ACTA CHEMICA SCANDINAVICA 11 (1957) 1365—1370

Isolation of Disaccharides from Golden Apple Gum

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The structure of Golden Apple gum has been studied by the method of partial hydrolysis. After a mild acid treatment 3-*O*-α-[4(?)-*O*-methyl-D-glucuronopyranosyl]-L-arabinose was obtained. This acid thus is an integral part of the easily split side-chains of the gum molecule. The partially hydrolysed material yielded after further hydrolysis the following three disaccharides: 3-*O*-β-D-galactopyranosyl-D-galactose, 6-*O*-β-D-galactopyranosyl-D-galactose, and 6-*O*-β-[4(?)-*O*-methyl-D-glucuronopyranosyl]-D-galactose. Consequently, these acids are parts of the more resistant, pyranoside framework of the gum.

A previous investigation [1] of the gum exuded from Golden Apple tree (*Spondias cytheria* Sonner; family *Anacardiaceae*) showed that it was composed of residues of arabinose, galactose, a uronic acid (probably 4-*O*-methyl-D-glucuronic acid), xylose, and traces of rhamnose and fucose. The isolation of 3-*O*-β L-arabopyranosyl-L-arabinose from a hydrolysate of the gum showed that some of the arabinose units were present in the pyranose form. At least some of the xylose units must be linked to arabinose since 3-*O*-α-D-xylopyranosyl-L-arabinose was also obtained from the hydrolysate.

This paper reports a further study of the gum. The sample examined contained 51 % of pentose anhydride units and 15 % of uronic anhydride units. Its equivalent weight was 1 070. $[\alpha]_D$ — 5° (*c* 1.1; water).

Many gums are considered to be built up of a framework of galactopyranose units to which are attached side chains, composed largely of arabofuranose residues. The gum was therefore first hydrolysed under conditions such that practically all of the furanoside bonds, but only a small part of the pyranoside ones should be split. This was done in the usual way by heating a solution of the gum in its acidic form (autohydrolysis) until the reducing power did not further increase (80 h at 98°C).

The hydrolysate obtained was divided by conventional methods into i) the acidic mono- and oligosaccharides, ii) the neutral mono- and oligosaccharides, and iii) the degraded gum which constituted the pyranoside framework of the gum.

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i) The weight of the acidic sugars was 13 % of that of the gum. The main component was an aldobiuronic acid, which is called acid A in the following. If formed about a third of the fraction. The rest was composed mainly of higher oligosaccharides, as revealed by paper chromatography of the fraction.

The hydrolysate of acid A showed on a paper chromatogram spots of arabinose and of a monomethyl-uronic acid, which probably was 4-*O*-methyl-D-glucuronic acid. The high positive rotation of A, $[\alpha]_D$ + 123°, suggested that its components were linked by an *alpha*-glycosidic bond.

The structure of A was determined by degradation in the following steps: complete methylation, reduction of the ester group to a carbinol group, methylation of the carbinol group, and hydrolysis of the glucosidic bond. A paper chromatogram of the hydrolysate obtained showed three spots. Two of the components were isolated in pure form by paper sheet chromatography. One of them was identified as 2,4-di-*O*-methyl-L-arabinose by conversion into the corresponding *N*-phenyl-glycosyl amine. The other was shown to be 2, 3, 4, 6-tetra-*O*-methyl-D-glucose by comparing its infra-red spectrum with that of an authentic sample.

The third component did not move when subjected to paper electrophoresis using a borate buffer. This implied that the hydroxyl group on C_2 was methylated since the α-glycol group on C_1 and C_2 should otherwise form a borate complex. (For instance, the rate of movement of 3, 4-di-*O*-methyl-L-arabinose was found to be 40 % of that of glucose.)

On paper chromatograms, the component gave a brown spot with *p*-anisidine hydrochloride, and had an R_F greater than that of 2, 4-dimethyl-arabinose. Two of the dimethyl-arabinoses, 2, 3- and 2, 5-dimethyl-arabinose, move faster than the 2, 4-derivative [1]. Only the 2, 5-derivative, however, gives a brown spot with anisidine; that of the other is coloured red [1]. The third component was therefore probably 2,5-di-*O*-methyl-L-arabinose.

From this followed that acid A should be 3-*O*-α-(4-*O*-methyl-D-glucurono-pyranosyl)-L-arabinose, which has not earlier been reported as having been isolated from a polysaccharide. The fact that *two* arabinose methyl ethers were obtained obviously indicated that the completely methylated disaccharide contained both arabopyranose and arabofuranose residues.

The identification of 4-*O*-methyl-glucuronic acid as the acidic component of A was, however, made only by paper chromatography. As the rates of the movement of the other monomethyl-glucuronic acids are not known, the position of the methoxyl group in acid A is not definitely established. 4-*O*-Methyl-D-glucuronic acid is, however, a common component in polysaccharides while the other monomethyl-glucuronic acids are comperatively rare in nature.

ii) The weight of the neutral sugars was 59 % of that of the gum. The fraction was composed mainly of arabinose (about 58 %). The other constituents were xylose (7 %), oligosaccharides (32 %) and small amounts of galactose and rhamnose (1—2 % each). This fraction had already been examined in the earlier investigation [1], which led to the isolation of 3-*O*-β-L-arabopyranosyl-L-arabinose and 3-*O*-α-D-xylopyranosyl-L-arabinose, and was therefore not examined further.

iii) The degraded gum constituted 28 % of the total gum. It contained 30.5 % of pentose anhydride units and 20 % of uronic anhydride units. $[\alpha]_D$—41° (*c* 1.0; water).

Its hydrolysate contained mainly galactose, arabinose, and an aldobiuronic acid, which is called acid B in the following. Acid A was not present.

From the hydrolysate of another sample of degraded gum, acid A was isolated (see experiment B in the experimental part). This sample of degraded gum had not been prepared by such a prolonged hydrolysis as the above mentioned sample and was obtained in a higher yield. It was therefore probable that it still contained considerable amounts of arabofuranose residues.

The difficulty of removing all of the arabinose units from the gum by autohydrolysis has already been pointed out[1]. It follows from the above that practically all of the remaining arabinose units in the degraded gum most probably are in the pyranose form.

The aldobiuronic acid isolated from the degraded gum, acid B, was built up of galactose and monomethyl-uronic acid, probably 4-*O*-methyl-D-glucuronic acid. It yielded, on methylation, the crystalline methyl ester of hepta-*O*-methyl-6-*O*-*β*-D-glucuronopyranosyl-D-galactose. Consequently, B should be 6-*O*-*β*-(4-*O*-methyl-D-glucuronopyranosyl)-D-galactose, which has previously been isolated from gum myrrh [2]. As in the case of acid A, the position of the methyl group in acid B is not definitely established. The rate of movement of B on a paper chromatogram and its $[\alpha]_D$ value agreed, however, with the corresponding figures for the acid from gum myrrh.

From the hydrolysate of the degraded gum small amounts of two disaccharides were isolated. They crystallised readily from methanol. One of them, disaccharide C, had m.p. 156—159 °C. The crystals of the other, disaccharide D, rapidly liquefied when kept in air. They probably contained methanol of crystallisation, which easily evaporated causing the destruction of the crystal lattice. Both disaccharides on hydrolysis gave only galactose, which was identified by paper chromatography.

Disaccharide C was found to be identical with 3-*O*-*β*-D-galactopyranosyl-D-galactose, which has been isolated from *Acacia pycnantha* gum[3].

Disaccharide D yielded no formaldehyde on periodate oxidation in slightly alkaline solution, which is characteristic for hexose containing disaccharides with 1,5 or 1,6-linkages [4]. The low positive optical rotation, $[\alpha]_D$ + 30°, indicated a *beta*-glucosidic link between the sugar units. D should accordingly be 6-*O*-*β*-D-galactopyranosyl-D-galactose, and this was confirmed by a comparison of the infra-red spectrum of D with that of a synthetic sample. This disaccharide has recently been obtained from the water-soluble arabogalactan which is present in the wood of *Larix occidentalis* [5].

EXPERIMENTAL

Analyses etc.

Paper chromatography. Solvents:

a. Ethyl acetate:acetic acid-formic acid:water (18:3:1:4 v/v).
b. Ethyl acetate:acetic acid:water (7:2:1: v/v) .
c. *n*-Butanol:ethanol:water:conc. ammonia (200:50:244:5 v/v, the top layer).

d. *n*-Butanol:pyridine:water (10:3:3 v/v).
Paper: Whatman No. 1.
Spray: *p*-Anisidine hydrochloride dissolved in butanol.
R_{gal}: the rate of movement relative to galactose.
R_G: the rate relative to 2,3,4,6-tetra-*O*-methyl-D-glucose.

Paper electrophoresis. The experiment was performed according to Foster [6]. The pH of the borate buffer was 10. Voltage 900 V. Time 2 h. The correction for electroendosmosis was determined by running parallel tests with 2,3,4,6-tetra-*O*-methyl-D-glucose.

M_G: the rate of movement relative to glucose.

Infra-red spectra. If not otherwise stated, they were determined on potassium bromide pellets with a Perkin-Elmer double beam spectrophotometer. The maxima in the most characteristic part of the spectra, the region 700 – 1 000 cm^{-1}, are recorded in this paper.

Pentose anhydride. This analysis was carried out according to a Tappi standard method [7], by which the amount of furfural formed is determined by oxidation with bromate-bromide solution. As the predominant pentose sugar in the gum was arabinose, the yield of furfural was assumed to be 74 %, which is the yield obtained from arabinose [7]. The correction for the amount of furfural formed from the uronic acid units was applied by assuming that the yield was one half of that from arabinose [8]. No correction was made for the small amount of hydroxymethylfurfural formed from the galactose units.

Uronic anhydride. The determinations were carried out according to Browning [9].

Reducing power. This was determined iodometrically according to Ingles and Israel [10]. The oxidation was carried out at pH 11.5.

Hydrolysis

Autohydrolysis. An aqueous solution of the gum (5.00 g) was passed through a column of a cation exchanger (Amberlite IR-120) saturated with hydrogen ions. The eluate (150 ml) was heated in a water bath at 98°C, The consumption of 0.1 N iodine solution per ml of the solution was after 0 h, 0.04 ml; after 3 h, 0.74 ml; after 9 h, 1.25 ml; after 21 h, 1.57 ml; after 45 h, 1.77 ml; after 68 h, 1.79 ml, and after 75 h, 1.75 ml.

After 80 hours' heating a part of the solution (78 ml) was passed through a column (diameter 4.5, height 7.5 cm) of a "weak" anion exchanger (Amberlite IR-4B) saturated with acetate ions. The column was then washed with water until the water gave a negative Molisch test.

The column was eluted with N formic acid. The residue after evaporation of the eluate (334 mg, corresponding to 13 % of the weight of the gum) showed on a paper chromatogram (solvent a) a faint, red spot at R_{gal} = 2.70 (probably 4-methyl-glucuronic acid), a strong, red spot at R_{gal} = 1.3 (acid A), and a series of spots having lower R_{gal} values (different oligosaccharides).

The residue (38 mg) was separated on a paper sheet using solvent a. The strip containing acid A was extracted with water. Evaporation of the extract yielded 12 mg of acid A.

The solution which passed directly through the anion exchanger column was neutralised with barium carbonate and concentrated to 20 ml. Ethanol (100 ml) was added and the precipitate of degraded gum (728 mg, corresponding to 28 % of the weight of the gum) was removed by centrifugation.

The residue on evaporation of the alcohol-water solution weighed 1 525 mg (corresponding to 59 % of the weight of the gum). A part of the residue (124 mg) was separated on paper sheets using solvent a. The strips containing the different components were extracted with water and the extracts were evaporated to dryness. The weight of the residue from the arabinose strips was 71 mg; from the xylose strips, 8 mg; from the rhamnose and galactose strips, 2 mg each; from the strips with 3-xylopyranosyl-arabinose, 9 mg; from the strips with 3-arabopyranosyl-arabinose, 15 mg; and from the strips containing the remaining oligosaccharides, 15 mg. Total 122 mg.

Hydrolysis of degraded gum. Experiment A. The degraded gum (681 mg obtained from the experiment described above) dissolved in N sulphuric acid (10 ml) was heated on a water bath for 5 h. The solution was neutralised with barium carbonate, passed through a column of a cation exhanger (Amberlite IR-120) saturated with hydrogen ions, and

evaporated to dryness. The weight of the residue was 480 mg. A paper chromatogram (solvent a) showed at R_{gal} = 2.70 a red, faint spot (probably 4-methyl-glucuronic acid), at R_{gal} = 1.63 a strong, red spot (arabinose), at R_{gal} = 1.00 a strong, brown spot (galactose), and at R_{gal} = 0.58 a strong, brown spot (acid B). No spot was observed at R_{gal} = 1.3 (which would be due to acid A). With solvent d spots were obtained at R_{gal} = 1.53 (arabinose), at R_{gal} = 1.00 (galactose), and at low R_{gal} values (acidic components).

Experiment B. The degraded gum for this experiment was obtained by heating an aqueous solution (2 000 ml) of the acidic gum (38.4 g) at 83°C for 24 h. The product (15.3 g, 40 % of the weight of the gum) was precipitated from the neutral part of the hydrolysate by addition of alcohol as described above.

The degraded gum (15.0 g) dissolved in 2 N sulphuric acid (200 ml) was heated at 53°C for 8 days. The solution was neutralised with barium hydroxide, and passed through a column of a cation excanger (Amberlite IR-120) saturated with hydrogen ions. The components of the solution were fractionated into *a*) neutral and *β*) acidic compounds by passing the solution through a column (diameter 4.5, height 11 cm) of a "weak" anion exchanger (Amberlite IR-4B) in its acetate form.

a) Evaporation of the solution which passed through the column yielded a syrup of the neutral components (4.9 g). The syrup was passed through a carbon-Celite column 1:1 w/w, diameter 3, height 27 cm). The column was eluted with water containing stepwise increasing amounts of ethanol in the usual way. The fractions obtained were examined by paper chromatography.

0 % ethanol. Weight 3.65 g. Contained: galactose, arabinose, and traces of rhamnose.

2 % ethanol. Weight 0.02 g. Contained: galactose, arabinose, 3-xylopyranosyl-arabinose (?), and traces of rhamnose.

5 % ethanol. Weight 0.18 g. Contained: 3-xylopyranosyl-arabinose (?), 3-arabopyranosyl-arabinose (?), disaccharides C and D.

The last fraction was chromatographed on paper sheets using solvent a. C (39 mg) and D (16 mg) were obtained.

b) The acidic fraction absorbed on the column was eluted with N formic acid. The eluate was continuously extracted with ether for 24 h (to remove formic acid), then evaporated to dryness, residue 6.1 g.

The residue (3.0 g) was fractionated on a cellulose column using solvent b. The following fractions were collected and examined by paper chromatography.

Fraction a) contained 4-methyl-glucuronic acid (?); weight, 67 mg. b) contained acid A; weight, 165 mg. c) contained acid B; weight 402 mg.

Structures of the disaccharides

Acid A. R_{gal} = 1.3 (solvent a). $[\alpha]_D$ +123° (*c* 1.0; water). Paper chromatograms of the hydrolysate of A showed three spots: i) unchanged A, ii) arabinose, and iii) a sugar, which moved at the same rate as 4-methyl-glucuronic acid in solvents a and d.

A (300 mg) was methylated with dimethyl sulphate and sodium hydroxide, and then with Purdie reagent. The infra-red absorption curve of the product (in carbon tetrachloride solution) showed no maximum in the vicinity of 3 400 cm^{-1} indicating the absence of hydroxyl groups.

The completely methylated A was then dissolved in ether (about 5 ml, distilled from lithium aluminium hydride). Lithium aluminium hydride (0.5 g) was added to the solution and the mixture was left at room temperature for 24 h. After addition of ice and water, the solution was continuously extracted with chloroform.

The extract was dried (Drierite) and evaporated to dryness. After methylation with Purdie reagent the product was hydrolysed (N hydrochloric acid, steam bath, 20 h). The hydrolysate was neutralised with silver carbonate, filtered, and evaporated to dryness. Residue 140 mg.

Paper chromatograms of the residue showed three spots with the R_G values (solvent c): 0.63 (red), 0.86 (brown), and 1.00 (red). Paper electrophoresis showed only one spot: M_g = 0.0.

The components of the hydrolysate were separated on paper sheets. By a mishap most of the components were lost during the separation. Only about 5 mg each of the fastest moving and the slowest moving components were obtained. The fast moving component

crystallised, m.p. 60—70°C. Its infra-red spectrum and that of 2,3,4-6-tetra-*O*-methyl-D-glucose were identical. They showed maxima at 700, 768 (broad), 853 (strong), 887 (weak), 905 (weak), 917 (weak), 964, and 990 (strong) cm^{-1}.

The slow moving component (5 mg), aniline (5 mg), and methanol (0.5 ml) were heated together on a water bath for 2 h. Evaporation of the solvent yielded crystals, m.p. 128—130°C. The infra-red spectrum of the crystals and that of *N*-phenyl-2,4-di-*O*-methyl-L-arabinosyl amine were identical. In the region 600—1 100 cm^{-1} they showed maxima at: 660, 695 (strong), 746 (strong), 760 (strong), 820, 825 (masked), 860, 880, 900, 928 (strong), 973, 990, 1 037 (strong), 1 055, 1 080 (strong, broad) cm^{-1}.

Acid B. R_{gal} = 0.58 (solvent a). $[\alpha]_D$ −1° (*c* 0.4; water). Paper chromatograms of the hydrolysate of B gave three spots; i) unchanged B, ii) galactose, and iii) a sugar which moved with the same rate as 4-methyl-glucuronic acid in solvents a and d.

Methylation of B (380 mg) with dimethyl sulphate and sodium hydroxide, and after that with Purdie reagent yielded the crystalline methyl ester of hepta-*O*-methyl-6-β-D-glucuronopyranosyl-D-galactose (195 mg). After recrystallisation from light petroleum, the compound had m.p. 92—93°C, undepressed on admixture with an authentic sample. (Found: C 51.2; H 7.8; OCH_3 52.8. Calc. for $C_{20}H_{36}O_{12}$: C 51.2; H 7.7; OCH_3 52.8.)

Disaccharide C. R_{gal} = 0.27 (solvent a), and 0.25 (solvent d). Paper chromatograms of the hydrolysate showed only one spot, that of galactose. C crystallised readily from methanol, m.p. 156—159°C, undepressed by addition of 3-β-D-galactopyranosyl-D-galactose. The behaviour of C with lead-tetracetate [11] and its X-ray powder diagram were identical with those of this disaccharide. (The oxidation and the determination of the X-ray diagram were kindly carried out by Dr. A. S. Perlin.)

Disaccharide D. R_{gal} = 0.16 (solvent a), and 0.11 (solvent d). $[\alpha]_D$ +30° (*c* 1.2; water). Paper chromatograms of the hydrolysate showed only one spot, that of galactose. D crystallised in needles from methanol. If kept in a dessiccator or in air, the crystals decomposed.

The periodate oxidation in slightly alkaline solution was carried out as described by Hough and Perry [4]. The pH of the periodate solution was 8. Oxidation time, 24 h. Formaldehyde was determined colorimetrically using the chromotropic acid method. In the test D gave the same reading as a blank. (This experiment was kindly performed by Mr. M. B. Perry.)

6-β-D-galactopyranosyl-D-galactose was synthesised according to Freudenberg and co-workers [12]. Its infra-red spectrum had maxima at 703, 775, 790 807, 876, 894, 916, 950, 973, 998 cm^{-1} in the region 700—1 000 cm^{-1}. Outside this region the spectrum had distinct maxima at 1 150, 1 213, and 1 248—1 252 (broad) cm^{-1}.

Since there was insufficient amount of D available the distinct maxima only showed up in the comparison diagram for D. They were at 703, 775, about 890, 916, 950, 1150, 1 213, and about 1 250 cm^{-1}.

Acknowledgement. I would like to express my thanks to Dr. J. K. N. Jones for suggesting this subject and for his kind interest. I am also indebted to the *Swedish America Foundation* for a fellowship.

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Received June 17, 1957.