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METHYLATED CHERRY GUM

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ABSTRACT

The constituent sugars of methylated cherry gum have been identified and quantitatively determined by g.l.c.–mass spectrometry of the methylated alditol acetates formed from the methylated gum, the methylated gum after reduction with lithium aluminium hydride, and the carboxyl-reduced, methylated gum after remethylation. The significance of these results is assessed in the light of partial hydrolysis studies.

INTRODUCTION

Detailed studies on complex methylated polysaccharides, such as those derived from *Prunus* gums, were initiated before modern separation and characterization techniques were available. Through the kindness of Professor J. K. N. Jones, a sample of methylated cherry gum[1] was made available to us and we report here an examination of the cleavage products from the methylated gum and its derivatives using g.l.c.–mass spectrometry for identification of the substitution patterns of the methylated sugars and for quantitative determination of the major constituents. In addition, information has been obtained on the location of aldobiouronic acid residues in the polysaccharide structure.

RESULTS AND DISCUSSION

The neutral sugar constituents of methylated cherry gum, including all of those characterized earlier by Jones[1], have been recognized by g.l.c. of the methyl glycosides formed on methanolysis. Their identities have been confirmed and their relative proportions determined by g.l.c.–mass spectrometry of the derived methylated alditol acetates[2], and the results are summarized in Table I. One newly recognized constituent

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TABLE I

NEUTRAL SUGARS FROM METHYLATED CHERRY GUM, AND ITS CARBOXYL-REDUCED AND REMETHYLATED DERIVATIVES

Sugar	*Proportions (%) of alditol acetates from*							*Composition of parent gum*	
	methylated cherry gum		*carboxyl-reduced methylated gum*		*remethylated reduced gum*			*previous work*[3]	*present methylation data*
2,3,4-Me$_3$-Xyl[a]	3.3	14.9	2.85	12.8	2.6	11.8	*xylose*	2.8	2.7
2,3,4-Me$_3$-Ara[a]	11.6		9.95		9.2				
2,3,5-Me$_3$-Ara	29.2		24.95		24.5				
2,5-Me$_2$-Ara	14.2		11.6		11.6[c]		*arabinose*	52.0	51.4
2,3-Me$_2$-Ara	6.0		5.45		5.5				
2,4,6-Me$_3$-Gal	9.1		7.6		9.5				
2,4-Me$_3$-Gal	5.4		4.55		4.5				
2,6-Me$_2$-Gal	10.5		9.1		9.1[d]				
2-Me-Gal	7.4		6.1[b]		6.1		*galactose*	25.6	28.3
4,6-Me$_2$-Man	3.2		5.95		5.9		*mannose*	n.d.	5.9
2,3,4-Me$_3$-Glc			4.7						
2,3-Me$_2$-Glc			1.45						
2-Me-Glc			5.75[b]						
2,3,4,6-Me$_4$-Glc					4.3[c]		*glucuronic acid*	11.9	11.7
2,3,6-Me$_3$-Glc					1.8				
2,6-Me$_2$-Glc					5.4[d]				

[a–d] The alditol acetates from the designated pairs of methylated sugars were not separated by g.l.c. and were estimated together. [a]Estimated as methylated aldonolactones. [b]The proportion of 2-*O*-methylgalactose is assumed to be the same as from the remethylated, reduced gum. [c]The proportion of 2,5-di-*O*-methylarabinose is assumed to be the same as from the carboxyl-reduced, methylated gum. [d]The proportion of 2,6-di-*O*-methylgalactose is assumed to be the same as from the carboxyl-reduced, methylated gum.

of the methylated gum, namely, 2,3,4-tri-*O*-methylarabinose*, was estimated as the alditol acetate in admixture with the corresponding derivative of 2,3,4-tri-*O*-methylxylose. However, an estimate of the relative proportions of these two sugars was obtained after their conversion into the corresponding aldonolactones, which were separated sufficiently on g.l.c. (Table II). This analysis of the sugar constituents of the methylated polysaccharide omitted the acidic constituent-sugars, but probably also underestimated those neutral sugars to which uronic acids were glycosidically attached, because of the resistance of glycosiduronic acid linkages to complete hydrolysis by acid. The methylated gum was, therefore, reduced with lithium aluminum hydride and a sample of the carboxyl-reduced methylated gum was remethylated. These two methylated polysaccharides were hydrolyzed and the derived methylated alditol acetates were quantitatively analyzed by g.l.c. (Table I), and the identities of the components were confirmed by g.l.c.–mass spectrometry. 2,3,4-Tri- and 2,3-di-*O*-methylglucuronic acid were recognized as constituents of the methylated gum as their methyl glycosides formed by methanolysis, and the corresponding derivatives of glucose were analyzed as methylated alditol acetates from the carboxyl-reduced samples of methylated gum. Evidence for the presence of 2-*O*-methylglucuronic acid as a constituent of the methylated gum was obtained less directly (see later). 2-*O*-Methyl- and 2,6-di-*O*-methylglucose, formed respectively from the

TABLE II

G.L.C. OF DERIVATIVES OF METHYLATED SUGARS FROM METHYLATED CHERRY GUM AND DERIVED POLYSACCHARIDES

Sugar	*Methyl glycosides on column* c			*Alditol acetates on column* a	*Aldonolactones on column* b
2,3,4-Me_3-Xyl	0.40	0.49		0.63	3.52
2,3,4-Me_3-Ara	0.83			0.63	3.72
2,3,5-Me_3-Ara	0.49	0.63		0.46	1.74
2,5-Me_2-Ara	1.50	2.69		1.06	14.7
2,3-Me_2-Ara	1.18	1.50		1.30	8.05
2,4,6-Me_3-Gal	3.48	4.02		2.26	
2,4-Me_2-Gal	13.1	15.0		6.60	
2,6-Me_2-Gal				3.66	
2-Me-Gal				8.30	
4,6-Me_2-Man	9.6			3.30	
2,3,4-Me_3-GlcA	2.28	2.99			
2,3-Me_2-GlcA	6.5	7.3	8.9		
2,3,4-Me_3-Glc				2.48	
2,3-Me_2-Glc				5.55	
2-Me-Glc				8.3	
2,3,4,6-Me_4-Glc				1.00	
2,3,6-Me_3-Glc				2.48	
2,6-Me_2-Glc				3.66	

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*No chiral attributions are given in the text, but are assigned as D for all components except arabinose, which is L, by reference to the previous work[1].

reduced, methylated polysaccharide and the reduced polysaccharide after further methylation, were in each case inseparable (by g.l.c. of the alditol acetates) from the corresponding galactose derivatives, but the presence of the glucose derivatives may be inferred from the quantitative data.

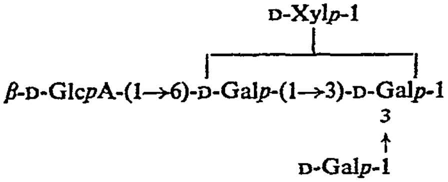
Table I summarizes the results obtained and indicates the proportions of the currently recognized constituents of the methylated gum. In earlier studies, Jones[1] identified 2,3,5-tri- and 2,5-di-*O*-methyl-L-arabinose. 2,4,6-tri- and 2,4-di-*O*-methyl-D-galactose, and 2,3,4-tri- and 2,3-di-*O*-methyl-D-glucuronic acid as constituents of methylated cherry gum, but derivatives of D-mannose and D-xylose, and possibly additional derivatives of D-galactose were present although not characterized. The identities of these and other additional sugar constituents have been now established. Jones characterized 2,3,4-tri- and 2,4-di-*O*-methyl-D-xylose as minor constituents of the methylated, degraded (arabinose-free) gum[3]. We have also obtained evidence that the latter sugar is a constituent of a methylated acidic oligosaccharide, but being unable to detect this or any other non-terminal xylose derivative on direct hydrolysis of the methylated gum we thus conclude that this sugar is of minor quantitative importance. The sum totals of the methyl ethers of the sugar constituents of the gum are in reasonable agreement with the composition of the parent gum (Table I).

Attempts to isolate partially methylated acidic oligosaccharides that were homogeneous in sugar composition and degree of methylation, from the partial depolymerization of the methylated gum, were not fully successful. However the following observations threw some light on the location of residues of the two aldobiouronic acids, 2-*O*-β-D-glucopyranosyluronic acid-D-mannose[4] and 6-*O*-β-D-glucopyranosyluronic acid-D-galactose[5], in the polysaccharide structure. Methanolysis products from the methylated gum were saponified, whereupon separation of the acidic sugars furnished two acidic oligosaccharide fractions whose cleavage products were examined directly and after remethylation. The results showed that the first fraction contained the methyl glycoside of 4,6-di-*O*-methyl-2-*O*-(2,3-di-*O*-methylglucopyranosyluronic acid)mannose and that the second fraction was a higher oligosaccharide, possibly a tetrasaccharide, containing the same partially methylated sugars. In these experiments, derivatives of the partially methylated aldobiouronic acid, 4,6-di-*O*-methyl-2-*O*-(2-*O*-methylglucopyranosyluronic acid)mannose, would have escaped detection, but subsequently this aldobiouronic acid was observed as a product of partial hydrolysis. Furthermore, compositional analysis of the methylated gum showed that residues of 2-*O*-methyl- and 2,3-di-*O*-methylglucuronic acid were present in the approximate ratio of 3:1. In an attempt to obtain reducing, acidic oligosaccharides whose sugar sequences might be established, the methylated gum was partially hydrolyzed in aqueous 1,4-dioxane, but only acidic oligosaccharides containing glucuronic acid linked to galactose residues were isolated. Separation of the acidic sugars furnished 2,3,4-tri-*O*-methylglucuronic acid, a trace of 2,3-di-*O*-methylglucuronic acid, and three partially methylated, oligosaccharide fractions. The cleavage products formed from the oligosaccharides directly, and after further methylation, were analyzed qualitatively. The results showed the fractions to consist of a partially methylated aldobiouronic acid, 2-*O*-methyl-6-*O*-(2,3,4-tri-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose, a higher oligosaccharide containing glucuronic acid end groups attached to a branched galactan chain, and a similarly branched oligosaccharide that also carried xylopyranose end-groups.

The significance of the present results, taken together with those from previous studies by Jones and his collaborators, may be assessed in terms of three regions in the structure of cherry gum, (*A*) the outer residues of L-arabinose (which may be selectively removed on controlled acid hydrolysis), (*B*) branched chains of D-galactopyranose residues mutually joined by (1→3) and (1→6) linkages and having D-glucuronic acid residues attached, and (*C*) residues of the aldobiouronic acid, 2-*O*-β-D-glucopyranosyluronic acid-D-mannose. 3-*O*-β-L-Arabinopyranosyl-L-arabinose was previously characterized as a partial-hydrolysis product from the gum[6]. The methylation data are consistent with this disaccharide's arising from terminal β-L-arabinopyranosyl-L-arabinofuranosyl groups, which, together with single L-arabinofuranosyl groups, comprise the majority of units in the peripheral regions. 5-*O*-Substituted L-arabinofuranose residues have yet to be placed. D-Xylopyranose residues are attached to interior chains of D-galactose instead of terminating peripheral chains of L-arabinose residues, as in cholla gum[6], or being linked to D-mannose in 2-*O*-β-D-glucopyranosyluronic acid-D-mannose residues, as in leiocarpan A[7,8].

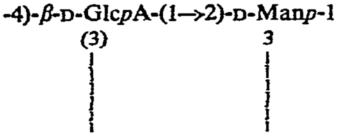
Residues of the two aldobiouronic acids occur in different regions of the polysaccharide, as glucuronic acid residues linked to galactose are present entirely as non-reducing end-groups, whereas residues of 2-*O*-β-D-glucopyranosyluronic acid-D-mannose are present in interior chains having branches carried through mannose and some glucuronic acid residues. These latter residues probably occur in longer sequences, but there is no evidence as yet to indicate whether residues of the two sugars alternate, as in leiocarpan A[7] and gum ghatti[9], or to show unambiguously whether cherry gum may be classified in the glucuronomannan group of polysaccharides[1], in which branched galactan chains and attendant peripheral arabinose residues are exterior to the basal chains.

$$\left.\begin{array}{r}\text{L-Ara}f\text{-1}\\ \beta\text{-L-Ara}p\text{-}(1\rightarrow3)\text{-L-Ara}f\text{-1}\end{array}\right\}\quad(A)$$



$$\begin{array}{c}\text{D-Xyl}p\text{-1}\\ \big|\\ \beta\text{-D-Glc}p\text{A-}(1\rightarrow6)\text{-D-Gal}p\text{-}(1\rightarrow3)\text{-D-Gal}p\text{-1}\\ 3\\ \uparrow\\ \text{D-Gal}p\text{-1}\end{array}$$

(*B*)



$$\begin{array}{l}\text{-4)-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow2)\text{-D-Man}p\text{-1}\\ \quad(3)\qquad\qquad\qquad 3\end{array}$$

(*C*)

EXPERIMENTAL

General methods. — Optical rotations were measured with a Perkin–Elmer model 141 polarimeter at 20 ±2° in chloroform solution. Paper chromatography was performed on Whatman Nos. 1 and 3MM papers with the following solvent systems (v/v): (*A*) 4:1:5 (upper layer) 1-butanol–ethanol–water; (*B*) 18:3:1:4 ethyl acetate–acetic acid–formic acid–water; (*C*) 200:17:1 butanone–water–formic acid; and (*D*) 3:2 solvents *B*–*C*.

G.l.c. was performed with a Hewlett–Packard model 5750 chromatograph by using columns of dichlorodimethylsilane-treated Celite coated with (*a*) 3% of silicone-polyester copolymer ECNSS-M (180°), (*b*) 5% of ethylene glycol adipate polyester (175°), and (*c*) 3% of neopentylglycol adipate polyester (150–170°). Retention times of methyl glycosides are given relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside, and those of partially methylated alditol acetates relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. For g.l.c.–mass spectrometry, column *a* was used in a Perkin–Elmer–Hitachi RMU-6 mass spectrometer; the latter was operated with an inlet temperature of 250°, an ionization potential of 70 eV, and an ion-source temperature of ~250°.

Evaporations were carried out under diminished pressure at bath temperatures of 40° or less.

Methylated cherry gum and derivatives. — Methylated cherry gum[1] was further methylated with methyl iodide and silver oxide in *N,N*-dimethylformamide[10] to give methylated polysaccharide, $[\alpha]_D$ −30° [Found: OMe, 41.9%], which showed no hydroxyl absorption in the i.r. spectrum. Methylated gum (200 mg) was reduced with lithium aluminum hydride (80 mg) in boiling tetrahydrofuran for 20 h. The excess of hydride was decomposed by the dropwise addition of moist ethyl acetate, insoluble salts were removed by filtration and washed thoroughly with chloroform, and the combined filtrate and washings were concentrated. The residue was dissolved in chloroform, whereupon addition of light petroleum afforded carboxyl-reduced, methylated cherry gum (180 mg), $[\alpha]_D$ −28° [Found: OMe, 39.9%]. Reduced, methylated gum (75 mg) was further methylated with methyl iodide and sodium hydride in methyl sulfoxide[11] to give methylated, carboxyl-reduced cherry gum (60 mg), $[\alpha]_D$ −21° [Found: OMe, 42.4%], which showed no hydroxyl absorption in the i.r. spectrum.

Identification of cleavage products from methylated polysaccharides. — (*1*) *Methylated alditol acetates.* Samples (10 mg) of methylated polysaccharides were heated in 90% formic acid (2 ml) for 1 h at 100°, the acid was removed by evaporation, and the residue was further hydrolyzed in 0.5M sulfuric acid for 12 h at 100°. The cooled solutions were neutralized with barium carbonate, and the reducing sugars were reduced with sodium borohydride, and then acetylated[2]. The resulting alditol acetates were examined by g.l.c. on column *a* and by g.l.c.–mass spectrometry. In all instances, the partially methylated alditol acetates derived from methylated cherry gum and carboxyl-reduced methylated cherry gum showed primary fragment-ions characteristic of their substitution patterns[2].

(*2*) *Methyl glycosides.* Samples (10 mg) of methylated cherry gum in methanolic 4% hydrogen chloride (1 ml) were heated in a sealed tube for 16 h at 100°. The cooled solutions were neutralized with silver carbonate, centrifuged, and the supernatant liquids were concentrated. The resulting methyl glycosides were examined by g.l.c. on columns *b* or *c*.

(*3*) *Methylated aldonolactones.* The hydrolyzate from methylated cherry gum (as just described) was treated with saturated bromine water for 7 days in the dark. The solution was aerated to remove the excess of bromine, neutralized with silver carbonate, and the suspension filtered. The filtrate was treated with Amberlite IR-120(H^+) resin and evaporated. The resulting methylated aldonolactones were examined by g.l.c. on column *b*.

Table II summarizes the relative retention-times of the various methylated sugar derivatives.

Partial depolymerization of methylated cherry gum. — (*1*) *Methanolysis.* Methylated cherry gum (0.50 g) was heated in methanolic 5% hydrogen chloride (40 ml) for 5 h in a boiling-water bath. The cooled solution was neutralized (silver carbonate) and evaporated to a syrup that was kept in aqueous sodium hydroxide (50 ml) at pH 12 for 2 h. The solution was extracted with chloroform, neutralized by treatment with Amberlite resin IR-120(H^+), concentrated, and adsorbed on a column of *O*-(2-diethylaminoethyl)-Sephadex A-25 (formate form). The glycosides of neutral sugars were removed by elution with water, whereas acidic sugars were desorbed with 0.5M formic acid (300 ml). The acidic eluate was concentrated (to 50 ml) and then continuously extracted overnight with ether. The ether and water solutions were each evaporated to yield respectively syrup A (150 mg) and syrup B (310 mg). A sample of syrup A was methanolyzed, and it furnished only methyl glycosides of 2,3,4-tri-*O*-methylglucuronic acid (g.l.c. on column *c*). Methanolysis of a sample of syrup B gave a complex mixture of acidic and neutral sugars. Filter-sheet chromatography of syrup B (110 mg) with solvent D gave two main fractions: B(*i*) (38 mg), R_{Glc} 0.60 in solvent *D*), and B(*ii*) (12 mg), R_{Glc} 0.33. Methanolysis of fraction B(*i*) gave methyl glycosides of 2,3,4-tri- (trace), 2,3-di-*O*-methylglucuronic acid and 4,6-di-*O*-methylmannose. Methylation[10] of the fraction followed by methanolysis gave methyl glycosides of 2,3,4-tri-*O*-methylglucuronic acid and 3,4,6-tri-*O*-methylmannose in approximately equimolar proportions. Methanolysis of fraction B(*ii*) gave methyl glycosides of 2,3-di-*O*-methylglucuronic acid and 4,6-di-*O*-methylmannose. Methylation[10] of the fraction followed by methanolysis gave methyl glycosides of 2,3,4-tri- and 2,3-di-*O*-methylglucuronic acid and 3,4,6-tri-*O*-methylmannose in the ratio of 1.0:0.4:1.8.

(*2*) *Hydrolysis.* Methylated cherry gum was heated under reflux (30 g) in a mixture of 0.5M sulfuric acid (1,500 ml) and 1,4-dioxane (900 ml) for 5 h at 96°. The cooled solution was neutralized (barium carbonate), treated with Amberlite resin IR-120(H^+) to remove barium ions, and adsorbed onto a column (3 x 30 cm) of *O*-(2-diethylaminoethyl)-Sephadex A-25 (formate form). Elution with water removed neutral sugars, and stepwise elution with water containing formic acid (up to 0.15M) gave a series of fractions (total, 3.35 g) containing overlapping mixtures of acidic sugars. Approximately half of each fraction was separated by filter-sheet chromatography in solvent *D*, and fractions of the same mobility were combined giving fractions 1 (905 mg), R_{Glc} 0.96 in solvent *D*, 2 (52 mg), R_{Glc} 0.70, 3 (54 mg) R_{Glc} 0.58, 4 (23 mg), R_{Glc} 0.26, and 5 (40 mg), R_{Glc} 0.08. The cleavage products from each fraction were examined qualitatively by g.l.c. of the methyl glycosides formed on methanolysis, and by paper chromatography of the reducing sugars formed on hydrolysis. The fractions were methylated[10] and the cleavage products from the methylated derivatives were similarly examined. The results showed that fractions 1 and 2 contained 2,3,4-tri- and 2,3-di-*O*-methylglucuronic acid respectively, and that fractions 3–5 were partially methylated, acidic oligosaccharides whose cleavage products are shown in Table III.

TABLE III

CLEAVAGE PRODUCTS FROM PARTIALLY METHYLATED OLIGOSACCHARIDE FRACTIONS FROM METHYLATED CHERRY GUM

Fraction	*Cleavage products*	*Cleavage products from fully methylated derivative*
3	2,3,4-Me$_3$-GlcA 2-Me-Gal	2,3,4-Me$_3$-GlcA 2,3,4-Me$_3$-Gal 2,3,5-Me$_3$-Gal
4	2,3,4-Me$_3$-GlcA 2,4,6-Me$_3$-Gal 2,4-Me$_2$-Gal 2-Me-Gal	2,3,4-Me$_3$-GlcA 2,3,4,6-Me$_4$-Gal 2,4,6-Me$_3$-Gal 2,3,4-Me$_3$-Gal 2,3,5-Me$_3$-Gal 2,4-Me$_2$-Gal
5	2,3,4-Me$_3$-GlcA 2,3,4-Me$_3$-Xyl 2,4-Me$_2$-Xyl[a] 2,4,6-Me$_3$-Gal 2,3,4-Me$_3$-Gal[a] 2,4-Me$_2$-Gal 2-Me-Gal	2,3,4-Me$_3$-GlcA 2,3,4-Me$_3$-Xyl 2,3,4,6-Me$_4$-Gal 2,4,6-Me$_3$-Gal 2,3,4-Me$_3$-Gal 2,4-Me$_2$-Gal

[a] These sugars are probably minor constituents of gum and incomplete separation of their derivatives from those of other sugars did not permit their inclusion in the compositional analysis of the methylated gum (Tables I and II).

(*3*) *Hydrolysis*. In an attempt to isolate acidic oligosaccharides containing residues of 4,6-di-*O*-methylmannose, methylated cherry gum (350 mg) was hydrolyzed successively with 90% formic acid for 1 h at 100° and with 0.5M sulfuric acid for 6 h. The hydrolyzate was processed in the usual way and separated by chromatography on *O*-(2-diethylaminoethyl)-Sephadex A-25 (formate form). Methanolysis of the fraction eluted with 0.1M formic acid gave methyl glycosides of 4,6-di-*O*-methylmannose and a sugar suspected of being 2-*O*-methylglucuronic acid (*T* 20 and 26.5 min on column *c*). The fraction was treated with methanolic hydrogen chloride, the methyl ester methyl glycosides were reduced with lithium aluminum hydride in tetrahydrofuran, and the reduction product was hydrolyzed. The reducing sugars were reduced with sodium borohydride, and acetylated, whereupon g.l.c. showed the presence of acetates of 4,6-di-*O*-methylmannitol and 2-*O*-methylglucitol.

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