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# Linkage Analysis in a Trisaccharide from *Dalbergia* by Methylation and Periodate Oxidation Methods

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**Abstract:** *Dalbergia* is a large genous with over hundred species of trees, shrubs and woody climbers, widely distributed throughout the tropics of the world. Nearly 27 species of this genus are found in different parts of India. Among Indian species *Dalbergia sissoo* has a wide distribution in North India. It grows gregariously in alluvial forests, characteristics of the river beds from Indus to Assam and in the Himalayan vallies. A mucilaginous polysaccharide was isolated from *Dalbergia sissoo* Roxb. Leaves and purified. Purified *sissoo* polysaccharide has been partially hydrolysed to get the structural oligosaccharides. An oligosaccharide has been isolated from the hydrolysed product of *Dalbergia sissoo* leaf polysaccharide. On completed hydrolysis it furnishes two monosugars, rhamnose and glucose in the molar ratio of 1:2. On methylation and periodate oxidation study it indicated the presence of 1→2 and 1→4 linkages among structural monosaccharide units.
**Keywords:** *Dalbergia*, Trisaccharide, Methylation, D-Glucose.

## Introduction

*Dalbergia sissoo* Roxb., a leguminous tree, occurs throughout the Sub-Himalayan tract from the Indus to Assam, and in the Himalayan valleys, ascending upto about 1500 m. The timber is valued not only for its finest quality of wood for different uses but also its resistance to attack by insects and microorganisms. Different parts of *Dalbergia sissoo* are of medicinal value. Bark is alterative, antiemetic, astringent and aphrodisiac, whereas roots are constipating. A number of medicinal properties of the leaves are also reported. The leaves are bitter, stimulant, ophthalmic, styptic, digestive, anthelmintic, and diuretic [1]. In a recent study, Hajare et al.[2] reported the marked antipyretic and analgesic activity of leaf extract of *Dalbergia sissoo*.

Different parts of *Dalbergia sissoo* have been examined for their phytochemistry. Mainly a novel group of compounds called 'Neoflavonoids' and its glycosides, coumarins, essential oils, phenols, terpenes [3-5] are reported from different parts of the plant. Mucilage of *sissoo* has been found to be very useful in acute stage of diarrhea, dyspepsia, hemorrhoids and burning sensation [1] and when it is mixed with sweet oil, used in skin excoriations [6]. A decoction of the

leaves is given in acute stage of gonorrhea [6]. Some reports are also available on the use of leaves mucilage for eye diseases [6]. All these properties of leaf mucilage are solely dependent on the polysaccharide present in the mucilage. Increased knowledge of the chemical nature of this type of polysaccharide is of interest to the plant chemist for its further utilization.

*Sissoo* polysaccharide produces one tri-, one hepta- and one nonasaccharide on partial hydrolysis. In the present paper only methylation and periodate analysis results of trisaccharide has been discussed. The present paper is a part of complete structure elucidation of *Dalbergia sissoo* polysaccharide.

## Experimental and General Methods of analysis

The leaves of *Dalbergia sissoo* were collected from Forest Research Institute campus and dried in shade. Solutions were concentrated at or below 40°C in a rotary evaporator under reduced pressure. All melting points are uncorrected. Optical rotation was determined on Autopol-II, automatic polarimeter (Rudolph Research, Flanders, New Jersey) at 589 nm, D-lines of sodium. Paper chromatography was carried on Whatmann 1 and 3 mm filter paper sheets using the following solvent systems; n-Butanol: Ethanol: Water (4:1:5, $S_1$) upper layer; n-Butanol: Pyridine: Water (6:4:3, $S_2$) and Ethyl acetate: Acetic acid: n-Butanol: Water (4:3:2:2, $S_3$). Detection was effected with Acetonical silver nitrate ($R_1$) and Aniline phthalate spray ($R_2$). Detection with Acetonical silver nitrate was done by treatment with the reagents: (a) To silver nitrate solution (12.5 g in 10 ml water), one litre acetone was added with continuous shaking. Distilled water was added drop wise with stirring until the white precipitate completely dissolved to form a clear solution. (b) Sodium hydroxide (20 g) was dissolved in 400 ml of ethanol. (c) Aqueous ammonia solution.

The dried chromatograms were dipped and passed through reagent solution (a) for about 5 min, dried at room temperature and passed through reagent (b); when the dark brown spots were visualized the paper was dipped in reagent (c) for some time with shaking (5–10 min.). Finally the chromatograms were washed with water and dried in air [7].

Gas liquid chromatography of the sugar mixtures was carried out on a Shimadzu Gas Chromatograph GC-9A fitted with a flame ionization detector (FID). The samples were analysed in the form of their alditol acetates on ECNSS-M (3%) on Gas Chrom Q (100–120 mesh) packed into 5'× 1/8'' stainless steel column under the following operating conditions Column temperature 170°C, nitrogen flow rate 35–40 ml/min ($C_1$).

**Isolation of polysaccharide**

The fresh leaves of *Dalbergia sissoo* were collected from Forest Research Institute campus in the month of April. The color of the leaves was green at the time of harvest. The moisture content of the leaves was found to be 80%. The leaves were air dried in shade to moisture content of 8%. The air dried leaves were made into small pieces using laboratory grinder. The leaf material [50 g] at this stage was stirred vigorously in distilled water [1000 ml] for 6 h at room temperature and centrifuged to remove water-insoluble part. The supernatant solution was decanted off and concentrated under vacuum using rotary evaporator. The concentrated aqueous extract [100 ml] was poured into three times its volume of ethanol [300 ml] with constant stirring. The polysaccharide was precipitated out in the form of a fluffy precipitate. The precipitate was again dissolved in water and added to ethanol. Precipitate was treated successively with dry solvent ether and acetone. It was filtered under vacuum and dried in vacuum desiccator at room temperature. The polysaccharide [8.5 g] so obtained was deionised by passing the aqueous solution successively through the columns of freshly regenerated cation [Dowex-50-W-X8] and anion [Seralite- SRA-400] exchange resins. The columns were washed with distilled water until the washings showed a negative Molisch test for carbohydrates. The combined eluents were concentrated to small volume [1/4th] and subjected to further purification by dialysis. In the process, the concentrated product was transferred into a cellophane bag and dialyzed for 72 h in running water. The dialyzed product was concentrated and re-precipitated with a large volume of ethanol to obtain finally the pure polysaccharide. It was kept overnight, alcohol was decanted off, and the precipitated polysaccharide treated with solvent ether, acetone and absolute ethanol. It was filtered and lyophilised at -40°C to obtain finally the pure polysaccharide in the form of a light brown amorphous powder [6.45g].

**Partial hydrolysis**

Pure polysaccharide (2.5 g) was heated with sulphuric acid (0.1 N, 100 ml) on a steam bath for 5 h. The hydrolysate was cooled, neutralized with saturated solution of barium carbonate till neutral pH and filtered. The combined filtrate was concentrated to yellowish syrup (1.15 g). The mixture of oligosaccharides and monosaccharide was resolved into its components by preparative chromatography on Whatmann No. 3 mm filter paper sheets using solvent system $S_2$. The strips corresponding to individual oligosaccharides were eluted with water, elutes were concentrated separately, to obtain the three oligosaccharides. Homogeneity of the oligosaccharides

was checked by paper chromatography in solvent system $S_1$, $S_2$, and $S^3$ using $R_1$ and $R_2$ as spray reagent.

**Methylation of oligosaccharide**

Oligosaccharide (0.0406 g) was methylated completely according to the method of Hakomori [8] using sodium hydride-dimethyl sulphoxide. Methylated oligosaccharide was recovered by chloroform extraction. After evaporation of the chloroform extracts to dryness, the residues were hydrolysed with formic acid (90%, 10 ml) for one hour on steam bath, the solutions evaporated and treated with aqueous sulphuric acid (0.13 M, 15 ml) for 18 h on a steam bath.

**Preparation of alditol acetates**

Alditol acetates of the hydrolysed methylated material were prepared by the method as described [9]. Sodium borohydride (0.020 g) was added to hydrolysates, and the mixture was kept for 18 h at room temperature. The mixture was neutralized by slow addition of dilute acetic acid (6 ml), and concentrated to dryness in the vacuum rotator at 40°C. Sodium was removed by passing it through cation exchange resin (Dowex-50 W-X8). Boric acid was removed by co-distillations, in the vacuum rotator with methanol (3 × 5 ml). The residue was treated with redistilled acetic anhydride and pyridine, 1:1 (4 ml) and refluxed for 6 h. Toluene (6 ml), which gave an azeotrope with acetic anhydride, was added, and the mixture was distilled as above, until the rate of distillation decreases. A new portion of toluene (6 ml) was added and the solution was concentrated to dryness. It was dissolved in water (10 ml) and the acetylated sugars separated by shaking with dichloromethane (4 × 25 ml). Traces of water present in dichloromethane were removed by adding anhydrous sodium sulphate followed by filtration and washing with dichloromethane before concentration.

**Periodate oxidation**

To a solution of oligosaccharide in water (0.05 g in 25 ml for each) an aqueous solution of sodium metaperiodate (0.2 g in 50 ml) was added and the volume of the resultant solution was made upto 100 ml. A blank solution of sodium metaperiodate (0.2 g in 100 ml water) was also prepared. These were kept in dark at room temperature (25°C) for 192 h. To determine periodate consumed, an aliquot (5 ml) of the periodate reaction mixture was added to a solution containing distilled water (20 ml), potassium iodide (20%, 2 ml) and sulphuric acid (0.5 N, 3 ml). The liberated iodine was immediately titrated with 0.1 N sodium thiosulphate solution using starch as an indicator [10-12].

Liberation of formic acid was determined by the methods reported earlier [11, 13-15]. To an aliquot (5 ml) of the periodate reaction mixture was added acid free ethylene glycol (0.5 ml), followed by an excess of potassium iodide (20%, 5 ml) after 10 min. To the above solution an excess of 0.01 N sodium thiosulphate (10 ml) was added, and the unused sodium thiosulphate was back titrated with 0.01 N iodine solution using starch as an indicator. A blank solution was titrated concurrently.

## Result and discussion

The polysaccharide was isolated from air dried leaves in 13% yield as detailed in experimental section.

**Partial hydrolysis**

*Dalbergia sissoo* leaf polysaccharide upon hydrolysis with dilute sulphuric acid (0.1 N, 5 h) furnished a mixture of oligosaccharides along with a monosaccharide. Preliminary paper chromatographic examination of the hydrolysates revealed the presence of three oligosaccharides along with one monosaccharide, L-rhamnose. The Rgal values of oligosaccharides were 0.84, 0.65 and 0.57, respectively, in solvent system $S_2$. From this mixture, the oligosaccharides were resolved by preparative chromatography on Whatmann No. 3 mm sheets and each oligosaccharide eluted separately and the eluates combined to isolate pure oligosaccharides. The homogeneity of the oligosaccharides was checked by paper chromatography using organic solvent systems $S_1$, $S_2$ and $S_3$ and spray reagents $R_1$ and $R_2$. The degree of polymerisation of three oligosaccharides corresponded to one tri, one hepta and one nonasaccharides. The nature and sequence of glycosidic linkages in trisaccharide was determined by methylation analysis and periodate oxidation assignment of the oligosaccharide. Linkage sequences in other two oligosaccharides will be discussed in future communication.

**Linkage analysis of trisaccharide**

This trisaccharide, $[\alpha]_D^{18}$ +53.3o (c 0.0375%, $H_2O$), m.p. 124–126°C (d), was found homogenous and gave single spot [Rgal 0.84] using organic solvent system $S_2$ and spray reagents $R_1$ and $R_2$ on chromatographic examination. Upon complete acid hydrolysis, it gave D-glucose and L-rhamnose on paper chromatogram in solvent systems $S_1$, $S_2$ and $S_3$. The alditol acetates of the hydrolysate of trisaccharide on GLC analysis under conditions $C_1$ showed two peaks corresponding to L-rhamnose and D-glucose in the molar ratio of 1:2.

The trisaccharide was completely methylated by Hakomori method [8]. Complete methylation was confirmed by IR spectrum of the methylated trisaccharide which showed complete absence of –OH band (3590–3225 $cm^{-1}$). It was hydrolysed and transformed into its alditol acetates according to the method of Jansson et al [9]. GLC of the resulting alditol acetate under conditions $C_1$, furnished, 3-O-methyl-L-rhamnose and 2,3,4,6-tetra- O-methyl-D-glucose in the molar ratio of 1:2, respectively.

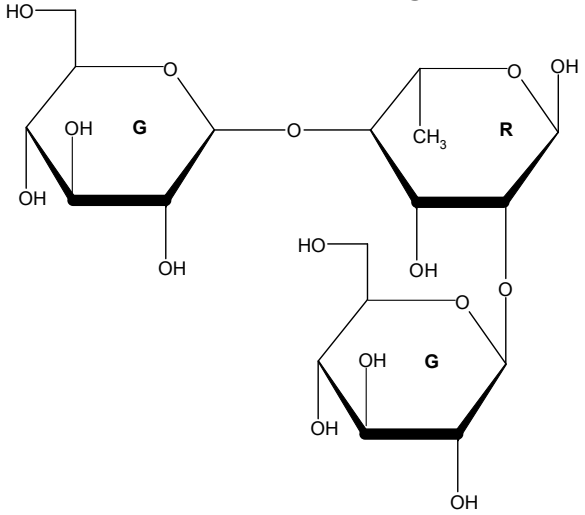
On the basis of methylation study it was found that out of two units of 2,3,4,6-tetra-O-methyl-D-glucose (non reducing end), one is linked to the C-4 and second unit to the C-2 position of 3-Omethyl- L-rhamnose. These results indicate the presence of 1→4 and 1→2 linkages in trisaccharide.

Evidence supporting the presence of 1→4 and 1→2 linkages in the framework of trisaccharide has also been obtained from the results of periodate oxidation [10-15]. The trisaccharide when subjected to oxidation with sodium meta periodate, consumed 1.28 mol of periodate per anhydrohexose unit and released 0.64 mol of formic acid per anhydrohexose unit. As the trisaccharide structure consists of 3 sugar residues, the above results can be interpreted in terms of the consumption of 3.84 mol of periodate with simultaneous liberation of 1.92 mol of formic acid by the repeating unit of the oligosaccharide. Thus, periodate oxidation data of oligosaccharide as calculated on the basis of proposed structure and obtained experimentally are found to be in close agreement with each other.

## Conclusion

From the results of methylation and periodate oxidation study of the oligosaccharide obtained from *D. sissoo* leaf polysaccharide, it can be concluded that it contain two type of linkage i.e. 1→4 and 1→2 in its structure. This linkage sequence also indicated that in the trisaccharide, two non reducing glucose units linked to rhamnose by 1→4 and 1→2 linkages, respectively. On the basis discussion on the results of methylation and periodate oxidation study, structure represented in figure-1 justify the linkage position in *Dlabergia* trisaccharide.

**Figure 1: Structure of trisaccharide showing 1→4 and 1→2 linkages**




**G: β-D-Glucose; R: α-L-Rhamnose**

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