Structure of a New Acid-polysaccharide from the Bark of *Pterospermum acerifolium*

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A new acid-polysaccharide isolated from the bark of *Pterospermum acerifolium* N.O. Sterculiaceae has been shown to be composed of residues of D-galacturonic acid, D-galactose, and L-rhamnose in the molar ratio 5 : 3 : 3, repectively. Partial acid hydrolysis gave three oligosaccharides, 2-*O*-(α-D-galactopyranosyl uronic acid)-L-rhamnose, 4-*O*-(α-D-galactopyranosyl uronic acid)-D-galacturonic acid, and *O*-D-galactopyranosyl uronic acid-(1 → 2)-*O*-L-rhamnopyranosyl-(1 → 4)-D-galactose. The various glycosidic linkages have been determined by the products from hydrolysis of the methylated polysaccharide and confirmed by periodate oxidation of the polysaccharide.

INSPECTION of the chemical literature reveals that no significant work on the medicinally important plant *Pterospermum acerifolium* has been done except for the isolation of a few flavonoids from its flowers [1] and bark.[2] A few amino acids in the trunk bark [3] and malvalic acid and the major cyclopropenoid components in the seed oil [4] have also been reported together with a phytochemical study of the flowers.[5] The bark, being rich in mucilage, has now been chosen for a study of its mucilagenous content.

The bark was collected locally by the Department of Botany at the University of Allahabad. The polysaccharide was isolated by extraction with 1% acetic acid followed by precipitation with ethanol. It was purified by dissolution in 4% sodium hydroxide followed by precipitation from acidified solution. The purified polysaccharide, in contrast to the crude product, was readily soluble in water. Its homogeneity was shown by fractional precipitation and zone electrophoresis. Effervescence occurred upon treatment with sodium hydrogencarbonate showing the acidic nature of the polysaccharide.

The purified polysaccharide, $[\alpha]_D^{27}$ +108°, did not undergo autohydrolysis, thus showing the absence of labile sugar residues. It was completely hydrolysed only with 2N-sulphuric acid resulting in the liberation of D-galacturonic acid, D-galactose, and L-rhamnose and in a decrease in specific rotation from +108° to +55° indicating that the linkages are mostly of the α-D-type. The sugars were identified by paper chromatography and other tests. On quantitative estimation D-galacturonic acid (48.92%), D-galactose (26.86%), and L-rhamnose (24.18%) (calculated as the anhydro units originally present in the polysaccharide) were found in a ratio of 5 : 3 : 3, respectively. Graded hydrolysis of the polysaccharide with 0.5N-sulphuric acid showed that galactose was liberated first followed by a trace of rhamnose and an oligosaccharide. This can be explained by showing that galactose and an oligosaccharide are present as end-groups in the side chain of the polysaccharide.

The polysaccharide was exhaustively methylated with dimethyl sulphate and sodium hydroxide followed by methyl iodide and silver oxide. The fully methylated polysaccharide, $[\alpha]_D^{28}$ +58.2°, was also resistant to hydrolysis, similar to the original polysaccharide, showing a behaviour that may be related to the presence of uronic acid units in pyranose forms. Complete hydrolysis with 2N-sulphuric acid yielded a mixture of neutral and acidic methylated sugars which were separated by treatment with ether. The neutral methylated sugars were separated by paper chromatography into 3,4-di-*O*-methyl-L-rhamnose and 2,3,6-tri-*O*-methyl- and 2,3,4,6-tetra-*O*-methyl-D-galactose in the molar ratio 3 : 1 : 2, respectively. These sugars were further characterised by their specific rotations, melting points, and derivatives. The acidic methylated sugars were separated into 2-*O*-methyl-, 2,3-di-*O*-methyl-, and 2,3,4-tri-*O*-methyl-D-galacturonic acids, confirmed by reduction of their methyl ester methyl glycosides with sodium borohydride into 2-*O*-methyl-, 2,3-di-*O*-methyl-, and 2,3,4-tri-*O*-methyl-D-galactoses in the ratio 3 : 1 : 1, respectively.

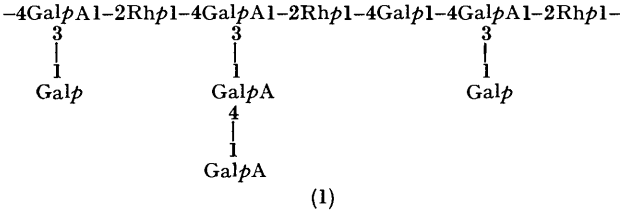
End-group analysis by periodate oxidation showed that three moles of formic acid were liberated, together with the consumption of eleven moles of periodate, per average repeating unit of eleven sugar residues. Considering the linkages found by methylation analysis, the liberation of three moles of formic acid with the consumption of six moles of periodate indicates the presence of three non-reducing terminal sugar residues. Therefore, it follows that five moles of periodate are consumed by five sugar residues without the production of formic acid. This accounts for the cleavage of eight sugar residues per equivalent of the polysaccharide and hence the remaining three sugar residues, being stable to periodate oxidation, must be highly branched.

On hydrolysis the oxidised polysaccharide gave only D-galacturonic acid, indicating the presence of uronic acid units as branch points in the main chain of the polysaccharide. However, on prolonged oxidation, the amount of liberated formic acid increased slowly, due to over-oxidation, suggesting the presence of at least one unit of uronic acid as the non-reducing terminal group (according to methylation results there are no reducing end groups).[6] These results are found to be in close agreement with those obtained from methylation studies.

Partial hydrolysis of the polysaccharide with N-sulphuric acid for six hours afforded a mixture of an aldobiouronic acid, 2-*O*-(α-D-galactopyranosyl uronic

acid)-L-rhamnose, a diuronic acid, 4-*O*-(α-D-galactopyranosyl uronic acid)-D-galacturonic acid, and an aldotriouronic acid, *O*-D-galactopyranosyl uronic acid-(1 → 2)-*O*-L-rhamnopyranosyl-(1 → 4)-D-galactose. The structures were elucidated by hydrolysis, methylation, reduction, and remethylation followed by hydrolysis and quantitative estimation of the products. Further confirmation was afforded by their specific rotations and melting points.* The fully methylated derivative of aldotriouronic acid, on reduction and hydrolysis, gave 2,3,4- and 2,3,6-tri-*O*-methyl-D-galactose and 3,4-di-*O*-methyl-L-rhamnose in equimolar amounts. 2,3,4-Tri-*O*-methyl-D-galactose must have been formed from the uronic acid component, namely 2,3,4-tri-*O*-methyl-D-galacturonic acid and should be at the non-reducing end of the trisaccharide. On periodate oxidation, one mole of the trisaccharide consumed five moles of periodate with the liberation of two moles of formic acid indicating the galactose to be at the reducing end. For rhamnose to be at the reducing end, four moles of periodate should have been consumed with the liberation of one mole of formic acid. This was also shown as only rhamnose was detected after oxidation of the trisaccharide with bromine water followed by hydrolysis. These aldouronic acids have been previously isolated from *Cochlospermum gossypium*,[7] *Khaya grandifolia*,[8] flax [9] seed, and okra [10] mucilage.

On partial methanolysis [11] the fully methylated polysaccharide liberated an oligosaccharide identified as 4-*O*-(2,3,4-tri-*O*-methyl-α-D-galactopyranosyl uronic acid)-2,3-di-*O*-methyl-D-galacturonic acid on the basis of its hydrolysis products. The oligossacharide must have arisen from the second aldobiouronic acid, *i.e.* 4-*O*-(α-D-galactopyranosyl uronic acid)-D-galacturonic acid, and must be present in the side chain of the polysaccharide as it is linked only through position 1. Therefore,



(1)

the uronic acid, which is present as the non-reducing group, is not directly linked, but is rather linked to the main chain through another uronic acid residue. On the basis of the observations and studies made so far, the probable structure (1) for a repeating unit of the polysaccharide is proposed, where Gal*p*A is D-galactopyranosyl uronic acid, Gal*p* is D-galactopyranose, and Rh*p* is L-rhamnopyranose.

Although no unique structure can be put forward for the polysaccharide, the above structure explains almost all of its physical and chemical properties. The structure resembles those of *Sterculia setigera* [12] gum and tragacanth gum in the respect that the branching occurs at uronic acid units. It also bears a similarity to the gums of *Sterculiaceae* family; these are characterised by a high proportion of D-galacturonic acid and by the presence of residues of L-rhamnose and D-galactose.[7,12]

* The glycosidic linkages were assigned to be α due to the high positive specific rotations.

EXPERIMENTAL

Specific rotations are equilibrium values. Paper chromatography was carried out by descending technique on Whatman No. 1 filter paper using the non-aqueous phase of the following solvent systems: A, n-butyl alcohol–isopropyl alcohol–water (11 : 6 : 3); B, n-butyl alcohol–acetic acid–water (4 : 1 : 5); C, n-butyl alcohol–ethanol–water (4 : 1 : 5); D, benzene–ethanol–water (169 : 47 : 15), and E, n-butyl alcohol–acetic acid–water (8 : 2 : 5). Aniline hydrogen phthalate was used as spraying reagent.

Isolation of the Polysaccharide.—The defatted bark (1 kg) was extracted with ethanol, dried, and then extracted with 1% aqueous acetic acid by stirring mechanically. The procedure was repeated four times and the combined extracts were poured with continuous stirring into ethanol (4 vols.). The fibrous precipitate was redissolved in 1% acetic acid, filtered, and precipitated with ethanol. The procedure was repeated six times when a pink crude product (36.8 g) was obtained.

Purification of the Polysaccharide.—The crude product was dissolved with stirring in 4% aqueous sodium hydroxide and the solution filtered. The faintly coloured viscous filtrate was chilled and acidified by careful addition of concentrated hydrochloric acid. The pale yellow mobile solution so obtained was poured, with stirring, into absolute ethanol (4 vols.). The fibrous precipitate was decanted off leaving the precipitated sodium chloride at the bottom. The precipitate was immediately dissolved in 20% aqueous ethanol and the polysaccharide was reprecipitated with absolute ethanol. The procedure was repeated till the filtrate gave negative test for chloride ions. The polysaccharide was dried *in vacuo* to a white amorphous powder (31.2 g), $[\alpha]_D^{27}$ +108° (*c*, 0.5 in water); ash 0.08%; N, S, halogen, nil; acetyl, methoxy, nil; equivalent wt. 361 (by direct titration).

Homogeneity of the Polysaccharide.—The homogeneity of the polysaccharide was checked by the following methods.

(a) *Fractional precipitation.* The polysaccharide (2 g) was dissolved in the minimum quantity of distilled water and poured into ethanol (100 ml). The precipitate was washed with absolute ethanol followed by ether and dried (Sample I). The filtrate, on treatment with ethanol (200 ml), gave Sample II and similarly the remaining filtrate with ethanol (300 ml) gave Sample III. Paper chromatography of the hydrolysates of each sample showed identical spots in the same molar ratio (see later) with those given by the hydrolysate of the original polysaccharide.

(b) *Zone electrophoresis.*[13] This was carried out on Whatman No. 1 filter paper using borate buffer solution (0.05M-sodium tetraborate, pH 9.2) at 320 V and 3.7 mA for *ca.* 6 h. The paper was cut into equal segments and eluted with water. To each eluate (5 ml), aqueous phenol (1 ml; 8.5%) and concentrated sulphuric acid (10 ml) was added. The mixture was allowed to cool to room temperature and the absorbance of each was measured in a Klett–Summerson Photoelectric colorimeter using filter no. 50. The absorbance of a blank was also measured. The corrected Klett reading, when plotted against the segment numbers, showed a single sharp peak.

Complete Hydrolysis of the Polysaccharide.—The polysaccharide (20 mg) was refluxed with 2N-sulphuric acid (20 ml) at 100 °C for 24 h. The hydrolysate was neutralised with barium carbonate and treated with Amberlite resin IR-120 and filtered. The filtrate on paper chromatography in solvents A and B showed three spots of D-galacturonic acid, D-galactose, and L-rhamnose, identified by co-chromatography with authentic samples. D-Galacturonic acid was also confirmed by the brick red colour [14] given with basic lead acetate and by oxidation to mucic acid, m.p. 213°.

Quantitative Estimation of Sugars.—The uronide content (48.92%) was determined by decarboxylation of the polysaccharide with 12% hydrochloric acid by the method of Dickson *et al.*[15] The neutral sugars, galactose (26.86%) and rhamnose (24.18%), were separated by paper chromatography in solvent A for 40 h and were estimated as anhydro sugar units originally present in the polysaccharide by periodate oxidation by the method of Hirst and Jones.[16]

Graded Hydrolysis of the Polysaccharide.—The polysaccharide (20 mg) was hydrolysed with 0.5N-sulphuric acid at 100 °C under reflux. Samples of the hydrolysate (0.1 ml) were removed at fixed intervals and analysed by paper chromatography against authentic samples. After ½ h a spot corresponding to galactose appeared followed by, after 3 hours, a faint spot due to rhamnose. After 4 h a spot near the base-line appeared, which was later identified as the fraction I of partial hydrolysis.

Methylation of the Polysaccharide.—The polysaccharide (8 g) was fully methylated four times using dimethyl sulphate and sodium hydroxide by the method of Parikh *et al.*[17] followed by four times using methyl iodide and silver oxide by Purdie's method.[18] The fully methylated polysaccharide was a pale yellow solid (5.6 g), $[\alpha]_D^{25}$ +58.2° (*c*, 1.32 in chloroform); OMe, 35.2%.

The methylated polysaccharide was hydrolysed with 2N-sulphuric acid at 100 °C for 18 h and the neutralised hydrolysate was extracted with ether to separate the neutral and acidic methylated sugars. The concentrated ethereal extract was a yellow syrup.

Identification and Estimation of Neutral Methylated Sugars.—The ethereal extract was separated into three fractions by paper chromatography in solvent system C and each sugar was eluted with hot methanol. The last two fractions, II and III, were separated in solvent D. Fraction I was a syrup, $[\alpha]_D^{25}$ +96° (*c*, 1.2 in water); R_{TMG} 0.70 (TMG is 2,3,4,6-tetra-*O*-methyl-D-glucose) (Found: OMe, 42.3. Calc. for $C_9H_{18}O_6$: OMe, 41.9%); in 4% methanolic hydrochloric acid, $[\alpha]_D^{35}$ +45° (initial) ⟶ −24° (20 h) ⟶ −46° (48 h) (ref. 19); identified to be 2,3,6-tri-*O*-methyl-D-galactose by oxidation with bromine–water to its γ-lactone, $[\alpha]_D^{25}$ −35° (water), m.p. 96° (lit.,[20] 99°). Fraction II was crystallised from ether–light petroleum to give needles, m.p. 97—98° (lit.,[12] 98°), $[\alpha]^{25}$ +24° (*c*, 0.54 in water); R_{TMG} 0.83 (Found: OMe, 31.7. Calc. for $C_8H_{16}O_5$: OMe, 32.26%); identified as 3,4-di-*O*-methyl-L-rhamnose by its γ-lactone, m.p. 76—78° (lit.,[10] 78—79°). Fraction III was a syrup, $[\alpha]_D^{25}$ +114° (*c*, 1.14 in water); R_{TMG} 0.88 (Found: OMe, 52.0. Calc. for $C_{10}H_{20}O_6$: OMe, 52.5%); identified as 2,3,4,6-tetra-*O*-methyl-D-galactose by its aniline derivative (by refluxing with aniline and ethanol), m.p. 193—194°, $[\alpha]_D^{25}$ +30° (acetone) (ref. 21).

These sugars, trimethylgalactose (7.94%), dimethylrhamnose (20.2%), and tetramethylgalactose (16.97%), corresponding to an average molar ratio of 1 : 3 : 2, respectively, were estimated as anhydro sugars by alkaline hypoiodite method.[22]

Identification and Estimation of Acidic Methylated Sugars.—The aqueous part of the hydrolysate, after treatment with Amberlite resin IR-120, showed on paper chromatography (solvent B) spots due to 2-*O*-methyl-, 2,3-di-*O*-methyl-, and 2,3,4-tri-*O*-methyl-D-galacturonic acids with R_{TMG} values 0.16, 0.35, and 0.63, respectively. These acidic sugars were left overnight in 4% methanolic hydrogen chloride and then refluxed for 7 h. After neutralisation with silver carbonate, the dry residue was dissolved in water and reduced with sodium borohydride by the method of Bose and Dutta.[23] The reduced sugars were identified by separation into three fractions by paper chromatography in solvent C. Fraction I, m.p. 146—147° (lit.,[24] 145—148°), had $[\alpha]_D^{25}$ +80° (water); R_{TMG} 0.23 (Found: OMe, 16.4. Calc. for $C_7H_{14}O_6$: OMe, 15.9%); identified as 2-*O*-methyl-D-galactose by its *N*-phenylglycosylamine derivative, m.p. 164°. Fraction II, a syrup had $[\alpha]_D^{25}$ +114° (water); R_{TMG} 0.43 (Found: OMe, 28.3. Calc. for $C_8H_{16}O_6$: OMe, 29.8%); identified as 2,3-di-*O*-methyl-D-galactose by its aniline derivative, m.p. 129—130° (lit.,[25] 130—131°), $[\alpha]_D^{25}$ +118° (ethanol). Fraction III, m.p. 82—83°, had $[\alpha]_D^{25}$ +117° (water); R_{TMG} 0.65 (Found: OMe, 40.7. Calc. for $C_9H_{18}O_6$: OMe, 41.9%); identified as 2,3,4-tri-*O*-methyl-D-galactose by its *N*-phenylglycosylamine derivative, m.p. 167° (lit.,[26] 169—170°); $[\alpha]_D^{25}$ +44° (methanol). The products, monomethyl- (15.8%), dimethyl- (5.7%), and trimethyl-galactose (6.12%), were found in the molar ratio of 3 : 1 : 1, respectively.

Partial Hydrolysis of the Polysaccharide.—The polysaccharide (5 g) was hydrolysed with N-sulphuric acid (150 ml) at 100 °C for 6 h and fractionated on a cellulose column (n-butyl alcohol saturated with water plus 10% ethanol as eluant) to give galactose. The column was then eluted with water. The water eluate was washed with methanol, treated with Amberlite resin IR-120, and separated into three fractions having R_{gal} values (rate of movement relative to galactose) 0.12, 0.37, and 0.81 by paper chromatography (solvent E). Fraction I, a syrup, $[\alpha]_D^{27}$ +115° (water), on hydrolysis gave only D-galacturonic acid; the fully methylated derivative,[27] m.p. 117—118°, $[\alpha]_D^{25}$ +160° (water), on reduction and remethylation followed by hydrolysis, gave 2,3,6-tri-*O*-methyl- (49.4%) and 2,3,4,6-tetra-*O*-methyl-D-galactose (50%) in a 1 : 1 ratio. Fraction II, a syrup, showed $[\alpha]_D^{27}$ +15.4° (water), equivalent wt. 490; the fully methylated derivative, on reduction and hydrolysis gave 3,4-di-*O*-methyl-L-rhamnose and 2,3,4-tri-*O*-methyl- and 2,3,6-tri-*O*-methyl-D-galactose. The syrup, on hydrolysis after oxidation with bromine water, gave only rhamnose. On periodate oxidation in the dark at low temperature, formic acid (2 mol per mol) was liberated with the consumption of periodate (5.2 mol per mol). Fraction III, a syrup, had $[\alpha]_D^{27}$ +70° (*c*, 1.1 in water); the fully methylated derivative,[9] m.p. 90—92°, $[\alpha]_D^{25}$ +125° (*c*, 0.8 in water), on reduction and hydrolysis, gave 2,3,4-tri-*O*-methyl-D-galactose (55.4%) and 3,4-di-*O*-methyl-L-rhamnose (44.2%) in the ratio 1 : 1.

Partial Methanolysis of the Methylated Polysaccharide.—The methylated polysaccharide (2 g) was refluxed with dry hydrochloric acid (1 ml) and absolute methanol (20 ml) at 100 °C for 8 h. The neutralised hydrolysate was refluxed with 0.2N-barium hydroxide for 2 h to saponify the uronide esters. The excess of barium hydroxide was destroyed with carbon dioxide and the mixture filtered. The

filtrate was concentrated and extracted with ether. The residue was dissolved in water, treated with Amberlite resin IR-120, and precipitated with ethanol. The precipitate, m.p. 116—119°, $[\alpha]_D^{28}$ +159° (water), on hydrolysis gave 2,3-di-*O*-methyl- and 2,3,4-tri-*O*-methyl-D-galacturonic acids.

Periodate Oxidation of the Polysaccharide.—The polysaccharide (104.2 mg) was oxidised with 0.25M-sodium metaperiodate (10 ml) in the dark at low temperature. The liberated formic acid [28] (0.166 2 mol) and consumed periodate [29] (0.690 7 mol) per 100 g of the polysaccharide were estimated after 48 h.

The reaction mixture (2 ml) was taken out and ethylene glycol added to stop the reaction. Hydrolysis revealed the presence of uronic acid only.

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