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SELECTIVE CLEAVAGE OF β-D-GLUCOPYRANOSIDURONIC ACID LINKAGES IN METHYLATED POLYSACCHARIDE ACIDS FROM *Drosera* SPECIES

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ABSTRACT

Permethylated derivatives of the polysaccharide mucins from the leaves of *Drosera binata* and *Drosera capensis* have been subjected to the following sequence of reactions: (*i*) saponification; (*ii*) decarboxylation–acetoxylation with lead tetraacetate; and (*iii*) reductive cleavage of the derived 5-acetoxypentopyranosidic linkages with sodium borohydride to give series of 4-*O*-D-mannopyranosyl-D-xylitol derivatives having side-chain substituents (where present) attached. The oligosaccharide alditols have been separated and characterized to provide evidence for alternating sequences of D-glucuronic acid and D-mannose residues in the main chains, and for the nature and sites of attachment of sugar residues in side chains in both polysaccharides, and, for *D. capensis*, some evidence for anomeric configurations of glycosidic linkages.

INTRODUCTION

Recent structural studies on the polysaccharide mucins of the carnivorous plants *Drosera binata*[1] and *Drosera capensis*[2] have shown them to belong to the D-glucurono-D-mannan family[3] of which rather few members have been fully authenticated. The results pointed to two polysaccharides (**1**) of only slightly different composition, each containing main chains of alternating 4-linked β-D-glucuronic acid and 2-linked α-D-mannopyranose residues, with α-D-galactopyranose residues attached to O-3 of some mannose residues and L-arabinofuranose, and infrequently α-D-xylopyranose, residues attached to approximately half of the glucuronic acid residues[1]. Anomeric configurations of glycosidic linkages were assigned on the basis of susceptibility or lack thereof towards oxidation with chromium trioxide[4]. In polysaccharides of this type it is often not possible to isolate, as products of partial depolymerization, oligosaccharides in which outer-chain units

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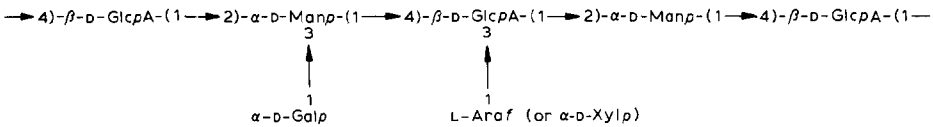
remain attached to sugar residues in the relatively acid-resistant inner chains. In these two polysaccharides[1,2], base-catalyzed degradation of the permethylated derivatives using 1,5-diazabicyclo[5.4.0]undec-5-ene and acetic anhydride[5] afforded a 3-*O*-D-galactopyranosyl-D-mannose derivative. However, only indirect evidence was obtained for the site of attachment of L-arabinofuranose residues. Recent studies have shown that treatment of permethylated glucuronans with lead tetraacetate effects structural modification by decarboxylation–acetoxylation. Treatment of the altered polysaccharides with sodium borohydride then results in reductive cleavage of the derived 5-acetoxypentopyranosidic linkages[6]. The reaction sequence furnishes partially methylated oligosaccharide alditols in which pentitol termini arise from the former uronic acid residues, with exposure of aglyconic hydroxyl groups to which the uronic acid residues had been attached. This nonhydrolytic degradation has been applied successfully to the permethylated derivative of the glucuronomannan leiocarpan A, which is structurally similar to the *Drosera* polysaccharides, to furnish two oligosaccharide alditols in which a mannopyranosylxylitol unit carries one or two pentose residues attached as side-chains to each disaccharide segment[7]. We describe now the application of the same reaction-sequence to the permethylated derivatives of *D. binata* and *D. capensis* mucins.



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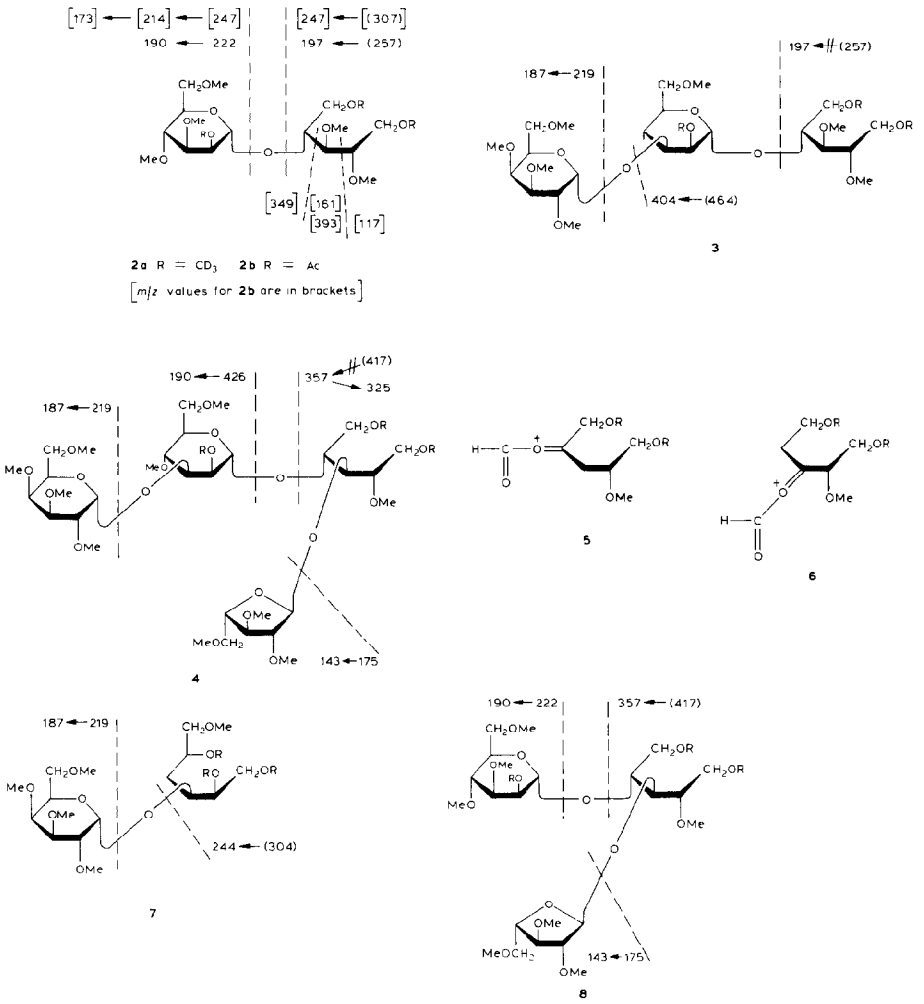
RESULTS AND DISCUSSION

Methylated *D. binata* glucuronomannan was treated twice with dilute aqueous alkali with saponification of ~ 80% of the uronic ester residues. The methylated polysaccharide acid was treated with lead tetraacetate until no ionizable carboxyl groups could be detected. That a high degree of structural modification had been achieved was shown by treatment of a portion of the methylated polysaccharide product with sodium borohydride followed by methylated sugar analysis of the hydrolyzate after conversion into partially methylated alditol acetates. The results showed the formation of derivatives of 2,3,5-tri-*O*-methylarabinose, 2,3,4,6-tetra-*O*-methylgalactose, 3,4,6-tri-, and 4,6-di-*O*-methylmannose, which had been shown previously to be constituents of the parent methylated polysaccharide[1], together with 2,3-di- and 2-*O*-methylxylitol arising from the corresponding glucuronic acid residues originally present.

The modified, methylated polysaccharide was treated with sodium borohydride to give a mixture of partially methylated oligosaccharide alditols, which was

examined in two ways. The major portion of the oligosaccharide mixture was benzoylated and the derivatives were separated by preparative l.c. to give three major fractions from which oligosaccharides B1′, B2′, and B3′ were obtained by *O*-deacylation. These three fractions were each trideuteriomethylated to give peralkylated oligosaccharide alditols B1, B2, and B3 for examination by mass spectrometry (direct insertion) and for methylated sugar analysis by g.l.c.–m.s. after conversion into partially methylated alditol acetates. The assigned structures **2a, 3**, and **4** for the oligosaccharides B1, B2, and B3, which are shown together with the major fragment-ions of structural significance, are in accord with the methylated



Scheme 1. In all formulas other than **2b**, R = CD_3. Salient features of mass-spectral fragmentations are shown with the convention that (257)→197 or (257)↔197 indicates that an ion of the J_1 series is or is not observed.

sugar analysis. The main features of the mass spectra of these methylated oligosaccharides are the ions arising from cleavage on either side of the glycosidic oxygen atoms, together with J_1 fragments arising from neighboring sugar residues carrying methoxyl groups[8] at C-3. In the case of the branched oligosaccharide alditol **4**, the appearance of an ion of the V series[9] at *m/z* 211 (**5** or **6**) supports the presence of vicinal glycosyl substituents in the xylitol residue. The remaining portion of the mixture of partially methylated oligosaccharide alditols was trideuteriomethylated, and the more-volatile components (di- and tri-saccharide alditols only) were analyzed directly by g.l.c.–m.s. The mass spectra of the two major components (B4 and B7) were identical to those of fractions B1 and B2 for which structures **2a** and **3** had been previously assigned. Structures **7** and **8** may be proposed for the two other components (B5 and B6) of the mixture on the basis of the fragment ions shown and of the known sugar constituents of the methylated polysaccharide. In the case of the branched trisaccharide alditol **8**, the same V ion was observed as for the tetrasaccharide **4**.

Methylated *D. capensis* glucuronomannan was subjected to the same series of reactions as the *D. binata* polysaccharide. In this case, the mixture of partially methylated oligosaccharide alditols formed on reductive cleavage of the modified polysaccharide was acetylated; preparative t.l.c. of these derivatives furnished three major components C1′, C2′, and C3′. Oligosaccharide C1′ as the acetylated derivative was examined by mass spectrometry (direct insertion) and structure **2b** was in accord with the methylated sugar analysis. Oligosaccharides C2′ and C3′ were each *O*-deacetylated and then trideuteriomethylated to give the peralkylated oligosaccharide alditols C2 and C3 for examination by mass spectrometry and ^{1}H-n.m.r. spectroscopy, and for methylated sugar analysis. These two compounds were indistinguishable from oligosaccharides **8** and **3** from the *D. binata* polysaccharide. In addition, the ^{1}H-n.m.r. spectrum of oligosaccharide alditol C3 (**3**) showed anomeric protons at δ 4.75 (doublet, $J_{1,2}$ 3.5 Hz) and 5.10 (doublet, $J_{1,2}$ 1.4 Hz) which may be assigned to α-D-galactopyranose and α-D-mannopyranose residues, respectively. The ^{1}H-n.m.r. spectrum of oligosaccharide alditol C2 (**8**) showed two signals for anomeric protons at δ 5.17 (not resolved) and 5.07 (doublet, $J_{1,2}$ 1.6 Hz). Although the signals could not be individually assigned, they may be attributed to α-D-mannopyranose and α-L-arabinofuranose residues. Neither signal shows a coupling constant similar to that of H-1 of a β-L-arabinofuranose residue ($J_{1,2}$ 4.3 Hz) in a methylated oligosaccharide alditol similarly derived[7] from methylated leiocarpan A.

The results of this investigation, which confirm and extend previous structural conclusions, establish the close similarity between the two *Drosera* polysaccharides other than in the actual proportions of sugar residues in side-chains. The oligosaccharide alditols formed from one or both glucuronomannans, for which structures **2, 3, 4**, and **8** are proposed, are derivatives of 4-*O*-α-D-mannopyranosyl-D-xylitol in which O-2 of mannose residues carry trideuteriomethyl substituents. The isolation of these oligosaccharides from the degradative sequence involving

selective cleavage of glucosiduronic acid linkages confirms the presence of alternating glucuronic acid and mannose residues in the inner chains of both polysaccharides. The characterization of oligosaccharides **3**, **4**, and **8** provides direct evidence for the sites of attachment of side-chains in confirmation of galactopyranose and in support of previous indirect evidence for the location of arabinofuranose residues. The ^{1}H-n.m.r. data obtained for oligosaccharide alditols **3** and **8** from *D. capensis* mucin confirms earlier assignments of anomeric configurations for α-D-galactopyranose and α-D-mannopyranose residues and gives the first configurational evidence for α-L-arabinofuranose residues in the polysaccharides. α-L-Arabinofuranose residues have been reported as substituents of the arabinoglucuronomannan from suspension-cultured tobacco cells[10], and this conclusion has been confirmed in this laboratory (unpublished results) using the lead tetraacetate degradation of the permethylated polysaccharide. In contrast, the arabinofuranose residues in leiocarpan A have the β-L-configuration.

Oligosaccharide **7** is the only degradation product in these studies not containing a terminal xylitol residue, but it is obviously structurally related to oligosaccharides **3** and **4**. No oligosaccharide of this type was encountered in the corresponding studies[7] on leiocarpan A, where no 3,4-di-*O*-glycosylglucuronic acid residues were present. In view of the susceptibility of 3-*O*-glycosyl sugars to base-catalyzed degradation, it is possible that reaction intermediates leading to the branched oligosaccharide **4** may have undergone degradation with loss of both 3-*O*- and 4-*O*-glycosyl substituents (compare ref. 11). No information was obtained on the specific location of α-D-xylopyranose residues.

This investigation gives further examples of the selective cleavage of glucosiduronic acid linkages in permethylated glucuronans by the decarboxylation–acetoxylation reaction followed by reduction. Full success in the overall sequence is dependent on completeness of reaction at each stage including (*i*) methylation of the polysaccharide with minimum accompanying base-catalyzed β-elimination from 4-*O*-substituted glucuronic acid residues (compare ref. 12) (*ii*) saponification of methyl glucuronate residues, often under more extended conditions than are required for uronic esters of low molecular weight, but without base-catalyzed degradation, (*iii*) the lead tetracetate reaction as judged by disappearance of ionizable carboxyl groups, and (*iv*) reductive treatment with sodium borohydride. These reaction sequences generate partially methylated oligosaccharide alditols of generally similar structure. Preparative separations of mixtures of such compounds or their *O*-acyl derivatives, for which there is little precedent in the literature, is not easy to achieve without loss of material. As the present investigations started with not more than 50 mg of polysaccharides, the quantities of oligosaccharide derivatives obtained after several steps were inadequate to explore extensively a range of possible separation methods in order to obtain both sufficient quantities of individual components for complete characterization and an accurate quantitative assay.

The results show that 4-*O*-α-D-mannopyranosyl-D-glucuronic acid segments

of the *D. binata* polysaccharide have substituents attached to neither residue, to one or the other, or to both. Our failure to detect oligosaccharide alditol **4** as a degradation product from the *D. capensis* polysaccharide does not rule out the presence of segments in which both residues carry side-chains since a different separation system was employed. Even for an estimate of the statistical distribution of side-chains along the glucuronomannan core, it would be necessary to perform a complementary non-hydrolytic depolymerization specific for mannopyranosidic linkages.

EXPERIMENTAL

General methods. — Acidic polysaccharides from the mucins of *Drosera binata* and *D. capensis* were isolated as described previously[13]. Evaporations were performed under diminished pressure at 40° or less. The i.r. spectra were measured with a Unicam SP 200 spectrophotometer. The ^{1}H-n.m.r. spectra were measured with a Bruker WH 400 spectrophotometer. G.l.c. was performed with a Perkin–Elmer Sigma 3B chromatograph using (*A*) a packed column of 3% of silicone–polyester copolymer ECNSS-M on Gas-Chrom Q or (*B*) a S.C.O.T. column coated with silicone gum OV-225. Mass spectra were determined for samples introduced by direct insertion or from g.l.c. capillary columns of (*C*) silicone gum OV-225 or (*D*) silicone DB5-15N (permanently bonded OV-54) attached by a jet separator to a VG Micromass 16F mass spectrometer, which was operated with an inlet temperature of 250°, an ionization potential of 70 eV, and an ion-source temperature of ~260°. High-performance liquid chromatography (l.c.) was performed on a Waters Associates μ-Porasil column (3.9 × 300 mm, 10μm silica), the solvent gradient was delivered by two model 6000A pumps controlled by a Waters Associates model 660 programmer, and a model 440 u.v.-absorbance detector set at 254 nm was used. Elution at a flow rate of 2 mL/min was with chloroform for 8 min, followed by a linear gradient over 20 min to a final solvent composition of 7:3 chloroform–acetonitrile, and isocratic elution at that solvent composition for a further 10 min. Preparative t.l.c. was performed on precoated silica plates (20 × 20 cm) [Baker t.l.c. plate silica gel 60-F (250 μm)].

Methylations by the Hakomori procedure were performed as described by Jansson *et al.*[14]. Acidic polysaccharides, if present in salt form, were treated in aqueous solution with Amberlite IR-120(H^+) resin and the filtered solutions were freeze-dried to give polysaccharide acids, which were further dried over phosphorus pentaoxide for 24 h. In order to minimize base-catalyzed β-elimination during methylation, the base (sodium methylsulfinylmethanide) was added in 30% excess of the quantity required to ensure complete ionization of carboxyl and hydroxyl groups, and the presence of a sufficient excess of base was checked by using triphenylmethane[15]. Methylated sugar analyses were conducted as described by Jansson *et al.*[14].

Degradation of methylated D. binata *polysaccharide acid by lead tetraacetate.*

— Permethylated *D. binata* polysaccharide (from 50 mg of parent polysaccharide) was treated with aqueous sodium hydroxide at pH 12 for 4 h at 0° and 15 h at room temperature to give methylated polysaccharide acid (35 mg) whose i.r. spectrum showed slight residual absorption at 1750 cm^{-1} in the presence of triethylamine. The polysaccharide was unchanged on further treatment with alkali. Methylated polysaccharide acid (35 mg) in benzene (4 mL) containing pyridine (0.2 mL) and lead tetraacetate (200 mg) was boiled under reflux for 24 h. The modified methylated polysaccharide was isolated as described previously, γ_{max} 1750 cm^{-1} but no absorption at 1600 cm^{-1} with triethylamine, indicating (within the limits of detection) that all ionizable glucuronic acid residues had been modified. A portion of the product was treated with sodium borohydride, hydrolyzed, and converted into partially methylated alditol acetates for g.l.c. analysis on column *A* (isothermal, 180°). The remainder of the mixture was filtered, the precipitate was washed with benzene, the combined filtrate and washings were concentrated, and the residue was purified by chromatography on Sephadex LH-20 in dichloromethane to give modified, methylated polysaccharide (30 mg). The modified, methylated polysaccharide in oxolane–water (1:1, 2 mL) was treated with sodium borohydride (50 mg) for 15 h to give a mixture of partially methylated oligosaccharide-alditols. A portion of the mixture was trideuteriomethylated. Examination of the resulting peralkylated oligosaccharide-alditols by g.l.c.–mass spectrometry on column *D* (180°, 4°/min to 230° and hold) showed oligosaccharide fractions B4–B7, having retention times* of 6.0, 7.8, 16.6, and 26.3 min. The remaining portion of the partially methylated oligosaccharide-alditols was treated with benzoyl chloride (0.05 mL) in pyridine (0.5 mL) at 0° and then kept for 24 h at room temperature. The resulting benzoates were separated by l.c. to give three chromatographically homogeneous fractions, each of which was *O*-deacylated to give fractions B1′–B3′ and then trideuteriomethylated to furnish peralkylated oligosaccharide-alditols B1, B2, and B3, which were examined by mass spectrometry (direct insertion) and by methylated sugar analysis (g.l.c.–m.s. on column *C* at 180°). The following partially methylated alditols were identified as their acetates: B1, 1,5-di-*O*-($^2H_3$)methyl-2,3-di-*O*-methylxylitol and 2-*O*-($^2H_3$)methyl-3,4,6-tri-*O*-methylmannitol; B2, 1,5-di-*O*-($^2H_3$)methyl-2,3-di-*O*-methylxylitol, 2,3,4,6-tetra-*O*-methylgalactitol, and 2-*O*-($^2H_3$)methyl-4,6-di-*O*-methylmannitol; and B3, 1,5-di-*O*-($^2H_3$)methyl-2-*O*-methylxylitol, 2,3,5-tri-*O*-methylarabinitol, 2,3,4,6-tetra-*O*-methylgalactitol, and 2-*O*-($^2H_3$)methyl-4,6-di-*O*-methylmannitol, with a small amount of 4,6-*O*-methylmannitol that probably resulted from incomplete re-alkylation.

Degradation of methylated D. capensis *polysaccharide acid by lead tetraacetate.* — Permethylated *D. capensis* polysaccharide (42 mg) was successively saponified (no residual ester) and treated with lead tetraacetate, as already described to afford modified methylated polysaccharide (33 mg), which was treated

*Under these conditions, permethylated disaccharide-alditols have retention times in the range of 5–8 min.

with sodium borohydride (50 mg) for 15 h to give a mixture (25 mg) of partially methylated oligosaccharide alditols. The oligosaccharide derivatives were acetylated, and preparative t.l.c. (6:1 chloroform–acetone), gave fractions C1′–C3′ (R_F 0.55, 0.4, and 0.3, respectively). Fraction C1′ was examined directly by m.s. and methylated sugar analysis showed derivatives of 3,4,6-tri-*O*-methylmannose and 2,3-di-*O*-methylxylitol. Fractions C2′ and C3′ were each *O*-deacetylated and trideuteriomethylated to give peralkylated oligosaccharide-alditols C2 and C3, which were examined by ^{1}H-n.m.r. spectroscopy, mass spectrometry (direct insertion), and methylated sugar analysis (g.l.c.–m.s. on column C at 180°). The following partially methylated alditols were identified as their acetates: C2, 1,5-di-*O*-($^2H_3$)methyl-2-*O*-methylxylitol, 2,3,5-tri-*O*-methylarabinitol, and 2-*O*-($^2H_3$)methyl-3,4,6-3,4,6-tri-*O*-methylmannitol; and C3, 1,5-di-*O*-($^2H_3$)methyl-2,3-di-*O*-methylxylitol, 2,3,4,6-tetra-*O*-methylgalactitol, and 2-*O*-($^2H_3$)methyl-4,6-di-*O*-methylmannitol.

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REFERENCES

1 D. C. GOWDA, G. REUTER, AND R. SCHAUER, *Phytochemistry*, 21 (1982) 2297–2300.
2 D. C. GOWDA, G. REUTER, AND R. SCHAUER, *Carbohydr. Res.*, 113 (1983) 113–124.
3 A. M. STEPHEN, in G. O. ASPINALL (Ed.), *The Polysaccharides*, Vol. II, Academic Press, New York, 1983, pp. 97–193.
4 J. HOFFMAN, B. LINDBERG, AND S. SVENSSON, *Acta Chem. Scand.*, 26 (1972) 661–666.
5 G. O. ASPINALL AND A. S. CHAUDHARI, *Can. J. Chem.*, 53 (1975) 2189–2193.
6 G. O. ASPINALL, H K. FANOUS, N. S. KUMAR, AND V. PUVANESARAJAH, *Can. J. Chem.*, 59 (1981) 935–940.
7 G. O. ASPINALL AND V. PUVANESARAJAH, *Can. J. Chem.*, 61 (1983) 1964–1968.
8 J. LONNGREN AND S SVENSSON, *Adv. Carbohydr. Chem. Biochem.*, 29 (1973) 41–106.
9 B. FOURNET, J.-M. DHALLUIN, G. STRECKER, J. MONTREUIL, C. BOSSO, AND J. DEFAYE, *Anal. Biochem.*, 108 (1980) 35–36.
10 M. MORI AND K. KATO, *Carbohydr. Res.*, 91 (1981) 49–81.
11 G. O. ASPINALL AND K. M. ROSS, *J. Chem. Soc.*, (1961) 3674–3677.
12 K. SHIMIZU, *Carbohydr. Res.*, 92 (1981) 219–224.
13 K. ROST AND R. SCHAUER, *Phytochemistry*, 16 (1977) 1365–1368.
14 P.-E. JANSSON, L. KENNE, H. LIEDGREN, B. LINDBERG, AND J. LONNGREN, *Chem. Commun. Univ. Stockholm*, 8 (1976) 1–75.
15 H. RAUVALA, *Carbohydr. Res.*, 72 (1979) 257–260.