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THE CARRAGEENANS OF *GIGARTINA SKOTTSBERGII* S. ET G.

PART III. METHYLATION ANALYSIS OF THE FRACTION PRECIPITATED WITH 0.3–0.4M POTASSIUM CHLORIDE*

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ABSTRACT

The fraction of carrageenan from the red seaweed *Gigartina skottsbergii* that is precipitated with 0.3–0.4M potassium chloride has been studied by methylation analysis. The results agree qualitatively with the structure previously suggested, except that 3-linked D-galactose 4-sulfate residues are present rather than the corresponding 2-sulfate. For every ten D-galactose residues linked at C-3, there are, on the average, six residues of 3,6-anhydro-D-galactose linked at C-4 and ten sulfate groups (five as 3,6-anhydro-D-galactose 2-sulfate and five as D-galactose 4-sulfate residues).

INTRODUCTION

In earlier work, a fraction of carrageenan was obtained from *Gigartina skottsbergii* by precipitation with 0.3–0.4M potassium chloride. It was studied by the periodate oxidation technique and by measurement of the kinetics of acid hydrolysis of the 3,6-anhydro-D-galactosyl residues and of the sulfate ester groups. It was suggested that, for every ten D-galactose residues linked at C-3 there were six corresponding 3,6-anhydro-D-galactose residues linked at C-4 and ten sulfate groups (five as 3,6-anhydro-D-galactose 2-sulfate and five as D-galactose 2-sulfate residues)[1]. We have verified this conclusion by methylation analysis.

RESULTS AND DISCUSSION

The fraction precipitated with 0.3–0.4M potassium chloride (previously referred to as fraction A)[1] was considered most suitable for these experiments because it does not contain alkali-labile sulfate ester groups which could be released during the methylation procedure[1].

After reduction with sodium borohydride to minimize alkaline degradation, the polysaccharide was methylated by repeated additions of dimethyl sulfate and

*Dedicated to Professor V. Deulofeu, in honor of his 70th birthday.

sodium hydroxide, first at room temperature and then at 35–40°. After hydrolysis and separation, the products obtained in greater yield were shown to be 2,6-di-*O*-methyl-D-galactose (isolated in crystalline form and characterized by its anilide) and 2,4,6-tri-*O*-methyl-D-galactose (characterized by the crystalline anilide). A small proportion of another tri-*O*-methylated D-galactose which shows the chromatographic behavior of 2,3,6-tri-*O*-methyl-D-galactose was detected. No tetra-*O*-methyl-D-galactose was found.

2,6-Di-*O*-methyl-D-galactose has been obtained previously[2–6] from methylated carrageenans and, when supported by other evidence[3–5], the isolation of this derivative indicates the presence of D-galactose 4-sulfate residues linked at C-3.

The isolation of 2,4,6-tri-*O*-methyl-D-galactose shows unambiguously that the original polysaccharide contained nonsulfated D-galactose residues linked at C-3. In this respect, at least, this sample differs from the κ-carrageenans from *Chondrus* and other *Gigartina* species in which virtually all the 3-linked residues are present as D-galactose 4-sulfate[7]. Certain κ-carrageenans from other sources, such as *Furcellaria*[8], have not all 3-linked residues as 4-sulfated esters, however.

It is difficult to isolate 3,6-anhydro-D-galactose derivatives quantitatively after scission of the methylated polysaccharides, because these derivatives are degraded readily under the usual conditions of acid hydrolysis. Several procedures have been tried[3–9] but only one was successful[6]; it consists of an initial protective hydrolysis in the presence of bromine. After removal of the bromine, the solution was heated at higher temperature to complete the hydrolysis. The 3,6-anhydro-D-galactonic acids were separated from the neutral sugars by ion-exchange chromatography and further fractionated by cellulose-column chromatography. Two pure products were isolated: in crystalline form 3,6-anhydro-2-*O*-methyl-D-galactonic acid, which was further characterized as the methyl ester, and 3,6-anhydro-D-galactonic acid which was converted into crystalline methyl 3,6-anhydro-2,4,5-tri-*O*-*p*-nitrobenzoyl-D-galactonate.

Paper chromatography in the five-component system specially developed for the separation of mixtures of anhydroaldonic acids[6] indicated the presence of small quantities of 3,6-anhydro-2,4-di-*O*-methyl-D-galactonic acid and showed that 3,6-anhydro-4-*O*-methyl-D-galactonic acid was virtually absent from the products of hydrolysis of the methylated carrageenan.

These results can only be considered qualitatively because hydrolysis of the 3,6-anhydro-D-galactoside linkages was stopped before completion of the protective step, in an attempt to avoid excessive contamination with D-galactonic acid derivatives. It is known that anhydro residues that are sulfated at position 2 stabilize the adjacent glycoside linkage[10], which complicates the interpretation of the results. In polysaccharides like the carrageenan described in this study[1], where a high percentage of the 3,6-anhydro units are sulfated at O-2, conditions of protective hydrolysis must be chosen to compromise between contamination with D-galactonic acids and loss of some sulfated anhydro residues.

The isolation of 3,6-anhydro-2-*O*-methyl- and 3,6-anhydro-D-galactonic acids

from the products of the oxidative acid hydrolysis confirm the existence of sulfated and nonsulfated 3,6-anhydro residues in the original polysaccharide and (accepting that position 4 is engaged in a galactoside linkage) that the sulfate ester is located at position 2.

When these results are compared with those obtained previously[1], it becomes obvious that the presence of galactose 2-sulfate residues previously suggested is incompatible with the isolation, in major proportion, of 2,6-di-*O*-methyl-D-galactose from the methylated carrageenan. The other results agree, at least qualitatively, with the structure proposed in the previous study[1].

EXPERIMENTAL

General methods. — Paper chromatography was performed on Whatman No. 1 paper and t.l.c. on microcrystalline cellulose (Avicel) with the following solvents: (A) Butyl alcohol half saturated with water, (B) butanone–water azeotrope, (C) 34:6:2:30:5 ethyl acetate–acetic acid–formic acid–butanone–water. The spray reagents were: (a) aniline phthalate in butyl alcohol saturated with water, (b) the hydroxylamine–ferric chloride reagent, and (c) the periodate–benzidine reagent. Infrared spectra were recorded with a Perkin–Elmer 137 B spectrometer. G.l.c. was performed with a Pye Argon Chromatograph on columns of neopentylglycol adipate (5%) on Gas Chrom P. All solvents were evaporated in a rotatory evaporator, under reduced pressure, at 35–40° (bath temperature). Optical rotations are equilibrium values.

Methylation of fraction A. — Fraction A was obtained from *Gigartina skottsbergii* carrageenan by precipitation with 0.3–0.4M potassium chloride as previously described[1]. The polysaccharide (10.0 g) was dissolved in water (300 ml) and reduced overnight with sodium borohydride (1.0 g). The solution was made alkaline with sodium hydroxide (30%, w/v, 40 ml), and then dimethyl sulfate (70 ml) and sodium hydroxide (30%, w/v, 210 ml) were slowly and simultaneously added under vigorous stirring over 6 h at room temperature, the stirring being continued for another 18 h. Three more additions were made in the same way, followed by a further three at 35–40°. The solution was dialyzed against running tap water until neutral, then concentrated, and freeze-dried. Yield 10.5 g, Found: OCH_3, 15.2%.

The i.r. spectrum showed a peak at 850 cm^{-1} which extended over 830 cm^{-1}, but no absorption at 820 cm^{-1}. Hydrolysis of a small sample (0.5M sulfuric acid, 6 h at 100°) and paper chromatography of the mixture (solvent A) showed nonmethylated D-galactose to be absent, thus indicating that the methylation was essentially complete. Nevertheless, another cycle of six methylations was carried out, after which the product was again isolated as just described. Yield 8.8 g. Found: OCH_3, 14.3%. Hydrolysis and paper chromatography showed a pattern identical to that just described.

Hydrolysis of the methylated polysaccharide and identification of the acid-stable product. — Methanolysis and g.l.c. indicated the presence of a minor proportion of methyl 2,4,6-tri-*O*-methyl-D-galactoside together with approximately equal pro-

portions of methyl 2,6-di-*O*-methyl-D-galactosides and 3,6-anhydro-2-*O*-methyl-D-galactose derivatives. Methylated fraction A (4.0 g) was dissolved in aqueous formic acid (45%, v/v, 300 ml) and heated for 16 h at 100°. Formic acid was removed by distillation *in vacuo*, with addition of distilled water followed by distillation to remove the last traces of formic acid. Sulfuric acid formed by hydrolysis of the sulfate esters was neutralized with sodium hydroxide.

After extraction of the sugars from the residue with abs. ethanol, t.l.c. (solvent A) showed two spots of R_F 0.41 and 0.74 together with degradation products derived from 3,6-anhydro-D-galactose derivatives. The hydrolyzate was applied to a column (60 × 4.5 cm) of cellulose and eluted with Solvent A. Fractions (10 ml) were collected automatically and examined by paper chromatography (solvent B), and combined into four large fractions.

Fraction 1. The syrup (0.48 g) was a mixture of degradation products.

Fraction 2. The syrup (0.15 g) had $[\alpha]_D^{20}$ +85.3° (*c* 0.6, water). Paper chromatography in solvent B indicated the presence of a major compound (R_F 0.35) and a small proportion of another having R_F 0.48. They were identified (by paper chromatography with standards) as 2,4,6-tri-*O*-methyl-D-galactose and 2,3,6-tri-*O*-methyl-D-galactose, respectively. Reaction of a solution of the mixture in ethanol with aniline yielded crystalline 2,4,6-tri-*O*-methyl-*N*-phenyl-D-galactopyranosylamine, m.p. 178–179° (recrystallization from ethanol), $[\alpha]_D^{20}$ +36.8° (*c* 0.4, acetone); lit.[11]: m.p. 179°, $[\alpha]_D^{19}$ +38° (acetone).

Fraction 3. The syrup (0.36 g) was a mixture of 2,4,6-tri-*O*-methyl-D-galactose and another product later identified as 2,6-di-*O*-methyl-D-galactose.

Fraction 4. The syrup (0.68 g), which crystallized on being kept at room temp., was pure 2,6-di-*O*-methyl-D-galactose and was recrystallized from ethyl acetate, m.p. 103–105°, $[\alpha]_D^{20}$ +83.2° (*c* 0.6, water); anilide, m.p. 120–122°, $[\alpha]_D^{18}$ +17.1° (*c* 0.6, ethanol); lit. values for 2,6-di-*O*-methyl-D-galactose: m.p.[6] 104–105°, $[\alpha]_D^{15}$ +88.5° (water)[3]; lit. values for the anilide: m.p.[6] 122°, $[\alpha]_D^{17}$ +15° (ethanol)[3].

Oxidative hydrolysis of the methylated polysaccharide. — The methylated polysaccharide (4.0 g) was dissolved in 0.25M sulfuric acid (300 ml) and bromine was added (1 ml). The mixture was kept for 30 h at 60° with occasional shaking and addition of fresh bromine when necessary. Although analysis for combined 3,6-anhydro-D-galactose residues showed that the hydrolysis was not complete, the reaction was stopped to minimize hydrolysis of the galactosyl linkages. The solution was cooled and bromine was removed by aeration. Concentrated sulfuric acid (12.45 ml) was added carefully with cooling to increase the acid concentration to 0.5M and the solution was heated for 18 h at 95° to hydrolyze the galactosyl and sulfate ester linkages. After reduction with sodium borohydride a small sample gave a negative test with the phenol–sulfuric acid reagent. The hydrolyzate was neutralized (barium carbonate), and silver carbonate was added to the suspension which was kept in the dark for 48 h at room temperature with occasional shaking. After filtration and treatment with Dowex 50W (H^+), the solution was added to a column of diethylaminoethyl-Sephadex (18 × 1.8 cm, A-25, HCO_3^-) which was washed with water to

elute nonacidic components. Elution with formic acid (2%) displaced all the acids. No further material was eluted by 5% aqueous formic acid. After evaporation under diminished pressure, water was repeatedly added to and distilled from the residue until all formic acid had been removed.

The mixture of aldonic acids was separated on a column (60 × 4.5 cm) of cellulose (solvent C). Fractions of 10 ml were collected automatically, and those of similar composition (by paper chromatography in solvent C) were combined and evaporated to dryness to give the following large fractions:

Fraction 1 (0.05 g). This gave a negative reaction with the hydroxylamine–ferric chloride reagent and the periodate–benzidine reagent and is presumed to be a mixture of degradation products formed from the unhydrolyzed 3,6-anhydro-D-galactose residues in the first step of the oxidative hydrolysis.

Fraction 2 (0.08 g). Paper chromatography (solvent C) developed with reagent b showed a strong spot with R_F 0.83 together with traces of another one with R_F 0.92. With reagent c, a yellow spot (R_F 0.43) appeared slowly with small amounts of another of R_F 0.74. These last two substances were chromatographycally identical to those of 3,6-anhydro-2-*O*-methyl-D-galactonic acid[6] (R_F 0.46) and 3,6-anhydro-2,4-di-*O*-methyl-D-galactonic acid[6]* (R_F 0.72), respectively. The syrup was dissolved in 0.1M sodium hydroxide and kept overnight at room temperature. After neutralization (Dowex 50W, H^+), paper chromatography indicated that the lactones had disappeared as shown by a strong spot for 3,6-anhydro-2-*O*-methyl-D-galactonic acid and a lesser spot for 2,6-di-*O*-methyl-D-galactonic acid[6] (R_F 0.38).

Fraction 3 (0.12 g). The syrup contained the same compound of R_F 0.82 as fraction 2 together with traces of another compound of R_F 0.72 (reagent b). After the same treatment with 0.1M sodium hydroxide as just described, 3,6-anhydro-2-*O*-methyl-D-galactonic acid and small proportions of 2,6-di-*O*-methyl-D-galactonic acid and 3,6-anhydro-2,4-di-*O*-methyl-D-galactonic acid were revealed by paper chromatography.

Fraction 4. The syrup (0.48 g), $[\alpha]_D^{22}$ +79.2° (*c* 0.53, methanol), crystallized on being kept at room temp. and was recrystallized from ethyl acetate, m.p. 139–141°. The methyl ester was recrystallized from benzene, m.p. 89–91°, $[\alpha]_D^{20}$ +71.2° (*c* 0.83, methanol). These values correspond to those reported in the literature[6] for 3,6-anhydro-2-*O*-methyl-D-galactonic acid and its methyl ester.

Fraction 5 (0.023 g). This fraction was composed of a mixture of two compounds having R_F 0.46 and R_F 0.39 corresponding to 3,6-anhydro-2-*O*-methyl-D-galactonic acid[6] (R_F 0.46) and 2,6-di-*O*-methyl-D-galactonic acid[6] (R_F 0.38), respectively.

Fraction 6 (0.05 g). This fraction contained only one major component (R_F 0.38) which had a mobility and color reactions identical with thoses of 2,6-di-*O*-methyl-D-galactonic acid.

*3,6-Anhydro-2,4-di-*O*-methyl-D-galactonic acid cannot be oxidized by periodate, and presumably the reason for its reaction with the periodate–benzidine spray is that its acidity inhibits the formation of Benzidine Blue. The author is indebted to Dr. D. Rees for this suggestion.

Fraction 7 (0.06 g). This fraction contained a small proportion of 2,6-di-*O*-methyl-D-galactonic acid and a new product of R_F 0.32 corresponding to 3,6-anhydro-D-galactonic acid[6] (R_F 0.32).

Fraction 8 (0.24 g). This fraction was composed of pure 3,6-anhydro-D-galactonic acid. The methyl ester was prepared and treated with *p*-nitrobenzoyl chloride, according to the procedure described elsewhere[6], to give methyl 3,6-anhydro-2,4,5-tri-*O*-*p*-nitrobenzoyl-D-galactonate which was crystallized from ethanol and recrystallized from acetone–ethanol, m.p. 186–189°, $[\alpha]_D^{21}$ −15.8° (*c* 0.9, acetone); lit.[6]: m.p. 187–191.5°, $[\alpha]_D$ −14° (acetone).

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REFERENCES

1 A. S. CEREZO, *J. Chem. Soc., C*, (1967) 2491.
2 J. BUCHANAN, E. E. PERCIVAL, AND E. G. V. PERCIVAL, *J. Chem. Soc.*, (1943) 51.
3 E. T. DEWAR AND E. G. V. PERCIVAL, *J. Chem. Soc.*, (1947) 1622.
4 R. JOHNSTON AND E. G. V. PERCIVAL, *J. Chem. Soc.*, (1950) 1994.
5 A. L. CLINGMAN AND J. R. NUNN, *J. Chem. Soc.*, (1959) 493.
6 N. S. ANDERSON, T. C. S. DOLAN, AND D. A. REES, *J. Chem. Soc., C*, (1968) 596.
7 N. S. ANDERSON, T. C. S. DOLAN, A. PEUMAN, D. A. REES, G. P. MUELLER, J. D. STANCIOFF, AND N. F. STANLEY, *J. Chem. Soc., C*, (1968) 602.
8 T. J. PAINTER, *Can. J. Chem.*, 38 (1960) 112.
9 S. PEAT, J. R. TURVEY, AND D. A. REES, *J. Chem. Soc.*, (1961) 1590.
10 D. A. REES, *J. Chem. Soc.*, (1963) 1821.
11 D. J. BELL AND E. BALDWIN, *J. Chem. Soc.*, (1941) 125.