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IDENTIFICATION OF LINKAGES OF A GALACTOMANNAN ISOLATED FROM SEED OF *Caesalpinia pulcherima*

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ABSTRACT

A galactomannan, purified by repeated complex formation with copper acetate, was shown to be composed of D-mannose and D-galactose in a molar ratio of 3 1 From methylation experiments, the following partially methylated sugars were identified 2,3,4,6-tetra-*O*-methyl-D-mannose, 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,3,6-tri-*O*-methyl-D-mannose, 2,4,6-tri-*O*-methyl-D-mannose, 3,4,6-tri-*O*-methyl-D-mannose, and 2,3-di-*O*-methyl-D-mannose (molar ratio 1 27 60 4 2 32) Ordinary and sequential (Smith) 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, the occurrence of small and equimolar quantities of 2-*O*-β-D-mannopyranosyl-D-erythritol and *O*-β-D-mannopyranosyl-(1→3)-*O*-β-D-mannopyranosyl-(1→2)-D-erythritol indicates a low frequency of isolated (1→3) linkages and consecutive (1→3) linkages A relatively simple structure may be assigned, based upon the experimental results

INTRODUCTION

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

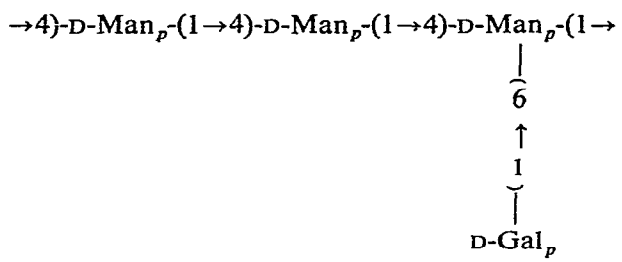
DISCUSSION

Extraction of fractured seed of *Caesalpinia pulcherima* with hot water resulted, upon cooling, in a highly viscous solution Precipitation with ethanol gave a characteristic greyish, stringy precipitate The polysaccharide was purified by repeated complex formation with copper acetate[1, 2], $[\alpha]_D^{22}$ +6° in 0 1 M sodium hydroxide

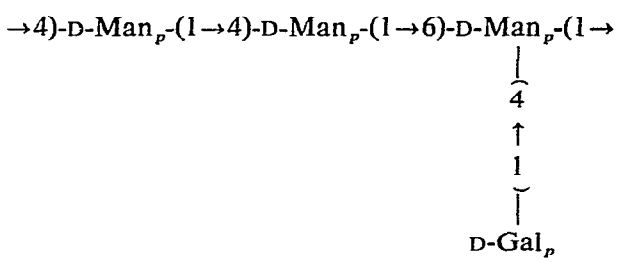
This low value suggests that the glycosidic linkages in the main chain have a β-D anomeric configuration Since the galactomannan was unaffected by prolonged treatment with emulsin, it would appear that the D-galactose units are attached by α linkages Ultracentrifugation showed a single peak, thus indicating that the polysaccharide was relatively homogeneous 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 gum (16% D-galactose), Carob bean (14–27% D-galactose), and Kentucky coffee bean (20% D-galactose), although some galactomannans are known to contain up to 50% D-galactose[3].

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*-methyl-D-mannose, 3,4,6-tri-*O*-methyl-D-mannose, and 2,3-di-*O*-methyl-D-mannose in the molar ratio 27 1 4 60 2 32 (by g l c) The isolation of these partially methylated sugars is in agreement with the periodate oxidation results (see next paragraph), namely approximately one out of every twenty D-mannose units is linked (1→3) In addition, the occurrence of a small number of (1→2) linkages was indicated by the identification of the requisite trimethyl sugar All of the D-galactose units occupy terminal non-reducing positions, since only 2,3,4,6-tetra-*O*-methyl-D-galactose was found, and the ratio of tetra-*O*-methyl-D-galactose to di-*O*-methyl-D-mannose was essentially 1 1 (the slightly lower proportion of tetra-*O*-methyl-D-galactose, as reported in 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 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 The behavior on ultracentrifugation of the polysaccharide indicated an approximate molecular weight of 60,000, hence three repeating units of an approximate D P of 120 each could account for one single polymeric molecule Further structural details were obtained from ordinary and sequential (Smith) periodate degradation studies.

Treatment of the polysaccharide with periodate at 4–5° resulted in periodate consumption of 1 2 mole per hexose unit Reduction of the polyaldehyde with borohydride, followed by mild acid hydrolysis (Smith degradation) gave glycerol, erythritol (no threitol), and two non-reducing components having paper chromatographic mobilities lower than that of D-galactose Analysis of the hydrolyzate by the phenol–sulfuric acid method[4] indicated that 4 to 6% of hexoses had survived the periodate attack The appearance of glycerol can be attributed to terminal, non-reducing D-mannose and D-galactose units, mostly the latter, since no D-threitol was detected D-Threitol would arise from internal D-galactose units linked (1→4) The major quantity of erythritol produced shows that the main chain of the polysaccharide is composed of D-mannopyranose units predominantly linked (1→4) D-Galactose units are linked (1→6) to approximately every third D-mannose unit of the main chain, *e g.*,



or (1→4) to a D-mannose unit linked at C-6, *e g* ,



However, structural studies of a considerable number of galactomannans have shown that the D-galactose units are ordinarily linked (1→6) to the D-mannose units of the main chain[3] The presence of two non-reducing oligosaccharide fractions in a molar ratio approximately 1 1 indicated that a very small proportion of linkages other than (1→4) and (1→4)–(1→6) existed Hydrolysis of component I (R_{Man} 0.64), gave D-mannose and erythritol in a molar ratio of 1 1 This finding indicates that a small proportion of D-mannose units were linked (1→3) and these units were flanked by (1→4)-linked units, *e g* ,

$$\text{-D-Man}_p\text{-(1}\rightarrow\text{4)-D-Man}_p\text{-(1}\rightarrow\text{3)-D-Man}_p\text{-(1}\rightarrow\text{4)-D-Man}_p\text{-}$$

Hydrolysis of oligosaccharide II (R_{Man} 0 35) gave D-mannose and erythritol in a ratio of 2 1 This indicated that a small proportion of D-mannose units were consecutively linked (1→3), *e g* ,

$$\text{-D-Man}_p\text{-(1}\rightarrow\text{4)-D-Man}_p\text{-(1}\rightarrow\text{3)-D-Man}_p\text{-(1}\rightarrow\text{3)-D-Man}_p\text{-(1}\rightarrow\text{4)-D-Man}_p\text{-}$$

Sequential (Smith) periodate degradation of II gave a non-reducing component (shown by hydrolysis to consist of D-mannose and glycerol in a 1 1 molar ratio), glycerol, and formaldehyde Formaldehyde would be expected from the C-4 fragment of the erythritol moiety of II, and glycerol from the terminal, "non-reducing" (1→3)-linked D-mannose unit while the internal (1→3)-linked D-mannose unit would survive

The low incidence of (1→2) linkages in the main D-mannose chain, as indicated by the identification of the requisite 3,4,6-tri-*O*-methyl-D-mannose (less than 2%) in methylation experiments is at least one factor which prevents a detailed representation of the possible molecular structure In the absence of detailed sequential linkage analysis, a highly randomized structure would appear the most appropriate consideration

EXPERIMENTAL

General methods — All evaporations were performed under reduced pressure Whatman No. 1 and 3M paper was used in qualitative and quantitative identifications The solvent systems used were *A*, 4 1 1 ethylacetate–acetic acid–water, *B*, 8 2 1 ethylacetate–pyridine–water, *C*, butanone–water (azeotrope), *D*, 4 1 5 butyl alcohol–ethanol–water, and *E*, 85 15 1 benzene–ethanol–water Chromatograms were sprayed with *p*-anisidine–trichloroacetic acid (*F*)[6] or Tollens solution (*G*)[7] The mobilities are reported relative to the front of the solvent (R_F) or to the mobility of 2,3,4,6-tetra-*O*-methyl-D-glucose (R_{TG}) Melting points reported are uncorrected

Isolation of the galactomannan — A slurry of fractured seed in boiling water was stirred for 3–4 h, after which the solids were removed by centrifugation The cooled, viscous solution was slowly poured, with vigorous stirring, into 4 vol of ethanol whereupon a light greyish, stringy precipitate formed The solid material was removed by filtration, washed with acetone, and dried in a desiccator

Purification by precipitation with copper acetate — To a portion (8 g) of polysaccharide in 0 5M sodium hydroxide (100 ml) was added with stirring 5% copper acetate (300 ml) The gelatinous precipitate that formed was removed by centrifugation Further addition of an equal volume of copper acetate solution to the supernatant and addition of an equal volume of ethanol (95%) resulted in precipitation of a copper complex which was not investigated at this time The gelatinous complex was suspended in 95% ethanol (200 ml) and acidified with 2M hydrochloric acid (20 ml), and after maceration in a Waring blender, the fluffy, greyish precipitate was removed by centrifugation and washed free of acid with alcohol Complex formation with Cu^{2+} was repeated twice more, as described, resulting in no observable change in the ratio of the sugar components (see succeeding sections), yield 5 g, $[\alpha]_D^{22}$ +6° (*c* 0 5, 0 1M sodium hydroxide)

Ultracentrifugation — Solutions of galactomannan (0 5 to 1%) in alkali (1 to 5M sodium hydroxide) were centrifuged for 2 h, at a rotor speed of 60,000 r p m The sedimentation pattern of 0 8% galactomannan in 5M sodium hydroxide indicated an approximate molecular weight of 60,000

Hydrolysis of polysaccharide — (*a*) *With* M *sulfuric acid* A sample (250 mg) of the polysaccharide in M sulfuric acid (25 ml) was heated for 8 h at reflux temperature After neutralization with barium carbonate, the filtrate was passed through weak anion (Duolite A-4) and cation (IR-120) exchange-resin columns, and the solution was evaporated The syrupy residue was chromatographed on Whatman No 1 paper in solvents *A* and *B* After spraying with reagent *E*, D-mannose and D-galactose were identified as the only sugars present Separation on Whatman No 3MM of a portion (100 mg) of the mixture with solvent *B* gave D-mannose (60 mg) which was characterized as the phenylhydrazone, m p 199°, $[\alpha]_D^{22}$ +33° (*c* 0 8, pyridine) and D-galactose (25 mg), $[\alpha]_D^{22}$ +80° (*c* 0 7, water) The molar ratio of the two sugars was determined by elution of each sugar from a single chromatogram followed by analysis with the phenol–sulfuric acid method[4], and by g l c of the mixture of trimethylsilyl derivatives[7]

on a 5 ft × ¼ in stainless steel column packed with 20% SE-30 on HMDS treated chromosorb W. A D-mannose to D-galactose ratio of 3 1 was obtained in all cases

(*b*) *Hydrolysis with emulsin* To a sterile solution of galactomannan (25 mg) (15 ml of phosphate buffer, pH 7.0) was added emulsin (10 mg) The solution was kept for 3 days at 25° 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 chromatography (Whatman No 1) in solvents *A* and *B* and using reagents *F* and *G*, to contain no detectable D-galactose (or D-mannose) and no evidence of smaller oligomers

Methylation analysis — A portion (2 0 g) of the galactomannan was initially treated with 30% sodium hydroxide and methyl sulfate, followed by treatment with sodium hydride in methyl sulfoxide and methyl iodide[8] The fully methylated polymer (1 5 g) (no OH-absorption evident in the i r spectrum of liquid film) was dissolved in cold 75% sulfuric acid (15 ml) The mixture was diluted with ice–water to approximately M acid concentration, and the solution was boiled for 7 h[9] A portion (900 mg) of the syrupy mixture (1 2 g), which was obtained after neutralization (barium carbonate) and evaporation of the filtrate, was analyzed by paper chromatography (Whatman No 1) using solvents *C*, *D*, and *E* The molar ratio of the partially methylated sugars was determined by the phenol–sulfuric acidproced ure and by g l c of the trimethylsilyl derivatives[10] (Table I) The mixture was separated on Whatman 3MM

TABLE I

GAS-LIQUID CHROMATOGRAPHY OF TRIMETHYLSILYL DERIVATIVES OF PARTIALLY METHYLATED SUGARS[a]

Trimethylsilyl derivatives	*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 9 (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

[a]Neopentylglycolsuccinate (20%) on Gas Chrom 900, 3/16 in by 12 ft copper column, operating temp 180°, flame-ionization detector

paper in solvent *C* (Table II), and the individual, partially methylated sugars were characterized as follows

Component 1 and synthetic 2,3,4,6-tetra-*O*-methyl-D-mannose showed identical R_F (solvent *C*, 0 79), R_{TG} (solvent *D*, 0 96), and retention time of the trimethylsilyl derivative (16 5 min)

Component 2 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

TABLE II
PAPER CHROMATOGRAPHY OF PARTIALLY METHYLATED SUGARS

Component	*Weight (mg)*	*Butanone–water azeotrope R_F*	*Butyl alcohol–ethanol–water R_{TG}*[a]	*$[\alpha]_D$ (degrees)*[b]	*Molar ratio*[c]	*Proposed compound*[c]
1	—	0 79	0 96	—	1	2,3,4,6-tetra-*O*-methyl-D-manno
2	120	0 69	0 88	+118		2,3,4,6-tetra-*O*-methyl-D-galacto
3	11	0 56	0 82	+150	2	2,4,6-tri-*O*-methyl-D-mannose
4	210	0 50	0 81	−9		2,3,6-tri-*O*-methyl-D-mannose a 3,4,6-tri-*O*-methyl-D-mannose
5	120	0 22	0 57	−16	1	2,3-di-*O*-methyl-D-mannose

[a]Mobility relative to 2,3,4,6-tetra-*O*-methyl-D-glucose [b]In water [c]From chromatographic results in butanon water azeotrope

190–191°, $[\alpha]_D^{22}$ +40° (*c* 0 5, acetone)[11] Component 2 and authentic 2,3,4,6-tetra-*O*-methyl-D-galactose showed identical R_F (solvent *C*, 0 69), R_{TG} (solvent *D*, 0 88), and retention time of trimethylsilyl derivative (15 2 min, α-anomer, 18 9 min, β-anomer)

Component 3 was treated with aniline, as described for component 2, to give 2,4,6-tri-*O*-methyl-*N*-phenyl-D-mannosylamine, m p 133–135°, $[\alpha]_D^{22}$ −8° (*c* 0 3, methanol)[12] The identity of component 3 was further established by the following treatments A small portion (about 6 mg) was reduced with sodium borohydride (24 h, room temp.) followed by treatment with methanol–hydrogen chloride (1%) to remove borate ions The product was treated with periodic acid (0 1M) for 2 days at 5° The solution (10 ml) was treated with barium carbonate to remove iodate and periodate ions, and then reduced with sodium borohydride as described in the preceding paragraph Borate ions were removed, and after evaporation, the residue was demethylated with 35% hydrobromic acid for 60 min at 100° D-Mannitol was identified by comparative paper chromatography and g l c of the corresponding trimethylsilyl derivative

A solution of component 4 in dry pyridine was treated with a 10% molar excess of *p*-nitrobenzoyl chloride After being heated for 30 min to 65°, the mixture was cooled, and after addition of a saturated solution of sodium hydrogen carbonate, extracted with chloroform Evaporation and recrystallization of the residue from methanol gave 1,4-di-*p*-nitrobenzoyl-2,3,6-tri-*O*-methyl-D-mannose (95% yield), m p 187–189°, $[\alpha]_D^{22}$ +33° (*c* 1 0, chloroform)[13] T l c on silica gel, showed 3 components in solvent *C* (R_{TG} 0 78, 0 76, and 0 68) and in solvent *E* (R_{TG} 0 52, 0 50, and 0.45), thus indicating three tri-*O*-methyl sugars G l c of trimethylsilyl derivatives showed the presence of 2,4,6-tri-*O*-methyl-D-mannose, 12 0 min (minor proportion), 3,4,6-tri-*O*-methyl-D-mannose, 10 4 min (minor proportion), and 2,3,6-tri-*O*-methyl-D-mannose, 13 4 min (major proportion) A portion (100 mg) of component 4 was subjected to the following series of reactions reduction with borohydride, oxidation with periodic acid, reduction with borohydride, demethylation with 35% hydrobromic

acid, deionization, and identification by paper and g l c of the degradation products Ethylene glycol and erythritol indicated the presence of 2,3,6-tri-*O*-methyl-D-mannose D-Arabinitol was identified by comparison with authentic D-arabinitol, the retention time of the trimethylsilyl derivative of authentic D-arabinitol and that obtained from component 4 were identical (3 4 min, 170°, other conditions as described previously) D-Arabinitol indicated the presence of 3,4,6-tri-*O*-methyl-D-mannose (or 2,3,4-tri-*O*-methyl-D-mannose, however, the absence of this sugar was established previously by g l c , see Table II) D-Mannitol was identified by comparison with an authentic compound, the trimethylsilyl derivatives of both having retention times of 4 4 min (170°, other conditions as described previously) D-Mannitol indicated the presence of 2,4,6-tri-*O*-methyl-D-mannose.

Component 5 gave upon treatment with *p*-nitrobenzoyl chloride, as previously described, 1,4,6-tri-*O*-*p*-nitrobenzoyl-2,3-di-*O*-methyl-D-mannose, m.p. 192–194°, $[\alpha]_D^{22}$ +65° (*c* 0 5, chloroform)[3]

Sequential periodate (Smith) degradation — To a portion (1 g) of galactomannan dissolved in water (400 ml) was added 0 1M periodic acid (100 ml), and the solution was kept at 5° Periodic acid consumption reached 1 2 mole per hexose unit in 3 days with little change thereafter To the polyaldehyde was added sodium borohydride (1 g), and the solution was kept overnight at room temperature Analysis of the solution of polyalcohol with phenol–sulfuric acid indicated an intact hexose content to the extent of 4–6%, in terms of D-mannose, of the original polysaccharide The solution was passed through a column of Amberlite IR-120 (H^+) and evaporated, and the borate ions were removed by repeated evaporation with methanol The residue was dissolved in 0 5M hydrochloric acid (50 ml), and the solution was kept for 8 h at room temperature The solution was deionized with ion-exchange resins Paper chromatography in solvents *A* and *B* indicated the presence of glycerol and erythritol, in the molar ratio 1 2 85 as determined by the periodate–chromotropic acid method[14], and of two non-reducing compounds having R_{Man} 0 64 (Compound I) and R_{Man} 0 35 (Compound II) The compounds were separated by paper chromatography (solvent *B*, Whatman 3MM paper) and characterized as follows

Glycerol (yield about 50 mg) was dissolved in dry pyridine A 10% molar excess *p*-nitrobenzoyl chloride was added, and the mixture was heated for 30 min to 75° After cooling to room temperature, an excess saturated sodium hydrogen carbonate solution was added, and the precipitate was collected and washed with water The tri-*p*-nitrobenzoate, after recrystallization from ethanol, had m p 191° (undepressed mixed m p)

Erythritol (yield about 140 mg) was treated in the same manner as glycerol. The tetra-*p*-nitrobenzoate had m p 250° (undepressed mixed m p)

Compound I (yield about 4 mg) was hydrolyzed in M sulfuric acid (10 ml) (reflux temp , 6 h) The hydrolyzate was shown by paper chromatography (Whatman No 1) in 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 the chromotropic acid procedure)

Compound II (yield about 7 mg) A portion (about 3 mg) was hydrolyzed as previously described for compound I, and the hydrolyzate was shown to contain D-mannose and erythritol in a 2 1 molar ratio The remaining portion of II was degraded with periodate (Smith degradation, as previously described) whereupon 1 molar proportion of formaldehyde was produced (analyzed before borohydride reduction of polyaldehyde) Glycerol and a non-reducing compound III having a slightly faster mobility than I were formed upon mild acid hydrolysis of the polyalcohol Complete hydrolysis of III, as described for I, gave D-mannose and glycerol in a 1 1 molar ratio (glycerol determined as formaldehyde by the chromotropic acid procedure)

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