Carbohydrate Polymers 6 (1986) 349–359

Substitution Reactions of Galactomannans in Organic Media

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(Received: 28 October 1985)

SUMMARY

The methylation of galactomannans in organic media produced patterns of substitution which did not depend on the relative reactivity of the hydroxyls, but that were governed by the state of aggregation of the polysaccharides. Similar results were observed on acetylation of these polymers dispersed in formamide, as shown by study of the products of oxidation of the acetylated derivatives with chromium trioxide and the analysis of an acetylated galactomannan-like oligosaccharide.

INTRODUCTION

Although the reactions of low molecular weight compounds depend mainly on their primary structure, those of polymers may be influenced by their secondary and tertiary structures.

We have therefore embarked on a study of the methylation of galactomannans in aqueous and organic media, and determined the degree of methylation, and the distribution of the methoxyl groups in the sugar units through the analysis of the mixture of methylated sugars produced by hydrolysis of the partially methylated galactomannans derived from each step of methylation.

The results so obtained were compared with those arising from the analysis of another derivative obtained by a substitution reaction in an organic medium; this involved, first, the acetylation of a galacto-

Carbohydrate Polymers 0144-8617/86/$03.50 — © Elsevier Applied Science Publishers Ltd, England, 1986. Printed in Great Britain

mannan dispersed in formamide, followed by oxidation with chromium trioxide. Experience has shown that this reagent would oxidize the peracetylated β-D-mannopyranose units to 5-hexulosonates and would also attack partially acetylated β-D-mannopyranose and α-D-galactopyranose residues, while the last would not react if they were completely substituted. Anomalous behaviour led us to study other acetylated galactomannans and galactomannan-like oligosaccharides.

The results suggested that, when both substitution reactions are carried out in organic media, the patterns of substitution are not controlled entirely by the relative reactivity of the hydroxyls but are strongly influenced by higher-order structures of the polysaccharides.

MATERIALS AND METHODS

The seeds of *Gleditsia triacanthos* were obtained from ripe pods collected at the Ciudad Universitaria (Buenos Aires). The milled endosperm was extracted exhaustively with water, successively, at room temperature, 50°C and 95°C. Each extract was precipitated by a stepwise addition of ethanol; 14 galactomannan fractions were obtained, distributed between four groups as previously described (Manzi *et al.*, 1984). Galactomannan from *Gleditsia amorphoides* was obtained as described elsewhere (Cerezo, 1965). Neutral sugar analyses were performed by the gas–liquid chromatographic method of Albersheim *et al.* (1967). Absolute amounts of each sugar, before and after the oxidation step, were determined by adding *meso*-inositol as inernal standard. Methylated galactomannans from *Gleditsia triacanthos* (0·5–1·5 mg) were hydrolyzed with trifluoroacetic acid and the partially methylated sugars obtained converted into the alditol acetates as described elsewhere (Manzi *et al.*, 1984). The mixtures of partially methylated alditol acetates were analysed by GLC and GLC–MS as previously described (Manzi *et al.*, 1984). Details of the permethylation of the galactomannans by the Haworth and Hakomori procedures have been published elsewhere (Manzi *et al.*, 1984).

The methylation of the galactomannans by the method of Hakomori (1964) with the modifications of Sandford & Conrad (1966) was carried out as follows. Anhydrous, distilled DMSO (0·1–0·5 ml) was added to the polysaccharide (1·0–15·0 mg) in a reac-

tion vial. The system was warmed at 50°C for 1 h, and further sonicated for the same time, the samples thereby forming a fine suspension. A solution (0·1–0·5 ml) of 2M methylsulphynyl carbanion in DMSO was added to the vial which was flushed with nitrogen. After further sonication for 1 h, methyl iodide (0·1–0·5 ml) was slowly added with simultaneous cooling in an ice-water bath and sonication was continued for 2 h. The procedure was repeated twice and the reaction mixture was poured into water (1·3–3.0 ml). The insoluble material (17–59% of the starting sample) was separated leaving a turbid solution which was dialyzed and freeze-dried.

The partially methylated galactomannan 5, obtained by the above procedure, was submitted again to the same method and the final product was isolated as above.

Partially methylated glactomannans 4 and 5, obtained by the treatment of Hakomori described above, were submitted to a further methylation in DMF by the technique of Kuhn (1957). Anhydrous DMF (0.3 ml) was added to the samples (3.0 mg) in closed vials which were sonicated until turbid solutions were obtained. Barium oxide (60 mg) was added. Sonication was continued during the addition (over 30 min) of methyl iodide (0·1 ml), and thereafter for another 2 h. The solution was poured into water (1·0 ml) and the methylated product recovered as above.

The galactomannan from *Gleditsia triacanthos* was acetylated and submitted to oxidation as described by Hoffman *et al.* (1972) for guar galactomannan; oxidized product A was thus obtained. In a second batch, the oxidized product was not extracted with chloroform, but isolated by dialysis and freeze-drying (B).

The galactomannan from *Gleditsia amorphoides* was dispersed in formamide, and acetylated as above. The acetone purified product contained 40·73% acetyl; further acetylation in formamide/pyridine (2:1) did not increase the percentage of acetyls (30·81%) (Cerezo, 1965).

The acetylated galactomannan (15·9 mg) in anhydrous acetic acid (0·5 ml) solution was oxidized with 54·0 mg of dry chromium trioxide for 2 h, at 50°C, with continuous stirring. After the oxidation, 2 ml water were added and the product was extracted with chloroform (5 × 1·5 ml). The chloroform was distilled off, giving 5·47 mg of I. The aqueous layer was dialyzed, producing an insoluble material (14·10 mg, II) and a solution, which by freeze-drying gave 14·25 mg of III.

Both products from the aqueous layer were highly contaminated with chromium salts.

Acetylation of a galactomannan-like oligosaccharide (N-2, see Manzi & Cerezo (1984); 69·90 mg) was performed in formamide (2·5 ml). Pyridine was added (1 ml), with constant stirring, followed by acetic anhydride (1 ml). The system was kept for 19 h at room temperature, and the acetylated derivative, AF1, was isolated by extraction with chloroform as above: yield, 40·9 mg; acetyl, 36·0%. TLC on a silicagel-G developed with chloroform/methanol 9:1 (plus a drop of acetic acid) showed that part of the sample moved with the front of the solvent, while the other part formed a tail from the starting point to the middle of the plate.

The product of the first acetylation was reacetylated in similar conditions, but at 50°C. The final product, AF2, was isolated by extraction with chloroform as above: yield, 12·10 mg; acetyl, 39·0%.

Infrared spectra of the acetylated samples were recorded from Nujol mulls, in a Perkin-Elmer 710 B infrared spectrophotometer. The ^{1}H- and ^{13}C-NMR spectra were recorded in an XL-100 Varian spectrometer, at room temperature, using 0·4 ml of solutions of the acetylated samples in Cl_3CD (30 mg of AF1, and 10 mg of AF2) with tetramethylsilane as an external standard.

RESULTS

The compositions in terms of partially methylated sugars of the galactomannans methylated by the different techniques are given in the following tables: (a) Table 1 — Hakomori method, sequence of two methylations by the Hakomori method, and sequence of a Hakomori methylation and a further Kuhn methylation; and (b) Table 2 — Haworth method.

The results indicate that methylation by the Hakomori procedure was successful in producing a high degree of substitution, about 13% higher than that obtained with sodium hydroxide and dimethyl sulphate, even if the reactions were not complete (Tables 1 and 2). Nevertheless, the patterns of methylation were different from the usual ones as they showed a major proportion of totally methylated units together with significant amounts of mono- or non-methylated residues (Table 1); the lack of residues with an intermediate

TABLE 1

Relative Proportions of Methylated Sugars from the Galactomannan Fractions of the Endosperm of the Seeds of *Gleditsia triacanthos* after Different Methylation Procedures (mol %)[a]

Galacto-mannans[b]	*Mannose*					*Galactose*				$-OCH_3$[c] (%)
	2,3,4,6-Tetra-methyl	*2,3,6-Tri-methyl*	*2,3-Di-methyl*	*2,6-+4,6-Di-methyl*	*Mono-methyl*[d]	*2,3,4,6-Tetra-methyl*	*2,3,6-Tri-methyl*	*2,4-Di-methyl*	*Mono-methyl*[d]	
1	0·7	27·8	19·6	2·9	24·4	9·2	1·5	1·4	9·8	33·9
2	1·5	38·1	32·1	2·1	1·8	24·4	—	Tr[e]	—	44·1
4	1·5	19·4	13·7	22·6	9·7	14·5	11·5	Tr[e]	Tr[e]	38·9
5	0.9	30·7	21·2	1·9	17·0	18·4	1·3	1·7	7·0	38·9
7	Tr[e]	15·9	19·8	2·5	39·1	3·1	1·0	0·9	13·4	27·3
9	Tr[e]	39·3	18·0	1·1	13·5	8·0	2·1	14·2	3·5	38·0
10	0·9	51·4	20·3	1·5	6·9	11·6	1·4	1·2	4·5	41·4
11	0·8	49·3	18·4	2·0	11·5	10·9	1·5	2·1	2·0	40·3
12	1·2	41·6	34·3	3·6	1·7	12·1	1·6	—	—	40·7
13	0·5	21·0	25·9	10·6	19·9	15·6	—	9·6	16·9	33·2
14	1·1	33·9	11·7	2·1	22·9	11·9	2·4	1·3	12·9	36·3
5[f]	2·4	33·7	17·7	Tr[e]	8·6	23·0	3·6	5·3	5·0	43·9
4[g]	0·8	31·0	20·0	1·4	19·2	18·0	1·3	Tr[e]	7·1	39·7
5[g]	0·9	37·1	25·0	0·5	11·5	24·4	—	0·6	Tr[e]	42·5

[a] Unless otherwise stated the samples were submitted to a methylation step by the Hakomori procedure.
[b] For the nomenclature see Manzi *et al.* (1984).
[c] Percentages of methoxyl (g of methoxyl per 100 g of methylated galactomannan) were calculated from the percentages of methoxyls for each 100 structural units.
[d] Monomethylated units include free monosaccharides.
[e] Tr = trace.
[f] The sample was submitted to a sequence of two methylations by the Hakomori method.
[g] The methylation was carried out by a two-step sequence of Hakomori and Kuhn methods.

TABLE 2

Relative Proportions of Methylated Sugars from the Galactomannan Fractions of the Endosperm of the Seeds of *Gleditsia triacanthos* after a Methylation Step by the Haworth Procedure (mol %)

Galacto-mannans[a]	*Mannose*						*Galactose*						*$-OCH_3$*[b] *(%)*
	2,3,4,6-Tetra-methyl	*2,3,6-Tri-methyl*	*2,3-Di-methyl*	*2,6-Di-methyl*	*3,6-Di-methyl*	*Mono-methyl*[c]	*2,3,4,6-Tetra-methyl*	*2,3,6-Tri-methyl*	*2,6-+ 4,6-Di-methyl*	*2,3-Di-methyl*	*2,4-Di-methyl*	*Mono-methyl*[c]	
1	0·5	8·4	14·0	4·0	9·7	36·6	1·8	Tr[d]	2·2	1·2	1·0	19·7	26·3
2	0·5	22·0	28·2	6·1	—	14·5	6·1	1·2	3·0	1·3	5·0	10·4	33·8
3	Tr[d]	13·6	26·9	7·1	—	27·5	2·8	0·7	3·2	0·7	3·1	13·7	29·6
4	0·5	13·5	19·3	5·3	6·4	19·8	2·7	0·5	2·4	0·9	1·9	12·0	26·8
5	1·1	24·2	23·5	5·9	3·4	10·4	7·2	1·4	3·2	1·2	4·7	7·3	34·4
7	0·6	13·6	19·2	7·6	10·3	28·9	1·9	Tr[d]	2·8	Tr[d]	0·5	14·4	29·3
8	Tr[d]	13·2	15·5	4·6	8·3	33·8	3·4	Tr[d]	1·9	0·8	1·1	16·2	28·3
9	1·3	32·8	22·5	—	—	10·3	5·7	2·0	9·8	1·2	2·3	5·2	35·3
10	0·6	37·0	17·9	8·2	8·2	12·3	4·0	0·6	1·7	1·0	2·2	5·3	36·3
11	1·6	38·7	15·3	4·2	4·1	9·2	11·4	1·0	0·9	Tr[d]	1·5	6·1	37·9
12	0·6	35·3	19·2	8·4	6·9	12·3	4·9	1·0	2·4	0·7	2·1	5·4	36·2
13	1·5	16·8	17·1	6·7	13·5	25·9	2·6	Tr[d]	2·2	0·9	—	11·8	33·0
14	0·8	21·0	16·2	8·2	12·4	25·0	2·1	Tr[d]	2·3	0·9	—	10·0	29·5

[a] For the nomenclature see Manzi *et al.* (1984).

[b] Percentages of methoxyl (g of methoxyl per 100 g of methylated galactomannan) were calculated from the percentages of methoxyls for each 100 structural units.

[c] Monomethylated units include free monosaccharides.

[d] Tr = trace.

degree of methylation was particularly noticeable. This is observed in all the samples, and is specially clear in the case of the galactose units.

In contrast, the patterns of methylation obtained with the method of Haworth are similar to those usually found in intermediate steps of the substitution reaction and are consistent with the degree of methylation produced (Table 2), and with the relative reactivity of hydroxyls (Haines, 1976).

The results of further methylation depend on the starting materials which are partially methylated. If a galactomannan, previously methylated in aqueous sodium hydroxide, is submitted to a further reaction by the Hakomori procedure, the permethylated polysaccharides are easily obtained (Manzi *et al.*, 1984). On the contrary, if the substitution reaction is carried out by a further Hakomori procedure (Table 1) on a product previously methylated in DMSO, even if the degree of methylation is increased to nearly theoretical values, no permethylation was obtained. Significant amounts of mono- or non-methylated units, were still observed and the nonstatistical distribution of methoxyls was maintained.

The oxidation of a peracetylated galactomannan should destroy the β-D-linked mannopyranose units without attacking most of the α-D-galactopyranose residues, leading to a reduction in the Man:Gal molar ratio. This is the case for the chloroform-soluble oxidation products (Table 3), but not for the major amounts of oxidized acetylated galactomannans, which remain soluble in water after the extraction with chloroform. In the case of these last products, the Man:Gal ratio increased showing that more α-D-galactose units were oxidised than β-D-mannose residues. This was confirmed by determining the absolute amounts of these sugars surviving oxidation (Table 3) which showed that: (a) significant percentages of the β-D-mannose units were not oxidized; and (b) major amounts of α-D-galactoses were destroyed by the chromium trioxide (Table 3).

An oligosaccharide ($DP=15$) with a galactomannan-like structure (Manzi & Cerezo, 1984) was acetylated, dissolved in formamide, and the acetylated derivative was isolated by extraction with chloroform. Even in these favourable conditions for the production of a peracetylated derivative, the product obtained contained only 36·0% acetyl and TLC showed that it was composed of derivatives with different amounts of substitution. The ^{1}H-NMR spectrum, carried out in Cl_3CD, showed broad and ill-defined bands. Further acetylation in the

TABLE 3
Composition in Neutral Sugars of the Oxidized Galactomannans

Galactomanns from:	*Man : Gal (molar ratio)*	*Total Man (μg)*	*Total Gal (μg)*
Cyamopsis tetragonolobus			
Non-oxidized	1·75	—	—
Oxidized[a]	0·55	—	—
Gleditsia amorphoides			
Non-oxidized	2·6	2916·0	1127·3
Oxidized product I	1·5	47·0	31·2
Oxidized product II	3·1	25·8[b]	8·3[b]
Oxidized product III	3·0	54·9[b]	18·5[b]
Gleditsia triacanthos			
Non-oxidized	2·0	—	—
Oxidized product A	0·3	—	—
Oxidized product B	3·8	—	—

[a] Fraction of the oxidized acetylated galactomannan extracted with chloroform from Hoffman *et al.* (1972).
[b] These figures represent μg of sugar per mg of oxidized product because contamination with chromium salts did not allow the total amounts to be obtained.

above conditions increased the percentages of acetyls to 39·0%. The infrared spectrum still showed a broad absorption band corresponding to the hydroxyl groups, the ^{1}H-NMR spectrum showed broad and ill-defined bands, and the ^{13}C-NMR spectrum showed sharp absorption peaks only in the zone corresponding to C-4 and C-5 of the D-mannose units.

DISCUSSION

In aqueous solution the galactomannan molecules have extended, ribbon-like structures (McCleary & Matheson, 1975). These entropically unfavourable conformations are promoted by the spontaneous, non-covalent binding of the molecules producing lower free-energy aggregates (Dea *et al.*, 1972; Dea &Morrison, 1975). A strong alkaline medium would disaggregate the molecules due to the ionization of the hydroxyl groups and accompanying rupture of hydrogen bonds and

electrostatic repulsion. The molecules would lose some of their elongated shape and become isolated, a situation that would be stabilized by solvation with the water molecules. Not surprisingly, then, rotation of the galactomannans is strongly decreased in highly alkaline situations (Hui & Neukom, 1964) which can be explained by a change from an ordered to a disordered conformation. The decrease in viscosity and in the sedimentation coefficient in such conditions (Hui & Neukom, 1964) is consistent with a rupture of the molecular interactions and with a change in the shape of the molecules to a less ordered conformation (Dea & Morrison, 1975). In such conditions, methylation of the isolated galactomannan molecules would produce a statistical distribution of the methoxyl groups, depending only on the different reactivity of the hydroxyls. Such partially methylated galactomannans with a high degree of methylation and a homogeneous distribution of the methoxyl groups are soluble in DMSO, due to the difficulty of aggregation; hence, when the further Hakomori methylation is carried out on isolated molecules, the permethylated derivatives are easily produced (Manzi *et al.*, 1984).

The direct methylation of the galactomannans in dimethyl sulphoxide faces a different problem. The methylation is carried out in an heterogeneous medium and, in those conditions, the galactomannan molecules are not isolated but packed into aggregates. In such aggregates, the mannose backbone and the galactose lateral chains are arranged in sheets with the side chains and the planes of the pyranose rings lying in the planes of the sheets as in the solid state (Palmer & Ballantyne, 1950; Aisenberg *et al.*, 1974). The ribbon-like chains of the mannose backbone are laid on top of each other, in stacks, as found in cellulose (Warwicker & Wright, 1967) and these stacks must be joined to each other by hydrogen bonding between equatorial hydroxyl groups. This aggregate is difficult to penetrate by reagents during the substitution reaction since the interior is highly packed. Once reaction has occurred at a particular site, the structure is opened for further reaction in the vicinity which explains the tendency for substituents to be grouped unevenly, with some units completely substituted and others poorly or non-substituted. This is in agreement with the results obtained in the first Hakomori methylation. The high degree of methylation produced in this reaction solubilizes the aggregates, but total dispersion is prevented by the unsubstituted zones; hence, further methylations by the Hakomori or Kuhn procedures are

carried out in an homogeneous medium but still on aggregates and not on isolated molecules. In agreement with this view, even if the percentages of methoxyl groups increased to nearly theoretical values, the analysis of the methylated galactomannans still showed substantial amounts of undermethylated units, indicating that some ordered regions of the aggregates have retained their microcrystallinity (Rees, 1969) until the last stages of the reaction.

Similar conclusions may be drawn for the acetylation which is carried out on the galactomannan aggregates dispersed in dimethylformamide or formamide. The results of the oxidation with chromic acid (Hoffman *et al.*, 1972) on the pathwise acetylated galactomannans can be rationalized as follows: the extraction with chloroform selects the peracetylated molecules, in which the reagent had attacked only the glycosidic carbon atoms producing a lower Man:Gal ratio than that of the starting galactomannan, as expected from the differences in the rate of oxidation. When all the products of the oxidation are taken into account, the Man:Gal ratio is higher, indicating that some galactose units were oxidized at a higher rate than the mannose units. This would happen in partially acetylated galactoses. The absolute amounts of mannose and galactose, remaining after the oxidation, confirm the above results and show also that, in some oxidized galactomannans, percentages of mannose units as high as 30% remain unaltered, suggesting a steric impediment which is consistent with the structure of the aggregates. Analysis of an acetylated galactomannan-like oligosaccharide suggests that these aggregates are formed also between oligomers of low molecular weight.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Consejo Nacional de Investigaciones Científicas y Técnicas. The authors are indebted to Dr J. Kavka for recording the GLC–MS spectra, and to UMYMFOR (FCEyN-CONICET) for technical assistance.

REFERENCES

Aisenberg, E., Smolko, E. E. & Cerezo, A. S. (1974). *An. Asoc. Quím. Argent.* **62**, 113.

Albersheim, P., Nevins, D. J., English, P. D. & Karr, A. (1967). *Carbohydr. Res.* **5**, 340.
Cerezo, A. S. (1965). *J. Org. Chem.* **30**., 924.
Dea, I. C. M. & Morrison, A. (1975). *Adv. Carbohydr. Chem. Biochem.* **31**, 241.
Dea, I. C. M., McKinnon, A. A. & Rees, D. A. (1972). *J. Mol. Biol.* **72**, 153.
Haines, A. H. (1976). *Adv. Carbohydr. Chem. Biochem.* **33**, 11.
Hakomori, S. (1964). *J. Biochem. (Tokyo)* **55**, 205.
Hoffman, J., Lindberg, B. & Svensson, S. (1972). *Acta Chem. Scand.* **26**, 661.
Hui, P. A. & Neukom, H. (1964). *Tappi* **47**, 39.
Kuhn, R., Low, I. & Trishmann, H. (1957). *Chem. Ber.* **90**, 203.
McCleary, B. V. & Matheson, N. K. (1975). *Phytochemistry* **14**, 487.
Manzi, A. E. & Cerezo, A. S. (1984). *Carbohydr. Res.* **134**, 115.
Manzi, A. E., Mazzini, M. N. & Cerezo, A. S. (1984). *Carbohydr. Res.* **125**, 127.
Palmer, K. J. & Ballantyne, M. (1950). *J. Am. Chem. Soc.* **73**, 736.
Rees, D. A. (1969). *Adv. Carbohydr. Chem. Biochem.* **24**, 267.
Sandford, P. A. & Conrad, H. E. (1966). *Biochemistry* **5**, 1508.
Warwicker, J. O. & Wright, A. (1967). *J. Appl. Polym. Sci.* **11**, 659.