CHAPTER III

GENERAL STRUCTURAL FEATURES OF A. pavonina SEED POLYSACCHARIDE.

In order to ascertain the precise form in which structural units occur in A. pavonina seed polysaccharide and to determine the nature of linkages between constituent sugars, the parent polysaccharide has been subjected to methylation analysis.

Methylation Studies of A. pavonina Seed Polysaccharide

Methylation of polysaccharides is a invaluable technique for elucidation of the types of linkage which occur between the different sugar residues. Characterisation of the individual methylated sugars in a hydrolysate of the methylated polysaccharide reveals the presence of any unmethylated hydroxyl groups, from which it may be deduced that the carbon atoms carrying these free hydroxyl groups were those involved in linking that particular unit in the polysaccharide. In addition, the number of residues in the average repeating unit, the nature of the terminal units, and the units at which branching occurs may also be deduced from methylation studies.

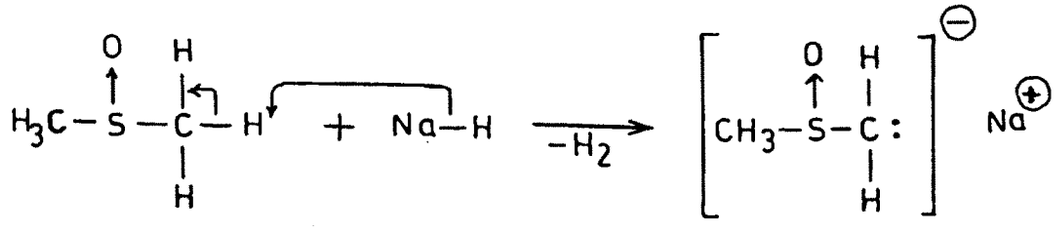
Monosaccharides being polyhydroxy compounds give rise to different types of glycosidic linkages such as (1→2), (1→3), (1→4) and (1→6). The type of substitution pattern prevailing in any polysaccharide is determined by methylation analysis[47-49]. In this procedure, the polysaccharide is first fully methylated by any one of the available methods[50] or by a

combination of the several methods to give a permethylated product. It is then completely hydrolysed and the identification of the resulting partially-O-methylated sugars is performed either by classical methods or by g.l.c.-m.s. analysis of their suitable derivatives. The critical pre-requisite for such a study is that all the free hydroxyl groups in the polysaccharide are fully methylated. The two conventional methods for permethylation involve treatment of the polysaccharide with dimethyl sulphate and sodium hydroxide (Haworth)[51] or Purdie and Irvine method[52], using methyl iodide and silver oxide. Since the polysaccharide is generally insoluble in organic solvents, they are usually methylated first with methyl sulphate and aqueous alkali. This yields a partially methylated product which, in many cases, becomes soluble in methyl iodide or a mixture of methyl iodide and methanol for further methylation by Purdie-Irvine method. In this method the function of freshly precipitated silver oxide is to neutralise the liberated hydroiodic acid. Since silver oxide is a mild oxidizing agent, it may also oxidise the reducing end of the sugar moieties to produce the corresponding carboxylic acid ester[53]. To avoid the secondary reaction, Bose and co-workers[54] have modified Purdie's method by using Al_2O_3 in place of silver oxide. An alternative method of methylation which has been developed by Muskat[55] and Freudenberg[56,57], is to dissolve the polysaccharide in liquid ammonia and treat it with methyl iodide and metallic sodium. This method has not been used extensively as it may lead to degradation of the polysaccharide and also to cause demethylation to some extent.

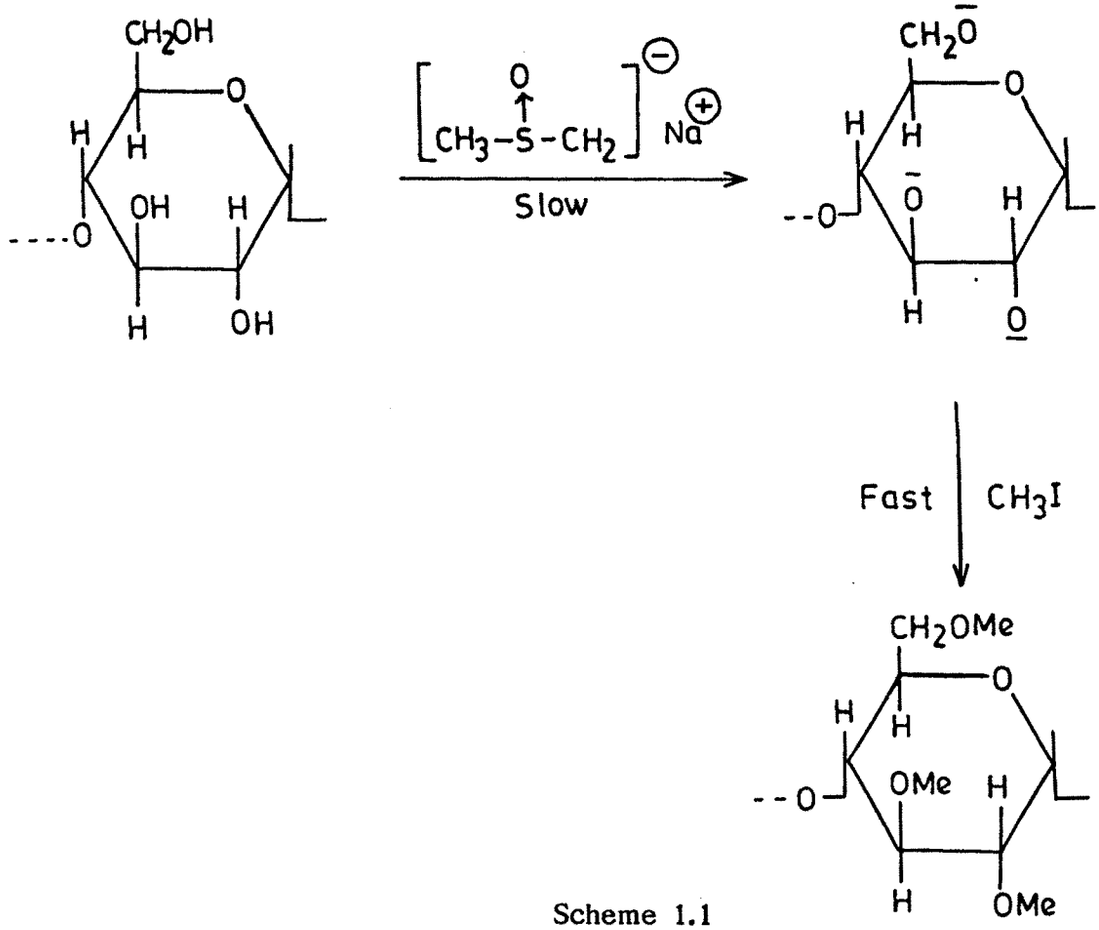
Difficulties are often faced with the methylation of polysaccharide containing uronic acids. One procedure that can be applied with some success is to convert the polysaccharide first into its thallium salt and then

methylate it by using methyl iodide and thallium hydroxide[58].

A number of variations in the above mentioned procedures have been developed recently that results in more efficient methylation[59]. The polysaccharide is dissolved in N,N-dimethyl formamide and then treated with methyl iodide in the presence of barium oxide or with methyl sulphate and barium oxide. Srivastava and coworkers[60] had reported a method of methylation using dimethyl sulphoxide as solvent instead of DMF and adding methyl iodide and barium oxide under constant stirring. Drierite (anhydrous $CaSO_4$) was also added to maintain anhydrous conditions. However, they later observed that better yield could be obtained by using dimethyl sulphate and powdered sodium hydroxide[61] instead of methyl iodide and barium oxide. This modification of Harworth has been found to be advantageous due to solvent effect of DMSO. The formation of methyl sulfinyl carbanion by reaction of dimethyl sulphoxide and sodium hydride was reported earlier by Corey and Chaykovsky[62]. Hakomori[63] applied this reaction to carbohydrates as a new development in the methylation technique. In this procedure, the polysaccharide is treated with a strongly basic solution of methyl sulfinyl-carbanion which is formed as a result of reaction of dimethyl sulphoxide with sodium hydride as shown below.



When the polysaccharide is added to this solution, the H of the hydroxyl groups is removed and the alkoxide ions react with greater facility with methyl iodide to form ether. The reaction of alkoxide ions with methyl iodide is much faster, thus causing complete methylation in one step as depicted below (Scheme 1.1).



Scheme 1.1

Methylation of Polysaccharide with Hakomori Reagent

In Hakomori methylation, the problem of dispersing polysaccharide in dimethyl sulphoxide, may be overcome by the use of ultrasonic treatment[64].

The methylation of the polysaccharide is usually followed by acid hydrolysis to obtain the individual partially methylated sugars. The depolymerisation method employed must be such as to minimise any demethylation

or destruction of the sugars. Sulphuric acid is the reagent usually employed although aqueous and methanolic hydrochloric, formic, oxalic and more recently trifluoroacetic acid have been used under certain conditions.

The separation of the methylated sugars by paper and column chromatographic techniques is a time consuming operation. Recent practice is to conduct gas-liquid chromatographic analysis for quick separation and identification of partially methylated sugars via their alditol acetates[65]. Majority of the methylated sugar derivatives can be tentatively identified by their relative retention times with reference to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol (Table 1.5).

Table 1.5

Retention Times of Alditol Acetates of Partially Methylated Sugars on the g.l.c. Column (3% OV-225)[66,67]

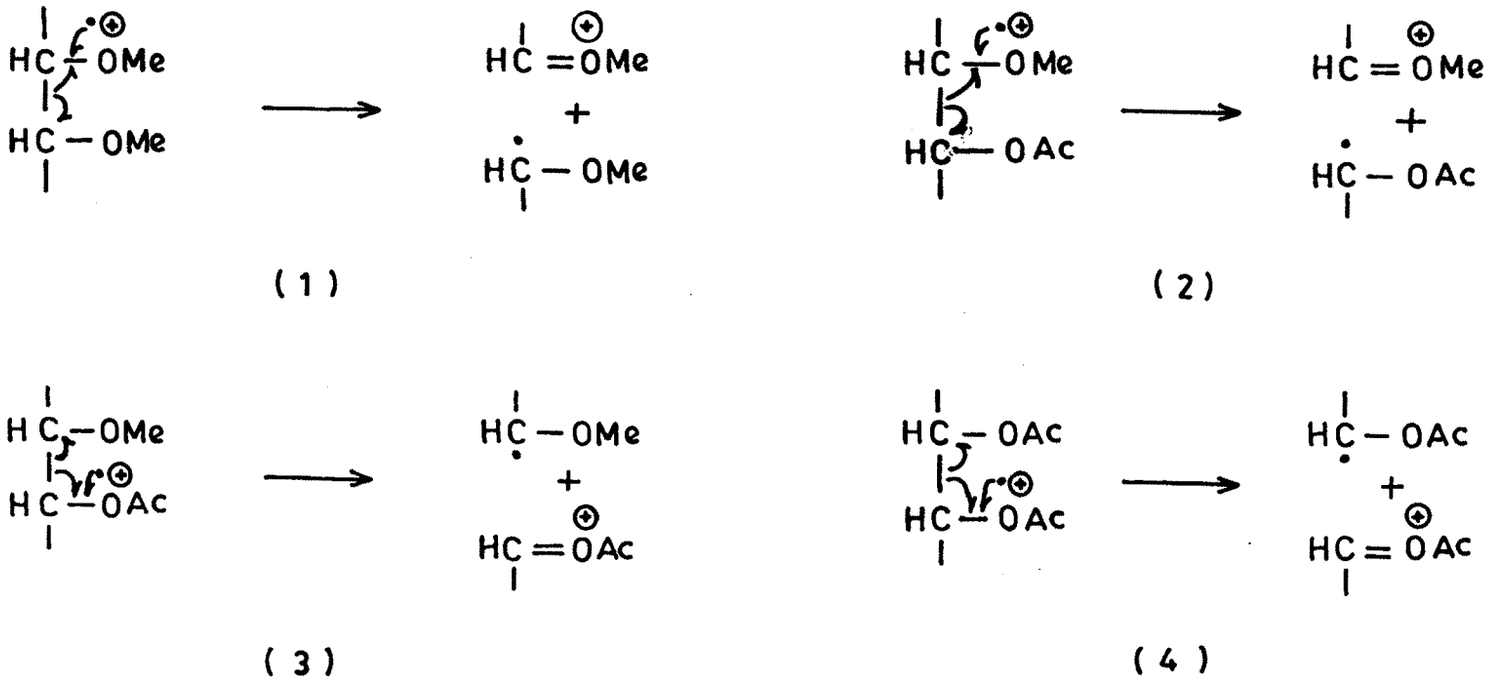
Alditol Acetates	Retention time (RT*) at 180° and flow rate of N_2, 30 ml/min.	Retention time (RT*) at 190° and flow rate of N_2, 30 ml/min.
2,3,4,6-tetra-O-methyl-mannose	0.98	0.97
2,3,4,6-tetra-O-methyl-galactose	1.17	1.13
2,3,6-tri-O-methyl-mannose	1.90	1.79
2,3,6-tri-O-methyl-galactose	2.04	-
2,3-di-O-methyl-mannose	3.62	3.21

* Retention Times relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol.

A combined g.l.c.-m.s. technique[68,69] can also be adopted for confirmation of the identity of partially methylated sugar derivatives. In this method, the sugar derivatives separated by g.l.c. are directly fed into the mass spectrometer. The electron impact mass spectra of the partially methylated alditol acetates give information about the position of the O-methyl and O-acetyl groups in the alditol. The mass fragmentation pattern of these types of partially methylated sugars derivatives do not carry any molecular ion peaks instead the spectra show primary mass spectral fragments formed by α-cleavage, resulting in fission between the carbon atoms in the alditol chain or by elimination of an acetoxyl group. The peak at m/z : 45 is due to a primary ion ($CH_2 = \overset{+}{O}Me$) and is less stable.

Secondary fragments are formed from the primary ones by single or consecutive eliminations of formaldehyde (30 m.u.), methanol (32 m.u.),acetic acid (60 m.u.), Ketene (42 m.u.), methyl acetate (74 m.u.), methoxy methyl acetate (104 m.u.) or acetoxy methyl acetate (132 m.u.). Lonngren and Svensson[69] have given four possibilities for the fragmentation of partially methylated alditol acetates as shown in Scheme 1.2.

As could be seen from the Scheme 1.2 during α-cleavage the charge is preferentially located on ether oxygen rather than an ester oxygen Thus fissions (1) and (2) are more important than fissions (3) and (4). The methoxylated radical formed in (1) seems to be more stable than the acetoxylated radical formed in (2).

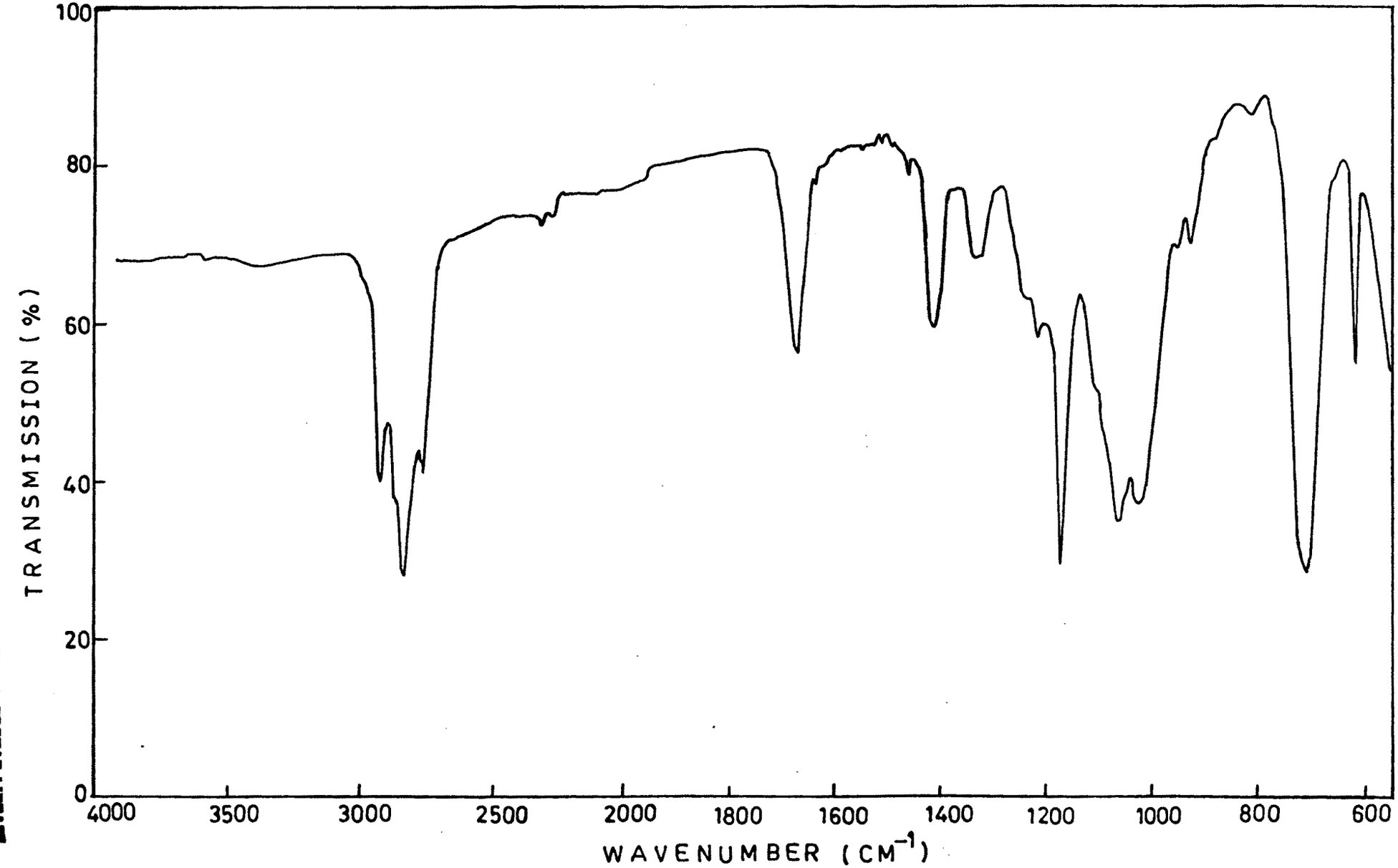


Scheme-1·2

MASS SPECTRAL FRAGMENTATION PATTERN OF PARTIALLY METHYLATED ALDITOL ACETATES ACCORDING TO LONNGREN AND SVENSSON[69]

Though methylation analysis is a standard procedure for elucidation of the structure of polysaccharide, it suffers from several limitations. The anomeric configuration of the glycosidic linkages and the mutual arrangement of monosaccharide units in the polymeric chains can not be ascertained by this method[47]. In case, complete methylation is not achieved, the hydrolysate may carry mono- and dimethyl sugars which could prevent unambiguous identification of interchain linkages.

In the present study, the nature and mode of linkage of the various constituent sugar moieties, occurring in A. pavonina seed polysaccharide were established by subjecting it to Haworth methylation followed by purdie procedure. The I.R. spectrum of the fully methylated polysaccharide (Fig. 1.9) showed no absorption band for -OH groups. It was next hydrolysed according to the procedure of Croon[70] by treating with 72% sulphuric acid at $0^{o}C$ temperature followed by heating with 12% sulphuric acid for 6 hours (Chart-1.5). The hydrolysis procedure was preferred since it has been reported that demethylation and degradation of methylated sugars occurs to minimum extent by this method. Preliminary paper chromatographic examination of the neutral methylated sugars mixture revealed two sharp spots having R_{TMG} values 0.87 and 0.54 along with three faint spots having R_{TMG} values 0.96, 0.80 and 0.71 respectively. Resolution of two methylated sugars having sharp spots by preparative partition chromatography resulted in the isolation of the homogeneous fractions (A_1 - A_2), which were characterised as 2,3,4,6-tetra-O-methyl-D-galactose (52), 2,3-di-O-methyl-D-mannose (53) from their migration rates, specific rotations, methoxyl values and melting point of their characteristic crystalline derivatives. The results are summarised in Table 1.6.



I.R. spectrum of fully methylated A.Pavonina seed polysaccharide

FIG. 1·9

METHYLATION STUDIES OF A. PAVONINA SEED POLYSACCHARIDE

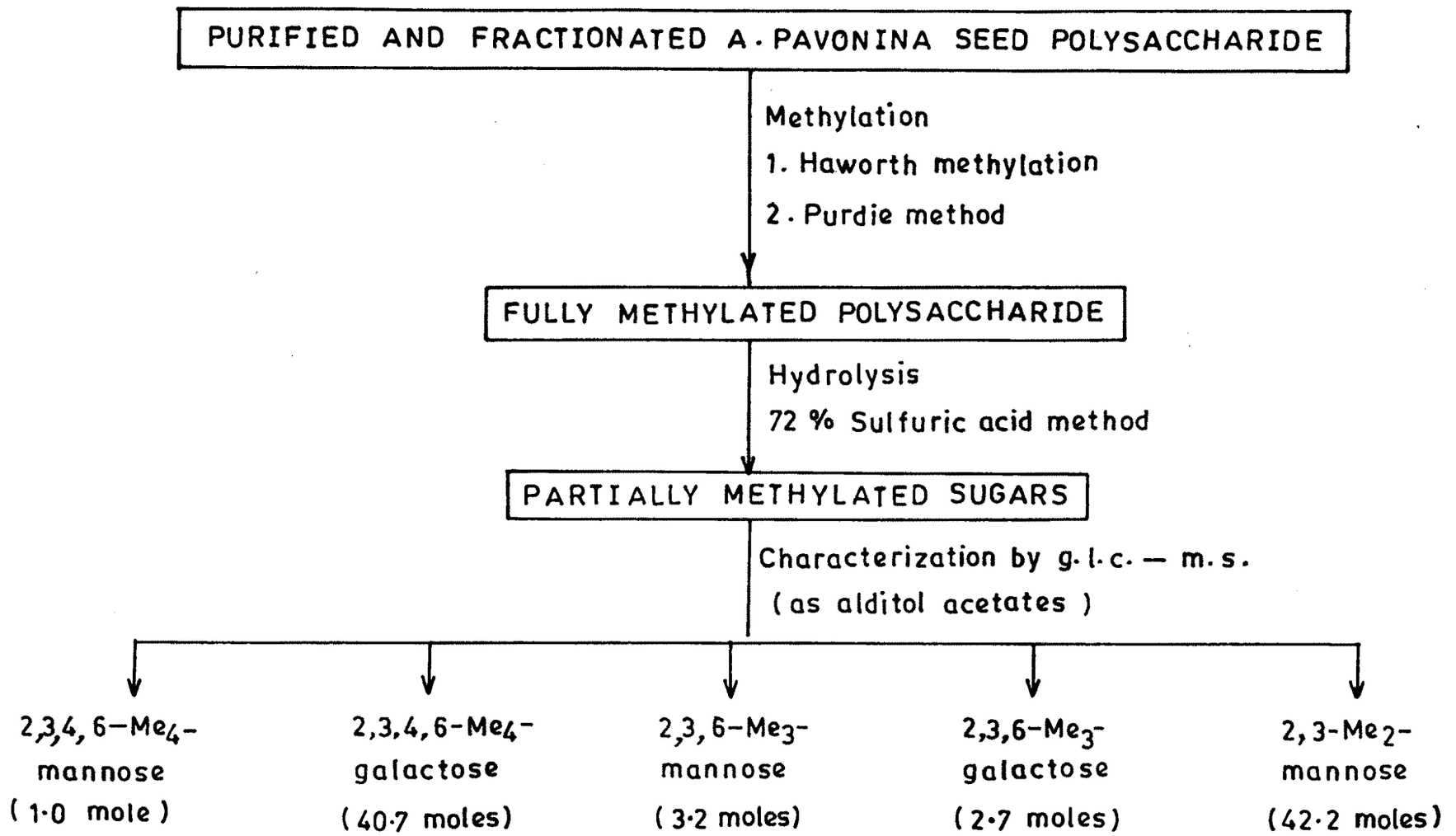


CHART 1·5

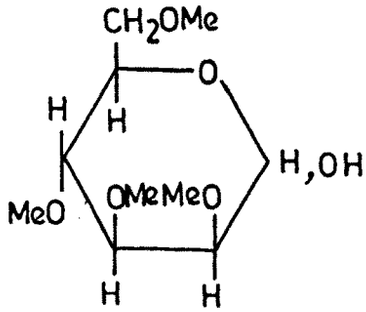
Table 1.6

Characterisation of Partially Methylated Neutral Sugars (A_1 - A_2) by Classical Methods

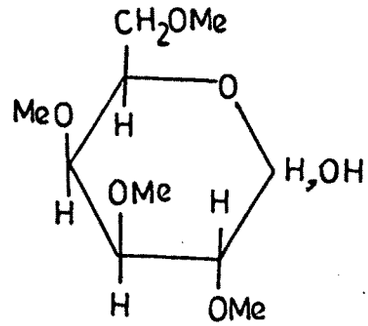
Fraction	O-methyl sugars	R_{TMG} (in solvent S_1)	$[\alpha]_D^{25}$ (H_2O)	Derivative	Mode of linkage
A_1	2,3,4,6-tetra-O-methyl-D-galactose	0.87	+112.2°	2,3,4,6-tetra-O-methyl-N-phenyl-D-galactosylamine, m.p. 191-92°C.	$Gal_p(1 \rightarrow$
A_2	2,3-di-O-methyl-D-mannose	0.54	- 15.7°	1,4,6-tri-p-nitrobenzoyl-2,3-di-O-methyl-D-mannose, m.p. 193-94°	$\rightarrow 4)\underset{\downarrow 6}{Man_p}(1 \rightarrow$

In order to characterise the minor partially methylated sugars, the aldital acetates of the methylated sugar mixture was subjected to g.l.c.-m.s. analysis under column condition C_2 (p. 97). The g.l.c. diagram is shown in Fig. 1.11. The retention time of alditol acetate of 2,3,4,6-tetra-O-methyl-D-glucose was also measured under the same column condition for the purpose of reference (Fig. 1.10). It can be observed from Fig. 1.11 that there are two major peaks (2 and 5) with relative retention times 1.15 and 3.27 and three minor peaks (1,3 and 4) with relative retention times 0.97, 1.79 and 2.01 respectively. These peaks (1 to 5) could be

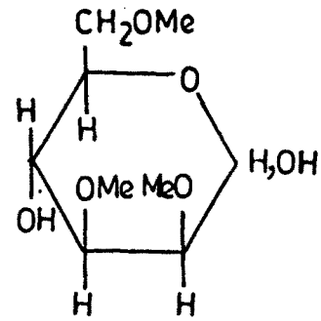
assigned to alditol acetates of 2,3,4,6-tetra-O-methyl-mannose (54), 2,3,4,6-tetra-O-methyl-galactose (52), 2,3,6-tri-O-methyl-mannose (55), 2,3,6-tri-O-methyl-galactose (56) and 2,3-di-O-methyl-mannose (53) which are present in an approximate molar ratio of 1:41:3;3:42 respectively. The partially methylated sugars were also confirmed by the m/z values of the fragmentation patterns as interpretated from their mass spectra. The summary of the analysis is given in Table 1.7.



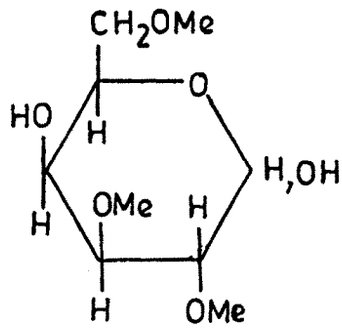
(54)



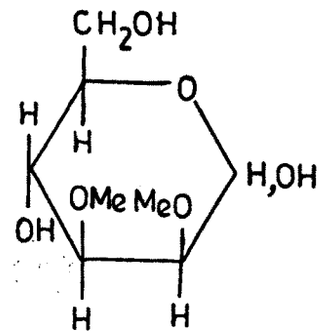
(52)



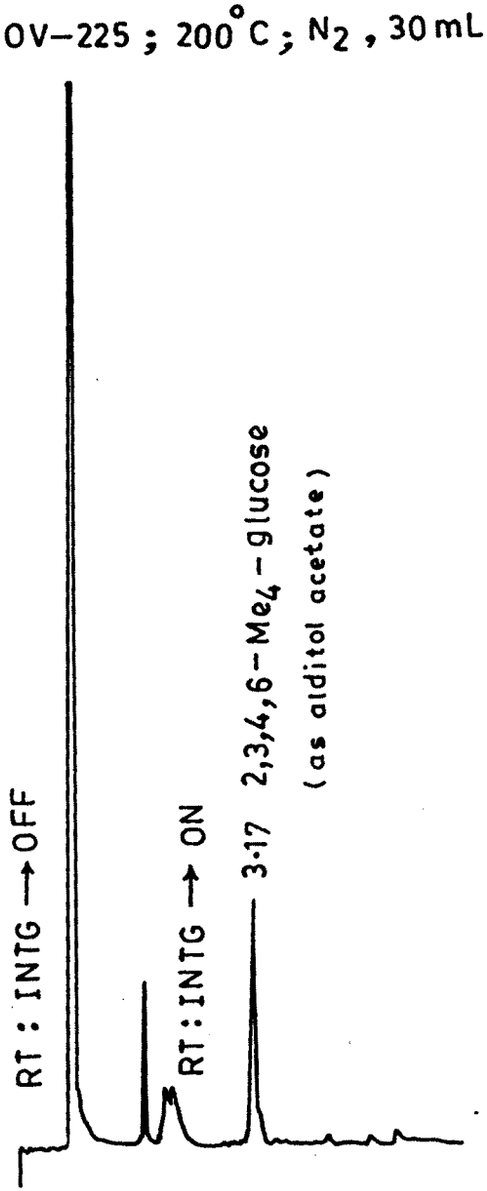
(55)



(56)

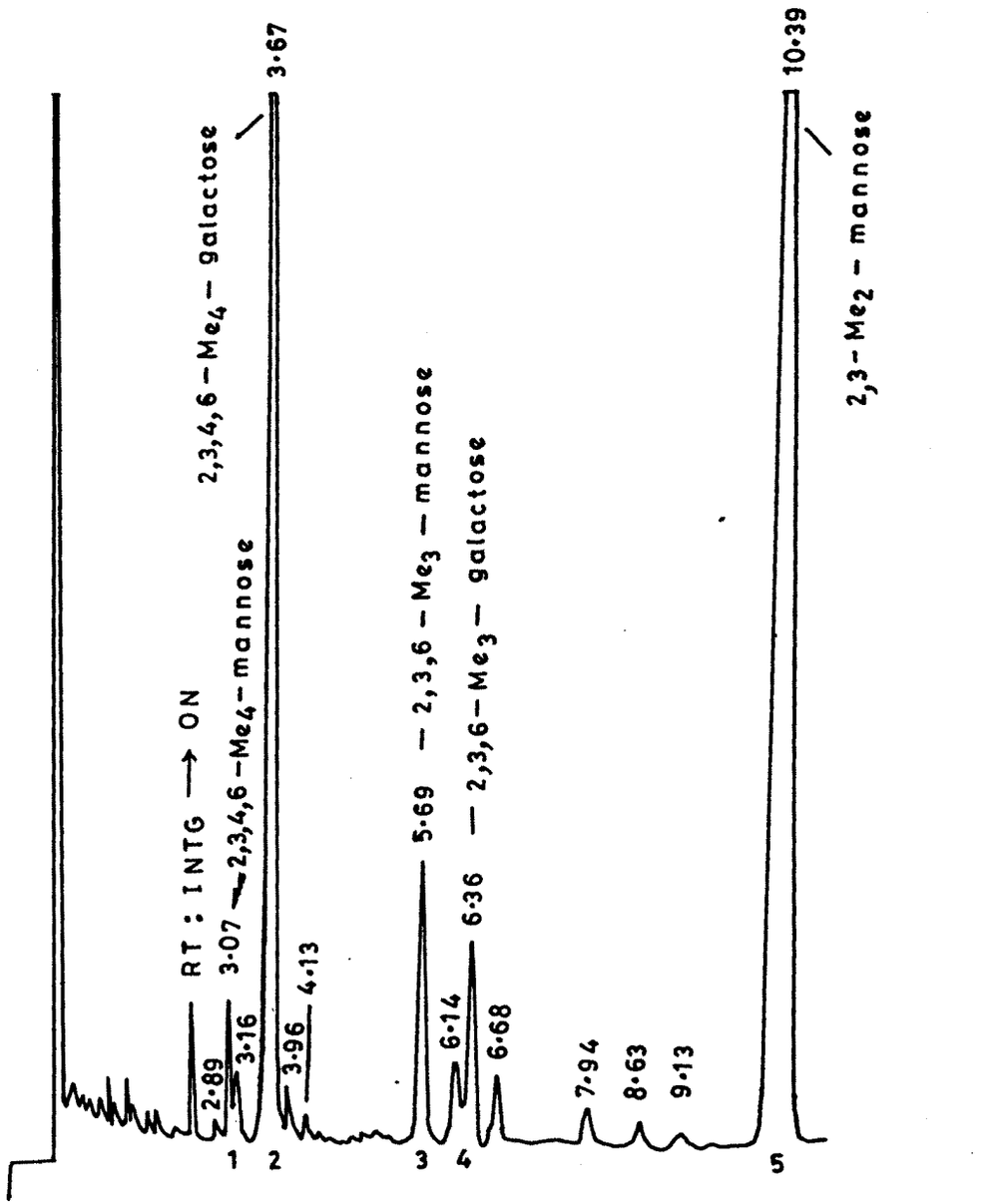


(53)



Gas chromatogram of 1,5–di–O– acetyl – 2,3,4,6 – tetra – O – methyl – D – glucitol

FIG. 1·10



Gas chromatogram of partially methylated alditol acetates derived from methylated A. pavonina seed polysaccharide

FIG. 1·11

Table 1.7

G.l.c.-m.s. Analysis of Partially Methylated Alditol Acetates

(Derived from Methylated Polysaccharide)

under Condition C_2 (3% OV-225)

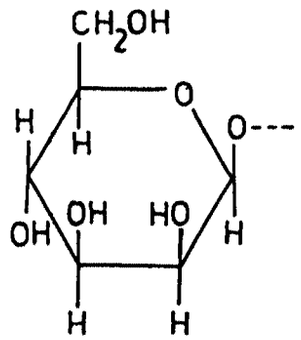
Peak No.	Retention Time (RT*)	Molar ratio**	Main fragments† (m/z)	Alditol acetates	Mode of linkage
1	0.97	1.0	43, 45, 71, 87, 101, 117, 129, 145, 161, 205.	2,3,4,6-tetra-O-methyl-mannose	$Man_p(1\rightarrow$
2	1.15	40.7	43, 45, 71, 87, 101, 117, 129, 145, 161, 205.	2,3,4,6-tetra-O-methyl-galactose	$Gal_p(1\rightarrow$
3	1.79	3.2	43, 45, 87, 99, 101, 113, 117, 233	2,3,6-tri-O-Methyl-mannose	$\rightarrow 4)Man_p(1\rightarrow$
4	2.01	2.7	43, 45, 87, 99, 101, 113, 117, 233	2,3,6-tri-O-methyl-galactose	$\rightarrow 4)Gal_p(1\rightarrow$
5	3.27	42.2	43, 101, 117, 261	2,3-di-O-methyl-mannose	$\rightarrow 4)Man_p(1\rightarrow$ (6 ↓)

* Retention time relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol; the values are identical with those of standard sugars, and agreement with literature values[66,67].

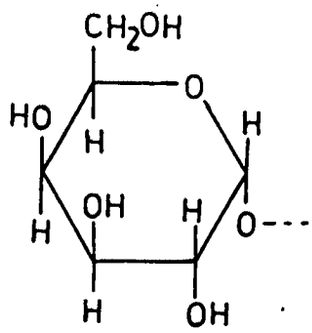
** Relative to 2,3,4,6-tetra-O-methyl-mannose.

† Characteristic mass fragments are identical with those reported in the literature[47,71].

On the assumption that methylation of A. pavonina seed polysaccharide was complete, the locations of the hydroxyl groups in the cleavage fragments indicated the position through which the monosaccharide units were involved in union with the other residues. Thus the isolation of 1 mole of 2,3,4,6-tetra-O-methyl-mannose (54) and 41 moles of 2,3,4,6-tetra-O-methyl-galactose (52) indicated that these residues constituted the 42 end groups in the average repeating unit of the molecular complex since they are linked only through the reducing groups at C_1 terminal as shown in the structures (57) and (58)

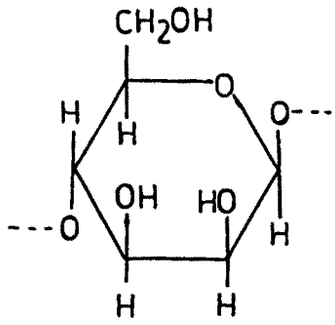


(57)

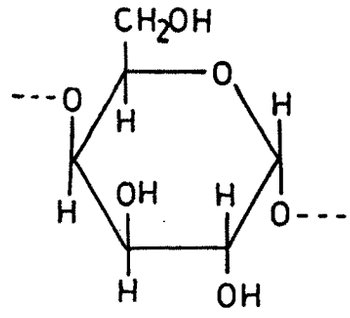


(58)

Similarly, the presence of 3 moles of 2,3,6-tri-O-methyl-mannose (55) and 3 moles of 2,3,6-tri-O-methyl-galactose (56) showed that these components originated from the mannose and galactose residues linked to other units in the complex through hydroxyl groups at C_1 and C_4 respectively as in the structures (59) and (60).

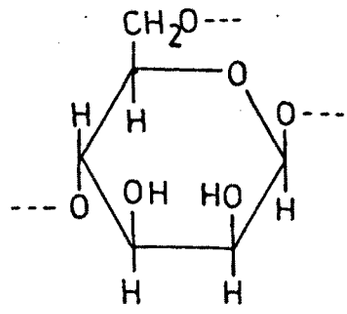


(59)



(60)

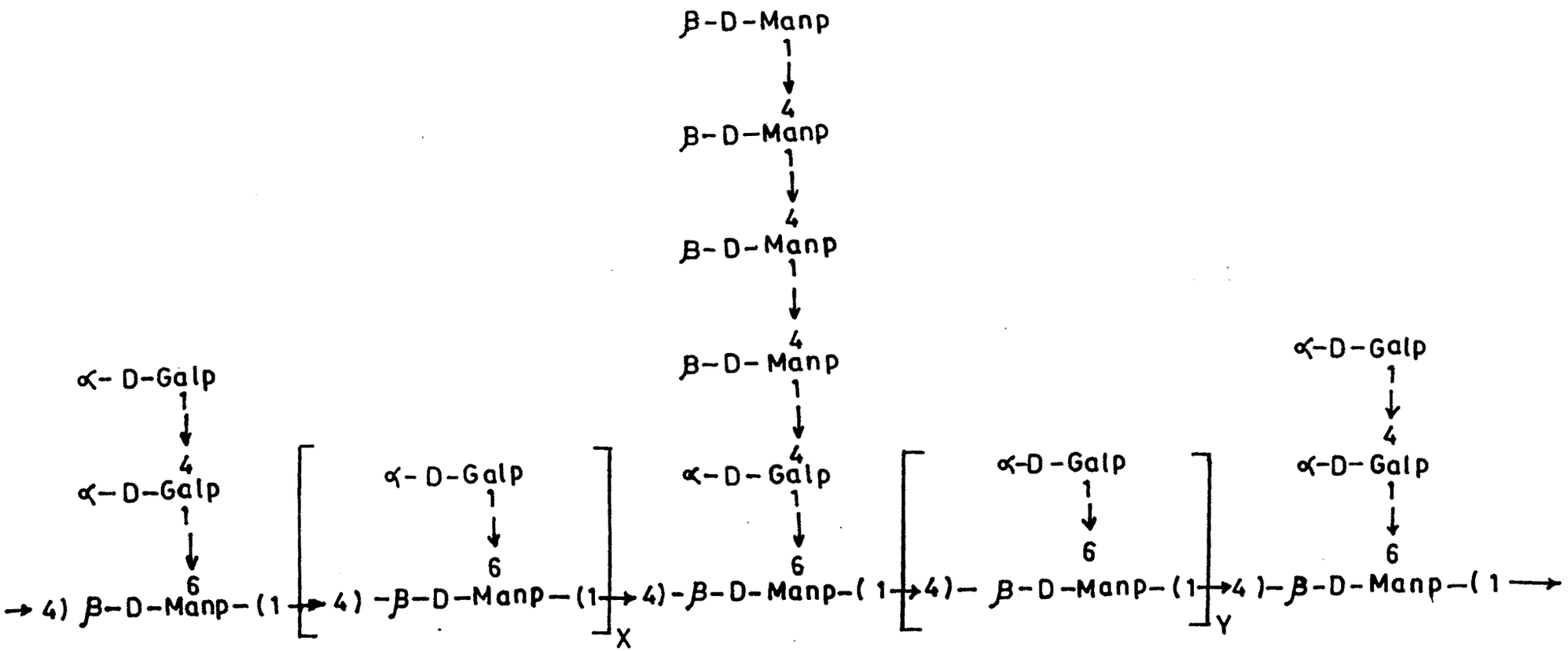
Further, the occurrence of 42 moles of 2,3-di-O-methyl-mannose (53) suggested that these mannose residues are joined to other monosaccharide unit through C_1, C_4 and C_6 as shown in structure (61).



(61)

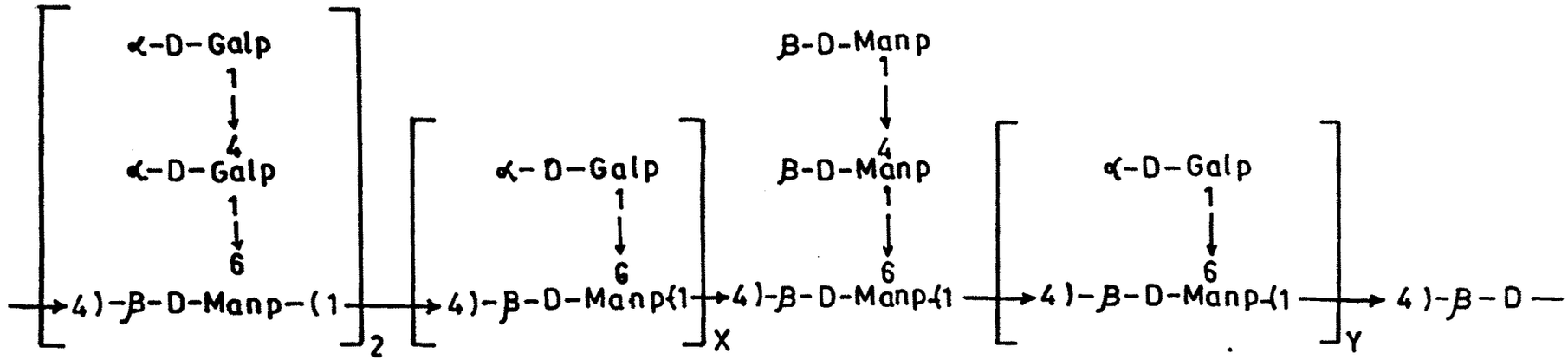
The foregoing results suggest a highly branched structure having 42 end groups in an average repeating unit consisting of 90 hexosyl residues for A. pavonina seed polysaccharide. The methylation results also demonstrate that all the galactose and mannose residues are in pyranose form. The I.R., ^{1}H-N.m.r. and ^{13}C-N.m.r. spectra of the parent polysaccharide as discussed earlier (in Chapter I) show the presence of α-linked-D-galactopyranosyl & ß-linked -D-mannopyranosyl residues in the polymer. The above findings (methylation data) suggest several variants of its structure with respect to the distribution of linkages and arrangement of sugar moieties in the average repeating unit of A. pavonina galactomannan. The variants (62-65) of its structure have been discussed as follows.

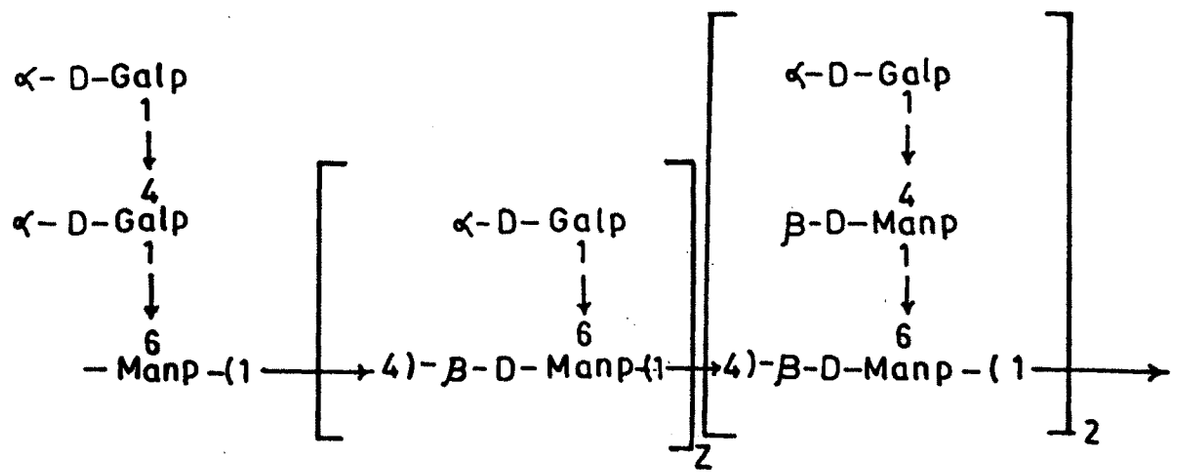
1) The basic structure of A. pavonina galactomannan, like that of the other water-soluble galactomannans, could consist of backbone of ß-D-(1 → 4)-linked D-mannopyranosyl residues with side chains formed by single α-D-(1 → 6)-linked D-galactopyranosyl residues. In addition to this, the infrequent, short chains of (1 → 4)-linked α-D-galactopyranosyl residues


β-D-Manp
1
4
β-D-Manp
1
4
β-D-Manp
1
4
β-D-Manp
1
4
α-D-Galp
1
4
α-D-Galp
1
6
→4) β-D-Manp-(1→
α-D-Galp
1
6
4)-β-D-Manp-(1→
X
α-D-Galp
1
6
4)-β-D-Manp-(1→
Y
α-D-Galp
1
4
α-D-Galp
1
6
4)-β-D-Manp-(1→

$$X + Y = 39$$

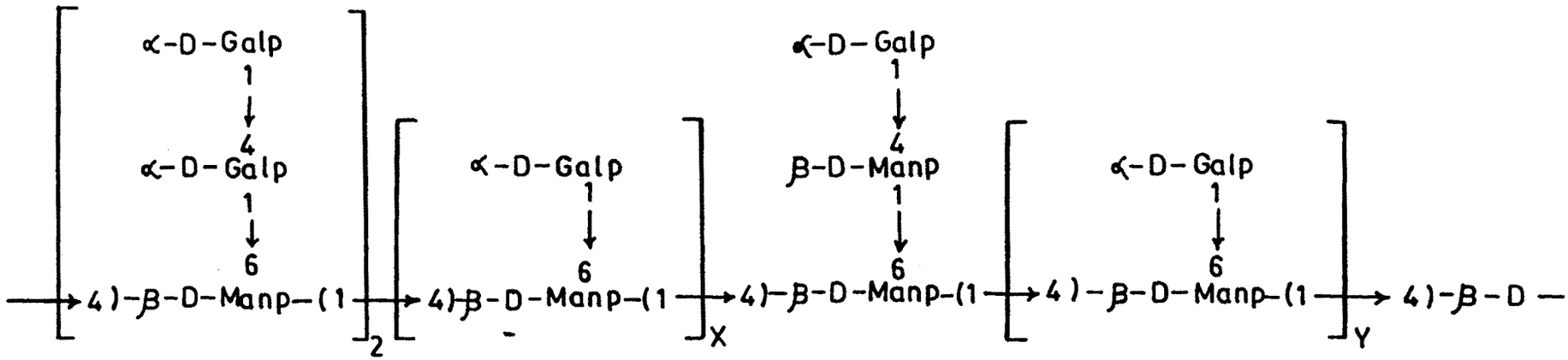
(62)

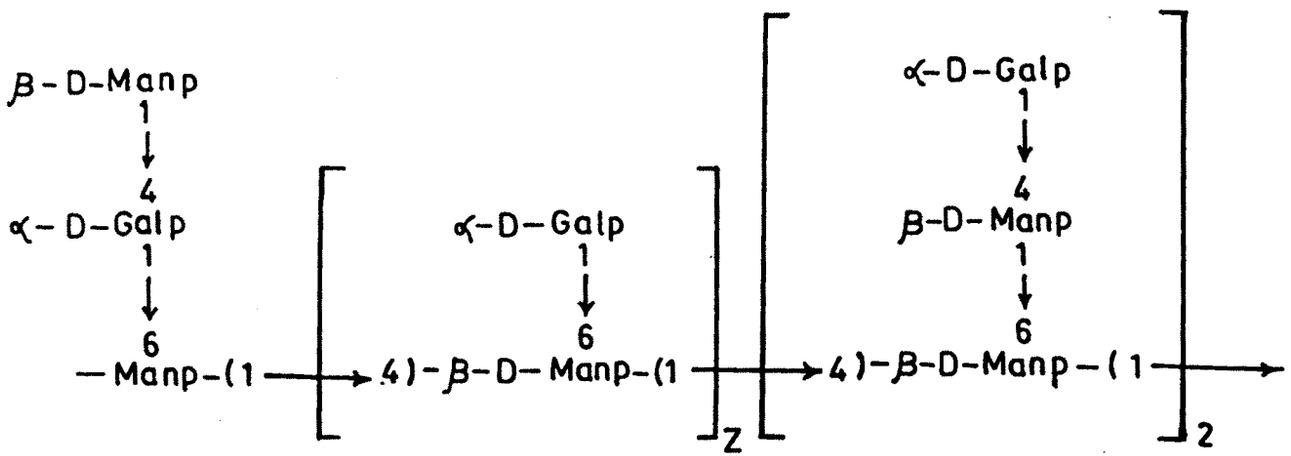

α-D-Galp
1
4
α-D-Galp
1
6
4)-β-D-Manp-(1
2
α-D-Galp
1
6
4)-β-D-Manp-(1
X
β-D-Manp
1
4
β-D-Manp
1
6
4)-β-D-Manp-(1
α-D-Galp
1
6
4)-β-D-Manp-(1
Y
4)-β-D—


α-D-Galp
1
4
α-D-Galp
1
6
—Manp-(1
α-D-Galp
1
6
4)-β-D-Manp-(1
α-D-Galp
1
4
β-D-Manp
1
6
4)-β-D-Manp-(1
Z
2

$$X + Y + Z = 36$$

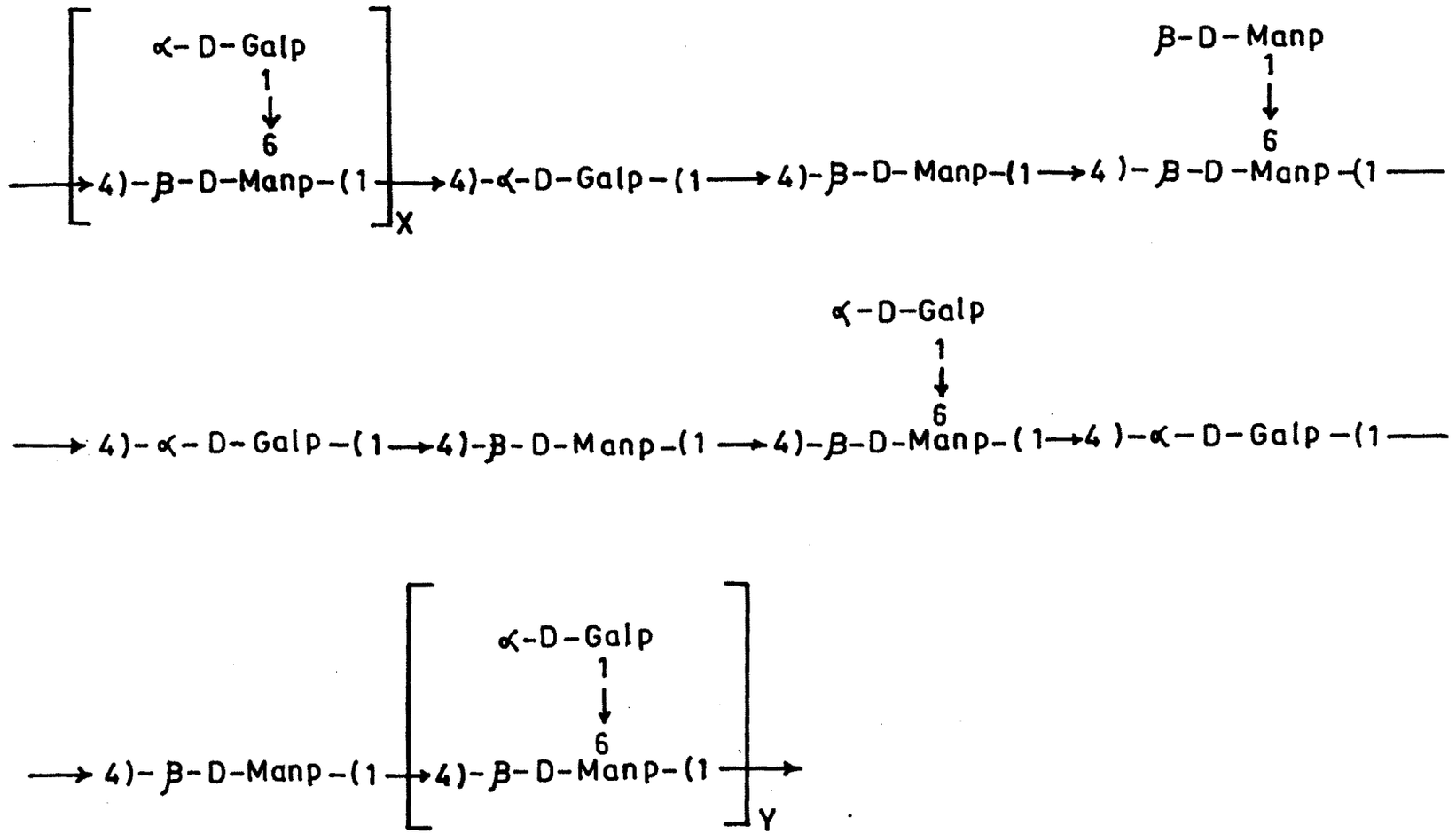
(63)





$$X + Y + Z = 36$$

(64)


α-D-Galp
1
6
4)-β-D-Manp-(1
X
4)-α-D-Galp-(1
4)-β-D-Manp-(1
β-D-Manp
1
6
4)-β-D-Manp-(1
α-D-Galp
1
6
4)-α-D-Galp-(1
4)-β-D-Manp-(1
4)-β-D-Manp-(1
4)-α-D-Galp-(1
4)-β-D-Manp-(1
α-D-Galp
1
6
4)-β-D-Manp-(1
Y

$$X + Y = 40$$

(65)

(composed of two galactose units) could be attached to the D-mannan backbone by (1→6)-linkages; one short chain of (1→4)-linked ß-D-mannopyranosyl units (consisting of four mannose residues) could also be attached at 0-4 of an α-D-galactopyranosyl single unit side chain, as shown in the tentative structure (62). The presence of such type of short chains attached in the similar fashion as described above, have been encountered in Melilotus indica galactomannan[66]. The presence of infrequent, short chains of (1→4)-linked α-D-galactopyranosyl residues attached to the mannan backbone by (1→6)-linkages have previously been reported in Gleditsia triacanthos galactomannan[72].

(2). It is also possible that in addition to galactose short chains as mentioned above, A. pavonina galactomannan could have short chain of (1→4)-linked ß-D-mannopyranosyl units (composed of two mannose units) and infrequent, short chains composed of both galactose and mannose units joined by (1→4)-linkage, and attached to D-mannan backbone by (1→6)-linkages, as shown in the another variant (63) of its structure.

(3). Another possibility is that in addition of galactose short chains as described above, A. pavonina galactomannan could consist of infrequent, short chains composed of both galactose and mannose units joined by (1→4)-linkage and attached to D-mannan backbone by (1→6)-linkages, as shown in the tentative structure (64).

This kind of mixed short chains in the form of branching as suggested in the tentative structures (63) and (64) have not been encountered before, in the galactomannans.

(4). It is also possible that (1 → 4)-linked D-galactose and D-mannose residues could form part of the backbone of A. pavonina galactomannan, as shown in tentative structure (65). The existence of such mixed type of main chain (backbone) has been reported in the galactomannans of Cassia grandis[73] and Crotalaria juncea[74].

Based on the results of methylation of the galactomannan from A. pavonina, four structures (62-65) were assigned as possible varients of A. pavonina seed polysaccharide structure, as discussed above. In order to decide which structure represents A. pavonina galactomannan under investigation, it would be necessary to degrade the polysaccharide by acid and/or an enzyme to obtain oligosaccharides of constitutional significance, and to perform periodate oxidation studies followed by Smith degradation of the polysaccharide in order to further confirm different types of linkage proposed on the basis of methylation data. However, A. pavonina galactomannan appears to have highly branched structure based on the above findings, and difers from the galactomannans which possess classical type of structure (containing a ß-D-(1 → 4)-linked D-mannan backbone to which are attached single α-D-galactosyl stubs at 0-6 of certain D-mannosyl residues).

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PART II

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