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Studies on the Polysaccharides of Lichens. II.[1] The Structure of Water-soluble Polysaccharides in *Umbilicaria pustulata* (L.) Hoffm. and *Umbilicaria spodochroa* (Ach.) Hoffm

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Water-soluble polysaccharides have been isolated from the lichens *Umbilicaria pustulata* (UP) and *Umbilicaria spodochroa* (US). Both species were found to contain partly *O*-acetylated pustulan, a linear β-1,6-glucan. Quantitative determination of the acetyl groups indicates one *O*-acetyl per 10–11 glucose units for both UP and US, the content being reduced to one per 14–15 glucose units after extraction of the crude lichens with methanol. Both the pustulans are acetylated in the 4-*O*-position. The molecular weight distribution determined by gel filtration gave for UP one fraction corresponding to approximately $3 \cdot 10^4$ ($\bar{M}_n$) and one fraction of very high molecular weight. US showed a very broad distribution curve.

All water-soluble polysaccharides so far isolated from the family *Umbilicariacea* (*Gyrophoraceae*) have been described as a β-1,6-glucan called pustulan. The first attempt to elucidate the structure of pustulan was carried out by Drake[2] who isolated a water-soluble polysaccharide from *Umbilicaria hirsuta* (Sw.) Ach. and from *U. pustulata* (L.) Hoffm. Based on specific rotation, enzymic and acid hydrolysis, a β-1,6-linked polysaccharide was suggested. Later methylation studies by Lindberg and his coworkers[3,4] have verified that pustulan isolated from *U. pustulata* was a β-1,6-glucan.

The water-soluble polysaccharides from *U. angulata* Tuck., *U. caroliniana* Tuck. and *U. polyphylla* (L.) Baumg.[5] were all found to be partly *O*-acetylated pustulan presumably containing one *O*-acetyl group per 10–12 glucose units, and the molecular weight found by the ultracentrifugation method corresponded to 120 anhydroglucose units. Water-soluble polysaccharides from *Gyrophora esculenta* Miyoshi, and *Lasallia papulosa* (Ach.) Llano[6,7,8] and from *Lasallia pensylvanica* (Hoffm.) Llano,[9] all belonging to the *Umbilicariaceae,* have been shown to be partly *O*-acetylated pustulan. The *O*-acetyl group was located at the 3-*O*-position[8] for every 10–12 glucose units. All these pustulans were shown to have a high degree of inhibiting effect on inplanted Sarcoma 180 in Mice. The antitumor activity seemed to decrease somewhat on deacetylation and showed a drastic decrease if all the OH-groups were blocked by acetyl, methyl or carbamate groups.[8]

In our work on fractionation and analysis of polysaccharides from different lichen species we now report on the investigation of a hot water-soluble polysaccharide from *Umbilicaria spodochroa* (Ach.) Hoffm. (hereafter called US-2) and a more detailed investigation of a pustulan isolated from *U. pustulata* (L.) Hoffm. (UP-2).

RESULTS AND DISCUSSION

In the earlier investigations on pustulan from *U. pustulata*[3] no acetyl content has been reported, possibly due to the pretreatment of the lichen (extraction with methanol for 14 days and with 2 % sodium carbonate for another 14 days) prior to the hot water extraction of the polysaccharide. Methanol, which has commonly been used as one of the extraction solvents for remov-

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ing lipids and coloring matter, might cause loss in the possible acetyl contents due to transesterification. In our investigation we have therefore divided the material after extraction with benzene and acetone. One part was extracted with hot water giving polysaccharides US-2 and UP-2 while another part was extracted with methanol (5 days) prior to the hot water extraction (USM-2 and UPM-2). By quantitative GLC-analysis [10] of *O*-acetyl in US-2 and UP-2, 3.8 and 3.6 % acetyl were found. After the lichens had been treated with methanol the *O*-acetyl contents were found to be 2.7 and 2.4 % in USM-2 and UPM-2, respectively. In other aspects, like carbohydrate content, monosaccharides present and specific rotation the polysaccharides USM-2 and UPM-2 were nearly identical with those of US-2 and UP-2. No distinction between the fractions will therefore be given.

By acid hydrolysis the fraction UP-2 was found to contain glucose with a trace of mannose, while US-2 in addition to glucose contained small amounts of mannose and galactose. The two latter were almost completely removed by a repeated freezing and thawing procedure. The total carbohydrate content in US-2 and UP-2 was 98.3 and 98.0 %, respectively, while the amount of protein was found to be 3.1 % in US-2 and 2.9 % in UP-2. Specific rotation, NMR and IR indicated that both the polysaccharides were β-D-glucosidically linked.

Gel filtration on Sepharose 4B gave a broad distribution curve for US-2 indicating a polymolecular polysaccharide, while in the case of UP-2 a main fraction corresponding to a molecular weight of approximately $3 \cdot 10^4$ ($\bar{M}_n$) and a small fraction of very high molecular weight were obtained. The molecular weight found might, however, differ somewhat from that which might be found by other methods as the dextrans used as standards for the calibration curve have some degree of branching and consist of another type of glucosidic bond.

Methylation analysis as described by Björndal *et al.* [11] and comparison with authentic samples, gave for both US-2 and UP-2 2,3,4-tri-*O*-methyl-D-glucose as the main methylated derivative together with small amounts of 2,3,4,6-tetra-*O*-methyl-D-glucose as analysed by GLC–MS. Small amounts of dimethylated D-glucose which was most probably a mixture of 2,4- and 3,4-di-*O*-methyl-D-glucose, were also detected. The di-*O*-methylated sugars might have been caused by some degree of demethylation during the acid hydrolysis or by a slight branching of the polysaccharide.

In order to get further information, the UP-2 was oxidised by periodate. When oxidised at 5 °C a consumption of 1.6 mol of periodate per mole of glucosyl residue was obtained after 6 d and 0.82 mol of formic acid had been formed. These values are too low for a completely oxidised linear 1,6-glucan. It has, however, been found [12] that under normal oxidation conditions the *O*-acetyl groups are not hydrolysed and therefore cause a lower periodate consumption. As adjustment for the *O*-acetyl groups does not account for the whole difference, a slight degree of branching, as also indicated by the methylation studies, might be the explanation. On the other hand, oxidation of a pustulan from *U. mammulata* [12] which also was found to contain one *O*-acetyl group per 10 glucose units, gave the same value for periodate oxidation. After deacetcylation of the latter polysaccharide a consumption of 1,97 units of periodate per unit of glycosyl residue was reported. Alternatively to branch points, the low oxidation value may therefore be due to a kind of steric hindrance caused by the *O*-acetyl group. Smith degradation [13] of UP-2 gave large amounts of glycerol but no glucose could be detected. Accordingly, branch points or acetyl groups are therefore not in the 3-*O*-position.

The ^{13}C NMR data obtained from US-2 and UP-2 were in good agreement with the data obtained by others [14,15] for pustulan. Due to the relatively low solubility and the small number of pulses used, no peak for the *O*-acetyl groups was found.

The position of the *O*-acetyl groups was determined using the method described by Belder and Norman.[16] By this method the *O*-acetyl groups are exchanged with *O*-methyl-groups and the partly methylated polysaccharides were analysed as alditol acetates using GLC separation and MS detection. In order to differentiate between 3-mono-*O*-methyl and 4-mono-*O*-methyl-D-glucose the hydrolysate was reduced with sodium borodeuterid. Based on retention times and fragmentation pattern hexaacetylglucitol (89 %, 90 %) and 1,2,3,5,6- penta-*O*-acetyl-4-mono-*O*-methyl-D-glucitol (5 %, 7 % in US-2 and UP-2, respectively) were identified. The 4-*O*-methyl substitution could be differentiated

from the 3-*O*-methyl by GLC and by the fragments *m/z* 262, 218, 189 and 129.

From the data obtained it can be concluded that the water-soluble polysaccharides isolated from *Umbilicaria spodochroa* and *U. pustulata* are of the pustulan type containing one *O*-acetyl group in the 4-position on every 10 glucose units. It is most likely that the two pustulans have a few branch points presumably β-(1→2) linked.

In the earlier investigations to locate the *O*-acetyl group in pustulans[8] the *O*-acetyl group was substituted with a *O*-methyl group, and the identification of the *O*-methylglucose was based on giving the same retention time as 3-*O*-methyl-D-glucose on TLC and GLC. No other authentic mono-*O*-methylglucoses were reported to have been used as reference. As the chromatographic behaviour of 3-*O*- and 4-*O*-methyl-D-glucose is very similar it might be a possibility that the *O*-acetyl groups in these pustulans also are located in the 4-position.

EXPERIMENTAL

General methods. Solutions were concentrated at reduced pressure below 40 °C. Paper chromatography was carried out on Whatman No. 1 filter paper with one of the following systems: A. Ethyl acetate–pyridine–water 10:4:3, B. Butanol–pyridine–water 6:4:3, C. Ethyl acetate–acetic acid–formic acid–water 18:3:1:4. As spray reagent either silver nitrate[17] or periodate/benzedine was used.[18] GLC was performed with a Hewlett Packard 5700 A instrument fitted with glass columns: G 1. (180×0.2 cm) containing 3 % SE-52 on 100/120 chromosorb WAW/DMCS, G 2. (200×0.2 cm) with 3 % OV 225 on 80/100 chromosorb WAW/DMSC, G 3. (400×0.2 cm) 10 % SP-1200 on 60/80 chromosorb WAW/HMS coated with 1 % phosphoric acid or G 4. glass capillary column (30 m×0.24 mm, I.D.) coated with SP-2340. Peak areas were determined with an Autolab Minigrator.

^{13}C NMR spectra were recorded with a Jeol JNM–FX 60 Fourier transform spectrometer (5–10 % in water at 20 °C with DMSO as internal standard). A recycle time of 3 s was used, GLC–MS was performed with a Micromass 7070 F equipped with Data System 2200 or a Finnigan MAT 4000. IR-spectra were recorded on a JASCO IRA-1 and specific rotation on a Perkin-Elmer 141 polarimeter.

Quantitative carbohydrate analysis was carried out by the Somogyi method[19] or by methanolysis[20] followed by trimethylsilylation and gas chromatography using column G 1 at 230 °C. *Isolation of the polysaccharides.* The dry pure lichen, US or UP (100 g; collected at Kragerø, Norway) after being ground in a mill and extracted in a soxhlet with (a) benzene (24 h) and (b) acetone (60 h) in order to remove waxy materials, was divided into two fractions. One fraction was extracted further with methanol (100 h). Samples (100 g) were stirred with water (2×1000 ml) at room temperature for 3 h. These fractions were found to contain mainly mono- and oligosaccharides. The residue after filtration was boiled with water (1000 ml) for 3 h filtered through muslin and centrifuged (3000 r.p.m., 30 min). The supernatant solutions were precipitated by adding ethanol (60 % v/v). Two further precipitation procedures were carried out before isolation of the crude polysaccharides by freeze drying. The fractions obtained were 6.4 g US-2, 11.5 g USM-2, 3.9 g UP-2 and 4.0 g UPM-2, respectively. The fractions were analysed after complete acid hydrolysis (1 M sulfuric acid for 2.5 h). By paper chromatography of the hydrolysate, using solvents A and B, only glucose with traces of galactose and mannose were detected. Quantitative determination by the methanolysis method gave for US-2 1.5 % galactose and 7.9 % mannose and for USM-2 2.0 % galactose and 8.0 % mannose.

Purification of the polysaccharides. By a repeated (3 times) freezing and thawing procedure all the galactose in US-2 was removed and only traces of mannose could be detected. The four fractions were found by the Somogyi method to contain 98.2 % (US-2), 95.4 % (USM-2), 98.0 % (UP-2), and 95.7 % (UPM-2) carbohydrate and 3.1 % (US-2), 2.9 % (USM-2), 2.9 % (UP-2) and 2.7 % (UPM-2) protein as analysed by the Lowry's method.[21] Infrared spectra (in nujol) gave for both US-2 and UP-2 absorption at 910 cm^{-1} (β-glucosidic linkage) and 1250 and 1735 cm^{-1} (ester). US-2: $[\alpha]_D^{20} = -32°$ (*C* 0.16, water), UP-2: $[\alpha]_D^{20} = -32°$ (*c* 0.16, water). ^{13}C NMR of US-2 (2230 pulses): and UP-2 (2000 pulses); were both in agreement with the reported data for pustulan.[14,15]

Gel filtration. Molecular weight determination of US-2 and UP-2 was carried out by gel filtration on a Sepharose 4B (45,3×2 cm) column with void volume V_o=64 ml using dextran T 500 ($\bar{M}_n$=1.93 · 10^5, V/V_o=2.09); T 250 ($\bar{M}_n$=1.13 · 10^5, V/V_o=2.16; T 70 ($\bar{M}_n$=3.72 · 10^4), V/V_o=2.33) and T 10 ($\bar{M}_n$=6.2 · 10^3 V/V_o=2.56) as calibration substances. As eluent TRIS-buffer (0.5 M) containing 0.02 % sodium azide was used. Elution of US-2 gave four peaks which were not baseline separated V/V_o=1.11, 1.59 (both $\bar{M}_n$>10^6), 2.17

($\bar{M}_n=1.06\cdot10^5$) and 2.42 ($\bar{M}_n=1.75\cdot10^4$) UP-2 gave a small peak $V/V_o=1.04$ separated from the main peak $V/V_o=2.34$ ($\bar{M}_n=3.02\cdot10^4$).

Methylation analysis. Methylation was carried out according to the Hakomori method[22] with the modifications described by Björndal and Lindberg.[11] The glucan (30 mg) dried in a vacuum over phosphorus pentoxide at 60° was dissolved in dimethyl sulfoxide (10 ml) by stirring in a nitrogen atmosphere, and methylated by treatment with methylsulfinyl carbanion (0.5 ml, 2 M) for 8 h, followed by methyl iodide (0.3 ml). After stirring for 8 h the methylation procedure was repeated by a new addition of the same amount of carbanion and methyl iodide. The methylated polysaccharide was dialysed, concentrated to a small volume, and extracted with chloroform (3×5 ml). The chloroform was evaporated and part of the methylated polysaccharide dried in vacuum (P_2O_5) at 60 °C prior to IR-spectroscopy. No IR-absorption in the region 3400–3600 cm^{-1} could be detected. The methylated polysaccharide (20 mg) was hydrolysed using the formic acid–dilute sulfuric acid method.[23] The dry hydrolysed material was dissolved in water (10 ml) and reduced with sodium borohydride (10 mg). Excess of borohydride was removed by addition of acetic acid, and the reduced material was acetylated as described by Björndal *et al.*[24] Separation of the mixture was carried out by gas chromatography (column G2 at 230 °C) giving three peaks, T: 1.00, 1.73 and 2.50 (T=time rel. to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. Peak 2, (90 %) and peak 1, (~5 %) were identified as 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol respectively, giving the same retention times and MS fragmentation pattern as authentic samples. The gas chromatographic behaviour of peak 3 (~5 %) and the fragmentation pattern *m/z* (% rel.int.) 189 (27), 129 (63), 117 (27), 99 (17), 87 (36), 43 (100) indicate a mixture of 2,4 and 3,4 dimethylated glucitol acetates.

Periodate oxidation. For determination of periodate consumption the oxidation was carried out in 0.2 M sodium acetate buffer. The polysaccharide (16 mg) was dissolved in water (10 ml), buffer (6.4 ml, pH 3.8), sodium metaperiodate (5 ml; 0.25 m mol) and water (to 25 ml) were added. The solution was kept in the dark at 5 °C. Samples were withdrawn and analysed for periodate as described[25] giving constant value after 6 d. Formic acid liberated during the oxidation (in unbuffered solution) was determined by titration with 0.01 M sodium hydroxide using pH 6.25 as end point. After reduction of the polyaldehyde the solution was hydrolysed in 0.025 M sulfuric acid,[26] and the hydrolysate analysed by paper chromatography (solvent C) and by gas chromatography after trimethylsilylation (G 1 at 130 and 230 °C). Only glycerol could be identified.

Acetyl determination. Quantitative determination of the ester groups were carried out by the hydroxylamine method[27] as well as by gas chromatography[10] (G 3 at 80 °C), both giving 3.8 % acetyl in US-2 and 3.6 % in UP-2, while USM-2 and UPM-2 were found to contain respectively 2.7 and 2.4 % acetyl. Dried US-2 and UP-2 (5 mg in reactivials) were dissolved in anhydrous DMSO (2 ml). *p*-Toluenesulfonic acid (8 mg) and methyl vinylether (1 ml) were added and the solutions kept with continuous stirring at 14 °C overnight. Dry nitrogen was bubbled through the solutions (2 h) before addition of DMSO-anion (0.5 ml; 2 M). After stirring (2 h) methyl iodide (1 ml) was added and the vial left at room temperature overnight with continuous stirring. The products were isolated, hydrolysed, reduced ($NaBD_4$) and acetylated as for methylation analysis. GLC–MS was performed on Finnigan MAT 4000 using column G 4 (150–250 °C, 6 °C/min).

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