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DEGRADATION OF GLUCURONIC ACID-CONTAINING EXOPOLYSACCHARIDES FROM *Rhizobium* BY THE HAKOMORI METHYLATION PROCEDURE

RANDI SØMME
Department of Chemistry, Agricultural University of Norway, N-1432 Ås-NLH (Norway)
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ABSTRACT

The uronic acid-containing polysaccharides secreted by some fast-growing, nodulating and non-nodulating strains of *Rhizobium* underwent marked degradation when methylated by the Hakomori procedure. The polysaccharide produced by *Rh. meliloti*, which lacks uronic acid, was not degraded.

INTRODUCTION

Methylation analysis based on the Hakomori procedure[1] has been widely applied to polysaccharides and glycoconjugates and has largely replaced the Haworth and Purdie methods[2]. With few exceptions, Hakomori methylation effects complete etherification in one step. Uronic acids are transformed into methyl esters and *O*-acetyl groups are cleaved, but acetal functions, *e.g.*, pyruvic acid acetals, are stable. Although it is not an ideal system, it effects complete methylation without degradation of the starting material[3–5], and it is now the most commonly used methylation procedure.

The Hakomori procedure involves the addition of dimsyl sodium to a solution of the carbohydrate in methyl sulfoxide, and methyl iodide is added subsequently. Attempts to increase the efficiency of the Hakomori procedure have involved, for example, the replacement of dimsyl sodium with dimsyl potassium[6]. Special precautions have to be taken with uronic acid-containing polysaccharides since repeated methylation results in degradation of the methyl esters by β-elimination[4,7,8]. It has been concluded that, for such polysaccharides, methylation and esterification are complete in one treatment and that β-elimination is insignificant, probably because of the rapid reaction between the anion and methyl iodide which is used in excess[9]. However, to our knowledge, only the non-dialysable methylated products from the uronic acid-containing polysaccharides produced by several strains of *Rhizobium* have been collected and examined.

Glucuronic acid-containing polysaccharides have been submitted to repeated Hakomori methylation and examined for degradation products of the acid formed

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by β-elimination[7], but not for the presence of methylated mono- and oligosaccharides.

Structural variations in the polysaccharides produced by different strains of *Rhizobium* bacteria are to be expected since there is strong evidence that these polysaccharides participate in the specific host-symbiont interaction, but so far no clear correlation has been found[10]. We now report on the degradation of glucuronic acid-containing *Rhizobium* polysaccharides during Hakomori methylation, which could have implications in the determination of structure.

EXPERIMENTAL

General methods. — The following *Rhizobium* strains were used: *Rh. trifolii*, U 226 (infective and co-operative, *i.e.*, nitrogen fixing in the host), Coryn (infective and non-co-operative, *i.e.*, merely parasitic), and Bart A (non-infective). The following infective and co-operative strains were also used: *Rh. meliloti*, U 27, and *Rh. leguminosarum*, U 311. The organisms were cultivated as described earlier[11]. The analytical procedures and chemical manipulations were performed as described elsewhere[12] except when otherwise indicated.

Alditol acetates were analysed by g.l.c. on a Hewlett–Packard 5380 A gas chromatograph with a flame-ionisation detector and a glass column (300 × 0.2 cm) filled with 0.3% of OV-275 and 0.4% of XF-1150 on 80/100 Supelcoport, programmed at 1°/min from 160 →195°. Mass spectra were recorded on a Micromass 12F instrument, using a glass column filled with the aforementioned phase.

T.l.c. was performed on Silica gel G, using chloroform–methanol (20:1) and detection with diphenylaniline–phosphoric acid[13].

H.p.l.c. was performed using a Spectra-Physics Model 3500B, fitted with a Valco valve loop injector (10 μL), a Spectromonitor III UV-detector (Laboratory Data Control) operated at 195 nm, and a Scintag SW 3/20 Chart Recorder. The column employed was a Chrompack C_{18} Cartridge (100 × 3 mm) and acetonitrile–water (50:50) was the mobile phase.

Methylation analysis. — Polysaccharides (10 mg) were methylated by the Hakomori method[10,14–16], the reaction times with dimsyl potassium being 24 and 48 h, and with methyl iodide 2 h. Each reaction mixture was dialysed against stirred, distilled water at 5° for 48 h. The water was changed three times during this period (total volume, 2.5 L). The combined dialysable material was concentrated to 25 mL and extracted with dichloromethane (4 × 10 mL), and the combined extracts were washed with water (4 × 10 mL) and concentrated to dryness. The non-dialysable material was also isolated.

RESULTS AND DISCUSSION

The polysaccharides studied contained *O*-acetyl and pyruvic acid acetal groups together with D-glucose, D-galactose, and D-glucuronic acid, except for that produced by *Rh. meliloti* which lacked glucuronic acid[11].

TABLE I

THE PROPORTION[a] OF THE METHYLATED PRODUCTS IN THE DIALYSABLE MATERIAL OBTAINED BY HAKOMORI METHYLATION OF THE POLYSACCHARIDE OF *Rh. trifolii*, BART A, RELATIVE TO THE NON-DIALYSABLE MATERIAL

Time[b] *(h)*	Rh. trifolii			Rh. legumin.	Rh. meliloti
	Bart A	*Coryn*	*U 226*	*U 311*	*U 27*
24	52	96	39	42	0
48	64	124	46	55	0

[a]Determined from peak areas in g.l.c. [b]Reaction time of the carbanion with the polysaccharide.

Each polysaccharide was methylated and the dialysable and the non-dialysable materials were separately hydrolysed; the products were converted into the alditol acetates and examined conventionally by g.l.c. and g.l.c.–m.s. The results are shown in Table I. The relative proportion of the dialysable material from the glucuronic acid-containing polysaccharides varied from 0.39–1.24 of that of the non-dialysable material. The degradation was greatest after methylation for 48 h.

No dialysable material was produced by the ultrasonic treatment necessary to dissolve the polysaccharide in methyl sulfoxide, but dialysable methylated carbohydrates were present after Hakomori methylation.

The non-dialysable methylated product from the *Rh. meliloti* polysaccharide, which lacks uronic acid, contained 2,4,6-tri-*O*-methyl-D-glucose, 2,4,6-tri-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-glucose, and 2,3-di-*O*-methyl-D-glucose in the molar ratios 2:1:1:2:2, which are the same as previously found[17,18], and no dialysable material was formed.

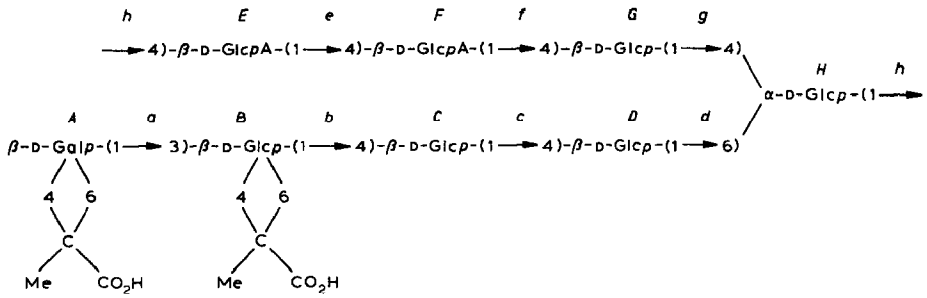
Both the dialysable and the non-dialysable products of the methylation of the uronic acid-containing polysaccharides of the other strains contained 2,3,6-tri-*O*-methyl-D-glucose, 2,3-di-*O*-methyl-D-glucose, 2,3-di-*O*-methyl-D-galactose, and 2-*O*-methyl-D-glucose in various amounts, but in molar proportions close to 50:15:15:15. The degradation did not result in the formation of new products.

T.l.c. and h.p.l.c. of the dialysable material revealed components with mobilities similar to those of the products of methylation of the mixture of oligosaccharides obtained by mild acid hydrolysis of the polysaccharide[14] of *Rh. trifolii*, Bart A. No monosaccharide derivatives were present in the dialysable material.

Repeated h.p.l.c. of the dialysable material obtained by Hakomori methylation of the acidic polysaccharide from *Rh. trifolii*, Bart A, gave eight fractions which were examined by g.l.c. A complete separation of the components was not obtained, but some conclusions can be drawn.

Six fractions contained the same products as the unfractionated material. Consideration of the postulated structure (**1**) of the repeating unit[10] indicates that these fractions must have been formed from the side-chains, since neither 2,3,4,6-tetra-*O*-methyl-D-glucose nor 2,3,4-tri-*O*-methyl-D-glucose was present. The lack

of the former product excluded cleavage at linkages **b**, **c**, and **f**, and the lack of the latter, cleavage of linkage **g**. Cleavage at linkage **a** can also be excluded since the product of methylation of the pyruvylated galactose molecule was not present. Thus, only linkages **e** and **h** could have been broken.



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The last two fractions both lacked 2,3-di-*O*-methyl-D-glucose, the product derived from the branched unit, which indicated cleavage at linkage **d**. This is unlikely since this linkage is stable during Hakomori methylation of the polysaccharide from *Rh. meliloti*. It is more likely that the two fractions resulted from cleavage of linkage **h** by β-elimination followed by alkaline degradation from the liberated reducing unit[19].

The eight fractions obtained by h.p.l.c. were not assayed for glucuronic acid because of lack of material. When the unfractionated mixture was carboxyl-reduced and then remethylated, the yield of 2,3,6-tri-*O*-methyl-D-glucose was increased and its ratio to a new component, 2,3,4,6-tetra-*O*-methyl-D-glucose, was 2:1. Borohydride reduction of the unfractionated mixture, omitting remethylation, increased the yield of 2,3-di-*O*-methyl-D-glucose.

The base-catalysed β-elimination of 4-*O*-substituted hexuronate derivatives is a useful reaction in structural studies of polysaccharides[19] which results in complete loss of the hexuronic acid residues[20]. The experimental procedure is similar to that of a normal Hakomori methylation using a methylated saccharide as the starting material[20,21].

The degradation observed in the above experiments can be explained by β-elimination reactions occurring during the Hakomori methylation of the polysaccharide. The repeating unit **1** of the polysaccharide contains the two contiguous (1→4)-linked glucuronic acid residues **E** and **F**. If linkage **h** is cleaved by a β-elimination reaction during the methylation, the increase in the amount of 2,3,6-tri-*O*-methyl-D-glucose in the carboxyl-reduced and remethylated dialysable material could originate from **F** in an early stage of the degradation with the partially degraded unit **E** still attached. This interpretation accords with the increase in the amount of 2,3-di-*O*-methyl-D-glucose when the dialysable material was reduced but not remethylated. The 2,3,4,6-tetra-*O*-methyl-D-glucose might originate from the glucuronic acid residue **F**, after liberation of degraded **E**. One would expect to find

2,3,4-tri-*O*-methyl-D-glucose among the products of the carboxyl-reduced dialysable material, but it was not detected, possibly because degraded **E** was liberated at a stage of the methylation process too late for methylation of the exposed HO-4 of **F** to occur.

The β-elimination might also start at linkage **e** which would result in the loss of both **E** and **F**, **F** by β-elimination and **E** by alkaline degradation from the reducing end.

The above degradation reactions explain the low content of uronic acid found in polysaccharides when determined by g.l.c. of products formed by carboxyl-reduction of a methylated polysaccharide, with and without subsequent remethylation, compared to colorimetric determination of the acid[10].

Although only polysaccharides produced by different strains of *Rhizobium* have been examined, it might be expected that polysaccharides containing 4-*O*-substituted uronic acid from other sources would be degraded in a similar way.

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