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# CHEMICAL STRUCTURE OF THE WATER-SOLUBLE POLYSACCHARIDE OF *Phellodendron amurense* RUPRECHT*

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ABSTRACT

Purified, water-soluble polysaccharide (P-WSPS) of *Phellodendron amurense* Ruprecht consists of residues of L-rhamnose, L-arabinose, D-galactose, and D-galacturonic acid in the ratios of 29.26:22.45:31.17:17.10 (% by weight). G.l.c.–m.s. analysis of methylated-reduced P-WSPS showed that it has a very complicated and highly branched structure, and consists mainly of (1→2)-linked L-rhamnose, (1→4)-linked L-arabinose, (1→4)-linked D-galactose, and (1→3)- and (1→4)-linked D-galacturonic acid. The presence of 2-*O*-(α-D-galactopyranosyluronic acid)-L-rhamnopyranose, 2-*O*-(β-D-galactopyranosyluronic acid)-L-arabinose, 4-*O*-(α-D-galactopyranosyluronic acid)-L-rhamnopyranose, and 4-*O*-(α-D-galactopyranosyluronic acid)-D-galactopyranose in the aldobiouronic acid fraction of the hydrolyzate of P-WSPS was also shown.

## INTRODUCTION

In Part I, it was reported[1] that the purified, water-soluble polysaccharide (P-WSPS) of *P. amurense* (Wobaku wood) has a highly branched structure. In general, plant gums have highly branched, complex structures, and many studies on plant polysaccharide have been made[2–6], but very few on the polysaccharide of *P. amurense*. Preliminary studies on this polysaccharide showed that it has a very complicated structure, and it was found necessary to make studies from various directions.

We now describe the gross structure of the whole polysaccharide, and elucidation of the structures of aldobiouronic acids derived therefrom.

*Studies on the Water-soluble Polysaccharide of *Phellodendron amurense* Ruprecht, Part II. For Part I, see ref. 1.

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RESULTS AND DISCUSSION

The water-soluble polysaccharide of *P. amurense*, which was purified by repeated precipitation with ethanol, and by chromatography on a column of DEAE-cellulose (to give P-WSPS), was hydrolyzed with 0.5M sulfuric acid. Liberation of galacturonic acid reached a maximum after 8 h, and, thereafter, the amount in the hydrolyzate decreased because of decomposition. Consequently, analysis of the sugar composition of P-WSPS was performed on the 8-h hydrolyzate. Paper chromatography of the hydrolyzate gave monosaccharide spots (*1*, $R_{Rha}$ 1.01; *2*, $R_{Rha}$ 0.68; *3*, $R_{Rha}$ 0.55; and *4*, $R_{Rha}$ 0.43) and spots of oligosaccharides in small proportions ($R_{Rha}$ 0.28, 0.14, and 0.05) in solvent system *A*. Spots *1*, *2*, and *4* were identified as those of rhamnose, arabinose, and galacturonic acid (from their $R_{Rha}$ values, and the colors given with the *o*-aminophenol reagent). Although the separation of spots *3* and *4* was unsatisfactory in solvent system *A*, good separation was achieved in solvent system *B* (*3*, $R_{Rha}$ 0.58; *4*, $R_{Rha}$ 0.05), and spot *3* was identified as galactose. Compound *3* was collected by preparative paper-chromatography, and treated with 1% methanolic hydrogen chloride; analysis by g.l.c. then showed that it was galactose.

These four sugars were separated by chromatography on a column of cellulose and the D or L configuration was ascertained. The rhamnose derivative examined was ethyl 2,3,4-tri-*O*-acetyl-1-thio-β-rhamnopyranoside, which had the L configuration. The positive rotation of the phenylosazones from the galactose and the galacturonic acid showed that they had the D configuration. The configuration of the arabinose was determined by fermentation with *Escherichia coli* $C_N$ strain; it was completely

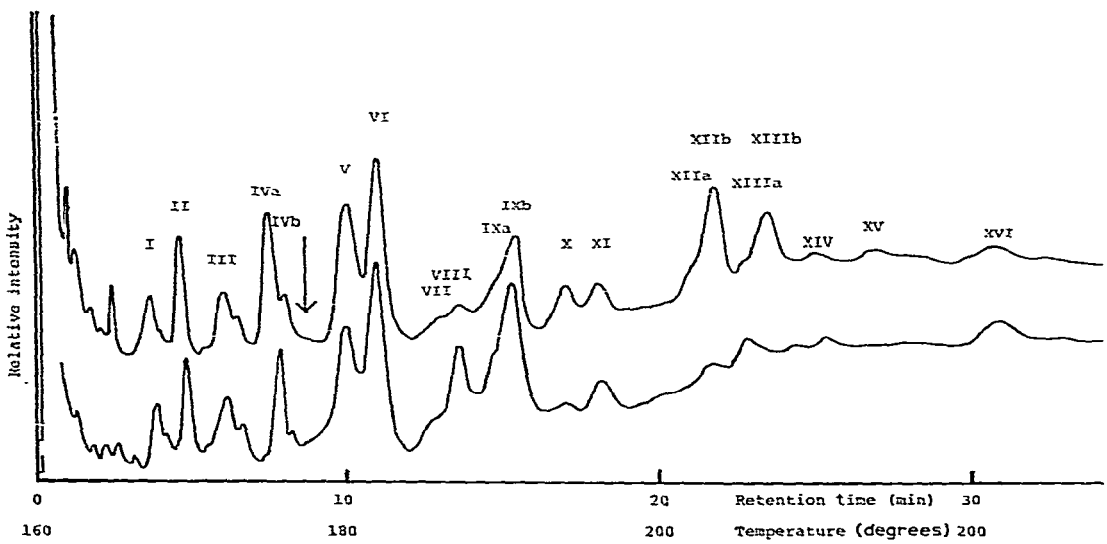


Fig. 1. G.l.c. patterns of (a) MR-P-WSPS (upper line) and (b) MMR-P-WSPS (lower line). [The temperature was raised from 160 to 200° at the rate of 2°/min. After reaching 200°, the temperature was kept there. A column (1.5 m) of 3% of ECNSS-M was used in the experiment. The arrow indicates the position of $R_T$ 1.00.]

TABLE I

SUMMARY OF THE RESULTS OF THE METHYLATION ANALYSIS OF P-WSPS

| *Peak*[b] | *Retention time*[a] | | | | *Fragment ion* (m/z) | | | | | | | | | | *Structure determined* | *Original sugar*[c] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *ECNSS-M* | | *OV-225* | | *45* | *117* | *131* | *161* | *175* | *189* | *203* | *205* | *233* | *261* | | |
| | *Found* | *Ref. 7* | *Found* | *Ref. 19* | | | | | | | | | | | | |
| I | 0.42 | 0.46 | 0.33 | 0.35 | | + | + | + | + | | | | | | 2,3,4-Me$_3$-Rha*p* | Rha*p* |
| II | 0.48 | 0.48 | 0.43 | 0.41 | + | + | | + | | | | | | | 2,3,5-Me$_3$-Ara*f* | Ara*f* |
| III | 0.68 | 0.73 | 0.56 | — | | + | | + | | | | | | | 2,3,4-Me$_3$-Ara*p* | Ara*p* |
| IVa | 0.90 | 0.92 | 0.79 | 0.86 | | | + | | | + | | | | | 3,4-Me$_2$-Rha*p* | Rha*p* |
| IVb | 0.99 | 0.98 | 0.86 | 0.87 | | + | | | | | + | | | | 2,3-Me$_2$-Rha*p* | Rha*p* |
| V | 1.20 | —<br>1.25 | 1.04 | 0.94<br>1.19 | + | + | + | + | | | + | + | | | 2,4-Me$_2$-Rha*p*<br>2,3,4,6-Me$_4$-Gal*p* | Rha*p*<br>Gal*p* |
| VI | 1.36 | 1.38 | 1.15 | 1.07 | | + | | | + | | | | | | 2,3-Me$_2$-Ara*p* | Ara*p* |
| VII | 1.86 | 1.94 | 1.64 | 1.67 | | | | | + | + | | | | | 3-Me-Rha*p* | Rha*p* |
| VIII | 2.24 | 2.28 | 1.90 | 2.03 | + | + | | + | | | | | + | | 2,4,6-Me$_3$-Gal*p* | Gal*p* |
| IX | 2.42 | 2.41 | 2.25 | 2.22 | + | + | | | | | | | + | | 2,3,6-Me$_3$-Gal*p* | Gal*p* |
| X | 3.05 | 3.41 | 2.80 | 2.89 | | | | | | | | | | | 2,3,4-Me$_3$-Gal*p* | Gal*p*A |
| XI | 3.40 | 3.64 | 3.15 | 3.14 | + | | | + | | | | | | + | 4,6-Me$_2$-Gal*p* | Gal*p* |
| XIIa | 4.89 | 4.35 | | | | | | | + | | | | + | | | |
| XIIb | 5.20 | 5.68 | | | | + | | | | | | | | + | 2,3-Me$_2$-Gal*p* | Gal*p*A |
| XIIIa | 5.90 | — | | | | | | | | | | | | | | |
| XIIIb | 6.30 | 6.35 | | | | + | | | | + | | | | | 2,4-Me$_2$-Gal*p* | Gal*p*A |
| XIV | 7.0 | 6.90 | | | | | | | | | | | | | 3,4-Me$_2$-Gal*p* | Gal*p* |
| XV | 8.3 | 8.10 | | | | + | | | | | | | | | 2-Me-Gal*p* | Gal*p*A |
| XVI | 10.6 | 11.1 | | | | | | | | + | | | | + | 3-Me-Gal*p* | Gal*p* |

[a]1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol: $R_T$ 1.00. [b]See Fig. 1. [c]Determined from the results of the analysis of MMR-P-WSPS.

fermented on incubation for 24 h. As *E. coli* ($C_N$ strain) ferments L-arabinose, but not D-arabinose, the arabinose had the L configuration.

Quantitative analysis by g.l.c. showed that P-WSPS consists of L-rhamnose, L-arabinose, D-galactose, and D-galacturonic acid in the ratios of 29.26:22.45:31.17:17.11 (% by weight).

Methylation analysis was performed on P-WSPS; as it was found difficult to methylate, three methylations were needed for complete methylation. The results of g.l.c. analysis of the methylated and reduced polysaccharide (MR-P-WSPS) are given in Fig. 1a. MR-P-WSPS gave a very complicated, g.l.c. pattern, and it was almost impossible to make assignment of each peak by relative retention-time ($R_T$) only. Therefore, the assignment of each peak was made by g.l.c.–m.s. as reported by Björndal *et al.*[7]. The results are summarized in Table I. From the rhamnose residues, the main product was the 3,4-di-*O*-methylrhamnopyranose derivative, but derivatives of 2,3,4-tri- and 2,3-di-*O*-methylrhamnose were also found. The results of the methylation analysis showed that rhamnose residues in the pyranoid form were built into the chain mainly with (1→2) linkages, and that considerable proportions of rhamnose residues were present at the nonreducing end and in (1→4) linkage. It is evident that there were also rhamnose residues at the branching points, in (1→2) and (1→4) linkage. Peak V contained two distinct components that were partially separated by chromatography at 150°. From the results of g.l.c.–m.s. at this temperature, it was concluded that one was the 2,4-di-*O*-methylrhamnose derivative and the other was the 2,3,4,6-tetra-*O*-methylgalactose derivative, in the ratio of ~1:3.

From the arabinose residues, the main product was the 2,3-di-*O*-methylarabinose derivative, showing that the arabinose residues in the polysaccharide chain were present in the pyranoid form with (1→4) linkages. The presence of considerable proportions of 2,3,5- and 2,3,4-tri-*O*-methylarabinose derivatives showed that the arabinose residues at the nonreducing ends were in both the pyranoid and furanoid forms.

It was presumed that the galacturonic acid residues were converted into partially methylated galactose derivatives by the reduction of the methylated P-WSPS with lithium aluminum hydride, but the derivatives obtained in relatively high proportions were the 2,3,4,6-tetra-, 2,3,6-tri-, 2,3-di, and 2,4-di-*O*-methylgalactose derivatives, and the 2,3,4-tri-*O*-methylgalactose derivative was present in considerable proportion. Remethylation of MR-P-WSPS (MMR-P-WSPS) caused complete disappearance of the 2,3- and 2,4-di-*O*-methylgalactose, and an increase in the proportions of the 2,3,6- and 2,4,6-tri-*O*-methylgalactose derivatives, showing that the galacturonic acid residues in P-WSPS were present in both (1→3) and (1→4) linkage. The results also showed that the galactose residues were mainly present in (1→4) linkage. The complete disappearance of the 2,3,4-tri-*O*-methylgalactose derivatives showed the presence of nonreducing (terminal) galactosyluronic acid groups. Relatively high proportions of 3-*O*- and 4,6- and 3,4-di-*O*-methylgalactose derivatives were present, and the sizes of these peaks did not change after remethylation, showing that the branching points on the galactose residues were (1→2, 1→3)-Gal, (1→2, 1→4, 1→6)-Gal, and (1→2,

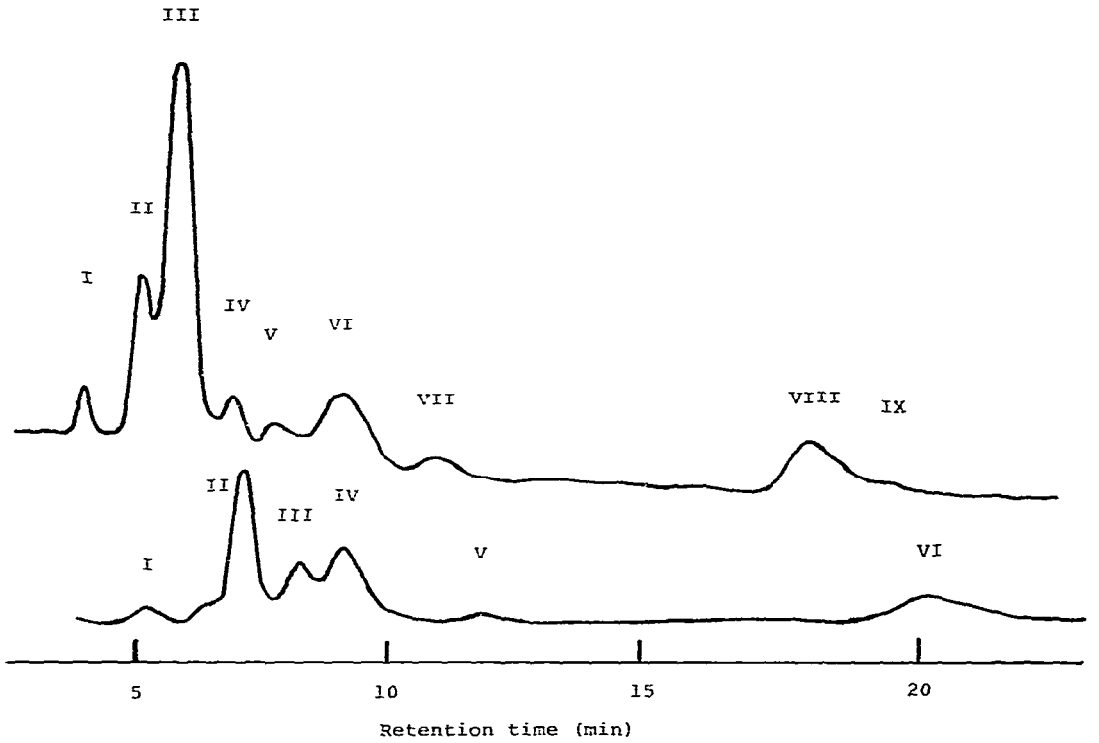


Fig. 2. G.l.c. patterns of methylated Fraction $E_5$ (upper line) and the methylation product of reduced Fraction $E_5$ in a column (1.5 m) of 5% of SE-30 at 210°.

1→6)-Gal. Additional peaks (XIIa and XIIIa) due to the branch points on galactose residues were also detected. The presence of only a small proportion of the 2-*O*-methylgalactose derivative showed that very few galacturonic acid residues were present in the branch points, and that the structures were (1→3, 1→4)-GalA.

These results showed that P-WSPS has an extremely unusual structure. It has many nonreducing-end sugars, including all four kinds of sugar, and relatively high proportions of various kinds of branching points, showing that P-WSPS has a highly branched structure, as shown in physicochemical studies[1], with the backbone structure containing mainly (1→2)-rhamnose, (1→4)-galactose, and (1→3 and 1→4)-galacturonic acid, and it is difficult to assume the presence of a simple repeating-unit. The positive value ($[\alpha]_D^{24.5}$ +94.2°) of the specific rotation of P-WSPS showed that the major parts are connected by α-D and β-L linkages.

In order to investigate the structure of P-WSPS further, the aldobiouronic acids fraction (Fraction $E_5$; ref. 8) of P-WSPS was methylated, complete methylation being achieved in one methylation, and the products were analyzed by g.l.c.–m.s. The results are given in Fig. 2 and Table II. Nine peaks of methylated aldobiouronic acids (peaks I–IX) were observed in g.l.c., and they were identified[9,10] as those of derivatives of the following aldobiouronic acids: D-Gal*p*A-(1→2)-L-Rha*p* (peaks II and

TABLE II

G.L.C.–M.S. ANALYSIS OF METHYLATED FRACTION $E_5$

| *Fraction* $E_5$ | | *Reduced fraction* $E_5$ | | *Oxidation*[b] | *Structure determined* |
|---|---|---|---|---|---|
| *Peak*[a] | *Fragment ion* (m/z) | *Peak*[a] | *Fragment ion* (m/z) | | |
| I | 175, 217, 219 | I | | + | non-sugar |
| II[c] | 59, 103, 161*w*[d], 201*b*[d], | II | 59, 103, 201*b*, 205, | − | α-GalA-(1→2)-Rha |
| III[c] | 233, 249, 319, 394 | II | 233, 319, 351, 363, 395 | | |
| IV | 161*w*, 169, 201b, 233, | III | not separated in | − | β-GalA-(1→2)-Ara |
| V | 249, 263, 319, 394 | III | a column of OV-1 | | |
| VI | 59*w*, 89, 203*b*, 233, 319, 379 | IV | | + | α-GalA-(1→4)-Rha |
| VII | 59, 89, 159, 201*b*, 233, 319, 394 | V | 59, 201*b*, 205, 233, 235, 249, 395 | + | α-GalA-(1→3)-Rha |
| VIII[e] | 159, 201*b*, 233, 289, | VI | 201, 233, 249*b*, 349, | − | α-GalA-(1→4)-Gal |
| IX[e] | 307, 331, 468 | | 425, 439, 484 | | |

[a]See Fig. 2. [b]Chromium trioxide oxidation. The symbol + means: resistant to oxidation. [c]See ref. 12. [d]The symbols *w* and *b* mean: weak and base peak, respectively. [e]See ref. 13.

III; **1**), D-Gal*p*A-(1→2)-L-Ara*p* (peaks IV and V; **2**), D-Gal*p*A-(1→4)-L-Rha*p* (VI: **3**) and D-Gal*p*A-(1→4)-D-Gal*p* (peaks VIII and IX; **4**). In the chromatogram of the permethylated, reduced Fraction $E_5$, peaks I and II, III and IV, and VII and VIII were converted into single peaks, showing that these pairs of peaks were those of anomeric pairs. James *et al.*[11] reported that sugar residues having equatorially oriented aglycons are readily oxidized by chromium trioxide to hexulosonic acids. The chromium trioxide oxidation of the permethylation product of Fraction $E_5$, which was reduced with sodium borohydride, revealed that only **2** had a β-linkage. Therefore, the oligosaccharides in Fraction $E_5$ were α-D-Gal*p*A-(1→2)-L-Rha*p* (**5**), β-D-Gal*p*A-(1→2)-L-Ara*p* (**6**), α-D-Gal*p*A-(1→4)-L-Rha*p* (**7**), and α-D-Gal*p*A(1→4)-D-Gal*p* (**8**). Of these aldobiouronic acids, the structures of **5** and **8** were confirmed by converting them into crystalline derivatives of the per-*O*-acetylated methyl esters of the methyl glycosides[12,13].

The intensity of peak VII was very small, and it was difficult to make identification, but the possibility of α-D-GalA-(1→3)-L-Rha for peak VII was indicated by m.s., retention time in g.l.c., and the results of chromium trioxide oxidation. In the depicted chromatogram in Fig. 1, the peak equivalent to this rhamnose residue (the 2,4-di-*O*-methylrhamnose residue) was not found, but this is reasonable in view of the low yield of Fraction $E_5$ (~8%).

The results of quantitative estimation of these peaks showed that galacturonic acid residues were present mainly combined to rhamnose by a (1→2) linkage, and considerable proportions of the galacturonic acid residues were in β-(1→2) and α-(1→4) linkage to arabinose and galactose residues, respectively.

## EXPERIMENTAL

*Extraction of water-soluble polysaccharide.* — Water-soluble polysaccharide was obtained from ground, commercial bark of *P. amurense* (1 kg, harvested in Miyazaki Pref., Japan) as previously described[8]; the yield was 51 g (~5%). The water-soluble polysaccharide obtained was purified by repeated precipitation with ethanol, and chromatography on a column of DEAE-cellulose as described[1]. The purified, water-soluble polysaccharide (P-WSPS) was essentially homogeneous, and had $[\alpha]_D^{24.5}$ +94.2° (*c* 0.149, water).

*Hydrolysis of P-WSPS.* — Complete hydrolysis was achieved at 1% concentration of P-WSPS by heating it in 0.5M sulfuric acid for 8 h at 100°. For partial hydrolysis, the reaction time was shortened to 5 h. After neutralization of the acid with barium carbonate, the precipitate was removed by filtration, and the filtrate was evaporated to a syrup.

*Paper chromatography.* — Paper chromatography was conducted on Whatman No. 3MM paper with the following solvent-systems: (*A*) 6:3:2 1-butanol–acetic acid–water and (*B*) 6:4:3 1-butanol–pyridine–water. Sugars were detected with alkaline silver nitrate reagent.

*Analysis of sugar composition.* — P-WSPS (10 mg) was hydrolyzed completely with 0.5M sulfuric acid (1 mL), the acid neutralized, the suspension filtered, and the filtrate evaporated to a syrup below 50°. The residue was dissolved in a small volume of water, and analyzed by paper chromatography. The sugar composition was also ascertained by g.l.c. according to the method of Perry and Hulyalkar[14] (consisting in reduction with sodium borohydride, and lactonization with concentrated hydrochloric acid). The g.l.c. analysis of the per(trimethylsilyl)ated derivatives was conducted in a column (1.5 m) of 5% of SE-30 on Chromosorb W at 180°.

*Determination of* D *or* L *configuration of component sugars.* — Polysaccharide P-WSPS (1 g) was hydrolyzed with M sulfuric acid for 12 h at 100°, the acid neutralized with barium carbonate, and the hydrolyzate placed on a column (2 × 30 cm) of Dowex-1 X8 (acetate) resin. Appropriate fractions were pooled, evaporated, placed on a column (3 × 40 cm) of cellulose, and eluted with water-saturated 1-butanol[15].

The rhamnose fractions were pooled and evaporated. The residue (162 mg) was treated with ethanethiol plus concentrated hydrochloric acid, and the product acetylated[8], to give pure, needle-like crystals (22 mg), identified as ethyl 2,3,4-tri-*O*-acetyl-1-thio-β-L-rhamnopyranoside by its optical rotation, $[\alpha]_D^{22.5}$ +58.1° (*c* 0.18, $CHCl_3$), and its i.r. and n.m.r. spectra.

The galactose fractions were evaporated, and the residue (134 mg) was converted into D-galactose phenylosazone (94 mg) as described[8]; m.p. 200.1–200.9°, $[\alpha]_D^{22}$ +1.03→+0.52° (24 h; *c* 0.2, methanol).

A portion of the arabinose fraction was added to a minimal-salts medium[16], inoculated with *Escherichia coli* $C_N$ (D-Ara-negative, L-Ara-positive), cultured for 24 h at 37°, and centrifuged at 6,000 r.p.m. for 10 min (to remove bacteria). The supernatant solution was analyzed by paper chromatography with solvent system *A*.

The acidic fraction was eluted with 0.5M acetic acid, the eluate evaporated (93 mg), and the residue converted into D-galacturonic acid phenylosazone (52 mg) as described[8]; m.p. 141.3–141.8°, $[\alpha]_D^{22}$ +32.2→+9.4° (24 h; *c* 0.41, methanol).

*Methylation analysis of P-WSPS.* — P-WSPS (500 mg) was stirred in dimethyl sulfoxide (40 mL) for 2 days at 50°; most of the P-WSPS had then dissolved, to give a cloudy suspension. Methylation was attempted by successive addition of 2.1M methylsulfinyl carbanion (10 mL) and methyl iodide[17] (3 mL), but was not complete, and so remethylation by the Kuhn method[18] was conducted. The solution of partially methylated P-WSPS was dialyzed against water, lyophilized, and the residue dissolved in *N,N*-dimethylformamide (50 mL), to give a clear, pale-yellow solution. Silver oxide (25 g) and methyl iodide (50 mL) were added, and the mixture was stirred overnight in the dark at room temperature, filtered, and the filtrate diluted with water (100 mL). The solids were dissolved by the addition of potassium cyanide solution, the solution was extracted with chloroform (50 mL), and the extract dried, and evaporated (430 mg). After three methylations, hydroxyl absorption was not observed in the i.r. spectrum. The completely methylated P-WSPS was dissolved in dry oxolane (50 mL) and reduced overnight with 1% lithium aluminum hydride–oxolane (150 mL), to afford MR-P-WSPS. Remethylated polysaccharide (MMR-P-WSPS, 395 mg) was obtained by addition of water, extraction with chloroform, evaporation of the extract, and remethylation with methylsulfinyl carbanion as before. The yield of MMR-P-WSPS was 282 mg.

Analysis of the methylation products was performed by the method of Björndal *et al.*[7]. Methylation products were hydrolyzed with 90% formic acid, and then with 0.5M sulfuric acid. The sugars in the hydrolyzates were converted into alditol acetates[8], and these were analyzed by g.l.c. on two columns (1.5 m): (*a*) 3% of ECNSS-M, and (*b*) 5% of OV-225, with a flow rate of 25 mL/min, at 180°. G.l.c.–mass spectra were obtained with a column of 5% of OV-1 at 170° at a chamber voltage of 20 eV and a chamber temperature of 180°.

The analysis of permethylated disaccharides was conducted by g.l.c. in a column (1.5 m) of 5% of SE-30. G.l.c.–mass spectra were obtained under the same conditions as before, except that the column temperature was 190°.

*Oxidation with chromium trioxide.* — Methylated oligosaccharides (10 mg) and chromium trioxide (5 mg) in glacial acetic acid (1 mL) were stirred for 1 h at 50° (the method of Hoffman *et al.*[11]). The reaction was stopped by the addition of water. Oligosaccharide derivatives were extracted with chloroform, and analyzed by g.l.c. in a column (1.5 m) of 5% of SE-30 as before.

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