*Carbohydrate Research*, 65 (1978) 320–325


## Note

# Identification of neutral oligosaccharides from a partial, acid hydrolysate of the water-soluble polysaccharide of the opium poppy*

BERIT SMESTAD PAULSEN, BJØRG JOHANSEN, AND JENS K. WOLD
*Institute of Pharmacy, University of Oslo, Box 1068 Blindern, Oslo 3 (Norway)*
(Received July 6th, 1977; accepted for publication in revised form, October 24th, 1977)

The capsule of the opium poppy contains a pectin-type polysaccharide[1,2]. When this polysaccharide was subjected to partial hydrolysis with 0.5M sulphuric acid at 100° for 3 h, several acidic oligosaccharides were obtained[3]. Only minute amounts of neutral oligosaccharides were formed under these conditions, and the yields were too low for any detailed structural investigation. We now report on the isolation, purification, and structural elucidation of the neutral oligosaccharides obtained when subjecting the polymer to milder conditions of hydrolysis.

After separation of neutral and acidic components on an ion-exchange resin, the neutral sugars were fractionated further by using an ethanol gradient on a carbon–Celite column. Of the four fractions obtained, only two contained sufficient amounts of oligosaccharides for separation and characterisation. Oligosaccharides **1**–**3** were present in the fraction eluted with 10% of ethanol in water, and oligosaccharides **4** and **5** were eluted with 15% of ethanol in water. The compounds were purified by paper chromatography. Oligosaccharide **1** was a mixture, whereas **2**–**5** are homogeneous oligosaccharides (Table I): **1**, *O*-L-arabinopyranosyl-(1→3)-L-arabinose (**a**) + *O*-L-arabinofuranosyl-(1→3)-L-arabinose (**b**); **2**, *O*-β-D-xylopyranosyl-(1→4)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose; **3**, *O*-α-L-rhamnopyranosyl-(1→4)-D-xylopyranose; **4**, *O*-β-D-xylopyranosyl-(1→4)-*O*-β-D-xylopyranosyl-(1→4)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose; and **5**, *O*-α-D-rhamnopyranosyl-(1→4)-*O*-D-xylopyranosyl-(1→4)-D-xylopyranose.

Analysis of oligosaccharide **1** by paper chromatography (solvents *A*, *B*, and *C*) and by electrophoresis in sodium bisulphite revealed only one compound, the migration indicating[4] a d.p. of 2. Electrophoresis in borate buffer also gave one spot only. However, analysis of the fully methylated product showed, after hydrolysis, reduction, and peracetylation, the presence of four compounds, namely, the alditol acetates corresponding to 2,3,5- and 2,3,4-tri-*O*-methylarabinose and to 2,4- and 2,5-di-*O*-

*The Water-Soluble Polysaccharide of Opium Poppy (*Papaver somniferum L.*): Part III. For Part II, see Ref. 3.

methylarabinose (Table II). This indicates that oligosaccharide **1** is a mixture of **1a** and **1b**. The g.l.c. peak areas of the partly methylated arabinitol acetates suggest that **1a** was the major component.

When the methyl glycosides of permethylated **1** were analysed by g.l.c. on column 3, five peaks were observed, indicating at least two different compounds. The mass spectra of the peaks $T_{TMG} = 5.28$ and 6.12 were identical (Table III), the

TABLE I

DATA OBTAINED FOR THE ISOLATED OLIGOSACCHARIDES

| *Oligosaccharide* | *Yield (mg)* | *Sugar composition*[a] | *Ratio*[a] | $R_{GAL}$ | | | $M_{GLC}$[b] |
|---|---|---|---|---|---|---|---|
| | | | | *Solvent* A | *Solvent* B | *Solvent* C | |
| 1 | 33 | L-Arabinose | | 0.55 | 0.77 | | 0.86 |
| 2 | 25 | D-Xylose | | 0.78 | 0.72 | | 0.69 |
| 3 | 74 | L-Rhamnose | 1 | 1.28 | 1.18 | | 0.81 |
| | | D-Xylose | 1 | | | | |
| 4 | 22 | D-Xylose | | | 0.28 | 0.32 | |
| 5 | 14 | L-Rhamnose | 1 | | 0.80 | 0.78 | 0.66 |
| | | D-Xylose | 2 | | | | |

[a]Determined by p.c. (free sugar) and g.l.c. (methyl glycosides). [b]Zone electrophoresis in 0.4M sodium bisulphite.

TABLE II

PRODUCTS OBTAINED AFTER HYDROLYSIS OF PERMETHYLATED OLIGOSACCHARIDES

| *Oligosaccharide* | T[a] | *Rel. peak area* | *Primary mass fragments* | *Alditol acetate corresponding to* |
|---|---|---|---|---|
| 1 | 0.48 | 1 | 45, 117, 161, 175 | 2,3,5-Tri-*O*-methylarabinose |
| | 0.58 | 3 | 117, 161 | 2,3,4-Tri-*O*-methylarabinose |
| | 0.90 | 2 | 45, 117, 233 | 2,5-Di-*O*-methylarabinose |
| | 1.09 | 1.8 | 117, 233 | 2,4-Di-*O*-methylarabinose |
| 2 | 0.59 | 1 | 117, 161 | 2,3,4-Tri-*O*-methylxylose |
| | 1.18 | 2 | 117, 189 | 2,3/3,4-Di-*O*-methylxylose |
| 3 | 0.49 | 1 | 117, 131, 161, 175 | 2,3,4-Tri-*O*-methylrhamnose |
| | 1.18 | 1 | 117, 189 | 2,3/3,4-Di-*O*-methylxylose |
| 4 | 0.59 | 1 | 117, 161 | 2,3,4-Tri-*O*-methylxylose |
| | 1.18 | 2.7 | 117, 189 | 2,3/3,4-Di-*O*-methylxylose |
| 5 | 0.49 | 1 | 117, 131, 161, 175 | 2,3,4-Tri-*O*-methylrhamnose |
| | 1.18 | 2 | 117, 189 | 2,3/3,4-Di-*O*-methylxylose |

[a]*T* is the retention value relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol.

mass fragments having *m/e* 143, 175, and 235 being obtained by degradation as shown in Fig. 1, routes 1 and 3. The authors assume the same pattern of degradation of ring a, whether linked through C-4 or C-3 of ring b.

TABLE III

G.L.C.–M.S. DATA FOR METHYL ETHER METHYL GLYCOSIDES OF OLIGOSACCHARIDES 1–3

| *Oligosaccharide* | T[a] | *Mass fragments (relative intensity)* |
|---|---|---|
| 1 | 2.76 | |
| | 3.25 Major | |
| | 4.02 | |
| | 5.18 Major | 71(14), 75(30), 88(76), 99(24), 101(100), 115(72), 143(24), |
| | 6.12 | 175(15), 235(32) |
| 2 | 2.87 Major | 71(34), 75(32), 88(100), 99(18), 101(76), 115(22), 143(37), |
| | 3.53 | 175(32), 235(20), 261(4) |
| 3 | 2.45 Major | 71(23), 75(26), 88(100), 101(28), 115(10), 157(6), |
| | 3.00 | 175(10), 189(16), 235(14), 249(5), 275(3) |

[a] *T* is the retention value relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol.

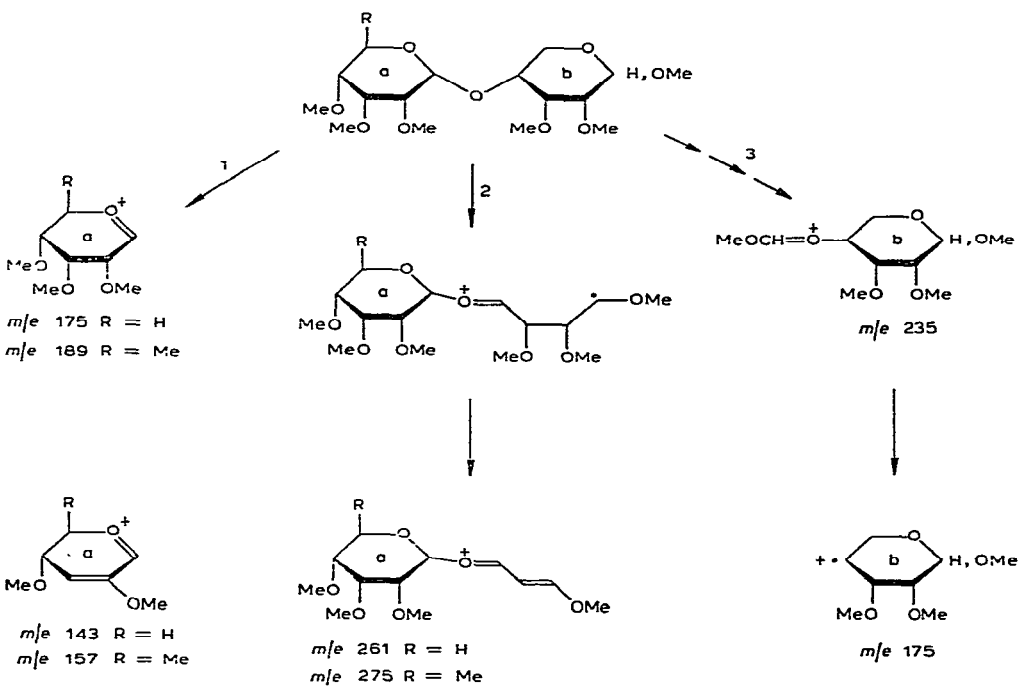


Fig. 1. Fragmentation of methyl ethers of (1→4)-linked disaccharide methyl glycosides[6,10].

In his studies on mass spectrometry of oligosaccharides, Kärkkäinen[5] observed that (1→3)-linked pentoses give relatively intense fragment ions having *m/e* 101 and 115. Table III shows that these are the predominant ions in the mass spectrum of oligosaccharide **1a**, and that their intensities are stronger than for oligosaccharides **2** and **3**. The mass spectra of the other three peaks were different, but no structural conclusions could be drawn.

The results obtained for oligosaccharide **1** confirm the structure of **1a**, and the methylation analysis indicates that **1b** is present. It has previously been shown that arabinofuranose is present in the polysaccharide[2]. The indications of more than two oligosaccharides, because of the presence of both 2,4- and 2,5-di-*O*-methylarabinose after methylation, are most probably due to the equilibrium between the pyranose and furanose forms of the reducing moiety.

The oligosaccharides **2** and **4** both gave D-xylose as the only sugar on hydrolysis. The $R_{GAL}$ values obtained by paper chromatography indicated that **2** is a tri- and **4** a tetra-saccharide. When subjected to electrophoresis in sodium bisulphite buffer, the migration value of **2** clearly indicated a trisaccharide.

The methylation studies showed that both oligosaccharides gave 2,3,4-tri-*O*-methylxylose and 2,3- or 3,4-di-*O*-methylxylose (Table II). As both compounds reacted with triphenyltetrazolium hydroxide, a (1→4) linkage is suggested. The ratios of tri-*O*-methylxylose to di-*O*-methylxylose in **2** and **4** were 1:2 and 1:3, respectively. Incubation of **2** or **4** with β-D-glucosidase or with cellulase led to the release of xylose, thus indicating β-D-glycosidic linkages.

The methyl glycoside of permethylated **2**, when analysed by g.l.c.–m.s. (column *3*), gave two peaks having identical mass spectra (Table III). The degradation routes to fragments *m/e* 143, 175, 235, and 261 are given in Fig. 1, routes 1, 2, and 3. The ion *m/e* 261 shows that the oligosaccharide is (1→4)-linked[6]. This is consistent with oligosaccharides **2** and **4** being tri- and tetra-saccharides consisting of (1→4)-linked β-D-xylopyranosyl residues.

On acid hydrolysis, oligosaccharides **3** and **5** gave L-rhamnose and D-xylose in the ratios 1:1 and 1:2, respectively. Migration values in paper chromatography and in paper electrophoresis indicated **3** to be a disaccharide, and **5** to be a trisaccharide. Only **3** was present in sufficient quantity for measurement of optical rotation, the value indicating an α-D-glycosidic linkage between the sugar units.

Methylation studies of oligosaccharides **3** and **5** showed the presence of 2,3,4-tri-*O*-methylrhamnose and 2,3- or 3,4-di-*O*-methylxylose in the ratios 1:1 and 1:2, respectively (Table II). However, a positive reaction with triphenyltetrazolium hydroxide suggested a (1→4) linkage.

The methyl glycoside of permethylated **3** gave two peaks when analysed by g.l.c.–m.s. (column *3*). The mass spectra obtained for the two peaks were identical (Table III). The fragments m/e 157, 175, 189, 235, and 275, formed through degradation routes 1, 2, and 3 (Fig. 1), confirm the structure proposed for oligosaccharide **3**. Neutral oligosaccharides of the type described above have apparently not been isolated previously from acidic polysaccharides.

EXPERIMENTAL

Extraction of opium poppy capsules was carried out as previously described[2]. The polysaccharide (25 g) was hydrolysed with 12.5mM sulphuric acid (2.5 l) at 100° for 1 h. After neutralization of the hydrolysate with the calculated amount of barium hydroxide, and treatment of the filtrate with Dowex 50 ($H^+$) resin, the resulting solution was concentrated *in vacuo*. The hydrolysate was then separated into neutral and acidic fractions by using De-Acidite FF-IP (SRA-67) ion-exchange resin in the formate form. The neutral fraction was eluted with water, and the acidic fraction with 2M formic acid. After concentration and freeze-drying, the fractions weighed 2.9 and 9.0 g, respectively. Studies of the acidic fraction will be reported elsewhere.

The neutral fraction was applied to a carbon–Celite column (2.5 × 12 cm), and fractionated by successive elution with water, and water containing 5%, 10%, and 15% of ethanol. The yields of the respective four fractions were 1080, 230, 570, and 120 mg. The fraction eluted with water contained only monosaccharides, and that eluted with 5% of ethanol in water contained monosaccharides and small proportions of oligosaccharides. The other two fractions contained oligosaccharides in sufficient amounts for purification and structural elucidation. The fractionation and purification was effected by preparative p.c. in systems *A* or *C*. Five oligosaccharides were isolated in yields sufficient for structural studies. Oligosaccharides **1–3** were eluted with 1:9 ethanol–water, and oligosaccharides **4–5** with 15% of ethanol in water.

Paper chromatography (p.c.) for analytical purposes was performed on Whatman No. 1 paper, and for preparative purposes on Whatman No. 3MM paper in the following systems: *A*, ethyl acetate–pyridine–water (8:2:1); *B*, ethyl acetate–acetic acid–formic acid–water (18:3:1:4); and *C*, 1-butanol–ethanol–water (40:11:19).

Sugars were detected on paper chromatograms with (*a*) aniline oxalate (saturated, aqueous solution) and (*b*) freshly prepared 1% 2,3,5-triphenyltetrazolium chloride in M sodium hydroxide. Zone electrophoresis was performed on Munktell No. 302 paper at ~20 V/cm in 0.4M sodium bisulphite[4] for 2 h. Oligosaccharide samples were left in contact with the buffer for 30 min prior to application. Sugars were detected on the electropherograms with spray reagent (*a*) containing 10% of acetic acid. $M_{GLC}$ is the migration distance of the compound relative to that of glucose.

Each oligosaccharide was hydrolysed in a sealed tube with 90% formic acid at 100° for 6 h. After dilution with 3 vol. of water, the mixture was heated for another 2 h. The formic acid was removed by repeated additions of methanol and subsequent evaporations to dryness, and the component sugars were analysed by p.c. Analysis of the sugars present in the oligosaccharides was also performed by g.l.c. with a Varian Model 1400 gas chromatograph. The dried sample (2–3 mg) was methanolysed with M methanolic hydrogen chloride (1 ml) at 85° for 20 h. The resulting methyl glycosides were converted into the per(trimethylsilyl) ethers, which were then separated on a glass column (400 × 0.3 cm) packed with 3% of GE SE-52 on Varaport 30 (column *I*); a temperature programme was used, starting at 140° with an increase of 2°/min for

10 min, followed by 4°/min to 240°. The yields, sugar composition, and $R_{GAL}$ and $M_{GLC}$ values are given in Table I.

Methylation of the oligosaccharides and conversion into partly methylated alditol acetates were performed as described previously[8]. The products were analysed by g.l.c. at 195° on a glass column (400 × 0.3 cm) filled with 3% of OV-225 on Varaport 30 (column *2*). G.l.c.–m.s. was performed on column *2* fitted in a Varian CH 7 low-resolution mass spectrometer. The instrumental details have been published elsewhere[9]. The results are given in Table II.

Methyl ether methyl glycosides of oligosaccharides **1**–**3** were analysed by g.l.c.–m.s. at 200° on a column (200 × 0.3 cm) filled with 2% of OV-225 on Varaport 30 (column *3*). Retention values ($T$) are given with reference to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol. The results are given in Table III.

Incubation with β-D-glucosidase (emulsin from almonds, Sigma) was carried out by digesting oligosaccharides **2** and **4** (0.5 mg), dissolved in 0.05M acetate buffer (pH 5.0; 0.8 ml), with 1 mg of enzyme at 37° for 5 h in the presence of 1 drop of toluene. The mixture was heated in boiling water for a few minutes and cooled. After deionization with Zerolit DM-F (carbonate form) mixed-bed resin, filtration, and concentration, the resulting product was analysed by p.c. (system *B*). Cellobiose and maltose were used as controls. Incubation with cellulase was performed as described above, but 2 mg of enzyme were used.

ACKNOWLEDGMENT

The authors thank the Institute of Clinical Biochemistry, Rikshospitalet, Oslo, for permission to use their g.l.c.–m.s. facilities.

REFERENCES

1 E. Ottestad, E. Brockmann-Hanssen, D. Øiseth, and A. Nordal, *J. Pharm. Pharmacol.*, 11 (1959) 689–693.
2 J. K. Wold, B. Smestad, R. Winsnes, and D. Resser, *Acta Chem. Scand.*, 24 (1970) 1262–1270.
3 J. K. Wold and B. Smestad, *Acta Chem. Scand.*, 24 (1970) 2472–2478.
4 J. L. Frahn and J. A. Mills, *Chem. Ind. (London)*, (1956) 1137–1138.
5 J. Kärkkäinen, *Carbohydr. Res.*, 17 (1971) 1–10.
6 J. Moor and E. S. Waight, *Biomed. Mass Spectrom.*, 2 (1975) 36–45.
7 V. N. Reinhold, *Methods Enzymol.*, 25 (1972) 244–248.
8 J. K. Wold, B. Smestad, and T. Midtvedt, *Acta Chem. Scand., Ser. B*, 29 (1975) 703–709.
9 E. Jellum, T. Stokke, and L. Eldjarn, *Clin. Chem.*, 18 (1972) 800–804.
10 N. K. Kochetkov and O. S. Chizhov, *Adv. Carbohydr. Chem.*, 21 (1966) 36–94.