

Studies on Arabogalactans

IV. A Methylation Study of Arabogalactan B from *Larix occidentalis* Nutt.

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Arabogalactan B, the lower molecular weight arabogalactan from Western larch (*Larix occidentalis*), was methylated and hydrolysed, giving 2-*O*-methyl-D-galactose, 4-*O*-methyl-D-galactose, 2,4-di-*O*-methyl-D-galactose, 2,6-di-*O*-methyl-D-galactose, an unknown methyl-galactose, 2,3,4-tri-*O*-methyl-D-galactose, 2,4,6-tri-*O*-methyl-D-galactose, 2,3,4,6-tetra-*O*-methyl-D-galactose, 2-*O*-methyl-L-arabinose, 2,3-di-*O*-methyl-L-arabinose, 2,5-di-*O*-methyl-L-arabinose, 2,3,4-tri-*O*-methyl-L-arabinose and 2,3,5-tri-*O*-methyl-L-arabinose.

The arabogalactans found in a number of species of *Larix* have been shown to consist of two main fractions (A and B)^{1,2}. B, which has a lower degree of polymerisation, usually forms about 5–10 % of the total arabogalactan fraction but in *L. occidentalis* Nutt. and *L. Lyalli* Parl. it is about 30 % of the arabogalactan. The isolation of arabogalactan B on a preparative scale from the arabogalactan of *L. occidentalis* was described in part II of this series¹. The polysaccharide was obtained as a white powder, $[\alpha]_D^{20} +10^\circ$, containing 21 % (molar) arabinose. It appeared from the results of paper electrophoresis and sedimentation analysis to be homogeneous.

Arabogalactan B was swollen in dimethyl formamide and then methylated with methyl iodide and silver oxide³ to yield a methylated polysaccharide containing 35.6 % methoxyl. A second methylation raised the methoxyl content to 42.6 % but four more methylations in the same way raised the methoxyl content only to 43.0 %, the theoretical value being 44.6 %. The formation of a precipitate of silver halogenide on hydrolysis of the methylated polysaccharide indicated that it was contaminated with complex silver salts formed during the methylation⁴. Careful examination of a hydrolysate of the methylated polysaccharide showed that unmethylated monomer was absent, which is a good criterion of exhaustive methylation.

The methylated polysaccharide was hydrolysed and the mixture of methyl ethers obtained was resolved on a carbon-Celite column. The amount of reduc-

ing sugar in each fraction was determined by hypiodite oxidation and the mixed fractions were further separated on filter papers both for preparative purposes and for quantitative estimation of the components present. The results are summarised in Table 1.

Table 1. Methylated sugars obtained from 2.0 g of methylated arabogalactan B.

Methyl ether	mmoles	molar %	M_G value
Unmethylated monomers	—	<0.1	
4-Methylgalactose	0.03	0.3	0.30
2-Methylgalactose	0.20	2.3	0.43
2-Methylarabinose	0.05	0.6	0.34
2,4-Dimethylgalactose	2.65	31.0	<0.05
2,6-Dimethylgalactose	0.33	3.9	0.38
2,3-Dimethylarabinose	0.04	0.5	<0.05
Unknown methylgalactose	0.06	0.7	0.30
2,3,4-Trimethylgalactose	1.01	11.8	
2,3,4-Trimethylarabinose	0.44	5.1	
2,5-Dimethylarabinose	0.69	8.1	
2,4,6-Trimethylgalactose	0.34	4.0	
2,3,4,6-Tetramethylgalactose	2.06	24.1	
2,3,5-Trimethylarabinose	0.65	7.6	

The appearance of 2-*O*-methyl-L-arabinose and 2,3-di-*O*-methyl-L-arabinose in approximately equimolecular quantities can be explained as due to the presence of about 1.7 % of an araban as an impurity in the original polysaccharide⁵. As arabofuranosidic residues should not form complexes with borate ions⁶ such an araban would not be precipitated in the fractionation with cetyltrimethylammonium hydroxide—boric acid. It should therefore be found only in the unprecipitated fraction, which is in agreement with the total absence of the corresponding ethers in the hydrolysate of methylated arabogalactan A¹.

The isolation of 2-*O*-methyl-D-galactose and 2,6-di-*O*-methyl-D-galactose from hydrolysates of the methylated derivatives of arabogalactan A and of the galactans obtained on mild hydrolysis of arabogalactan A was reported in parts II¹ and III² of this series. The argument presented there on their structural significance must also be valid in the present case. The possibility that these ethers originate from a branched galactan built up of 1,4-linked units cannot be disregarded. However they constitute 4,6—6,6 mole % of the hydrolysates which would correspond to at least 10 % of such a galactan in the original polysaccharide. Such a quantity would not easily have been overlooked in the fractionation and electrophoresis and it therefore seems more probable that these ethers are derived from the arabogalactans and not from a contaminating galactan. The isolation of 2-*O*-methyl-D-galactose from methylated European larch arabogalactan⁷ and of 2,6-di-*O*-methyl-D-galactose from the arabogalactans of White spruce⁸ and Jack pine⁹ suggests that a small proportion of 1,4-linkages is normally present in the arabogalactans from coniferous woods.

A comparison of the present results with those obtained on the methylated arabogalactan A shows that the chains of galactose residues forming the backbones of the two polysaccharides are very similar both in the types of linkage present and in their proportions. However, in making such a comparison the possibility must be born in mind that A may be heterogeneous. The amount of end groups as calculated from methylation data is 34.8 and 36.8 % for A and B, respectively. However, due to various deficiencies in the methods used these figures will be too low, especially in the case of A. Estimation of the amount of endgroups from the amount of branching points gives values of 42.3 and 39.0 % respectively. There is a higher proportion of 3-*O*- β -L-arabopyranosyl-L-arabofuranose units in fraction B than in A. The excess of 2,5-di-*O*-methyl-L-arabinose relative to 2,3,4-tri-*O*-methyl-L-arabinose shows that some arabinose must be present in 3-*O*-L-arabofuranosyl-L-arabofuranose residues. The existence of 3-*O*-L-arabofuranosyl-3- β -L-arabopyranosyl-L-arabofuranose is less probable as no 2,4-di-*O*-methyl-L-arabinose was found in the hydrolysate.

Tests, made by courtesy of Dr. M. Heidelberger, State University, New Jersey, USA, of the reactivity of the arabogalactans towards Type XIV antipneumococcal horse serum, showed differences between the two polysaccharides. Arabogalactan A not only reacted faster than B but precipitated considerably more antibody. It seems unlikely that the slight differences between A and B indicated by the methylation data are sufficient to account for the differences in their reactivity towards antipneumococcal serum and in their rate of migration on electrophoresis (M_G 0.76 for A and 0.63 for B). It is more probable that the two molecules are built up in a different manner with concomitant differences in the accessibility of those groups which are normally involved in the formation of borate complexes and of complexes with antipneumococcal serum (*cf.* Ref. ¹⁰).

Some reference should be made to possible deficiencies in the methods used to obtain the methylation data given in Parts II, III and IV of this series. As was pointed out in Part II estimates of the number of endgroups present from the yield of tetramethylgalactose and trimethylarabinose give lower values than those required by estimates of the numbers of branching points from the yield of monomethyl and dimethyl galactose. This is partly due to the slight volatility of the tetramethylgalactose and the trimethylarabinose. This discrepancy was greatly diminished by chloroform extraction of the distillates obtained on concentration of solutions of these highly methylated ethers and returning the recovered material to the ether mixture. The quantitative estimation of difficultly separated substances after fractionation on thick filter paper is of course subject to some inaccuracy. This was especially the case in the estimation of 2,6-di-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-galactose, 2,5-di-*O*-methyl-L-arabinose and 2,3,4-tri-*O*-methyl-L-arabinose but since the relative error probably lies within the range 5–10 %, this inaccuracy should not seriously affect the results.

It appears from the analytical data (*cf.* Table 4, Part II ¹ and Table 2 in the present paper) that some trailing occurred in the elution from the carbon column. It was possible to take this trailing into account only in the case of the most abundant components as the material trailing was only a few percent

of the total present in each case. In the investigation of degraded arabogalactan A² when a different batch of activated carbon was used in the column, practically no trailing occurred.

It was found that the mild hydrolysis of larch arabogalactan, in addition to removing the arabofuranose residues also splits off large amounts of galactose and 6-*O*- β -D-galactopyranosyl-D-galactose at the same time¹². It was assumed that these units were originally attached to the rest of the molecule through furanosidic linkages. That galactofuranose units are present in larch arabogalactan has already been suggested by Jones¹³. Normal paper chromatographic methods proved inadequate for the separation of the expected 2,3,5-tri-*O*-methyl-D-galactose from the accompanying 2,3,4,6-tetra-*O*-methyl-D-galactose and of 2,3,5,6-tetra-*O*-methyl-D-galactose from 2,3,5-tri-*O*-methyl-L-arabinose. However the chromatographic technique developed by Wickberg¹⁴ using paper impregnated with dimethyl sulphoxide as stationary phase and ether or benzene as mobile phase gave an excellent separation of these methyl ethers. This showed clearly that no methylated galactofuranoses were present in the hydrolysates from the methylated arabogalactans A and B.

The easy release of these galactose residues is unexpected. Estimation of the amounts of oligosaccharides formed on hydrolysis of unfractionated arabogalactan A in 0.01 N hydrochloric acid at 100°C¹⁵ showed that after 8 h about 80 % of the arabinose is split off and that the oligosaccharide part of the hydrolysate contains 55 % arabinose and 3- β -arabopyranosyl-arabinose, 35–40 % galactose and 6- β -galactopyranosyl-galactose and 5–10 % of larger fragments containing galactose. Detectable amounts of 3- β -galactopyranosyl-galactose were found only after 10 h. This disaccharide is usually present in mixtures of oligosaccharides obtained on stronger hydrolysis of the arabogalactan^{7,15}. These results indicate that a part of the galactopyranosidic linkages is hydrolysed with about the same rate as the arabofuranosidic linkages. Hydrolysis at those linkages leads to the loss of about 15 % of the galactose originally present.

A comparison of these results with those obtained from methylation studies on arabogalactans from *Larix decidua*⁷ and from *Pinus*^{9,16} and *Picea*⁸ shows that the *Pinus* and *Picea* arabogalactans are of a different, less branched type with the arabinose present as single, terminal furanose residues. The arabogalactans from *Larix decidua* are apparently very similar in structure to those from *L. occidentalis* except for a slightly different arrangement of the arabinose residues.

EXPERIMENTAL

All melting points are corrected. All evaporations were done under reduced pressure. *Chromatography*. Papers: Whatman No. 1 and 3 MM, Schleicher and Schüll 602 hP. Solvent: Butanol, ethanol, water, 10:3:5. Spray reagent: anisidine hydrochloride.

Methylation of arabogalactan B. Arabogalactan B ($[\alpha]_D^{20} + 10^\circ$ (c, 1.0 in water), 21 mole-% arabinose) was prepared as described in Part II. The polysaccharide (4.72 g) was swollen in dry dimethyl formamide (50 ml) overnight, silver oxide was then added and the mixture was stirred vigorously to give a suspension. Dry methyl iodide (25 ml) was distilled into the reaction flask in small portions over a period of 3 h and further silver oxide (14 g) was added during the same time in 2 g portions. The mixture was stirred for a further 15 h at room temperature and then worked up as described in Part II. The product

(5.30 g; OCH₃, 35.6 %) was then methylated five times in the normal way but the methoxyl content could not be raised above 43 % (theoretical value 44.6 %).

Fractionation of the hydrolysate of methylated arabogalactan B. Methylated arabogalactan B (2.0 g) was heated with formic acid (100 ml) at 100°C for 6 h. The solution was concentrated and the product was dissolved in aqueous 0.5 N hydrochloric acid (100 ml) and kept at 100°C for 14 h. This solution was then neutralised by filtering through an ion exchange column (IR 4-B), and concentrated to small volume. The aqueous distillate from this concentration was extracted three times with chloroform and the minute residue obtained on evaporation was dissolved in a few drops of water and combined with the concentrated hydrolysate. The same procedure was always used in the concentration of solutions containing free, highly methylated ethers.

The hydrolysate was adsorbed on a carbon-Celite column (3.5 × 43 cm) and separated by gradient elution with 5.5 l 6 – 24 % aqueous ethanol followed by 1 l 50 % acetone. 20 ml fractions were collected.

The results of the fractionation are summarised in Table 2. The amount of reducing sugar in each of the combined fractions was determined by hypoiodite oxidation¹². The mixed fractions were further separated by chromatography of 10–15 mg samples on Schleicher and Schüll 602 hP paper for quantitative estimation¹ and by chromatography of the remainder for preparative purposes on Schleicher and Schüll 602 hP or Whatman 3 MM papers.

Table 2. Fractionation of methylated hydrolysed arabogalactan B.

Fract.	mmoles (tot.)	Sugars	%	mmoles
25–27	<0.010	Arabinose Galactose		
28–40	0.280	4-Methylgalactose 2-Methylgalactose 2-Methylarabinose	10 73 17	0.028 0.204 0.048
50–81	2.52	2,4-Dimethylgalactose		
82–91	0.120	2,4-Dimethylgalactose 2,3-Dimethylarabinose	67 33	0.08 0.040
92–106	0.370	2,4-Dimethylgalactose 2,6-Dimethylgalactose Unknown galactose ether	11 73 16	0.041 0.270 0.059
107–119	0.715	2,4-Dimethylgalactose 2,6-Dimethylgalactose 2,3,4-Trimethylgalactose 2,3,4-Trimethylarabinose	2 8 58 32	0.014 0.057 0.415 0.229
120–136	0.876	2,3,4-Trimethylgalactose 2,3,4-Trimethylarabinose 2,5-Dimethylarabinose	55 24 21	0.482 0.210 0.184
137–179	0.613	2,3,4-Trimethylgalactose 2,5-Dimethylarabinose	18 82	0.110 0.503
180–246	0.344	2,4,6-Trimethylgalactose		
Residue	2.71	2,3,4,6-Tetramethylgalactose 2,3,5-Trimethylarabinose	76 24	2.06 0.65

Characterisation of the methyl ethers. 4-O-Methyl-D-galactose. The ether was separated from the accompanying 2-O-methyl-D-galactose and 2-O-methyl-L-arabinose on Schleicher and Schüll 602 hP papers. The small quantity obtained (about 3 mg) was crystallised from ethanol, m. p. 195° and mixed m. p. 195–205°. It was chromatographically and electrophoretically indistinguishable from an authentic specimen of 4-O-methyl-D-galactose.

2-O-Methyl-D-galactose. The ether was recrystallised from ethanol, m. p. and mixed m. p. 150–152°, chromatographically and electrophoretically indistinguishable from authentic 2-O-methyl-D-galactose.

2-O-Methyl-L-arabinose. This ether gave only arabinose on demethylation and travelled on chromatograms at about 90 % of the rate of 2,4-di-O-methyl-D-galactose. On electrophoresis it migrated faster (M_G 0.34) than 4-O-methyl-D-galactose (M_G 0.30) but slower than 2-O-methyl-D-galactose (M_G 0.43). These characteristics make a 2-O-methyl-L-arabinose structure very probable.

2,4-Di-O-methyl-D-galactose. The ether was recrystallised from chloroform-moist ethanol, m. p. 97°, sintering at 85°; aniline derivative, m. p. 210–212° undepressed on admixture with an authentic specimen of 2,4-di-O-methyl-D-galactosyl-N-phenylamine.

2,3-Di-O-methyl-L-arabinose. The ether was obtained by separation of fractions 82–91 on thick filter paper. It gave arabinose only on demethylation and travelled slightly faster than 2,4-di-O-methyl-L-arabinose on chromatography. It was immobile on electrophoresis and therefore a 2,3-di-O-methyl-L-arabinose structure is the most probable.

2,6-Di-O-methyl-D-galactose. The ether was separated from the accompanying unknown methylgalactose and 2,4-di-O-methyl-D-galactose by fractionation of fractions 92–106 on thick filter paper. M. p. and mixed m. p. 116–118°. It was chromatographically and electrophoretically (M_G 0.38) indistinguishable from authentic 2,6-di-O-methyl-D-galactose.

Unknown methylgalactose. This ether, probably an artifact, gave galactose on demethylation and on chromatography moved about 20 % faster than 2,4-di-O-methyl-D-galactose. It had M_G 0.30 which shows that either the 1,2- or the 3,4-glycol grouping was unsubstituted¹⁸. Of the possible ethers with this structural feature 3,4-di-O-methyl-D-galactose would probably be more easily desorbed from the column. This indicates the alternatives 4,6-di- or 3,4,6-tri-O-methyl-D-galactose, the latter being in best agreement with the high R_F value.

2,3,4-Tri-O-methyl-D-galactose. This ether and the next two were obtained from fract. 107–179 by fractionation on thick filter paper. It was characterised as the aniline derivative, m. p. 170–172°, undepressed on admixture with an authentic specimen of 2,3,4-tri-O-methyl-D-galactosyl-N-phenylamine.

2,3,4-Tri-O-methyl-L-arabinose. The ether was converted to the phenylhydrazide of the corresponding acid which was recrystallised from ethyl acetate-isopropyl-ether, m. p. 159–161°, undepressed on admixture with an authentic specimen of 2,3,4-tri-O-methyl-L-arabonic acid N-phenylhydrazide.

2,5-Di-O-methyl-L-arabinose. The ether was converted to the amide of the corresponding acid. After recrystallisation from ethyl acetate it had m. p. 133–134° in good agreement with values previously reported¹⁹.

2,4,6-Tri-O-methyl-D-galactose. The ether was converted to the aniline derivative, m. p. 181–182°, undepressed on admixture with an authentic specimen of 2,4,6-tri-O-methyl-D-galactosyl-N-phenylamine.

2,3,4,6-Tetra-O-methyl-D-galactose and 2,3,5-tri-O-methyl-L-arabinose. After removal of a suitable amount for quantitative estimation and chromatography, the fraction containing these ethers (550 mg) was boiled with aniline in ethanol. Crystalline 2,3,4,6-tetra-O-methyl-D-galactosyl-N-phenylamine (250 mg), m. p. and mixed m. p. 197–198° separated from the reaction mixture. The non-crystalline portion was heated with aqueous 0.5 N sulphuric acid (10 ml) for 3 h, neutralised and concentrated to dryness. After removal of a small quantity for chromatography, the remainder was fractionated on a small cellulose column which had previously been impregnated with the lower phase of the *n*-butanol-water system and then washed with the upper phase which was also used as eluant. The top fraction afforded pure 2,3,5-tri-O-methyl-L-arabinose which was converted to the amide of the corresponding acid, m. p. 138–139°, in good agreement with values previously reported¹⁹.

Chromatography on paper impregnated with dimethyl sulphoxide (DMSO). For an attempted detection of furanosidic galactose methyl ethers, fractions 120–136, 137–179, 180–245 and the residue were chromatographed according to the technique described by Wickberg¹⁴. 20 % DMSO in toluene was used for impregnation of the papers. Ethyl ether-DMSO 24:1 was used as mobile phase for tetramethylgalactose and trimethylarabinose ethers and benzene-DMSO 20:1 for trimethylgalactose and dimethylarabinose ethers. The following R_F -values were obtained when ether was used as mobile phase: 2,3,5,6-tetra-*O*-methyl-*D*-galactose 0.72, 2,3,5-tri-*O*-methyl-*L*-arabinose 0.64, and 2,3,4,6-tetra-*O*-methyl-*D*-galactose (two spots with trailing) 0.52 and 0.47. When benzene was used as mobile phase the chromatograms were run for 2.5 h giving the following migration lengths: 2,3,4-tri-*O*-methyl-*D*-galactose 2.2 cm, 2,4,6-tri-*O*-methyl-*D*-galactose 3.5 cm, 2,3,5-tri-*O*-methyl-*D*-galactose 4.4 cm, 2,5-di-*O*-methyl-*L*-arabinose 3.0 cm, and 2,3,4-tri-*O*-methyl-*L*-arabinose 10 cm. No furanosidic methylgalactoses were found in the fractions investigated.

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