STRUCTURAL STUDIES ON GALACTOMANNANS FROM SEED OF *CROTALARIA MUCRONATA* AND *CAESALPINIA PULCHERIMA* : PARTS I AND II.

PART III - ISOLATION AND STRUCTURES OF SOME OLIGOSACCHARIDES OF *VERTICILLIUM DAHLIAE*.

PART IV - SYNTHESIS OF 2,4,6-TRI-O-METHYL-D-MANNOPYRANOSE.

PART V - SYNTHESIS OF 2,3,6-TRI-O-METHYL-D-MANNOPYRANOSE.

by

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PART I

STRUCTURE STUDY OF A GALACTOMANNAN FROM SEED OF *CROTALARIA MUCRONATA*

INTRODUCTION

The so-called vegetable mucilages are derived from the barks, roots, leaves, seeds, and in some cases, the flowers of plants. They are the products of normal plant metabolism and may serve as food reserves in much the same manner as starch in many plants and glycogen in animals.

Excellent sources of mucilages composed of neutral sugar residues are the seeds of Leguminosae such as the locust bean or carob bean (Ceratonia siliqua L.), guar (Cyamopsis tetragonolobus), Kentucky coffee bean (Gymnocladus dioria), honey locust (Gleditsia tricanthos), and many others. Palm seeds also provide a neutral mucilaginous polysaccharide.

Polysaccharides composed of D-mannose and D-galactose are most commonly found in the seeds of the Leguminosae and are in particular localized in the endosperm. They are also found in the seeds of non-leguminous plants such as the African oil palm (Elaeis guinensis), date palm (Phoenix dactylifera) (1), Cocus mucifera and Coffee arabia. The relatively small seeds of clovers, common to the north and south temperate zone, for example, Trifolium pratense, Lotus corniculatus, Melilotus species, and alfalfa (Medicago sativa),

sometimes called lucerne and purple media, also contain galactomannan.

Although the industrial importance of galactomannans (gums) may have been partially offset by the introduction of specialty synthetic polymers, they still find considerable use in paper and cloth sizing, and emulsion stabilizers in the food industry (e.g., ice cream extender, puddings, thickeners). Huge quantities of gum were used, particularly during World War II (1939-45), in the flotation process for ore separations. The much restricted importation of gums and mucilages into the United States from the middle and far East during the war (1939-45), led to an investigation of the seeds of a number of leguminous plants that commonly grow in North and South America. The galactomannan concerned in the present investigation is isolated from seed of Crotalaria mucronata which can be grown in North America. The growing habit of this plant is rather similar to that of alfalfa although the stems are more highly lignified.

In a study of the endosperm of leguminous seeds, Anderson (2) examined 163 species and by visual inspection found that about three fourths of these contained mucilage-yielding endosperms in amounts up to 60 per cent of the seed. The galactomannan was isolated from the endosperm of several species by aqueous extraction

and its composition determined by hydrolysis with 2 per cent sulfuric acid followed by analysis of the D-mannose and D-galactose as their phenylhydrazones. The relative amounts of D-mannose and D-galactose varied from 81 per cent mannose and 16 per cent galactose in Sopora japonica to 59 per cent mannose and 38 per cent galactose in guar (3). In comparison, the galactomannan herein described contains about 75 per cent D-mannose and 25 per cent D-galactose.

LITERATURE REVIEW

The identification of a specimen of a gum or mucilage may be relatively simple if full use is made of the present knowledge of these substances and the available analytical techniques. The methods that have been developed for studying the fine structure of the polysaccharides have reached such a degree of precision that the purity of the starting products becomes increasingly more important. The presence of small amounts of other polysaccharides may considerably influence the structural deduction that can be made. It is therefore of the utmost importance to at least attempt to establish the homogeneity, or otherwise, of the starting material (4).

Polysaccharides, after extraction with water, can be purified by fractional precipitation with ethanol(5). Fehling solution has been widely employed for purifying polysaccharides, especially those containing a relatively high proportion of mannopyranose units joined by (1→4)-β-D-glycosidic bonds (6, 7, 8, 9, 10). It appears that the axial, equatorial, C_2 - C_3 hydroxyls are involved in complex formation. In addition to Fehling solution, cupric chloride (11), cupric sulfate (12), cupric acetate (13, 14), and cupriethylenediamine (9, 10, 15) have been employed for the same purpose. Most, if not all, the

galactomannan gums extracted from seeds of the Leguminosae give copper complexes with Fehling solution and this offers a means for their purification and identification. The homogeneity of the polysaccharide can further be shown by electrophoresis (glass paper or moving boundary) and by ultracentrifugation.

The purified gum is further characterized by the determination of the specific optical rotation either in water or dilute alkali and by hydrolysis to its individual sugar constituents by heating at 95-100°C with 1 to 2 N sulfuric acid.

Paper partition chromatography introduced in 1944 for the separation and determination of amino acids, has been highly sucessful for the separation of sugars and probably even more sucessful for their quantitative determination. It was demonstrated (16, 17) that closely related sugars could be separated from each other provided a suitable solvent or combination of solvents could be selected. Under a given set of conditions of temperature, solvent, type of paper, and pH (18, 19, 20, 21), the rate of movement of a particular sugar will be fairly constant. This rate of movement relative to the movement of the solvent front, the R_F value, is a measurement which now assumes in organic chemistry almost as much importance as the boiling point or melting

point. The sugars are located on the dried papers by various color reactions. The reagents selected make use of the reducing (aldehydic) properties of the sugar or sugar derivative. For most research purposes two or three reagents are usually sufficient. Not only has paper partition chromatography provided a means for the preliminary identification of the sugars, but by using thicker or larger sheets of paper it is possible to separate the components of the hydrolysate from a gum in sufficient amounts to enable the components to be characterized, either by direct crystallization or by transformation into a suitable crystalline derivative. Quantitative determination may for instance be accomplished by the phenol-sulfuric acid method (23). In addition to the above, vapor phase chromatography (22) has now been developed to the point where it may be generally employed for the qualitative and quantitative analysis of sugars and sugar derivatives.

After purification of the gum or mucilage by one or more fractionation methods, determination of the composition by paper chromatography and vapor phase chromatography, and characterization of the component sugars by crystallization or by preparation of derivatives, one is faced with the problem of determining (a) the mode of union of various component sugars,

(b) the sequential order of the components, (c) the anomeric nature of the glycosidic linkages uniting the components, and (d) the average number of individual sugar residues that make up the molecule of the polymer.

One approach adopted in the study of the constitution of gums is to apply methylation studies to the polysaccharides themselves. Generally the methylation proceeds normally with methyl sulfate and alkali (24). To obtain complete alkylation, this may be followed by treatment with sodium hydride in methyl sulfoxide and methyl iodide, a process which gives better methylation within reasonably short time (26, 27). The results obtained will show the composition of the gum, the nature of the building units, how they are joined together, and the number of the residues in the average repeating unit. The findings also enable the terminal units and the units at which branching occurs to be designated. However, by themselves, methylation studies on the polysaccharides provide little knowledge concerning the exact sequence of the building units unless the number of structural possibilities is limited by the fact that the structure of the polysaccharide is relatively simple, being built up of only one or two types of sugar units. Methylation results will become more diagnostic when considered in conjunction with periodate oxidation

studies.

Non-reducing terminal units in a polysaccharide or (1→6)-linked non-terminal units having three adjacent hydroxyl groups will be cleaved by two molecular proportions of periodate to give one molecular proportion of formic acid. Non-terminal units joined by (1→2) or (1→4) bonds undergo cleavage by one molecular proportion of periodate, but no formic acid is generated. Units which do not possess adjacent hydroxyl groups such as non-terminal units joined by (1→3) bonds or units involved in branching at C_2 and C_4 are not affected by periodate. Thus oxidation of a polysaccharide and quantitative determination of the proportion of the surviving sugar units will give information concerning the nature and proportion of the glycosidic linkages present in the polysaccharide (3, 28, 29, 30).

Smith et al (31, 32, 33) reported that periodate oxidation followed by reduction with either hydrogen and Raney nickel catalyst or with sodium borohydride (34) in aqueous solution represented a general analytical procedure which can be applied to polysaccharides. When a sugar residue of a polysaccharide is cleaved by periodate and reduced, the resulting alcoholic derivative, being a true acetal, is sensitive to mild acid hydrolysis, whereas, when a sugar unit which survives cleavage

is joined to a unit which is cleaved, the surviving unit appears as a glycoside which is relatively stable to mild acid hydrolysis. Because of the marked difference in stability between true acetals and glycosides, it is now possible to obtain glycosides of mono-, di-, and oligosaccharides from a wide variety of polysaccharides after the Smith periodate degradation. The analysis of the structures of these glycosides will throw light on the fine structure of the parent polysaccharides (3, 35, 36).

EXPERIMENTAL METHODS AND RESULTS

A. Isolation of Galactomannan

Broken seed of Crotalaria mucronata was slurried in boiling water for 3 to 4 hours after which the solids were removed by centrifugation. The cooled, viscous solution was slowly poured with vigorous stirring into 4 volumes of ethanol whereupon a light grey, stringy precipitate formed. The solid material was removed by filtration, washed with acetone and dried in a desiccator.

B. Purification of Galactomannan with Copper Acetate

To a portion (2 grams) of polysaccharide in 0.5 N sodium hydroxide (400 ml) was added with stirring 5% copper acetate (W/V) (75 ml). A gelatinous precipitate resulted which was removed by centrifugation. Addition of a further volume (75 ml) of copper acetate solution resulted in further precipitation of a copper complex which was not further investigated at this time. The gelatinous complex was suspended in alcohol and acidified with hydrochloric acid and after maceration in a Waring blendor, the light grey precipitate was removed by

centrifugation and washed free of acid with alcohol. Complexation with Cu^{++} was repeated twice more as described and resulted in a non observable change in sugar ratio (see succeeding section), yield 1.2 grams, $[\alpha]_D^{22}$ 9 ± 1° (c 0.3, 0.1 N sodium hydroxide).

C. Ultracentrifugation

Solutions of galactomannan (0.5 to 1% concentration) in alkali (1 to 5 N sodium hydroxide) were centrifuged for 2 hours, rotor speed 60,000 r.p.m. The sedimentation pattern (0.8% galactomannan in 5 N sodium hydroxide) (Figure IV) showed that the polysaccharide was homogeneous and that the molecular weight was about 60,000 when compared to a pattern obtained for β-amylase of known molecular weight (62,500).

D. Hydrolysis of Polysaccharide

(a) With 1 N sulfuric acid

A small portion (250 mg) of the polysaccharide was refluxed (6 hours) in 1 N sulfuric acid (25 ml) followed by neutralization (barium carbonate) and paper (Whatman No.1) chromatography of the syrupy residue left after evaporation of the filtrate. D-mannose and

D-galactose were identified as the only sugar components using solvents A (Ethyl acetate:acetic acid:water 8:2:2) and B (Ethyl acetate:pyridine:water 8:2:1), spray reagent F (p-anisidine-trichloroacetic acid (37)). Separation (Whatman 3MM) of a portion (100 mg) of the mixture using solvent B gave D-mannose, 60 mg, further characterized as the phenylhydrazone, m.p. 199°C, $[\alpha]_D^{22}$ 33° (c 0.9, pyridine) (lit. m.p. 199 - 200°C) and D-galactose, 28 mg, $[\alpha]_D^{22}$ 80° (c 0.7, water). The molar ratio of the two sugars was further confirmed by elution of each sugar from a single chromatogram followed by analysis employing the phenol-sulfuric acid method (23) : to 1 ml of the eluted solution of the corresponding sugar was added 1 ml 20% phenol solution followed by 5 ml concentrated sulfuric acid. The solution was shaken for a few minutes and allowed to stand for 30 minutes. The absorbance of the solution was measured at 490 mu in a spectrophotometer. The amount of sugar present was determined by reference to standard curves from D-mannose and D-galactose which are shown in Figure I and II.

The sugar ratio was also determined by V.P.C. of the mixture of trimethylsilyl derivatives (22) using a 5' x ¼" stainless steel column packed with 20% SE 30 on HMDS treated chromosorb W. A D-mannose:D-galactose ratio of 3:1 was obtained in all cases. (To prepare trimethyl-

silyl derivative : a few mg of sample was dissolved in a few drops of pyridine. Six drops of hexamethyldisilazane was then added followed by three drops of trimethylchlorosilane. The mixture was stirred for a few minutes).

(b) Hydrolysis with Emulsin

To a sterile solution (15 ml) pH 7.0, of galactomannan (25 mg) was added emulsin# (10 mg). The solution was stored at room temperature for 3 days. No observable change in the reducing power (3,5-dinitrosalicylic acid reagent) was observed. The solution was evaporated and the residue was shown, by paper (Whatman No.1) chromatography, solvents A and B, spray reagents F and G (Tollens solution (16)), to contain no detectable D-galactose (or D-mannose) and no evidence of small oligomers.

obtained from Nutritional Biochemicals; bacterial origin (Bacillus subtilus).

E. Methylation analysis

A portion (2 grams) of the galactomannan was initially treated with 30% sodium hydroxide and methyl sulfate (24) followed by treatment with sodium hydride in methyl sulfoxide and methyl iodide (26, 27). A fully methylated polymer (1.5 grams) (no OH- absorption evidence in the I.R. spectrum) was dissolved in cold 75% sulfuric acid (5ml)(39), the mixture was held at room temperature for 1 hour after which the solution was diluted with ice-water to approximately 1 M and boiled for 7 hours. The syrupy mixture (1.2 grams) which was obtained after neutralization (barium carbonate) and evaporation of the filtrate was analysed by paper chromatography (Whatman No.1) using solvents C (Butanone-water azeotrope), D (n-Butanol:ethanol:water (upper phase) 4:1:5), E (Benzene:ethanol:water 85:15:1). The molar ratio of the partially methylated sugars was determined by the phenol-sulfuric acid procedure (Table II) and by V.P.C. of trimethylsilyl derivatives (20% neopentylglycolsuccinate on Gas Chrom. 900, 3/16" by 12' column, 180°C, helium flow rate 100 ml/min., flame ionization detector) (40) (Table I). The mixture was separated using Whatman 3MM paper, solvent C, and the individual partially methylated sugars characterized

Table I

Vapor phase chromatography of trimethylsilyl derivatives of partially methylated sugars

Trimethylsilyl derivative	Retention time (min.)	Molar ratio
2,3,4,6-tetra-O-methyl-D-mannose	16.5	1
2,3,4,6-tetra-O-methyl-D-galactose	15.3 (α) 18.1 (β)	23
2,4,6-tri-O-methyl-D-mannose	12.2	11
3,4,6-tri-O-methyl-D-mannose	10.6	4
2,3,6-tri-O-methyl-D-mannose	13.5	28
2,3-di-O-methyl-D-mannose	8.5	26

Table II

Paper chromatographic separation of partially methylated sugars

Component	Butanone-water azeotrope R_F	n-Butanol-ethanol water R_G#	Proposed compound	Molar ratio ##
1	0.79	0.96	2,3,4,6-tetra-O-methyl-D-mannose	1
2	0.69	0.88	2,3,4,6-tetra-O-methyl-D-galactose	20
3	0.56	0.82	2,4,6-tri-O-methyl-D-mannose	10
4	0.50	0.81	2,3,6-tri-O-methyl-D-mannose and 3,4,6-tri-O-methyl-D-mannose	25
5	0.21	0.57	2,3-di-O-methyl-D-mannose	26

mobility with reference to 2,3,4,6-tetra-O-methyl-D-glucose.

from chromatographic results in butanone-water azeotrope using phenol-sulfuric acid method.

as follows:

Component 1, was identified as follows: Authentic 2,3,4,6-tetra-O-methyl-D-mannose was prepared by methylation of methyl α-D-mannopyranoside with sodium hydride in methyl sulfoxide and methyl iodide (26, 27) followed by hydrolysis (1 N sulfuric acid, 8 hours) to generate the free tetra-O-methyl sugar. Component 1 and the synthetic tetra-O-methyl sugar showed identical R_F, 0.79 in solvent C and R_G[#], 0.96 in solvent D and retention time of trimethylsilyl derivatives (16.5 minutes).

Component 2, 38 mg, $[\alpha]_D^{22}$ 118° (equil.) (c 0.4, water), gave upon treatment with aniline in dry ethanol containing a trace of glacial acetic acid, 2,3,4,6-tetra-O-methyl-N-phenyl-D-galactosylamine, m.p. 190 - 191°C, $[\alpha]_D^{22}$ 40° (equil.) (c 0.5, acetone) (lit. m.p. 192°C, $[\alpha]_D$ 41° in acetone) (41). Further confirmation of identity was established by synthesis, as described for component 1. Component 2 and authentic 2,3,4,6-tetra-O-methyl-D-galactose showed identical R_F, 0.69 in solvent C and R_G, 0.88 in solvent D and retention time of trimethylsilyl derivatives (15.3 minutes, α-anomer; 18.1 minutes, β-anomer).

mobility with reference to that of 2,3,4,6-tetra-O-methyl-D-glucose.

Component 3, 18 mg, $[\alpha]_D^{22}$ 15° (equil.) (c 0.3, water), was treated with aniline as above to give 2,4,6-tri-O-methyl-N-phenyl-D-mannosylamine, m.p. 133 - 135°C, $[\alpha]_D^{22}$ 8° (equil.) (c 0.4, methanol) (lit. m.p. 134°C, $[\alpha]_D$ -150→8°) (42). The identity of component 3 was further established by the following series of reactions: A small portion (about 10 mg) was subjected to sodium borohydride reduction (24 hours, room temperature) followed by treatment with methanol-hydrogen chloride to remove borate. The product was treated with periodic acid (0.5 N) at 5°C for 2 days. The solution (25 ml) was treated with barium carbonate to remove iodate and periodate and after evaporation, the residue was hydrolysed (100°C) with 35% hydrobromic acid for 30 minutes to affect demethylation. D-mannitol was identified by comparative a) paper chromatography, b) vapor phase chromatography.

Component 4, 51 mg, $[\alpha]_D^{22}$ -9° (equil.) (c 0.9, water), was dissolved in dry pyridine and 10% molar excess p-nitrobenzoyl chloride added followed by heating at 65°C for 30 minutes. The mixture was cooled, saturated solution of sodium bicarbonate was added followed by extraction with chloroform. After recrystallization from ethanol, the 1,4-di-p-nitrobenzoyl-2,3,6-tri-O-methyl-D-mannose had m.p. 187 - 189°C, $[\alpha]_D^{22}$ +33° (c 1.0, chloroform) (lit. m.p. 187 - 188°C, $[\alpha]_D$ +33° in chloroform) (43).

Component 4 was further analysed as follows: Thin layer chromatography on silica gel showed using solvent C, three components with R_G values of 0.78, 0.76 and 0.68. In solvent E, three components were also evident with R_G values of 0.52, 0.50 and 0.45 thus indicating three tri-O-methyl sugars. V.P.C. analysis of trimethylsilyl derivatives (40) (conditions described previously) showed the presence of 2,4,6-tri-O-methyl-D-mannose, 12.2 minutes (minor proportion), 3,4,6-tri-O-methyl-D-mannose, 10.6 minutes (minor proportion), and 2,3,6-tri-O-methyl-D-mannose, 13.5 minutes (major proportion). A portion of component 4 was subjected to the following series of reactions : (a) reduction with borohydride, (b) oxidation with periodic acid, (c) reduction with borohydride, (d) demethylation with 35% hydrobromic acid, (e) deionization and (f) identification by paper and gas chromatography of products. Ethylene glycol and erythritol were identified and would arise from 2,3,6-tri-O-methyl-D-mannose. D-arabinitol was identified by comparison with authentic D-arabinitol obtained via the above series of reactions carried out on 3,4,6-tri-O-methyl-D-mannose prepared by a published procedure (44). The retention time of authentic D-arabinitol and that obtained from component 4 was identical (3.4 minutes, 170°, otherwise conditions as described previously),

m.p. of pentaacetate 75 - 77°C (lit. m.p. 76°). D-arabinitol would arise from 3,4,6-tri-O-methyl-D-mannose (the same pentitol would arise from 2,3,4-tri-O-methyl-D-mannose; however, the absence of this sugar was established previously from V.P.C. results - see Table I). D-mannitol was identified by comparison with authentic compound, the trimethylsilyl derivatives of both having retention time of 4.4 minutes (170°C, otherwise conditions as described previously). D-mannitol would be derived from 2,4,6-tri-O-methyl-D-mannose via the above series of reactions.

Component 5, 51 mg, $[\alpha]_D^{22}$ -15° (equil.) (c 1.0, water), gave upon treatment with p-nitrobenzoyl chloride, as above, 1,4,6-tri-p-nitrobenzoyl-2,3-di-O-methyl-D-mannose, m.p. 192 - 194°C, $[\alpha]_D^{22}$ +65° (c 1.0, chloroform) (lit. m.p. 194°C, $[\alpha]_D$ +65° in chloroform) (3). Further confirmation of identity was established by synthesis via methyl 4,6-benzylidene-α-D-mannoside (38). Component 5 and authentic 2,3-di-O-methyl-D-mannose showed identical R_F, 0.21 in solvent C and retention time of trimethylsilyl derivatives (8.5 minutes).

F. Smith Periodate Degradation

To a portion (500 mg) of galactomannan dissolved in water (200 ml) was added 0.5 M periodic acid (10 ml) and the solution stored at 5°C. Periodate consumption reached 0.95 mole# per hexose unit in 2 days with little change thereafter. To the polyaldehyde was added sodium borohydride (500 mg) and the solution stored at room temperature for 16 hours. Analysis of the solution of polyalcohol with phenol-sulfuric acid indicated intact hexose to the extent of 18 - 20% (in terms of D-mannose) of the original polysaccharide. The solution was passed through a cation exchange column (Amberlite 120-H^+), the acidic solution evaporated and borate removed by repeated evaporation with methanol. The residue was dissolved in 0.5 N hydrochloric acid (50 ml), stored at room temperature for 8 hours, followed by deionization with cation and anion exchange resin.

To a flask containing 1.5 grams of sodium bicarbonate an excess of standard sodium arsenite solution was added, with shaking, a 5-ml aliquot of the periodate oxidation solution followed by 1 ml of 20% potassium iodide solution. After standing 15 minutes, the excess sodium arsenite was titrated with standard 0.1 N iodine solution using starch as the indicator. The amount of periodate consumed by the polysaccharide was calculated in moles per hexose unit.

Paper chromatography, solvents A and B, indicated the presence of glycerol and erythritol, molar ratio 1:2 as determined by the periodate-chromotropic acid method (45) (described in the following section) and two non-reducing components R_{Man} = 0.61 (component I) and R_{Man} = 0.33 (component II). The compounds were separated by paper chromatography (solvent B, Whatman 3 MM paper) and characterized as follows:

Glycerol (about 30 mg) - was dissolved in dry pyridine, 10% molar excess p-nitrobenzoyl chloride added and the mixture heated at 75°C for 30 minutes. After cooling to room temperature, excess saturated sodium bicarbonate solution was added and the insoluble precipitate collected and washed with water. The tri-p-nitrobenzoate, after recrystallization from ethanol had m.p. and mixed m.p. 191°C.

Erythritol (about 60 mg) - was treated in the same manner as glycerol. The tetra-p-nitrobenzoate had m.p. and mixed m.p. 250°C.

Component I (about 5 mg) - was hydrolysed in 1 N sulfuric acid (10 ml) and the hydrolysate shown by

paper chromatography, using solvents A and B and spray reagent G, to contain D-mannose and erythritol in a 1:1 molar ratio (D-mannose - by phenol-sulfuric acid; erythritol - determined as formaldehyde by periodate-chromotropic acid).

Component II (about 8 mg) - a portion (4 mg) was hydrolysed as I above and the hydrolysate shown to contain D-mannose and erythritol in 2:1 molar ratio. The remaining portion of II was degraded with periodate (Smith degradation) whereupon 1 molar proportion of formaldehyde was produced (analysed before borohydride reduction) and glycerol and a non-reducing component (III) with slightly faster mobility than I were formed upon mild acid hydrolysis. Complete hydrolysis of III, as described for I, gave D-mannose and glycerol in a 1:1 molar ratio.

Periodate-chromotropic acid procedure for quantitative determination of erythritol and glycerol

To 5 ml of the sample solution, 1 ml 0.1 M periodic acid was added and the solution was allowed to stand for 10 minutes. Barium carbonate was added to precipitate the iodate and periodate. To 1 ml of the

filtered solution, was added 5 ml chromotropic acid reagent (1 gram 1,8-dihydroxynaphthalene-3,6-disulfonic acid in 100 ml water plus 400 ml aqueous sulfuric acid (sulfuric acid:water 2:1)). The precipitate that formed was centrifuged off and the clear solution was heated in a boiling water bath for 30 minutes in the absence of light. The solution was cooled to room temperature and the absorbance measured at 560 mu in a spectrophotometer. The amount of formaldehyde present was determined by reference to a standard curve from authentic erythritol (Figure III). The procedure is sensitive to less than 1 ug/ml of formaldehyde.

DISCUSSION

A rather large number of gums of varying composition and varying degree of structural complexity have been isolated from seed of legumes. Ordinarily, the neutral gums most often found in the endosperm are less complex structurally (composed of D-mannose and D-galactose in varying ratios) than the acid polysaccharides which are more characteristically asscciated with seed coats and usually contain more than just two sugar building units.

A. Isolation and hydrolysis of the galactomannan

The polysaccharide was purified by repeated complexation with copper acetate (13, 14) to give a product with a low specific rotation ($[\alpha]_D^{22}$ 9 ± 1° in 0.1 N sodium hydroxide). This low value suggests that the glycosidic linkages in the main chain have the beta anomeric configuration which is commonly observed for most galactomannans that had been isolated (3). Since the galactomannan was unaffected by prolonged treatment with emulsin, it would appear that the D-galactose units are attached by alpha linkages (for most galactomannans that have been isolated it has been observed that the

D-galactose units are attached by alpha linkages (3)). Ultracentrifugation showed a single peak, indicating a monodisperse system and a relatively narrow molecular weight range for the polymer. Hydrolysis of the polysaccharide gave D-mannose and D-galactose in a molar ratio of 3:1. The D-galactose content is higher than that found in some of the more commonly known galactomannans such as guar (16% D-galactose), Carob bean (14 - 27% D-galactose), and Kentucky coffee bean (20% D-galactose), although some galactomannans are known which have up to 50% D-galactose (3).

B. Methylation Analysis

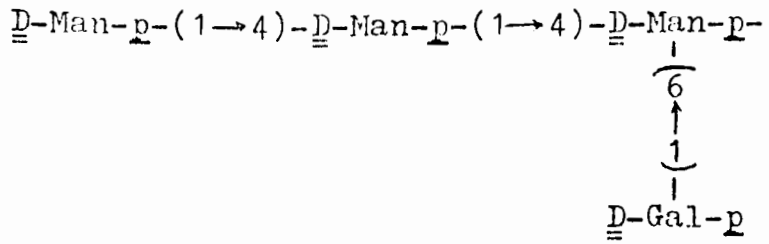
Hydrolysis of the fully methylated gum gave the following partially methylated sugars : 2,3,4,6-tetra-O-methyl-D-galactose, 2,3,4,6-tetra-O-methyl-D-mannose, 2,4,6-tri-O-methyl-D-mannose, 2,3,6-tri-O-mehtyl-D-mannose, 3,4,6-tri-O-methyl-D-mannose and 2,3-di-O-methyl-D-mannose, molar ratio 23:1:11:28:4:26 (from vapor phase chromatography). The presence of the above sugars is in agreement with the periodate oxidation results (see succeeding section), namely, approximately one of every six D-mannose units is linked (1→3). The occurence of a small number of (1→2)-linkages is also demonstrated. Although

(1→2)-linkages are not uncommon in yeast mannans (40), this glycosidic linkage has not been previously associated with legume seed galactomannans. All the D-galactose units occupy terminal, non-reducing "branch point" positions since the ratio of the tetra-O-methyl-D-galactose to di-O-methyl-D-mannose is essentially 1:1 (the slightly lower tetra-O-methyl-D-galactose quantity, Table I, is probably due to inadvertent loss during evaporation). The absence of 2,4-di-O-methyl-D-mannose indicated the absence of (1→3) - linked D-mannose units associated with branching at C_6. The approximate D.P. of the repeating unit based on the amount of tetra-O-methyl-D-mannose formed was found to be about 80. From the approximate molecular weight deduced from ultracentrifugation, it would appear that four such units constitute one molecule. It is assumed that the use of alkali as the solvent ensured that a monodisperse system was analysed in the ultracentrifuge.

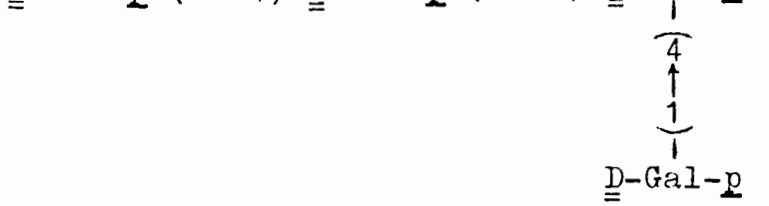
C. Smith Degradation

The distinguishing feature of the Smith periodate degradation is based on the relative lability to acid hydrolysis found for true acetal systems compared to glycosides, both of which may be formed in periodate

degradation of polysaccharides. Treatment of the polysaccharide with periodate (4 - 5°C) resulted in periodate consumption of slightly less than 1 mole per hexose unit. This indicates the presence of periodate resistant hexose units, e.g., (1→3)-linkages. Reduction of the polyaldehyde with borohydride followed by mild acid hydrolysis in which only acetal systems would be cleaved, gave glycerol, erythritol, (no threitol) and two non-reducing components (glycosides) with slower paper chromatographic mobilities than D-galactose. Analysis of the hydrolysis mixture for intact hexose by the phenol-sulfuric acid method indicated that 18 - 20% had survived periodate attack. The appearance of glycerol can primarily be attributed to terminal, non-reducing D-mannose and D-galactose units, mostly the latter since no threitol was detected. Threitol would arise from D-galactose units consecutively linked (1→4). The relatively major quantity of erythritol produced shows that the main chain of the polysaccharide is composed of D-mannopyranose units predominantly linked (1→4). The results also indicate that the D-galactose units are present as single unit branch points primarily linked (1→6) to approximately every third D-mannose unit of the main chain, for example,



or it can be linked (1→4) to the main chain, for example,

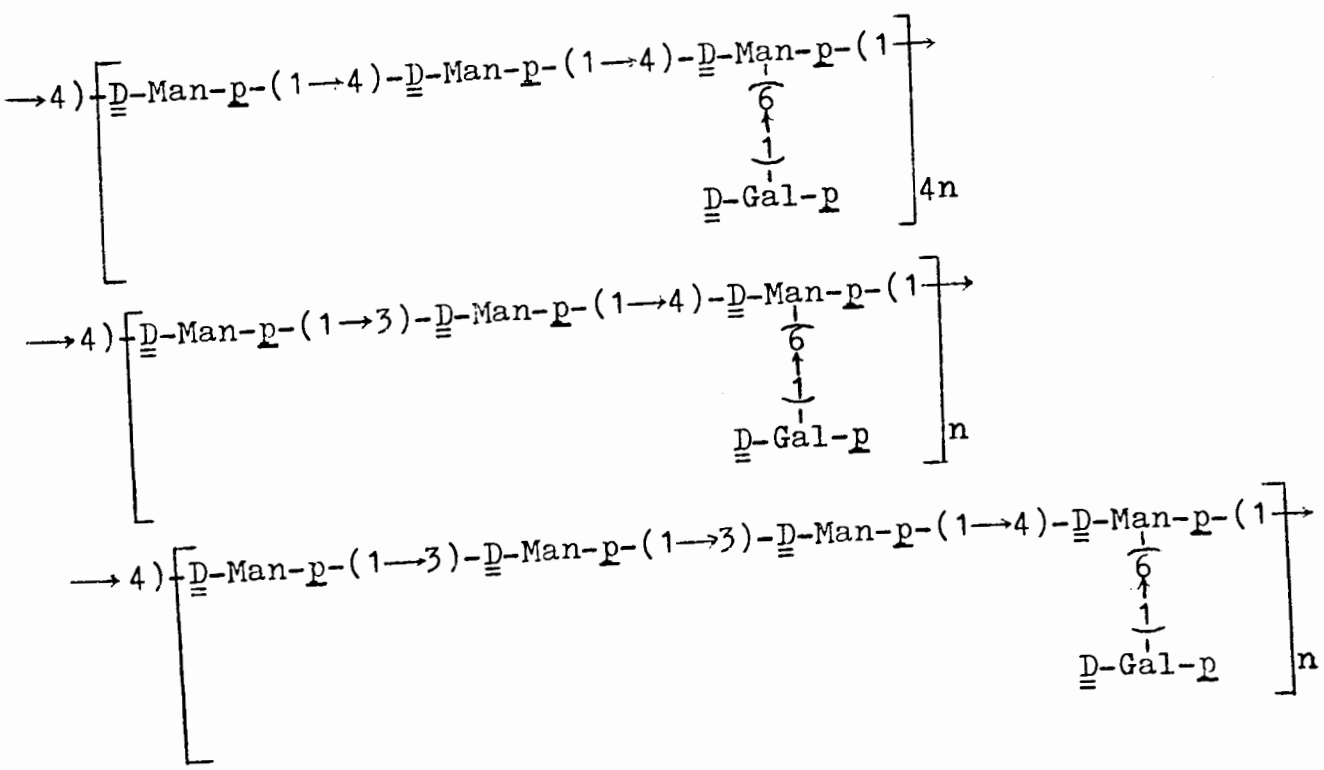


However, studies of a large number of galactomannans (3) have shown that the D-galactose units are usually linked (1→6) to the main chain. The presence of two non-reducing oligosaccharides fractions indicated that a small proportion of other than (1→4)- and (1→4) - (1→6)-linkages existed. Hydrolysis of component I, R_{Man} = 0.61, gave D-mannose and erythritol in a ratio of 1:1. This finding indicates that a minor proportion of D-mannose units were linked (1→3) and these were flanked by (1→4)-linked units, for example,
D-Man-p-(1→4)-D-Man-p-(1→3)-D-Man-p-(1→4)-.
Oligosaccharide II, R_{Man} = 0.33, the molar ratio of I and II being approximately 1:1, when hydrolysed gave D-mannose and erythritol in a ratio of 2:1 indicating a small proportion of D-mannose units consecutively linked (1→3), for example,

D-Man-p-(1→4)-D-Man-p-(1→3)-D-Man-p-(1→3)-D-Man-p-(1→4)-.
A further Smith type periodate degradation of II gave a non-reducing component (shown by hydrolysis to consist of D-mannose and glycerol, molar ratio 1:1), glycerol and formaldehyde. Formaldehyde would be expected from C_4 of the erythritol moiety of II, whereas glycerol would arise from the terminal, "non-reducing" (1→3)-linked D-mannose unit. The internal (1→3)- D-mannose unit would survive.

D. Structural notation

From the methylation and periodate oxidation experiments, a reasonably simple structure for the particular galactomannan may be assigned with relative certainty, since the polysaccharide was immune to action of emulsin and the identity of the methylated sugars and periodate degradation products was definitely established directly or through specific degradation. The following structure is consistent with the experimental results obtained.


→4)-D-Man-p-(1→4)-D-Man-p-(1→4)-D-Man-p-(1→
6
1
D-Gal-p
4n
→4)-D-Man-p-(1→3)-D-Man-p-(1→4)-D-Man-p-(1→
6
1
D-Gal-p
n
→4)-D-Man-p-(1→3)-D-Man-p-(1→3)-D-Man-p-(1→4)-D-Man-p-(1→
6
1
D-Gal-p
n

The low incidence of (1→2)-linkages in the main D-mannose chain (4 in 70) is, due to complexity of representation, not included in the above structural notation. Although the above notation provides the simplest structure that can be envisaged based on the experimental results obtained, it is in no way intended to indicate a highly regularized sequence. Such information could only be obtained from a sequential linkage analysis which is not as yet available. It seems reasonable to assume that the total structure of polysaccharides such as the one investigated arises from a rather orderly co-ordination of enzyme catalysed events and a rather regularized structure such as indicated by the above notation appears more attractive than a completely random sequence.

SUMMARY

(1) The major galactomannan isolated from seed of *Crotalaria mucronata* was found after purification via repeated copper complex formation, to be composed of D-mannose and D-galactose in a molar ratio of 3:1.

(2) Methylation and hydrolysis led to the identification of

	Molar ratio
2,3,4,6-tetra-O-methyl-D-mannose,	1
2,3,4,6-tetra-O-methyl-D-galactose,	23
2,3,6-tri-O-methyl-D-mannose,	28
2,4,6-tri-O-methyl-D-mannose,	11
3,4,6-tri-O-methyl-D-mannose, and	4
2,3-di-O-methyl-D-mannose	23

(3) Classical and Smith-type periodate degradation indicated that the D-galactose units occupied single unit branch points linked (1→6); approximately one in every six D-mannose units was resistant to periodate attack ((1→3)-linkage indicated); an approximately equimolar occurrence of 2-O-β-D-mannopyranosyl-D-erythritol and 3-O-β-D-mannopyranosyl-2-O-β-D-mannopyranosyl-D-erythritol indicated a significant frequency of isolated and consecutive (1→3)-linkages.

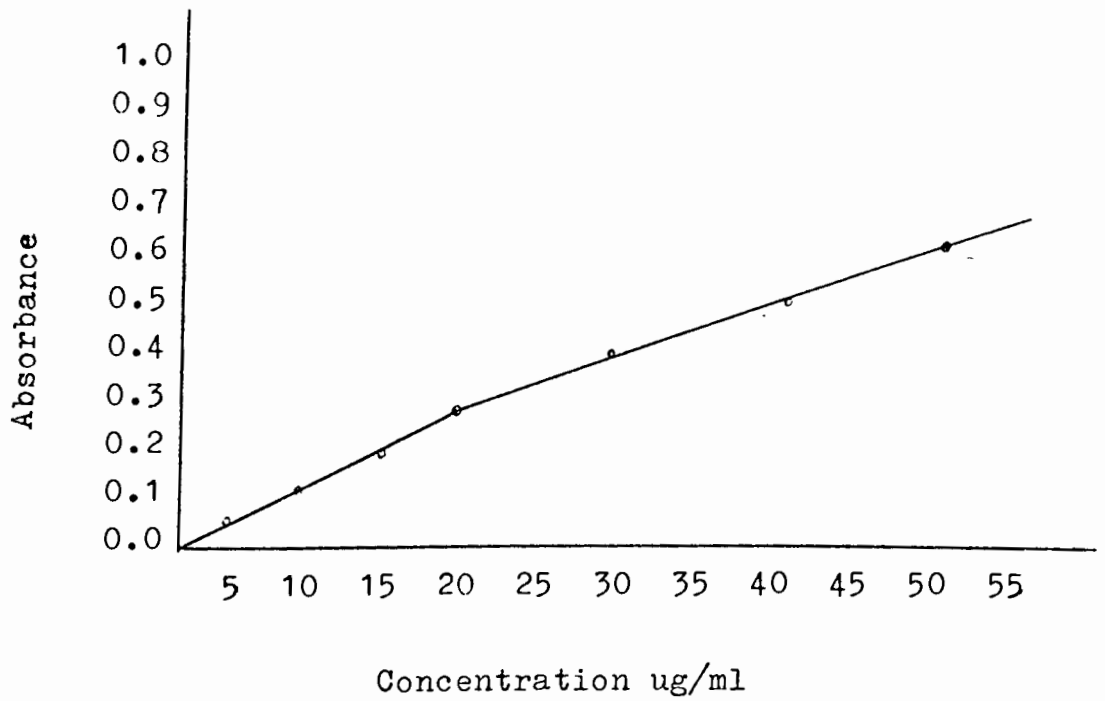


Figure I. Standard curve for D-mannose (phenol-sulfuric acid procedure).

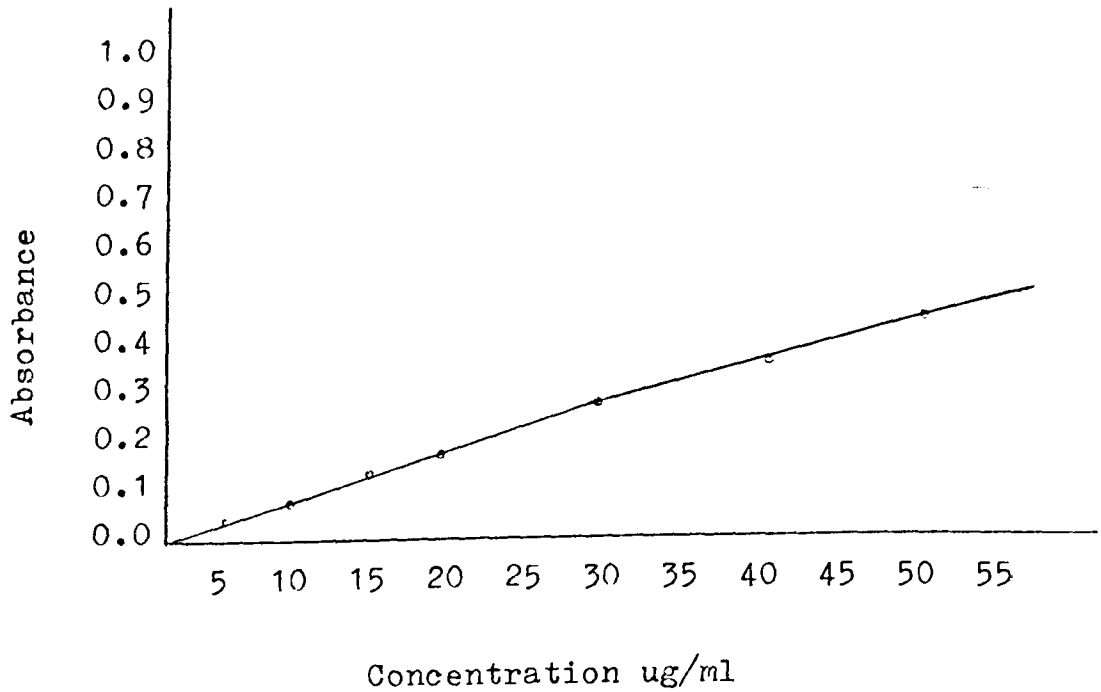


Figure II. Standard curve for D-galactose (phenol-sulfuric acid procedure).

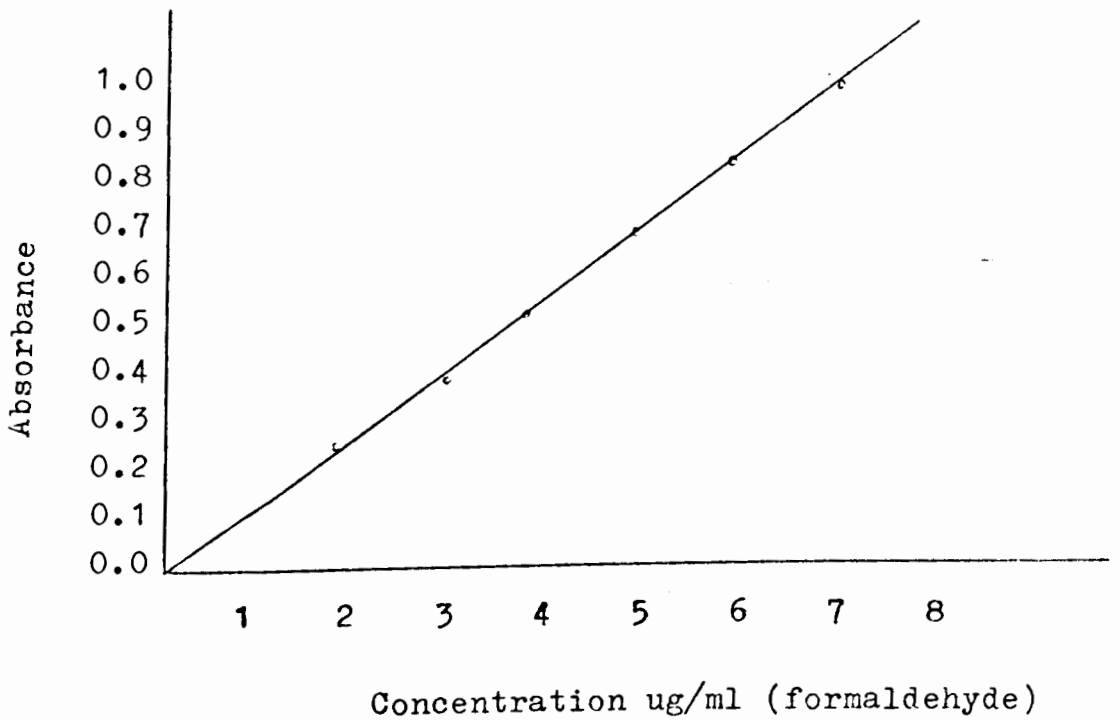


Figure III. Standard curve for erythritol (chromotropic acid procedure).

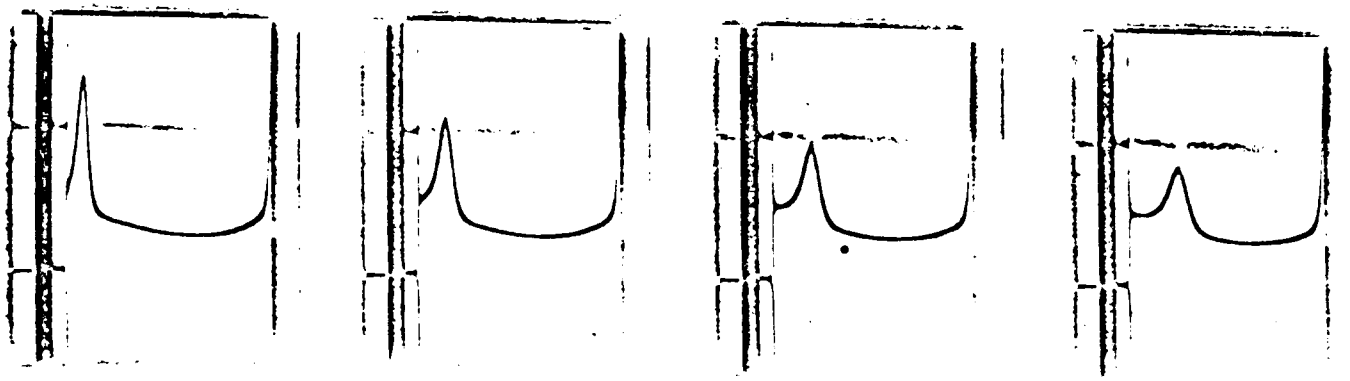


Figure IV. Ultracentrifuge sedimentation pattern of Crotalaria galactomannan (0.8% polysaccharide in 5 N sodium hydroxide, rotor speed 60,000 r.p.m., time - 2 hours, 16 minute exposure intervals).

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PART II

STRUCTURE STUDY OF A GALACTOMANNAN FROM SEED OF

CAESALPINIA PULCHERIMA

INTRODUCTION

Caesalpinia pulcherima is a thorny, bushy legume which is widely distributed in tropical and sub-tropical regions. Its thorny character (thorns are extremely hard and approximately one to two inches long) make this plant ideal for barriers. The mature plant produces an abundance of flowers and the pods which subsequently developed contain from two to eight large seeds (*ca* 0.7 to 1.0 cm. dia., 0.2 to 0.4 cm. thick). In contrast, the *Crotalaria mucronata* plant is small (eight to sixteen inches tall) and the seed pods resemble the spiral seed pod of alfalfa plants. Characteristic of legumes, the seeds of *Caesalpinia pulcherima* contain a liberal quantity of neutral gum (galactomannan) in the endosperm.

Whereas the galactomannan from mesquite is of tremendous commercial value due to ease of harvesting, the thorny character of the *Caesalpinia pulcherima* plant renders it relatively ineffective as a potential industrial source of neutral gum.

EXPERIMENTAL METHODS AND RESULTS

A. Isolation of Galactomannan

Galactomannan was extracted from coarsely ground seed as described in Part I. The grey, stringy precipitate was dried by solvent exchange.

B. Purification of Galactomannan with Copper Acetate

The crude galactomannan was purified by repeated complex formation with copper acetate as described in Part I, $[\alpha]_D^{22}$ +6° (c 1.0, 0.1 N sodium hydroxide).

C. Ultracentrifugation

Solutions of galactomannan (0.5 to 1% concentration) in alkali (1 to 5 N sodium hydroxide) were centrifuged for 3 hours, rotor speed 60,000 r.p.m. The sedimentation pattern (0.8% galactomannan in 5 N sodium hydroxide) showed that the polysaccharide was homogeneous (one single, relatively sharp peak) (Figure I). The molecular weight was estimated to be about 60,000 by comparison to the sedimentation rate observed for β-amylase with a known molecular weight of 62,500.

D. Hydrolysis of Polysaccharide

(a) With 1 N sulfuric acid

The galactomannan was hydrolysed and analysed as described in Part I. The ratio of D-mannose and D-galactose as determined by paper chromatography and gas-liquid chromatography was found to be 3:1. The individual sugars were further identified as described in the comparable section in Part I.

(b) Hydrolysis with Emulsin

The polysaccharide was treated (see Part I for methodology) with emulsin. After extended incubation, no D-galactose (or D-mannose) was detected by paper chromatography, neither was any evidence obtained for the formation of small oligomers.

E. Methylation Analysis

The polysaccharide was methylated and analysed as described in Part I. The molar ratio of the partially methylated sugars is shown in Table I and II. The mixture of sugars was separated using Whatman 3 MM paper with butanone-water azeotrope (1) and the individual partially methylated sugars characterized as follows :

Table I

Vapor phase chromatography of trimethylsilyl derivatives of partially methylated sugars

Trimethylsilyl derivative	Retention time (min.)	Molar ratio
2,3,4,6-tetra-O-methyl-D-mannose	16.5	1
2,3,4,6-tetra-O-methyl-D-galactose	15.2 (α) 18.0 (β)	27
2,4,6-tri-O-methyl-D-mannose	12.0	4
3,4,6-tri-O-methyl-D-mannose	10.4	2
2,3,6-tri-O-methyl-D-mannose	13.4	60
2,3-di-O-methyl-D-mannose	8.6	32

Table II

Paper chromatographic separation of partially methylated sugars

Component	Butanone-water azeotrope R_F	n-Butanol-ethanol water R_G#	Proposed compound	Molar ratio ##
1	0.79	0.96	2,3,4,6-tetra-O-methyl-D-mannose	
2	0.69	0.88	2,3,4,6-tetra-O-methyl-D-galactose	1
3	0.56	0.82	2,4,6-tri-O-methyl-D-mannose	
4	0.50	0.81	2,3,6-tri-O-methyl-D-mannose and 3,4,6-tri-O-methyl-D-mannose	2
5	0.21	0.57	2,3-di-O-methyl-D-mannose	1

mobility with reference to 2,3,4,6-tetra-O-methyl-D-glucose

from chromatographic results in butanone-water azeotrope using phenol-sulfuric acid method.

Component 1 and authentic 2,3,4,6-tetra-O-methyl-D-mannose showed identical R_F, 0.79, in butanone-water azeotrope, and R_G, 0.96, in n-butanol:ethanol:water (4:1:5) and retention time of the trimethylsilyl derivatives (16.5 minutes) (20% neopentylglycolsuccinate on Gas Chrom. 900, 3/16" by 12' column, 180°C, helium flow rate 100 ml/min., flame ionization detector) (7).

Component 2, 120 mg, $[\alpha]_D^{22}$ 118° (equil.) (c 0.7, water), gave upon treatment with aniline in dry ethanol containing a trace of glacial acetic acid, 2,3,4,6-tetra-O-methyl-N-phenyl-D-galactosylamine, m.p. 190-191°C, $[\alpha]_D^{22}$ 40° (equil.) (c 0.5, acetone) (lit. m.p. 192°C, $[\alpha]_D$ -83→41°) (2). Further confirmation of identity was established by comparative chromatography. Component 2 and authentic 2,3,4,6-tetra-O-methyl-D-galactose showed identical R_F, 0.69, in butanone-water azeotrope, and R_G, 0.88, in n-butanol:ethanol:water (4:1:5) and retention time of the trimethylsilyl derivatives (15.2 minutes, α-anomer; 18.0 minutes, β-anomer).

Component 3, 11 mg, $[\alpha]_D^{22}$ 15° (equil.) (c 0.3, water), was treated with aniline as above to give 2,4,6-tri-O-methyl-N-pheryl-D-mannosylamine, m.p. 133 - 135°C, $[\alpha]_D^{22}$ +8° (equil.) (c 0.3, methanol) (lit. m.p. 134°C, $[\alpha]_D$ -150→8°) (3). The identity of component 3 was further established by treatment with sodium borohydride,

periodic acid and demethylation as described in comparable section in Part I. D-mannitol was identified by comparative paper chromatography and vapor phase chromatography, R_{Man}, 0.94 in ethyl acetate:pyridine:water (8:2:1), retention time of trimethylsilyl derivatives, 4.4 minutes (170°C, otherwise conditions as described previously).

Component 4, 210 mg, $[\alpha]_D^{22}$ -9° (equil.) (c 0.7, water), was dissolved in dry pyridine, 10% molar excess p-nitrobenzoyl chloride added, followed by heating to 65°C for 30 minutes. The mixture was cooled, saturated solution of sodium bicarbonate added followed by extraction with chloroform. After recrystallization from methanol, the 1,4-di-p-nitrobenzoyl-2,3,6-tri-O-methyl-D-mannose had m.p. 187 - 189°C, $[\alpha]_D^{22}$ +33° (c 1.0, chloroform) (lit. m.p. 187 - 188°C, $[\alpha]_D$ -33° in chloroform) (4). Component 4 was further analysed as follows: Thin layer chromatography on silica gel showed, using butanone-water azeotrope, three components with R_G values of 0.73, 0.76 and 0.68. In benzene:ethanol:water (35:15:1), three components were also evident with R_G values of 0.52, 0.50 and 0.45 thus indicating three tri-O-methyl sugars. V.P.C. analysis of trimethylsilyl derivatives showed the presence of 2,4,6-tri-O-methyl-D-mannose, 12.0 minutes (minor proportion), 3,4,6-tri-O-methyl-D-mannose,

10.4 minutes (minor proportion), and 2,3,6-tri-O-methyl-D-mannose, 13.4 minutes (major proportion). A portion (100 mg) of component 4 was subjected to the following series of reactions : (a) reduction with borohydride, (b) oxidation with periodic acid, (c) reduction with borohydride, (d) demethylation with 35% hydrobromic acid, (e) deionization and (f) identification of products by paper and gas chromatography. Ethylene glycol and erythritol were identified and would arise from 2,3,6-tri-O-methyl-D-mannose. D-arabinitol was identified by comparison with authentic D-arabinitol obtained via the above series of reactions carried out on 3,4,6-tri-O-methyl-D-mannose prepared by a published procedure (5). The retention time of the trimethylsilyl derivatives of authentic D-arabinitol and that obtained from component 4 was identical (3.4 minutes, 170°C, otherwise conditions as described previously), pentaacetate derivative was not prepared due to insufficient quantity. D-arabinitol would arise from 3,4,6-tri-O-methyl-D-mannose (the same pentitol would arise from 2,3,4-tri-O-methyl-D-mannose; however, the absence of this sugar was established previously from V.P.C. results - see Table I). D-mannitol was identified by comparison with authentic compound, the trimethylsilyl derivatives of both having a retention time of 4.4 minutes (170°, conditions as described

previously). D-mannitol would be derived from 2,4,6-tri-O-methyl-D-mannose via the above series of reactions.

Component 5, 120 mg, $[\alpha]_D^{22}$ -16° (c 0.5, water), gave upon treatment with p-nitrobenzoyl chloride, as above, 1,4,6-tri-p-nitrobenzoyl-2,3-di-O-methyl-D-mannose, m.p. 192 - 194°C, $[\alpha]_D^{22}$ +65° (c 0.5, chloroform) (lit.m.p. 194°C, $[\alpha]_D$ +65° in chloroform) (6)

F. Smith Periodate Degradation

The polysaccharide (1 gram) was treated with periodic acid and analysed as described in Part I. Periodate consumption reached 1.2 moles per hexose unit in 3 days with little change thereafter. Analysis of the polyalcohol solution by the phenol-sulfuric acid method indicated intact hexose to the extent of 4 - 6% (in terms of D-mannose) of the original polysaccharide. Glycerol and erythritol (molar ratio 1:2.85) were detected and the molar ratio determined as described in Part I. Two non-reducing components R_{Man} = 0.64 (component I) and R_{Man} = 0.35 (component II) were also evident upon paper chromatographic analysis. The compounds were separated using Whatman 3 MM paper, ethyl acetate:pyridine:water (8:2:1) and characterized as follows :

Glycerol (about 50 mg) - The derived tri-p-nitrobenzoate had m.p. and mixed m.p. 191°C.

Erythritol (about 140 mg) - The derived tetra-p-nitrobenzoate had m.p. and mixed m.p. 250°C.

Component I (about 4 mg) - was hydrolysed in N sulfuric acid (10 ml) and the hydrolysate after the usual work up was shown by paper (Whatman No.1) chromatography, using ethyl acetate:pyridine:water (8:2:1), and silver nitrate spray, to contain D-mannose and erythritol in a 1:1 molar ratic (procedures described in Part I).

Component II (about 7 mg) - A portion (3 mg) was hydrolysed as I above and the hydrolysate shown by paper chromatography (Whatman No.1, ethyl acetate:pyridine:water (8:2:1)) to contain D-mannose and erythritol in a 2:1 molar ratio. The molar ratio was determined using phenol-sulfuric acid method in conjunction with the periodate-chromotropic acid reagent. The remaining portion of II was degraded with periodate (Smith degradation) whereupon 1 molar proportion of formaldehyde (assayed with chromotropic acid) was produced and a non-reducing component III with slightly faster mobility than I were formed upon mild acid hydrolysis. Complete hydrolysis

of III, as described for I, gave D-mannose and glycerol in a 1:1 molar ratio.

DISCUSSION

A. Isolation and hydrolysis of the galactomannan

The purified polysaccharide had $[\alpha]_D^{22}$ +6° (0.1 N sodium hydroxide) and was in this respect very similar to Crotalaria gum. This low specific rotation indicates that the glycosidic linkages in the main chain have the beta anomeric configuration. Since the galactomannan was unaffected by treatment with emulsin, the D-galactose units are evidently attached to presumably the main D-mannose chain by alpha linkages. This is strikingly similar to the linkage pattern for Crotalaria galactomannan.

B. Methylation analysis

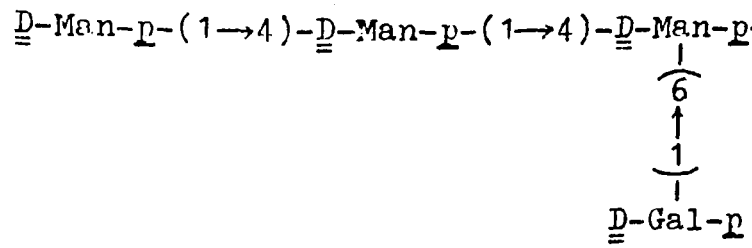
The results obtained from V.P.C. analysis of the partially methylated sugars generated by hydrolysis of the fully methylated galactomannan are essentially in agreement with the periodate oxidation results in that approximately one of every twenty D-mannose units is linked (1→3). The occurence of a small proportion of (1→2)-linkages was noted by the presence of the requisite sugar. All the D-galactose units occupy

terminal, non-reducing "branch point" positions since the ratio of tetra-O-methyl-D-galactose to di-O-methyl-D-mannose is essentially 1:1. The absence of 2,4-di-O-methyl-D-mannose indicated the absence of (1→3)-linked D-mannose units associated with branching at C_6. The approximate D.P. of the repeating unit, based on the amount of tetra-O-methyl-D-mannose found, was 120 which is considerably larger than the value for Crotalaria gum. From the results of ultracentrifugation, it is evident that three such units constitute one molecule.

C. Smith degradation

The appearance of glycerol can be attributed to terminal non-reducing D-mannose and D-galactose units, mostly the latter since no threitol was detected. Threitol would, as was indicated earlier, arise from D-galactose units consecutively linked (1→4). The relatively major quantity of erythritol produced shows that the main chain of the polysaccharide is composed of D-mannopyranose units predominantly linked (1→4). The above indicated, as was the case for the methylation results, that the D-galactose units are present as single unit branch points exclusively linked (1→6) to approximately every third D-mannose unit of the main

chain, for example,

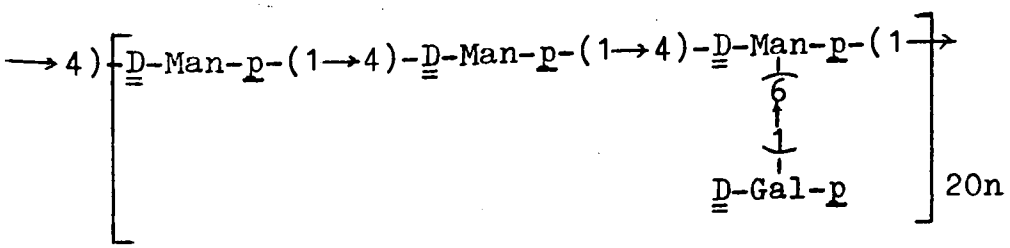


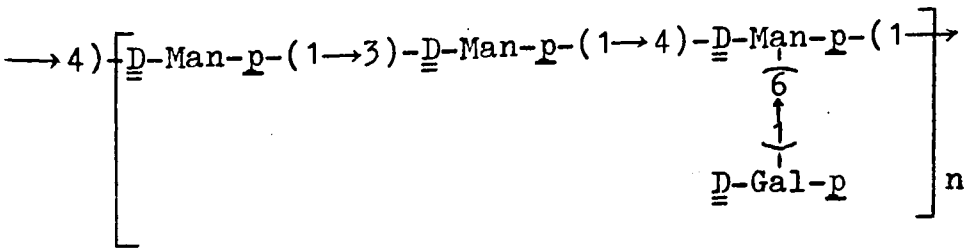
The presence of the two non-reducing components described in the experimental section indicated a significant incidence of isolated and consecutive (1→3)-linkages, for example,

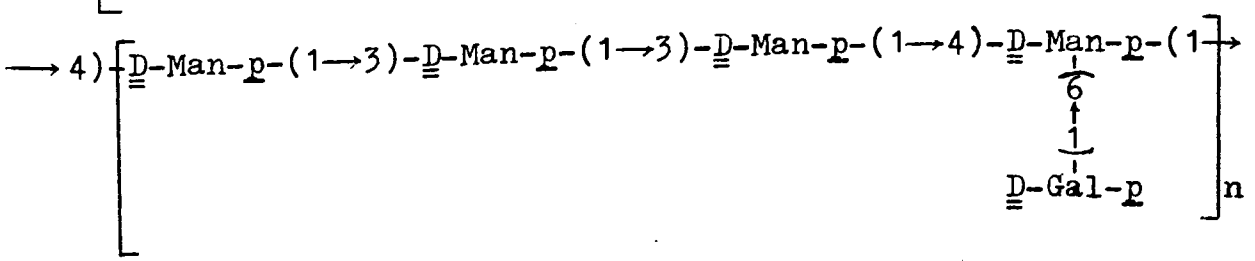
D-Man-p-(1→4)-D-Man-p-(1→3)-D-Man-p-(1→4)-
D-Man-p-(1→4)-D-Man-p-(1→3)-D-Man-p-(1→3)-D-Man-p-(1→4)-

D. Structural notation

From the methylation and periodate oxidation experiments, a reasonably simple structure for this particular galactomannan may be assigned with relative certainty. This is believed possible since the polysaccharide was immune to the action of emulsin and the identity of the methylated sugars was definitely established directly or through degradation. The following structure is consistent with the experimental results obtained :


⟶4)-D-Man-p-(1⟶4)-D-Man-p-(1⟶4)-D-Man-p-(1⟶
6
1
D-Gal-p
20n


⟶4)-D-Man-p-(1⟶3)-D-Man-p-(1⟶4)-D-Man-p-(1⟶
6
1
D-Gal-p
n


⟶4)-D-Man-p-(1⟶3)-D-Man-p-(1⟶3)-D-Man-p-(1⟶4)-D-Man-p-(1⟶
6
1
D-Gal-p
n

As in the case of the galactomannan from *Crotalaria*, the low incidence of (1→2)-linkages (2%) cannot be conveniently included in the structural notation. Again, the structural notation does not necessary imply a regularized sequence.

SUMMARY

(1) The galactomannan obtained from seed of *Caesalpinia pulcherima* is composed of D-mannose and D-galactose in a molar ratio of 3:1.

(2) Methylation and hydrolysis revealed the presence of

	Molar ratio
2,3,4,6-tetra-O-methyl-D-mannose,	1
2,3,4,6-tetra-O-methyl-D-galactose,	27
2,3,6-tri-O-methyl-D-mannose,	60
2,4,6-tri-O-methyl-D-mannose,	4
3,4,6-tri-O-methyl-D-mannose and	2
2,3-di-O-methyl-D-mannose	32

(3) Classical and Smith type periodate degradation indicated that the D-galactose units occupied single unit branch points linked (1→6); approximately one in every twenty D-mannose units was resistant to periodate attack ((1→3)-linkage indicated); an approximately equimolar occurrence of small quantities of 2-O-β-D-mannopyranosyl-D-erythritol and 3-O-β-D-mannopyrsnosyl-2-O-β-D-mannopyranosyl-D-erythritol indicated a slight frequency of isolated and consecutive (1→3)-linkages.

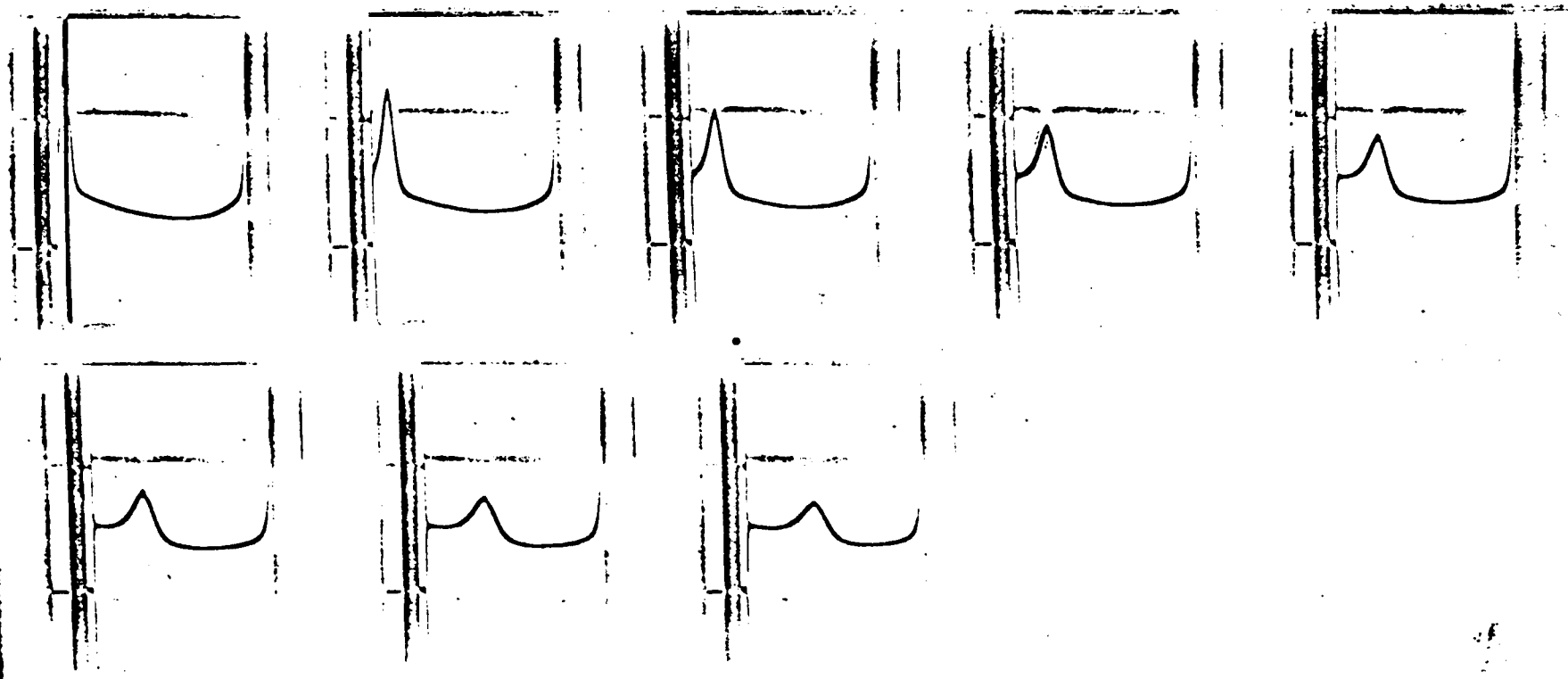


Figure I. Ultracentrifuge sedimentation pattern of Caesalpinia galactomannan (0.8% polysaccharide in 5 N sodium hydroxide, rotor speed 60,000 r.p.m., time - 3 hours, 16 minute exposure intervals).

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PART III

STRUCTURE STUDIES OF OLIGOSACCHARIDES

PRODUCED BY

VERTICILLIUM DAHLIAE

INTRODUCTION

Until quite recently, interest in oligosaccharides was largely confined to a limited number of compounds occuring free in nature or which could be easily prepared from simple sugar polymers such as cellulose and starch. The advent of chromatographic techniques has brought with it the rapid development of relatively easy methods for the effective fractionation of complex mixtures of oligosaccharides and many new compounds in the oligosaccharide series have now been isolated and characterized. The synthesis of oligosaccharides by either chemical or enzymic means has been receiving very considerable attention particularly in immunobiochemistry. Both types of syntheses have primarily been aimed at an understanding of the structure and biological formation of the physiologically important sugars occuring in nature. In the course of such work many other oligosaccharides have been prepared, often in the case of enzymic synthesis under conditions of doubtful physiological significance. These <u>in vitro</u> enzymic synthesis do, however, represent important preparative routes for a number of useful oligosaccharides.

The present investigation deals with the synthesis

of oligosaccharides by the plant pathogenic fungus, Verticillium dahliae, from sucrose. The structural analysis of several of a series of oligosaccharides (disaccharides) are presented.

LITERATURE REVIEW

A limited number of naturally occuring oligosaccharides may be isolated by relatively simple crystallization procedures. Of great concern, however, is the isolation in a pure state of the many compounds which are obtained in complex mixture and which are often only present as minor constituents of these mixtures. Such isolations involve the use of various methods of partition and adsorption chromatography. A detailed review of the application of chromatographic techniques to the preparation and purification of oligosaccharides is given by Bailey and Pridham (1).

Relatively large amounts of monosaccharides can be quite easily separated by adsorption chromatography on charcoal. Charcoal-celite (1) mixtures are commonly used although charcoal-cellulose (2) and stearic acid-treated charcoal (3) have also been recommended. The basis of charcoal fractionation is that di- and higher saccharides are adsorped on the charcoal and eluted with aqueous ethanol, the D.P. of the eluted oligosaccharide increasing as the ethanol concentration increases. In simple fractionation, elution is attained in a stepwise manner; monosaccharides being removed by water, while disaccharides are eluted in primarily one fraction

(using 5 - 7 per cent ethanol) (4, 5) followed by trisaccharides (9 - 15 per cent ethanol) (4, 5) and so on. This procedure is, therefore, most useful for separating the members of an homologous series, or by freeing a particular sugar from saccharides of higher and lower D.P.

Although adsorption chromatographic methods mentioned above are excellent for separating oligosaccharides of different D.P., they are not always suitable for separating isomeric compounds of the same D.P. In this latter case, partition methods often offer the only means of effectively separating closely related oligosaccharide isomers. Although the application of cellulose column chromatography has proved very effective for the resolution of monosaccharide mixtures, it has not proved so suitable for separating oligosaccharides, mainly because of slowness and the fact that many of the solvents which give good separations of oligosaccharide mixtures on papers (1) are not suitable for use with cellulose columns. Various modifications have therefore, been developed. By the use of thicker paper it is comparatively easy matter to isolate 30 to 50 mg of pure oligosaccharide. Such quantities are often ample for structural studies.

Although the above chromatographic procedures

generally separate individual oligosaccharides from mixtures or from traces of sugar impurities, the isolated compounds must often be freed from troublesome traces of non-sugar impurities and must always be checked for homogeneity so far as the sugar itself is concerned. Traces of inorganic material can usually be conveniently removed by dissolving the anhydrous sugar in dry methanol and filtering off the insoluble inorganic impurities. Tetra- and higher saccharides are often not very soluble in methanol, however, they may be purified by dissolving in anhydrous ethanol followed by addition of the minimum amount of water needed to effect solution (5). Inorganic ions may also be removed with ion-exchange resins which show little or no tendency for irreversible adsorption of oligosaccharides. It is particularly important that weak anion exchange resins be used. Homogeneity of the oligosaccharide should be checked by paper chromatographic analysis in a variety of solvents (acidic, basic and neutral) which are known to give good separations of these substances (1) when the D.P. is below four.

Certain chemical properties are common to the whole general class of oligosaccharides and are mainly relevent to the determination of structure. These are briefly reviewed below. Oligosaccharides are commonly divided into two main classes, reducing and non-reducing. It

should be mentioned at this point, however, that oligosaccharides with a substituent on C-2 of a reducing aldose unit are virtually non-reducing to the commonly used copper reagents (6).

The chromatographic techniques which have had such a major impact on preparative methods have also had a marked effect on the methods used in investigating structure. The classical chemical transformations used to establish the structures of compounds which were readily available in large amounts have to a large extent given way to methods requiring much smaller (1 - 10 mg) amounts of material. This means that the structure of a new oligosaccharide can now often be assigned with a fair degree of certainty when only a few milligrams of pure sugar are available (1, 7, 8).

There are two general aspects of structural studies. Firstly, establishment of proof of identity of an isolated compound with a compound of known structure and secondly, establishment of the structure of a hitherto unknown compound. The first may be largely achieved by paper chromatographic studies without necessarily isolating the compound. Such identifications should be based on a direct comparison with an authentic specimen both with respect to movement in a wide variety of solvents and the reactions with several spray reagents.

Sole reliance, in chromatographic studies, on published R_F and R_G values should be resorted to only if no authentic specimen can be obtained.

There are quite a number of classical methods used in the structural analysis of oligosaccharides, e.g., paper chromatography, partial linkage analysis and total acid hydrolysis, periodate oxidation, methylation analysis, enzymic hydrolysis, optical activity, etc. The present investigation is confined to the characterization of some disaccharides. Due to the very limiting quantities available, only a few methods for characterization could be employed.

The first task in ascertaining the constituents of oligosaccharides is the identification of the monosaccharides of which the oligosaccharide is composed. This is effected either by acid or enzymic hydrolysis. The identification of cleavage products by means of paper chromatography (9, 10) or V.P.C. (18) presents no difficulty.

Methylation analysis is an important technique for the study of oligosaccharide structure. The classical methylation methods are, however, unsuitable for microscale work on 1 - 5 mg of sugar. This presented a problem of some considerable magnitude since more often than not only very small amounts of oligosaccharide are

available. A recent methylation procedure using methyl iodide in N,N-dimethylformamide with silver or barium oxide, developed by Kuhn and associates (11), has largely overcome this difficulty. Coupled with identification and quantitive measurement of the methylated monosaccharides by gas-liquid chromatography this method has enabled Perila and Bishop (12) to assign structures to oligosaccharides using only 0.5 - 2.0 mg of compound. The methylation method, proved to be as effective as the one described, used in the present investigation involved the use of sodium hydride in methyl sulfoxide and methyl iodide (13, 14).

EXPERIMENTAL METHODS AND RESULTS

Solvents systems used in paper chromatography were:

A. Ethyl acetate:acetic acid:water (8:2:2)

B. Ethyl acetate:pyridine:water (8:2:1)

C. n-Propanol:ethyl acetate:water (7:1:2) (17)

D. Butanone-water azeotrope (20)

A. Isolation of oligosaccharides

Oligosaccharides which were mixed with a large quantity of D-glucose and D-fructose were eluted from a charcoal-celite column (1:1 ratio), 4 x 50 cm., by washing with 10 litres of water. A mixture of oligosaccharides was eluted by passage of 5% aqueous ethanol. On paper chromatography using solvent A, spray reagents E (Tollens solution) (15), F (p-anisidine-trichloroacetic acid) (16), four components were located. In solvent B, four components were also evident having $R_{Sucrose}$, 1.56, 1.22, 1.0 and 0.75 respectively (D_1, D_2, D_3, D_4). Another two fractions were again eluted by 15% and 50% aqueous ethanol, but were not further investigated at this time. The fraction eluted by 5% aqueous ethanol was further purified by dissolving in methanol, filtered and evaporated. Final resolution was affected by paper

Table I

Paper chromatography of hydrolysate of methylated D_4

Component	Paper chromatography Butanone-water azeotrope R_F	Thin layer chromatography Butanone-water azeotrope R_G#	Proposed compound
1	0.82	1	1,3,4,6-tetra-O-methyl-D-fructose
2	0.61	0.78	2,3,4-tri-O-methyl-D-glucose

Mobility with reference to 2,3,4,6-tetra-O-methyl-D-glucose.

Table II

Vapor phase chromatography of trimethylsilyl derivatives of partially methylated sugars

Component	Retention time (min.)	Proposed compound	Molar ratio
1	8.7	1,3,4,6-tetra-O-methyl-α-D-fructose	1
	10.7	1,3,4,6-tetra-O-methyl-β-D-fructose	
2	12.2	2,3,4-tri-O-methyl-D-glucose	1

chromatography (Whatman No.3), using solvent B, and the compounds (D_1 to D_4) were analysed as follows :

B. Analysis of component D_4

The compound, D_4, 13 mg, had $[\alpha]_D^{22}$ $+6^\circ$ (c 0.65, water), $[\alpha]_D^{22}$ -24° (c 0.65, methanol), was shown to be homogeneous by paper chromatography using the three solvent systems, A, B and C. It had a mobility slower than sucrose, $R_{Sucrose}$, 0.75, but slightly faster than maltose, $R_{Maltose}$, 1.03 in solvent B.

To a portion (2 mg), 0.5 ml alkaline Fehling solution was added. On heating, a reddish brown precipitate was formed indicating that D_4 was a reducing sugar. The approximate molecular weight was found to be 330 (Molecular weight machine, Model 115, Hitachi-Perkin-Elmer).

A portion (5 mg) of D_4 was hydrolysed ($100^\circ C$, $1\frac{1}{2}$ hours) with 1 N sulfuric acid (5 ml). After deionization, and evaporation, the hydrolysate was found by paper chromatography, solvents A, B, spray reagents E and F, to contain D-glucose and D-fructose. The hydrolysate was separated (Whatman No.1, solvent B) and the molar ratio was found to be 1:1 as determined by the phenol-sulfuric acid procedure (described in Part I). The ratio

was also determined by V.P.C. of the trimethylsilyl derivatives (18, 19), using 20% neopentylglycolsuccinate on Gas Chrom. 900, 3/16" by 12' column, 180°C, helium flow rate 100 ml/min., flame ionization detector, and was found again to be 1:1 (allowance was made for the loss of D-fructose during hydrolysis).

The remaining portion was methylated using sodium hydride in methyl sulfoxide and methyl iodide (13, 14). The fully methylated disaccharide was hydrolysed (100°C, 1½ hours) with 1 N sulfuric acid (5 ml). The hydrolysate after neutralization with barium carbonate was analysed as follows : Thin layer chromatography on silica gel using solvent D, showed two components, R_G# values 1.0 and 0.78. Paper chromatography in solvent D showed two components with R_F values 0.82 and 0.61. V.P.C. analysis of the trimethylsilyl derivatives showed three peaks with retention times 8.7 minutes, 10.7 minutes and 11.5 minutes. The results are summarized in Tables I and II.

mobility with respect to the mobility of 2,3,4,6-tetra-O-methyl-D-glucose.

Further confirmation of identity was established by preparing 1,3,4,6-tetra-O-methyl-D-fructose and 2,3,4-tri-O-methyl-D-glucose. These were conveniently obtained by hydrolysis of fully methylated raffinose. Component 1 and authentic 1,3,4,6-tetra-O-methyl-D-fructose showed identical R_F, 0.82 in solvent D (paper chromatography), R_G, 1.0 in solvent D (thin layer chromatography), and retention times of trimethylsilyl derivatives (8.7 minutes, α-anomer; 10.7 minutes, β-anomer).

Component 2 and authentic 2,3,4-tri-O-methyl-D-glucose showed identical R_F, 0.61, solvent D (paper chromatography), R_G, 0.78, solvent D (thin layer chromatography on silica gel), and retention time of trimethylsilyl derivatives (11.5 minutes). The molar ratio of the two components was found by V.P.C. to be 1:1.

C. Analysis of component D_3

D_3, 8 mg, after deionization was found to be non-reducing to alkaline Fehling solution. Paper chromatography using solvent systems A, B, C, indicated the presence of one component, which had the same R_F value as authentic sucrose. On hydrolysis by 1 N sulfuric

acid (100°C, 1½ hours), D-glucose and D-fructose were shown in a molar ratio of 1:1. The compound reacted more slowly with p-anisidine-trichloroacetic acid spray than typical reducing disaccharides, e.g., maltose and is believed to be sucrose. It should be noted that sucrose was the sole carbon source for the growing fungus.

D. Analysis of component D_2

Compound D_2, 9 mg, gave, after the usual work up as described for D_4, $[\alpha]_D^{22}$ -16° (c 0.6, water). It was found to be a reducing sugar (by positive test with alkaline Fehling solution) and homogeneous as shown by paper chromatography, using solvents A and B ($R_{Sucrose}$, 1.22 and $R_{Maltose}$, 1.8, in solvent B).

A portion (3 mg) was hydrolysed with 1 N sulfuric acid (10 ml) and after neutralization with barium carbonate, filtration and evaporation of solvent, only D-fructose was identified by paper chromatography, solvents A and B.

The remaining portion (4 mg) was methylated by sodium hydride in methyl sulfoxide and methyl iodide. After hydrolysis (10 ml 1 N sulfuric acid, 100°C, 30 minutes) and neutralization (barium carbonate), the hydrolysate was shown to contain three components by

thin layer chromatography on silica gel, solvent D, R_G values 1.0, 0.79 and 0.70. Paper chromatography, solvent D, three components were evident (I, II, III) with R_F values 0.82, 0.62, 0.51. Component I and authentic 1,3,4,6-tetra-O-methyl-D-fructose showed identical R_F value 0.82 (paper chromatography, solvent D) and R_G value 1.0 (solvent D, thin layer chromatography on silica gel). Components II and III were tentatively identified as a mixture of tri-O-methyl-D-fructoses which could not be analysed in detail due to insufficient quantity. A significant loss of methylated D-fructoses during hydrolysis may be attributed to the apparent inherent instability of fructose derivatives to hot mineral acid.

E. Analysis of component D_1 (12 mg)

Compound D_1, migrating faster than than sucrose in solvent B, gave after previously described isolation techniques, $[\alpha]_D^{22}$ -25° (c 0.6, methanol). Paper chromatography (Whatman No.1) using three solvent systems A, B, C, indicated one single component, $R_{Sucrose}$, 1.56, $R_{Maltose}$, 2.3 (solvent B).

To a portion (2 mg), 0.5 ml alkaline Fehling solution was added. On heating a reddish brown

precipitate was obtained indicating that D_1 is a reducing sugar. Another portion (4 mg) was hydrolysed with 1 N sulfuric acid (100°C, 1½ hours). The neutralized (barium carbonate) hydrolysate was found by paper chromatography, solvents A and B, to contain D-glucose and D-fructose in a molar ratio of 1:1 (phenol-sulfuric acid procedure).

The remaining portion was methylated by sodium hydride in methyl sulfoxide and methyl iodide. The fully methylated disaccharide was then hydrolysed by 1 N sulfuric acid (100°C, 1½ hours). After neutralization by barium carbonate, the hydrolysate was analysed as follows: Thin layer chromatography on silica gel using solvent D, showed the presence of two components having R_G values 1.0 and 0.71. Paper chromatography in solvent D, two components were again evident, R_F values 0.82 and 0.55. V.P.C. analysis of trimethylsilyl derivatives (19), three peaks were obtained, retention times, 8.7 minutes, 10.7 minutes and 12.2 minutes. The results are summarized in Tables III and IV.

Further confirmation of identities was established by preparing authentic samples of 1,3,4,6-tetra-O-methyl-D-fructose from raffinose (as described previously) and 2,3,6-tri-O-methyl-D-glucose from amylose by standard methylation, hydrolysis and isolation of major sugar. Component 1 and authentic 1,3,4,6-tetra-O-methyl-D-

Table III

Paper chromatography of hydrolysate of methylated D_1

Component	Paper chromatography Butanone-water azeotrope R_F	Thin layer chromatography Butanone-water azeotrope R_G#	Proposed compound
1	0.82	1	1,3,4,6-tetra-O-methyl-D-fructose
2	0.55	0.71	2,3,6-tri-O-methyl-D-glucose

Mobility with reference to 2,3,4,6-tetra-O-methyl-D-glucose.

Table IV

Vapor phase chromatography of trimethylsilyl derivatives of partially methylated sugars

Component	Retention time (min.)	Proposed compound	Molar ratio
1	8.7	1,3,4,6-tetra-O-methyl-α-D-fructose	1
	10.7	1,3,4,6-tetra-O-methyl-β-D-fructose	
2	12.2	2,3,6-tri-O-methyl-D-glucose	1

fructose showed identical R_F, 0.82 in paper (Whatman No1) chromatography (solvent D), R_G, 1.0 in thin layer chromatography on silica gel (solvent D), and retention times of trimethylsilyl derivatives (8.7 minutes, α-anomer, 10.7 minutes, β-anomer). Component 2 and authentic 2,3,6-tri-O-methyl-D-glucose showed identical R_F, 0.55 in paper chromatography (solvent D), R_G, 0.71 in thin layer chromatography on silica gel (solvent D) and retention time of trimethylsilyl derivatives (12.2 min.). The molar ratio of the two components was found by vapor phase chromatography to be 1:1.

DISCUSSION

While some 40 or more oligosaccharides are found free in Nature the majority are obtained either by synthesis or by hydrolysis of a higher polymers or glycosides. More recently the enzymic synthesis of oligosaccharides has interested a significant number of carbohydrate chemists as well as immunobiochemsits. The *in vitro* enzymic syntheses represent important preparative routes for a number of useful oligosaccharides. Usually, these enzymes are obtained from bacterial systems. In this investigation, bacterial synthesis was discounted in that no contamination was evident (microscopic examination of culture filtrates).

A. Isolation of oligosaccharides

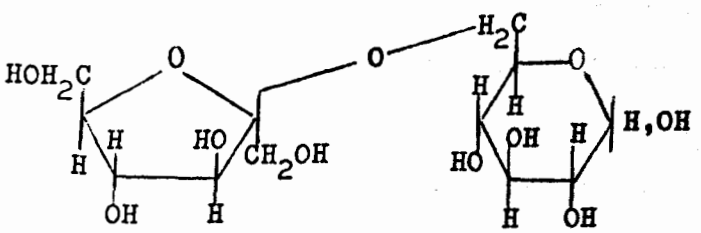
Before purification, it was difficult to effectively separate the oligosaccharides even by paper chromatography on Whatman 3 MM sheets since the solution contained a very large amount of D-glucose and D-fructose (over 99 per cent). However, charcoal-celite column chromatography provides a very effective means of separating the oligosaccharides from monosaccharides by elution with a large quantity of water. Water containing

5% ethanol eluted a mixture of disaccharides. Further separation of disaccharides could be accomplished by paper chromatography. Although both Whatman No.1 and 3 MM paper may be used, the former gives a somewhat clearer resolution and sufficient amounts of the individual compounds may be isolated for further structural investigations.

B. Structure of D_4

From the results of paper chromatography in which D_4 showed $R_{Sucrose}$ 0.75, $R_{Maltose}$ 1.03, and molecular weight determination, it became evident that D_4 was a disaccharide. This is in agreement with the results obtained by Whistler et al (4) and Whelan et al (5) in that 5 - 7% aqueous ethanol eluted disaccharides from a mixture of higher oligomers. The purified compound had $[\alpha]_D^{22}$ +6° (c 0.65, water), $[\alpha]_D^{22}$ -24° (c 0.65, methanol). The low specific rotation suggests that the glycosidic linkage has the beta anomeric configuration. Hydrolysis gave D-glucose and D-fructose in a molar ratio of 1:1. The reduction of alkaline Fehling solution indicated a reducing disaccharide in which either the D-glucose or D-fructose unit could occupy the reducing end position. On methylation and hydrolysis, 1,3,4,6-

tetra-O-methyl-D-fructose and 2,3,4-tri-O-methyl-D-glucose were obtained in a molar ratio of 1:1. These findings showed that the D-glucose unit is the reducing end while the D-fructose unit is joined to the D-glucose unit by a (2→6)-linkage. The following structure agrees with the results described above.



6-O-β-D-fructofuranosyl-D-glucose

The disaccharide, D_4, appears to be identical in all respects to a compound isolated by Whelan and Jones (21) from digests containing β-methyl fructoside, glucose and invertase solution and to a substance isolated by Bell and Edelman (22) from a sucrose-yeast invertase digest. The same type of disaccharide has also been isolated by Feingold et al (23) from a sucrose-levansucrase digest. The identification of large quantities of D-glucose and D-fructose in the original culture filtrate indicated that the fungus contains or produces an enzyme similar in hydrolytic character to that of

invertase. It is therefore not entirely unexpected that at least a limited synthetic ability is associated in which minor amounts of disaccharides and possibly other oligosaccharides are generated which are normally associated with sucrose-invertase or levansucrase systems.

C. Structure of D_3

Compound D_3 was a non-reducing disaccharide and was found to be identical with respect to R_F values to sucrose. On hydrolysis it gave D-fructose and D-glucose in the molar ratio of 1:1. It is of interest to note that since sucrose served as the sole carbon source for the fungus, the very small amount of D_3 (sucrose) which remained escaped a rapid hydrolysis to D-glucose and D-fructose or represented a slow resynthesis of the disaccharide.

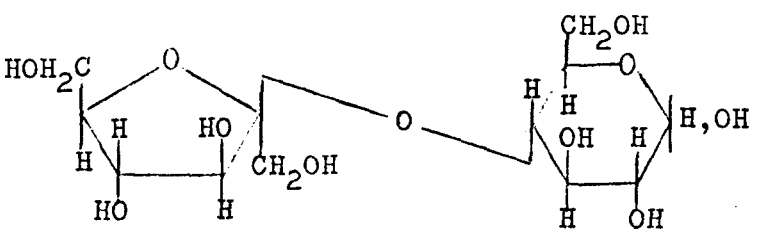
D. Structure of D_2

Compound D_2 had $[\alpha]_D^{22}$ -16° (c 0.6, water). This low value indicated, as in the case of D_4, that the glycosidic linkage has the beta anomeric configuration. It was found to be a reducing substance (disaccharide). On hydrolysis, D_2 gave only D-fructose. Methylation and

hydrolysis gave 1,3,4,6-tetra-O-methyl-D-fructose and a mixture of two tri-O-methyl-D-fructoses which could not due to lack of sufficient quantity be positively characterized. It would therefore appear likely that D_2 is composed of a minimum of two closely related disaccharides and the constituents are most probably fructosylfructoses. These could be two disaccharides which would be formed by a glycosyl transfer to a primary alcoholic group on fructose, viz., 1-O-β-D-fructofuranosyl-D-fructose and 6-O-β-D-fructofuranosyl-D-fructose. The difructoses isolated appear to be similar to those isolated by Bacon (24), Bell and Edelman (22) and Feingold et al (23). The relatively major proportion of 1,3,4,6-tetra-O-methyl-D-fructose could also indicate a difructose linked (2→2) while the reducing property could have arisen from two disaccharides giving rise to the small amount of tri-O-methyl-D-fructoses. Whereas in the glucobiose series, α,α-trehalose, α,β-trehalose and β,β-trehalose are known to occur in nature, the corresponding fructobioses, ((2→2)-linkage), have not been isolated.

E. Structure of D_1

The disaccharide was found to be reducing based on a positive Fehling's test. The low specific rotation, $[\alpha]_D^{22}$ -25° (c 0.6, methanol) of D_1 suggests that the glycosidic linkage had the beta anomeric configuration. Hydrolysis gave D-fructose and D-glucose in a molar ratio of 1:1. It would appear from this that as for D_4, either D-fructose or D-glucose occupied the reducing end position. On methylation followed by hydrolysis, D_1 gave 1,3,4,6-tetra-O-methyl-D-fructose and 2,3,6-tri-O-methyl-D-glucsoe in the molar ratio of 1:1. The above findings showed that the D-glucose unit is at the reducing end while the D-fructose unit is joined to the D-glucose unit by a (2→4)-linkage. The following structure is consistent with the results described.



4-O-β-D-fructofuranosyl-D-glucose

The disaccharides synthesized by Verticillium dahliae are quite similar if not identical to those obtained from levansucrase, yeast invertase, yeast fructosidase as well as disaccharides obtained from other species of fungi. The experiments described in the previous sections of this part gave similar results first noted by Edelman (25), Bacon and Edelman (26), Blanchard and Abon (27), and Bacon (28) in that the sucrase preparations from both yeasts and moulds are capable of synthesizing oligo- and hetero-saccharides apparently by the transfer of the fructose residue from sucrose to suitable glycosyl acceptors. Bealing and Bacon (29) suggested in 1953 that all the previously described invertase preparations from moulds had similar transferring ability. Pazur and Gordon (30) have shown by paper chromatographic techniques that the oligosaccharides produced enzymatically from sucrose contain the sucrosyl and inulobiosyl moiety. Later work (1956) by Feingold et al (23) has indicated that oligorepetitive transfructosylation is a property of ordinary mould and yeast fructosidases as well as levansucrase.

SUMMARY

(1) The disaccharide fraction eluted from a charcoal-celite column was found to contain a minimum of four components as deduced from paper chromatographic analysis.

(2) Compound D_4 was found to be 6-O-β-D-fructofuranosyl-D-glucose.

(3) Compound D_3 is sucrose.

(4) Component D_2 was found to contain a mixture of at least three difructoses. The major component is believed to be linked (2→2), probably β,β.

(5) Compound D_1 was shown to be 4-O-β-D-fructofuranosyl-D-glucose.

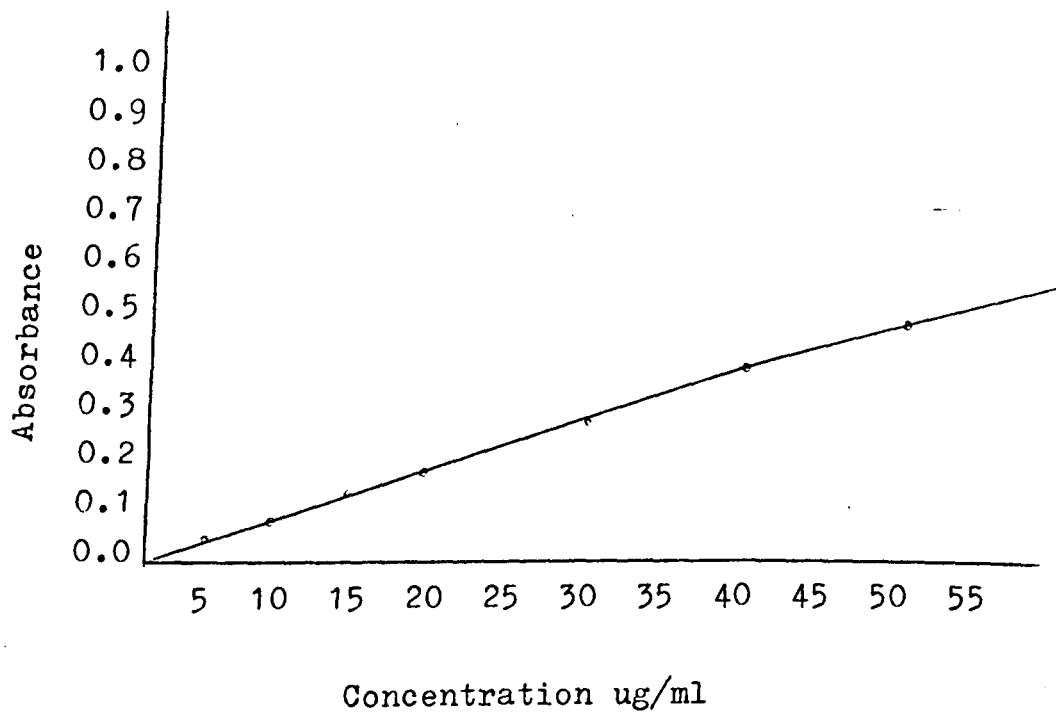


Figure I. Standard curve for D-glucose (phenol-sulfuric acid procedure).

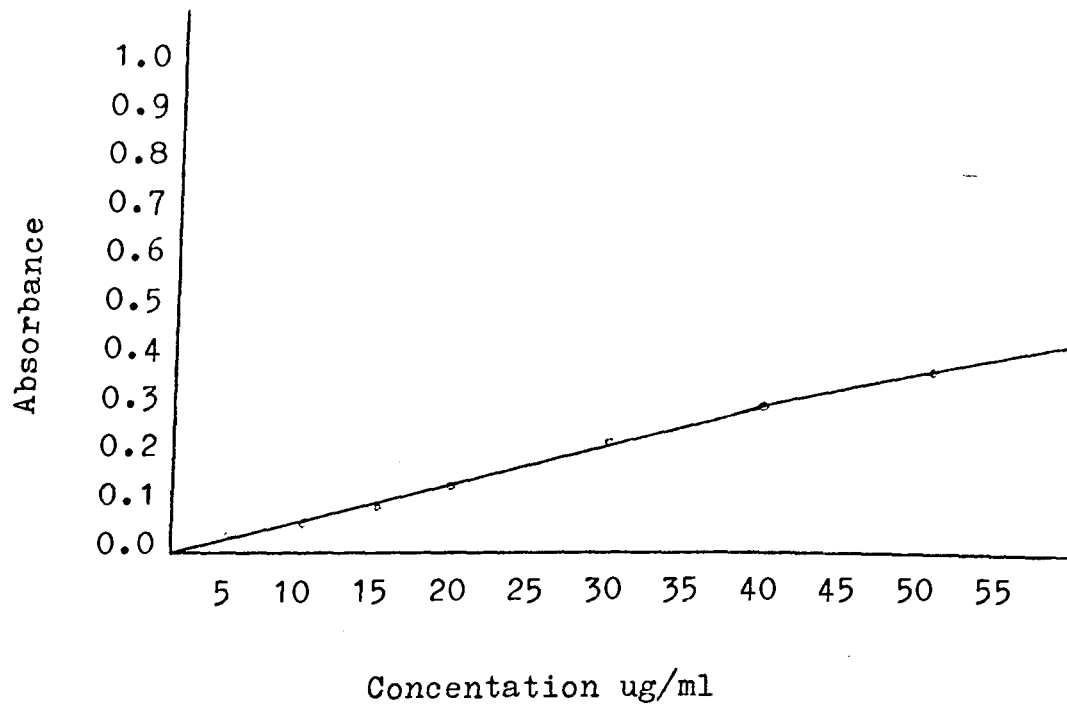


Figure II. Standard curve for D-fructose (phenol-sulfuric acid procedure).

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PART IV

A CONVENIENT AND UNAMBIGUOUS SYNTHESIS

OF

2,4,6-TRI-O-METHYL-D-

MANNOPYRANOSE

INTRODUCTION

The use of methylation techniques has been widely applied in the structural elucidation of oligosaccharides and polysaccharides in spite of some inherent limitations. In some early work, the isolation of unknown methyl ethers as cleavage fragments from the hydrolysis product of methylated polysaccharides frequently gave sufficient material to establish the positions of methoxyl groups by chemical means. In such cases, the proof of structure of these sugar ethers by synthesis was not critical. With the introduction of more delicate microanalytical procedures, such as g.l.c., for the analysis of complex mixtures of partially methylated sugars, it has become necessary, if not mandatory, to prepare known compounds in order to standardize the procedures both qualitatively and quantitively.

D-mannose is commonly found in the carbohydrate fraction of glycoproteins, glycolipids, and many polysaccharides. Reference samples of the various methyl ethers required for structural studies may be synthesized unambiguously by methylation of known derivatives of D-mannose, and from which the protecting groups can be finally removed to give the corresponding free methyl ethers. It is the object of the present investigation

to synthesize one to these sugars which had incidentally been isolated as early as 1941 from yeast mannan (1), i.e., 2,4,6-tri-O-methyl-D-mannose. Curiously, a simple, unambiguous synthesis of this compound has not been reported previously.

LITERATURE REVIEW

2,4,6-Tri-O-methyl-D-mannopyranose was isolated as its monohydrate from the hydrolysis products of methylated yeast mannan and its structure was proved by Haworth, Heath and Peat (1).

It is known that the secondary hydroxyl group at the 3-position of methyl α-D-mannopyranoside is more reactive towards p-toluenesulfonyl chloride than the hydroxyls at the 2 and 4-positions (2). Advantage has been taken of this characteristic to protect the 3-position of methyl α-D-mannopyranoside in further synthetic work.

The 4 and 6-positions of methyl α-D-mannopyranoside may be easily blocked by preparation of the corresponding methyl 4,6-benzylidene-α-D-mannopyranoside (3) and this may in fact be preferred over the formation of the 6-O-trityl derivative. Monotosylation should preferentially occur at the hydroxyl of the 3-position which is equatorial, while very little tosylation should occur at the 2-position as this hydroxyl function is axially oriented. Removal of the benzylidene group has been shown to readily occur in an aqueous acetone solution of very dilute hydrochloric acid (3).

EXPERIMENTAL METHODS AND RESULTS

Methyl 4,6-benzylidene-α-D-mannopyranoside (3) -

Finely powdered methyl α-D-mannopyranoside (20 grams) (m.p. 191°, $[\alpha]_D$ +80°) was heated with freshly distilled benzaldehyde (100 ml) under about 330 mm pressure in a current of dry carbon dioxide at 150° - 155°C. When the sugar had dissolved (2 - 3 hours), the excess benzaldehyde was distilled as completely as possible at the same temperature by further diminution of the pressure. The residue was poured while hot into 200 ml of absolute ethanol, and a crystalline precipitate which is known to be the methyl 2,3-4,6-dibenzylidene-α-D-mannopyranoside was obtained. The alcoholic filtrate containing the soluble 4,6-monobenzylidene derivative was evaporated to dryness under reduced pressure. The dry syrup was dissolved in ethyl acetate, the solution was filtered and evaporated to dryness. The residue crystallized from petroleum ether - absolute ethanol. The crystalline compound, 8.5 grams (30% yield), had m.p. 138°C. The product thus obtained was recrystallized from petroleum ether - absolute ethanol giving pure 4,6-benzylidene-α-D-mannopyranoside in the form of slender needles, m.p. 142°C, $[\alpha]_D^{22}$ +70° (c 1.0, chloroform),

Lit. m.p. 142°C, $[\alpha]_D$ +71.7° in chloroform (3).

Methyl 4,6-benzylidene-3-O-p-toluenesulfonyl-α-D-mannopyranoside -

Methyl 4,6-benzylidene-α-D-mannopyranoside (4.5 grams) in dry pyridine (70 ml) was esterified by addition of p-toluenesulfonyl chloride (3.5 grams) during a period of 12 hours at room temperature. The reaction mixture was poured into ice-water. The product was extracted with chloroform and the combined extracts were washed successively with dilute hydrochloric acid, sodium carbonate solution, water, and finally dried over anhydrous sodium sulfate and evaporated. The product was fractionated on a silica gel column, using chloroform as solvent, to remove traces of impurities. The syrupy product thus obtained was induced to crystallize by addition of petroleum ether, yield 6.1 grams (90%). When recrystallized from petroleum ether - chloroform, it gave lustrous crystals, m.p. 155 - 156°C, $[\alpha]_D^{22}$ +24.5° (c 1.0, chloroform).

Analysis for $C_{21}H_{24}O_8S$: C, 57.79; H, 5.54.
Found : C, 57.1; H, 5.56.

Methyl 3-O-p-toluenesulfonyl-α-D-mannopyranoside -

Methyl 4,6-benzylidene-3-O-p-tolunensulfonyl-α-D-mannopyranoside (5 grams) was dissolved in a mixture of acetone (90 ml), water (40 ml), and N hydrochloric acid (6 ml). The mixture was boiled for 2 - 3 hours (3). The hydrolysis was followed by t.l.c. on silica gel using acetone:chloroform (5:95). The reaction mixture was neutralized with barium carbonate, the insoluble material was removed by filtration, and solvent evaporated under diminished pressure. The aqueous residue was extracted twice with chloroform (10 ml) to remove benzaldehyde and traces of unreacted compound. The solvent was removed in the presence of a minor amount of dissolved carbonate. The dry syrupy residue was extracted with chloroform and the combined extracts, when evaporated and dried under vacuum, gave a syrup, yield 3.6 grams (92%). The product crystallized on standing in benzene solution, m.p. 72°C. When recrystallized from chloroform - petroleum ether, fine white needles, m.p. 87 - 90°C, $[\alpha]_D^{22}$ +51° (c 1.0, chloroform) were obtained.

Analysis for $C_{14}H_{20}O_8S$: C, 48.27; H, 5.79.
Found : C, 47.7; H, 5.74.

Methyl 3-O-p-toluenesulfonyl-2,4,6-tri-O-methyl-α-D-mannopyranoside -

Methyl 3-O-p-toluenesulfonyl-α-D-mannopyranoside (3 grams) was treated with methyl iodide (40 ml) and silver oxide (4 grams) (7) (acetone was added to affect complete solution). Filtration and evaporation gave a product which after six such further methylations yielded the fully methylated compound (no OH absorption in IR spectrum), 3.1 grams (94%). The product crystallized spontaneously and after recrystallization from ether had m.p. 118 - 120°C, $[\alpha]_D^{22}$ +44° (c 1.0, chloroform).

Analysis for $C_{17}H_{26}O_8S$: C, 52.29; H, 6.71; OCH_3, 31.7. Found : C, 52.1; H, 6.78; OCH_3, 31.3.

Methyl 2,4,6-tri-O-methyl-α-D-mannopyranoside -

Methyl 3-O-p-toluenesulfonyl-2,4,6-tri-O-methyl-α-D-mannopyranoside (2 grams) was dissolved in a solution of sodium methoxide (2.5 grams of sodium in 50 ml methanol) (5), and the solution was refluxed for 12 hours. Water (25 ml) was added to the cooled solution, organic solvents were distilled off, and the suspension was extracted with chloroform (5 x 25 ml). The extract was dried over anhydrous sodium sulfate and evaporated to

a syrup (1.1 grams, 92%). The product was chromatographically pure (t.l.c. on silica gel), having an R_F value of 0.55 (butanone-water azeotrope). It had the same retention time (14 minutes) as a sample of methyl 2,4,6-tri-O-methyl-α-D-mannopyranoside obtained by partial methylation of methyl α-D-mannopyranoside (9) with subsequent separation by V.P.C. (20% neopentylglycolsuccinate, column 3/16" by 10', 170°C, helium flow rate 100 ml/minutes) (8). The compound had $[\alpha]_D^{22}$ +51° (c 2.0, chloroform) and failed to crystallize.

Analysis for OCH_3, 52.7%; found, 51.7%.

2,4,6-Tri-O-methyl-D-mannopyranose -

Methyl 2,4,6-tri-O-methyl-α-D-mannopyranoside (1.0 grams) was heated with N sulfuric acid (20 ml) at 100°C, and the hydrolysis, the course of which was followed by t.l.c. on silica gel (butanone-water azeotrope), was complete in 12 hours. The solution was neutralized (barium carbonate), and the filtered solution evaporated to give a syrup (0.94 gram, 98%). The product crystallized upon addition of a small quantity of ethyl acetate. On recrystallization from ether, two lustrous crystalline forms were obtained (α and β-anomers), the m.p. of the mixture was 53 - 57°C, $[\alpha]_D^{22}$ +15.1° (equil.)(c 0.83, water).

Paper chromatography using Whatman No.1 paper, butanone-water azeotrope and p-anisidine spray, showed a single component, having R_F, 0.55, which had the same mobility as a sample of 2,4,6-tri-O-methyl-D-mannopyranose obtained by partial methylation of methyl α-D-mannopyranoside (both gave pink colour reactions with p-anisidine spray). Thin layer chromatography on silica gel, using butanone-water azeotrope, p-anisidine spray and sulfuric acid spray, also showed one single component having the same mobility (R_F, 0.48) as a previously described sample of 2,4,6-tri-O-methyl-D-mannopyranose. Moreover, V.P.C. of the trimethylsilyl derivative of the unambiguously synthesized compound also showed one component (the α and β-anomers do not separate under the conditions used). The compound had the same retention time (4.2 minutes) as 2,4,6-tri-O-methyl-D-mannopyranose obtained from partial methylation experiment previously described (20% neopentylglycolsuccinate, 3/16" by 10' column, 170°C, helium flow rate 100 ml/min.).

A portion (100 mg) was subjected to the following series of reactions : (a) reduction with borohydride, (b) oxidation with periodic acid, (c) reduction with borohydride, (d) demethylation with 35% hydrobromic acid, (e) deionization and (f) identification by paper and gas chromatography of degraded products. D-mannitol was

identified as the only product by comparison with authentic compound, the trimethylsilyl derivatives having a retention time of 2.5 minutes (conditions described previously). D-mannitol would be expected to arise from 2,4,6-tri-O-methyl-D-mannopyranose via the above series of reactions. Another portion (260 mg) was dissolved in 10 ml of absolute ethanol and 250 mg aniline and 1 drop of glacial acetic acid were added. The mixture was refluxed for 5 hours. After evaporation of ethanol, the syrupy compound crystallized on standing in a small volume of ether, yield 180 mg. The derived N-phenylglycosylamine on recrystallization from ether gave fine needles having m.p. 133 - 134°C, $[\alpha]_D^{22}$ -149°→+8° (c 1.0, methanol) (24 hours) (lit. m.p. 134°C, $[\alpha]_D$ -150°→+8° in methanol) (1).

DISCUSSION

2,4,6-Tri-O-methyl-D-mannopyranose is known to present in a large number of hydrolysed methylated mannans and galactomannans. The object of the present experiment was to unambiguously synthesize this compound which could subsequently be used in structural studies of mannose containing polysaccharides. The synthesis of this tri-O-methyl sugar can easily be achieved by blocking the 3-position of methyl α-D-mannopyranoside via the 4,6-benzylidene derivative.

The 4,6-benzylidene derivative of methyl α-D-mannopyranoside was prepared by the facile condensation of benzaldehyde with the methyl α-D-mannopyranoside. The yield of the desired monobenzylidene compound is not particularly high due to the fact that the hydroxyls on C_2 and C_3 are in the axial-equatorial cis conformation. This leads to a facile formation of the dibenzylidene derivative. However, the dibenzylidene derivative can be easily removed by precipitation in dry ethanol in which the monobenzylidene derivative is soluble.

Monotosylation of the 4,6-benzylidene derivative could be best performed in pyridine solution at room temperature. Higher temperatures are not recommended as it leads to ditosylation. Tosylation preferentially

occurs at the C_3 hydroxyl which is equatorial rather than the C_2 hydroxyl which is axially oriented. The 3-O-tosyl derivative is obtained in very high yield.

Removal of the benzylidene group is simple and easily affected by boiling the requisite compound in an aqueous acetone solution of dilute hydrochloric acid. This does not affect, as expected, the removal of the tosyl group.

The usual Purdie method of methylation produced methyl 2,4,6-tri-O-methyl-α-D-mannopyranoside in very good yield. The Hakomori procedure (10) cannot be used as it involves a strong base (sodium hydride) (4) which rather readily hydrolyses the tosyl group.

Detosylation cannot be done in either dilute aqueous acid or alkali, but was achieved by the use of a strong alcoholic solution of sodium methoxide. Walden inversion is not observed since the cleavage involves oxygen-sulfur fission (6). Since the tosyl group is generally rather stable to migration (6) in common organic reagents (the acetyl group in the C_3-position can readily migrate to C_6 if the latter is unsubstituted), it was found that a very pure preparation of 2,4,6-tri-O-methyl-D-mannopyranose was obtained in rather high yield after the hydrolysis of the mannoside derivative with mineral acid

2,4,6-Tri-O-methyl-D-mannopyranose crystallized in ether to give two lustrous forms (α and β-anomers). The mixture gave a depressed m.p. being about 30° lower than that reported for an apparently pure anomer (1). The report of the higher melting point involved a mechanical separation of the two crystal forms. The separation of the two anomers could not be affected by t.l.c. on silica gel, paper chromatography and even vapor phase chromatography.

Apart from the above, the analysis of the tri-O-methyl sugar by comparative t.l.c. on silica gel, paper chromatography and V.P.C. provided a useful means of positive characterization. The compound, after exposure to periodic acid and other reagents described above, showed the presence of D-mannitol which could only have arisen from 2,4,6-tri-O-methyl-D-mannopyranose. The derived N-phenylglycosylamine gave a sharp m.p. and a specific optical ratation ($[\alpha]_D$), consistent with values reported for this compound when obtained from hydrolysis of methylated mannans, galactomannans and other D-mannose containing natural products.

SUMMARY

(1) Condensation of methyl α-D-mannopyranoside readily affords the 2,3-4,6-dibenzylidene and 4,6-monobenzylidene derivatives. The dibenzylidene derivative is easily removed by precipitation in dry ethanol.

(2) Preferential tosylation of the hydroxyl on the C_3-position was shown to occur and can be rationalized on stereochemical grounds.

(3) Removal of the 4,6-benzylidene group is easily affected by acid hydrolysis.

(4) Methylation by the Purdie method, although somewhat time consuming, results in a high yield of tri-O-methyl derivative.

(5) Removal of the 3-O-tosyl group by a strong base (sodium methoxide in methanol) proceeded by oxygen-sulfur cleavage (no Walden inversion).

(6) Hydrolysis of the glycoside by mineral acid (sulfuric acid) gave the free tri-O-methyl sugar with little or no destruction.

(7) Identification of the product through comparative chromatography, degradation and formation of suitable derivative conclusively showed it to be 2,4,6-tri-O-methyl-D-mannopyranose.

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PART V

A CONVENIENT AND UNAMBIGUOUS SYNTHESIS

OF

2,3,6-TRI-O-METHYL-D-

MANNOPYRANOSE

INTRODUCTION

As previously mentioned in Part IV, D-mannose is commonly found in polysaccharides and other compounds which are widely distributed in Nature. As might be expected, the usual procedures employed in structural studies of polysaccharides lead to the formation of various isomeric O-methyl sugars and considerable reliance must therefore be placed in independent synthesis of these compounds preferrably through unambiguous routes. As in the case of 2,4,6-tri-O-methyl-D-mannose (Part IV), such a synthesis had, to the best of our knowledge, not been affected or reported. It is the object of this part of the thesis to describe a convenient and unambiguous synthesis of one of the tri-O-methyl ethers of D-mannose which occurs very frequently in methylation experiments concerned with galactomannans. The particular O-methyl sugar in question, namely 2,3,6-tri-O-methyl-D-mannose, arises from a linkage which is ordinarily associated with the D-mannose "backbone" of commonly occuring galactomannans. The synthesis was easily affected through the application of a few additional but simple steps in comparison to the synthesis of 2,4,6-tri-O-methyl-D-mannopyranose previously described.

LITERATURE REVIEW

2,3,6-Tri-O-methyl-D-mannopyranose is present in large proportion in methylated galactomannans (1). It is a constituent of the hydrolysis products of the methylated polysaccharides containing D-mannose units linked (1→4). The structural deduction of the sugar follows from the work of Haworth, Hirst and Streight (2).

Since the C_3 hydroxyl of methyl α-D-mannopyranoside is more reactive towards acylating agents (3), the direct blocking of the C_4 hydroxyl cannot be achieved by direct reaction of the 6-O-substituted α-D-mannopyranoside. Closer examination revealed that upon application of a few additional but easy steps should produce in high yield the desired compound, namely 2,3,6-tri-O-methyl-D-mannopyranose.

EXPERIMENTAL METHODS AND RESULTS

Methyl 4,6-benzylidene-α-D-mannopyranoside (4) -

Methyl 4,6-benzylidene-α-D-mannopyranoside was prepared as described in Part IV, m.p. 142°, $[\alpha]_D^{22}$ +70° (c 1.0, chloroform).

Methyl 4,6-benzylidene-2,3-di-O-methyl-α-D-mannopyranoside (4) -

Methylation was carried out with methyl iodide and silver oxide (6) with the addition of acetone to effect complete solution of the material (3 grams). Seven successive treatments were necessary to effect maximun methylation. The final product contained one single component on t.l.c. (silica gel), R_F, 0.80 (acetone: chloroform 5:95), $[\alpha]_D^{22}$ +62° (c 2.0, chloroform), yield 3.2 grams (97%).

Methyl 2,3-di-O-methyl-α-D-mannopyranoside (4) -

The product (3.2 grams) described in the previous section was dissolved in a mixture of acetone (50 ml), water (22 ml) and N hydrochloric acid (3 ml) (4). The

mixture was refluxed and the progress in the hydrolysis followed by t.l.c. on silica gel (acetone:chloroform 5:95). Complete hydrolysis was attained in 2 hours. The reaction mixture was neutralized with barium carbonate, filtered, and the solvent evaporated under diminished pressure. The wet residue was extracted twice with chloroform (2 x 5 ml) to remove benzaldehyde and traces of unchanged starting compound after which the solution was evaporated to dryness. The dry residue was extracted with chloroform followed by evaporation of the combined extract, yielding a syrup (2.2 grams, 96%) having R_F 0.15, t.l.c. on silica gel (acetone:chloroform 10:90), $[\alpha]_D^{22}$ +43° (c 1.0, chloroform).

Methyl 2,3-di-O-methyl-6-O-trityl-α-D-mannopyranoside (4) -

Methyl 2,3-di-O-methyl-α-D-mannopyranoside (2.1 grams) and triphenylchloromethane (2.8 grams) were dissolved in dry pyridine (20 ml) and heated on a boiling water bath for 2 hours. The reaction mixture was poured into ice water. The product which precipitated was fractionated by silica gel column chromatography. Triphenylcarbinol and trityl chloride were removed by elution with chloroform (1 litre). Pure methyl 2,3-di-O-

methyl-6-O-trityl-α-D-mannopyranoside (3.3 grams, 73%) was obtained by immediate elution with 5% ethanol in chloroform, R_p, 0.47, t.l.c. on silica gel (acetone: chloroform 5:95). The compound was recrystallized from ethanol (lustrous plates) and had m.p. 172 - 174°C (lit. m.p. 172 - 173°C) (4).

Methyl 2,3-di-O-methyl-4-O-p-toluenesulfonyl-6-O-trityl-α-D-mannopyranoside -

Methyl 2,3-di-O-methyl-6-O-trityl-α-D-mannopyranoside (3.2 grams) and p-toluenesulfonyl chloride (3.0 grams) were dissolved in dry pyridine and kept at 53°C for 3 days. The reaction mixture was poured into ice water. The precipitate that formed was washed thoroughly with water to give a homogeneous (t.l.c. on silica gel) product (4.1 grams, 94%), R_p, 0.70 (acetone:chloroform 5:95). Crystallization and recrystallization from ethanol gave coarse needles having m.p. 144 - 145°C $[\alpha]_D^{22}$ +36.5° (c 2.0, chloroform).

Analysis for $C_{35}H_{38}O_8S$: C, 67.94; H, 6.19; OCH_3, 15.05. Found : C, 67.99; H, 6.23; OCH_3, 14.80.

Methyl 2,3-di-O-methyl-4-O-p-toluenesulfonyl-α-D-mannopyranoside -

The product (4 grams) described in the above section was dissolved in 10 ml chloroform and kept at 0°C on an ice bath. The chloroform solution was quickly saturated with anhydrous hydrogen chloride and kept at the same temperature for 1 hour. A few drops of saturated solution of sodium bicarbonate were first added followed by a further addition of solid sodium bicarbonate to neutralize the hydrogen chloride. The residue was fractionated on a silica gel column by firstly elution with chloroform to remove triphenylmethanol followed by elution with ethanol:chloroform 5:95 to remove the desired compound. The syrupy product (2.2 grams, 91%) showed a single component, having on t.l.c. (silica gel), an R_F 0.15 (acetone:chloroform 5:95), $[\alpha]_D^{22}$ +41.3° (c 2.54, chloroform).

Analysis for OCH_3, 24.7%; found, 24.4%.

Methyl 4-O-p-toluenesulfonyl-2,3,6-tri-O-methyl-α-D-mannopyranoside -

Methyl 2,3-di-O-methyl-4-O-p-toluenesulfonyl-α-D-mannopyranoside (2 grams) was treated with methyl iodide (40 ml) and silver oxide (4 grams). Filtration

and evaporation gave a product which after seven such further treatments yielded the fully methylated compound (1.96 grams, 96%). The resulting compound crystallized on standing in ethyl acetate and petroleum ether. On recrystallization from petroleum ether, it gave lustrous cubes, m.p. 91 - 92°C, $[\alpha]_D^{22}$ +38° (c 1.0, chloroform). The compound was chromatographically pure, having R_F 0.3, t.l.c. on silica gel (acetone:chloroform 5:95).

Analysis for $C_{17}H_{26}O_8S$: C, 52.29; H, 6.71; OCH_3, 31.7. Found : C, 52.60; H, 6.76; OCH_3, 31.1.

Methyl 2,3,6-tri-O-methyl-α-D-mannopyranoside -

Methyl 4-O-p-toluenesulfonyl-2,3,6-tri-O-methyl-α-D-mannopyranoside (1.94 grams) was dissolved in a solution of sodium methoxide (from 2.5 grams of sodium in methanol (50 ml)) (7), and the mixture was refluxed for 12 hours. Water (25 ml) was added to the cooled solution, organic solvent was distilled off, and the suspension was extracted with chloroform (5 x 25 ml). The extract was dried over anhydrous sodium sulfate and evporated to a syrup (1.1 grams, 94%). The product was chromatographically pure (t.l.c. on silica gel), having R_F 0.52 (butanone-water azeotrope). It had the same retention time (17.2 minutes) as a sample of methyl

2,3,6-tri-O-methyl-D-mannopyranoside obtained via methylation of guar gum (a galactomannan) (20% neopentylglycolsuccinate, 3/16" by 10' column, 170°C, helium flow rate 100 ml/min.) (8). The compound had $[\alpha]_D^{22}$ +32° (c 2.8, chloroform).

Analysis for OCH_3, 52.7%; found, 52.2%.

2,3,6-Tri-O-methyl-D-mannopyranose -

Methyl 2,3,6-tri-O-methyl-α-D-mannopyranoside (1 gram) was heated with N sulfuric acid (20 ml) at 100°. The hydrolysis, which was followed by t.l.c. on silica gel (butanone-water azeotrope) was complete in 11 hours. The acid was neutralized (barium carbonate) and the filtered solution was evaporated to give a syrup (0.93 gram, 97%). Paper chromatography using butanone-water azeotrope, Whatman No.1 paper, and p-anisidine spray, showed a single component, having R_F 0.50, which was identical to the mobility of "authentic" 2,3,6-tri-O-methyl-D-mannopyranose obtained from guar gum via methylation and hydrolysis. Both gave reddish brown colour reactions with p-anisidine spray. Thin layer chromatography on silica gel using butanone-water azeotrope, p-anisidine spray and sulfuric acid spray, also showed one single component having the same mobility

as "authentic" compound (R_F 0.40). Moreover, V.P.C. (conditions described in the previous section) of the trimethylsilyl derivative of the synthesized compound showed one single peak and had an identical retention time (4.8 minutes) as the "authentic" specimen. The synthesized compound had $[\alpha]_D^{22}$ -11° (c 2.0, water).

A portion (220 mg) of the compound was dissolved in dry pyridine (12 ml) to which p-nitrobenzoyl chloride (900 mg) was added. The mixture was heated for 30 minutes at 65 - 75°C and left overnight at room temperature. A saturated solution of sodium bicarbonate was then added until no further effervescence of CO_2 was observed. The product was extracted with chloroform (3 x 50 ml). The combined extract was evaporated to about 5 ml. Petroleum ether was added to induce crystallization. The derived 1,4-bis-p-nitrobenzoate on recrystallization from methanol gave fine needles having m.p. and mixed m.p. 188°C, $[\alpha]_D^{22}$ +34° (c 1.0, chloroform).

DISCUSSION

2,3,6-Tri-O-methyl-D-mannopyranose is present in large quantities in the hydrolysis products of a large number of methylated galactomannans and mannans. The synthesis of this partially methylated sugar involves the effective and specific blocking of the C_4 hydroxyl function. Since the hydroxyl on the C_3 position has been shown to be more reactive towards p-toluenesulfonyl chloride (3), the direct blocking of the 3-O-position could not therefore be achieved by the action of p-toluenesulfonyl chloride on the methyl 6-O-trityl-α-D-mannopyranoside.

The hydroxyls on C_2 and C_3 can first be blocked by methylation of methyl 4,6-benzylidene-α-D-mannopyranoside. Removal of the benzylidene group can be readily affected followed by facile tritylation of the C_6 primary hydroxyl function leaving the C_4 hydroxyl free for further substitution. Substitution with tosyl chloride can readily be affected and is desirable because the 4-O-tosyl derivative is rather stable as such and shows no detectable tendency towards acyl migration. The bulkiness of the 6-O-trityl group made tosylation at the C_4 hydroxyl rather difficult at room temperature, however, reaction at higher temperature

(50^{o} or above), rendered the 4-O-tosyl derivative in very high yield.

After removal of the trityl group, methylation was carried out by the usual Purdie method to obtain methyl 4-O-p-toluenesulfonyl-2,3,6-tri-O-methyl-α-D-mannopyranoside in rather good yield. The very effective Hakomori methylation method (10) cannot be used in this instance since it involves the use of a very strong base (sodium hydride) (11) which readily hydrolyses the tosyl group. After the Purdie methylation, detosylation was readily achieved by using a strong alcoholic solution of sodium methoxide. Walden inversion was not observed as it involved the sulfur-oxygen fission (9). Epoxide formation would not occur since no free trans hydroxyl function is present on the neighboring carbon. After hydrolysis of the methyl glycoside with mineral acid, pure 2,3,6-tri-O-methyl-D-mannopyranose was obtained in high yield. The analysis of the sugar by comparative t.l.c. on silica gel, paper chromatography and V.P.C. showed that the synthetic compound was identical in all respects to 2,3,6-tri-O-methyl-D-mannopyranose obtained via methylation and hydrolysis of guar gum (a galactomannan). The derived 1,4-bis-p-nitrobenzoate had a sharp mixed m.p. and consistent specific optical rotation with

reported values for this compound (again obtained via a natural product).

SUMMARY

I. A simple, unambiguous synthesis of 2,3,6-tri-O-methyl-D-mannopyranose was affected by

(a) preparation of 4,6-benzylidene by condensation of methyl α-D-mannopyranoside with benzaldehyde

(b) methylation by Purdie procedure

(c) removal of the benzylidene group

(d) introduction of the trityl group at the C_6 hydroxyl

(e) blocking of the C_4 hydroxyl by p-toluene-sulfonyl chloride

(f) removal of the trityl group

(g) methylation by Purdie procedure

(h) removal of the tosyl group by hydrolysis with a strong base

(i) hydrolysis of the methyl glycoside with mineral acid.

II. The synthesized product was identified and characterized through comparative chromatography (t.l.c., paper, V.P.C.), optical rotation, and preparation of a suitable derivative.

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