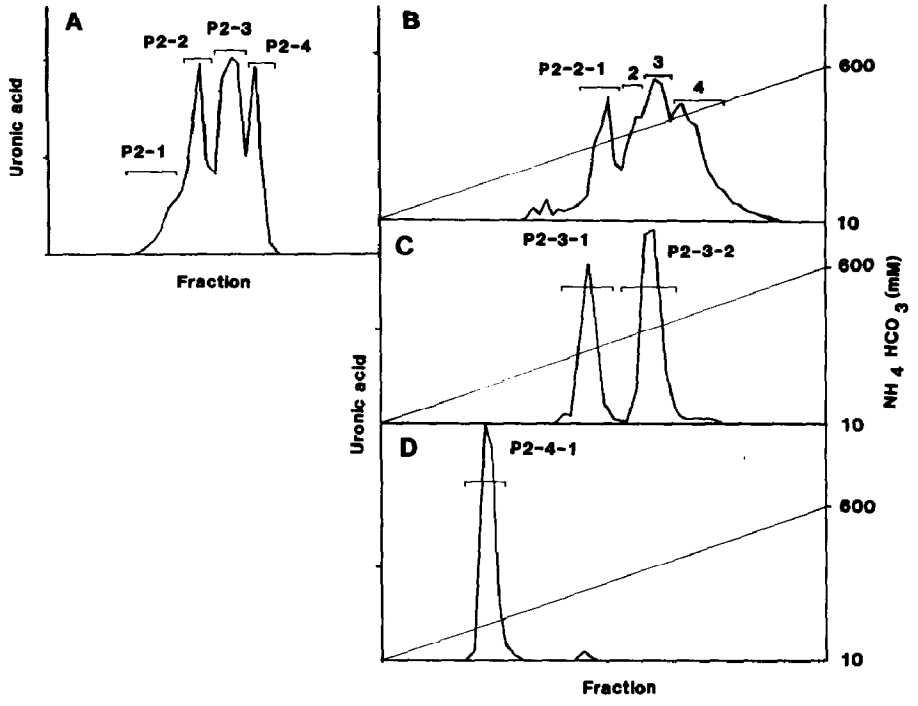


Fig. 3. *A*, Gel filtration of PG-4 on Bio-gel P2. Elution patterns on QAE-Sephadex of the galacturono-oligosaccharide fractions P2-2 (*B*), P2-3 (*C*), and P2-4 (*D*) from *A*.

Analysis of the neutral oligosaccharides from PG-1. — Methylation analysis showed that LMW-N contained mainly terminal Gal and Glc, small proportions of terminal Man and 4-linked Glc (Table VI), and also 3- and 6-linked hexitol-*1-d*. HMW-N mainly contained 4-linked Xyl, terminal and 4-linked Glc, and relatively small proportions of terminal and 4,6-linked Gal, and 6-linked Glc. G.l.c.–c.i.-m.s. of LMW-N revealed hexosyl-hexitol-*1-d* (**2N–5N**) (Table VII), and g.l.c.–e.i.-m.s. showed that **2N–5N** were Hex-(1→3)-Hex-ol-*1-d*, Hex-(1→3)-Hex-ol-*1-d*, Hex-(1→2)-Hex-ol-*1-d*, and Hex-(1→3)-Hex-ol-*1-d*, respectively (Table VIII). E.i.-m.s. also revealed Hex-(1→2)-Rha-ol-*1-d* (**1N**), Hex-(1→6)-Hex-ol-*1-d* (**6N**), and Hex-(1→6)-Hex-ol-*1-d* (**7N**). However, the hexose in these disaccharide-alditols could not be identified.

Isolation of the galacturono-oligosaccharides from PG-4. — Elution of PG-4 from Bio-gel P-2 gave fractions P2-1–4 (Fig. 3A). P2-2–4 each consisted of GalA, whereas P2-1 contained GalA and small proportions of Rha, Ara, and Gal (data not shown). P2-2–4 were each fractionated further on QAE-Sephadex, and P2-4-1 (from P2-4), P2-3-1 and -2 (from P2-3), and P2-2-1–4 (from P2-2) were obtained as galacturono-oligosaccharides (weight ratio P2-2-1:P2-2-2:P2-2-3:P2-2-4:P2-3-1:P2-3-2:P2-4-1 = 0.3:0.2:0.3:0.4:0.8:1.2:1.0) (Fig. 3B–D).

TABLE VI

Methylation analysis of the products obtained from PG-1 by partial acid hydrolysis

Residue	*Position of OMe groups*	*Position of deuterium*	*Deduced glycosidic linkages*	*Mol. %*			
				HMW-N	*LMW-N*	*HMW-A*	*LMW-A*
Ara	2,3,5		Terminal (furanosyl)			1.1	
	2,3		4 or 5			10.3	
	2		3,4 or 3,5			11.6	
Xyl	2,3		4 or 5	17.7			
Rha	3,4		2			4.3	
	3		2,4			1.9	
Hex[a]	1,2,3,4,5	1-*d*	6 (reducing terminal)		7.7		
	1,2,4,5,6	1-*d*	3 (reducing terminal)		10.6		
Gal	2,3,4,6		Terminal	7.4	39.9		
	2,4,6		3	4.5		2.8	
	2,3,6		4			5.8	
	2,3,4		6	4.1		5.3	
	2,3		4,6	8.9		1.2	
	2,4		3,6			0.6	
Glc	2,3,4,6		Terminal	12.6	31.1		
	2,4,6		3	3.7		1.6	
	2,3,6		4	19.5	6.0	10.0	
	2,3,4		6	9.3			
Man	2,3,4,6		Terminal	3.5	4.6		
	2,3,6		4	2.8			
	3,4		2,6	6.8			
GalA	2,3,4,6		Terminal			18.4	70.8
	2,3,6		4			11.5	
GlcA	2,3,4,6		Terminal			13.7	29.2

[a] The hexitol of the reducing terminal was not identified.

Analysis of the galacturono-oligosaccharides from PG-4. — P2-4-1 and P2-3-1 and -2 contained no methyl ester, and P2-2-1–4 contained 16.1, 12.1, 3.7, and 3.2%, respectively, of methyl ester. T.l.c. indicated P2-4-1 to be GalA and P2-3-1 and -2 to be galacturono-oligosaccharides. H.p.l.c. of the 2-aminopyridine-labelled derivatives from P2-3-1 and -2 gave peaks with retention times of those from galacturono-di- and -tri-saccharides, respectively (data not shown). In f.a.b.-m.s., P2-3-1 and -2 gave ions at *m/z* 369 and 545 $[(M - H)^-]$ and *m/z* 391 and 567 $[(M + Na^+ - 2H)^-]$, respectively (Table IX). ^{1}H-N.m.r. spectroscopy of P2-3-1 and -2 gave signals for anomeric protons at 4.95 ($J_{1,2}$ 5.2 Hz), 5.22 ($J_{1,2}$ 5.2 Hz), and 4.52 p.p.m. ($J_{1,2}$ 10.1 Hz) (ratios 1.0:0.5:0.5), and 4.97 ($J_{1,2}$ 5.4 Hz), 5.02 ($J_{1,2}$ 5.2 Hz), 5.22 ($J_{1,2}$ 5.2 Hz), and 4.51 p.p.m. ($J_{1,2}$ 10.1 Hz)

TABLE VII

Diagnostic c.i.-m.s. fragment ions of the disaccharide-alditols of the neutral (LMW-N) and acidic fractions (LMW-A) derived from PG-1 by partial acid hydrolysis

Peak	*Fragment ions* m/z (*relative abundance*)								*Oligosaccharide-alditol*
	$(M+H)^+$	$(M+H)^+ - MeOH$	aJ_1	aJ_2	$aJ_2OH^+_2$	bA_1	bA_2	bA_3	
LMW-A **1A**	444 (28.5)	412 (2.2)	266 (0.4)	206 (100)	224 (25.5)	221 (75.9)	189 (87.6)	157 (8.0)	Hexuronosyl→6-deoxyhexitol-*1-d*
2A	444 (21.9)	412 (1.8)	266 (0.7)	206 (100)	224 (27.0)	221 (73.7)	189 (92.0)	157 (18.8)	Hexuronosyl→6-deoxyhexitol-*1-d*
LMW-N **2N**	472 (32.1)	440 (16.1)	296 (6.6)	236 (26.3)	254 (80.3)	219 (75.2)	187 (100)	155 (4.4)	Hexosyl→hexitol-*1-d*
3N	472 (13.1)	440 (5.8)	296 (7.3)	236 (13.1)	254 (100)	219 (43.1)	187 (84.7)	155 (12.4)	Hexosyl→hexitol-*1-d*
4N	427 (38.7)		296 (10.2)	236 (21.9)	254 (100)	219 (43.1)	187 (84.7)	155 (12.4)	Hexosyl→hexitol-*1-d*
5N	427 (27.7)	440 (12.4)	296 (16.1)	236 (19.0)	254 (98.8)	219 (67.9)	187 (100)		Hexosyl→hexitol-*1-d*

TABLE VIII

Diagnostic e.i.-m.s. fragment ions of the disaccharide-alditols of the neutral (LMW-N) and acidic fractions (LMW-A) derived from PG-1 by partial acid hydrolysis

Peak	*Fragment ions* m/z (*relative abundance*)											*Oligosaccharide-alditol*
	aJ_1	aJ_2	bA_1	bA_2	ald							
LMW-A **1A**	266 (2.1)	206 (100)	221 (10.7)	189 (35.0)	308 (1.3) 134 (30.6)	340 (1.3) 277 (2.7)	352 (0.4) 309 (2.2)	384 (0.9)				GlcA-(1→2 and 4)-Rha-ol-*1-d*
2A	266 (1.8)	206 (100)	221 (10.9)	189 (33.6)	308 (1.4) 134 (29.2)	340 (0.9) 277 (3.4)	352 (0.4) 309 (1.7)	384 (0.7)				GalA-(1→2 and 4)-Rha-ol-*1-d*
LMW-N **1N**	266 (14.5)	206 (36.5)	219 (13.9)	187 (74.4)	306 (8.0)	350 (0.9)	363 (1.0)	382 (1.8)				Hex-(1→2)-Rha-ol-*1-d*
2N	296 (9.2)	236 (66.7)	219 (18.3)	187 (46.9)	134 (8.9)	306 (0.9)	338 (0.7)	349 (4.7)	381 (2.1)	382 (0.7)		Hex-(1→3)-Hex-ol-*1-d*
3N	296 (14.5)	236 (42.3)	219 (13.3)	187 (70.5)	133 (8.1)	306 (0.7)	338 (0.6)	349 (0.9)	350 (5.1)	381 (1.1)	382 (5.0)	Hex-(1→3)-Hex-ol-*1-d*
4N	296 (15.9)	236 (47.8)	219 (17.3)	187 (42.0)	133 (7.9)	306 (1.6)	338 (0.7)	350 (10.4)	382 (2.8)			Hex-(1→2)-Hex-ol-*1-d*
5N	296 (17.5)	236 (48.3)	219 (14.4)	187 (38.1)	306 (1.5)	338 (1.1)	349 (5.0)	381 (0.6)	425 (0.1)			Hex-(1→3)-Hex-ol-*1-d*
6N	296 (9.0)	236 (37.3)	219 (10.9)	187 (60.2)	134 (6.8)	146 (27.7)	178 (6.0)					Hex-(1→6)-Hex-ol-*1-d*
7N	296 (14.2)	236 (39.1)	219 (3.2)	187 (39.5)	134 (9.3)	146 (19.0)	178 (9.8)	337 (4.4)	349 (1.4)			Hex-(1→6)-Hex-ol-*1-d*

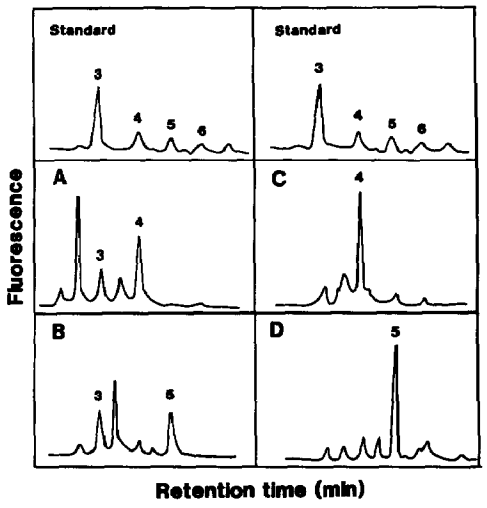


Fig. 4. H.p.l.c. of 2-aminopyridine-labelled galacturono-oligosaccharides: the standards (3–6) are (1→4)-linked α-galacturono-tri- to -hexa-saccharides; *A*, P2-2-1; *B*, P2-2-2; *C*, P2-2-3; *D*, P2-2-4 from Fig. 3B.

(ratios 1.0:1.0:0.5:0.5), respectively. These results indicated P2-3-1 and -2 to be (1→4)-α-galacturono-di- and -tri-saccharides, respectively. H.p.l.c. of P2-2-3 and -4 each gave major peaks with the same retention times as those of galacturono-tetra- and -penta-saccharides, respectively (Fig. 4C and D). In f.a.b.-m.s., P2-2-3 gave ions due to $(GalA)_4$ at *m/z* 721 $[(M - H)^-]$, 744 $[(M + Na^+ - H)^-]$, and 766 $[(M + 2Na^+ - H)^-]$, whereas P2-2-4 gave ions due to $(GalA)_5$ at *m/z* 919 $[(M + Na^+ - 2H)^-]$ and 941 $[(M + 2Na^+ - H)^-]$ (Table IX). The ^{1}H-n.m.r. spectra of P2-2-3 and -4 each contained signals (4.96–5.02 p.p.m.) for H-1α. Methylation analysis indicated P2-2-3 and -4 to be comprised mainly of terminal and 4-linked GalA together with 3,4- and 2,4-linked GalA (Table X). These results indicated P2-2-3 and -4 to consist mainly of (1→4)-α-galacturono tetra- and -penta-saccharides, respectively. H.p.l.c. indicated that P2-2-1 and -2 contained galacturono-tri- and -tetra-saccharides, and galacturono-tri- and -penta-saccharides, respectively, in addition to peaks of unknown compounds (Fig. 4A and B). F.a.b.-m.s. of P2-2-1 gave an ion at *m/z* 781 $[(M + 2Na^+ - H)^-]$ due to a galacturono-tetrasaccharide, one GalA of which was methyl-esterified, whereas P2-2-2 gave ions at *m/z* 939 $[(M - H)^-]$ and 962 $[(M + Na^+ - H)^-]$ due to a galacturono-pentasaccharide, three GalA of which were methyl-esterified (Table IX). The ^{1}H-n.m.r. spectra of P2-2-1 and -2 also each contained signals at 4.97–5.02 p.p.m. for H-1α. Methylation

TABLE IX

Negative-ion f.a.b.-m.s. of the galacturono-oligosaccharides obtained from PG-4

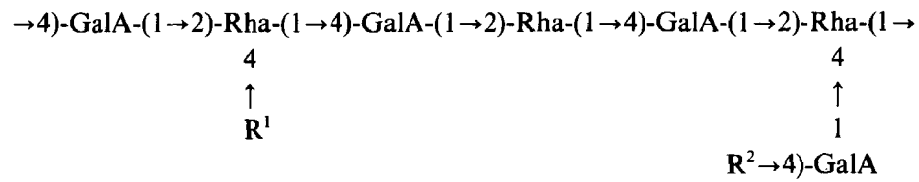
Fraction[a]	$(M-H)^-$	$(M+Na^+-H)^-$	$(M+Na^+-2H)^-$	$(M+2Na^+-H)^-$	*Sequence*
P2-2-1	n.d.[b]	n.d.	n.d.	781	$(GalA)_3(MeGalA)$
P2-2-2	939	962	n.d.	n.d.	$(GalA)_2(MeGalA)_3$
P2-2-3	721	744	n.d.	766	$(GalA)_4$
P2-2-4	n.d.	n.d.	919	941	$(GalA)_5$
P2-3-1	369	n.d.	391	n.d.	$(GalA)_2$
P2-3-2	545	n.d.	567	n.d.	$(GalA)_3$

[a] Fractions from Fig. 3. [b] Not determined.

TABLE X

Methylation analysis of the galacturono-oligosaccharides

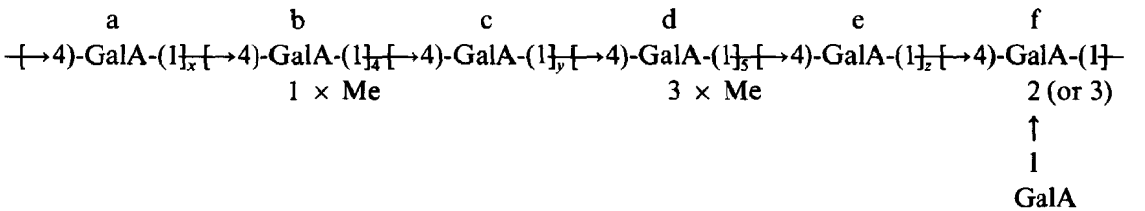
Linkage	*Molar ratio*			
	P2-2-1	*P2-2-2*	*P2-2-3*	*P2-2-4*
Terminal GalA	1.0	1.0	1.0	1.0
4-GalA	2.5	3.0	1.8	3.7
3,4-GalA	0.2	0.6	0.1	0.2
2,4-GalA	0.2	0.3	0.1	0.3



R^1 or R^2 = GlcA-(1→

R^1 or R^2 = neutral chains consisting of Xyl, Gal, Glc, or Man

1



a + c + e : b + d + f = 71.4 : 28.6

2

analysis indicated that P2-2-1 and -2 each contained mainly terminal and 4-linked GalA together with 3,4- and 2,4-linked GalA (Table X). These results indicated that P2-2-1 and -2 contained (1→4)-α-galacturono-tetra- and -penta-saccharides, one and three GalA, respectively, of which were methyl-esterified. P2-2-1 and -2 also each contained 2 or 3 branched (1→4)-α-galacturono-oligosaccharides, as inferred from the results of methylation analysis.

DISCUSSION

The present results indicated the anti-ulcer polysaccharide, Bupleuran 2IIc, to be a pectic polysaccharide. The general structure of pectic polysaccharides consists[31,32] of "ramified" (rhamnogalacturonan with neutral side chains) and (1→4)-α-galacturonan regions on the basis of the results of digestion with endo-α-(1→4)-polygalacturonase. Digestion of Bupleuran 2IIc with this enzyme gave small proportions of enzyme-resistant fractions (PG-1–3) and a large proportion of galacturono-oligosaccharides (PG-4) derived from galacturonans (yield ratios 8.6:2.3:3.3:85.8).

Base-catalysed β-elimination and partial acid hydrolysis strongly suggested that PG-1 was a rhamnogalacturonan $\{[\rightarrow 4)\text{-GalA-}(1\rightarrow 2)\text{-Rha-}(1\rightarrow 4)\text{-GalA-}(1\rightarrow]_n\}$ with side chains linked to position 4 of some Rha units. Partial acid hydrolysis also gave GalA-(1→4)-Rha, and it was inferred that some Rha in the rhamnogalacturonan core was attached together with 4-linked GalA at position 4 of Rha. This sequence has been found in pectic polysaccharides from the roots of *Angelica acutiloba*[33,34] and *Bupleurum falcatum*[3], and the leaves of *Artemisia princeps*[35]. Partial acid hydrolysis also indicated that PG-1 contained sequences such as GlcA-(1→4)-Rha-(1→, and neutral carbohydrate chains which consisted of Xyl, Gal, Glc, and Man. It was inferred that GlcA and the neutral carbohydrate chains were attached to the rhamnogalacturonan core at position 4 of Rha in the core directly, or through 4-linked GalA as side chains, respectively (**1**). Partial acid hydrolysis also gave GlcA-(1→2)-Rha-(1→; however, it is not known whether this sequence was present in the core or the side chains. The detailed structures of neutral side chains in PG-1 remain to be determined.

Digestion with endo-α-(1→4)-polygalacturonase and structural analysis of the resulting galacturono-oligosaccharides indicated that at least 71.4% of the galacturonan in Bupleuran 2IIc consisted of (1→4)-linked α-GalA and that some (1→4)-linked α-galacturono-tetra- and -penta-saccharide units had one and three methyl ester groups, respectively (**2**). Some (1→4)-linked α-GalA units possessed GalA side chains at position 2 or 3 (**2**).

Sugar analysis indicated that the other enzyme-resistant fragments (PG-2 and -3) each contained the unusual sugars 2-Me-Fuc, 2-Me-Xyl, Api, AceA, and Kdo, in addition to Rha, Ara, Gal, Glc, and GalA. These unusual sugars have been detected[29] in rhamnogalacturonan II (RGII)[30], a plant cell-wall polysaccharide, and it was assumed

that PG-2 and -3 each contained an RGII-like structure. RGII was purified from suspension-cultured cell walls of sycamore[30], rice[36], and Douglas fir[37] by digestion with endo-polygalacturonase, and Pectinol AC[38]. Digestion of plant cell walls with endo-polygalacturonase also gave rhamnogalacturonan I (RGI)[39], and it was suggested[31,32] that RGI and RGII are present as structural units and linked by galacturonans. However, the isolation of homogeneous pectic polysaccharides containing RGI and RGII has not been reported. RGI has been defined[31,32] as a rhamnogalacturonan with various neutral side chains, and PG-1 appears to have a similar structure. Bupleuran 2IIc was a homogeneous polysaccharide; therefore, it was suggested that the "ramified" region (PG-1) might be combined with RGII-like structural units (PG-2 and -3) through (1→4)-linked α-galacturonans.

Yamada *et al.* reported[1] that the anti-ulcer activity of Bupleuran 2IIc was decreased by treatment with endo-polygalacturonase. However, (1→4)-α-galacturonan does not express anti-ulcer activity as potent as Bupleuran 2IIc, and not all pectic polysaccharides have anti-ulcer activity. Therefore, it is suggested that the overall structure of Bupleuran 2IIc is important for the expression of anti-ulcer activity. Further work on the detailed structure of Bupleuran 2IIc is in progress.

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