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Note

Partial methanolysis of the agar-type sulfated galactan of the red seaweed *Laurencia gemmifera*

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This report describes the use of paper chromatography (PC), fast-flow liquid chromatography (FFLC) and reversed-phase high-performance liquid chromatography (RP-HPLC) for analysis of the products of mild methanolysis of the sulfated galactan of the red seaweed *Laurencia gemmifera*. The complex mixture of methyl glycosides of neutral monosaccharides, acetals of agarobiose, and acetals of 3,6-anhydro-L-galactose was completely resolved. The method provides an alternative to GLC for determining xylose and other minor constituents in red-seaweed galactans.

Partial methanolysis has been used for structural studies of galactans of red seaweeds[1–5], employing PC, TLC, and adsorption chromatography for separation and identification of the products. HPLC (normal phase) was particularly useful for the assignment of agar or carrageenan structures to red-algal polysaccharides[6], based on identification of the dimethyl acetals of agarobiose and carrabiose, respectively. No attempt has yet been made to analyze the methanolysis products of agar-type polysaccharides by RP-HPLC, although methanolysis studies of various complex carbohydrates have been carried out by RP-HPLC[7,8].

Chromatographic analysis of the products of mild methanolysis of agar-type polysaccharides is particularly complicated because of the complex pattern of substitution with non-carbohydrate[9] (sulfate, *O*-methyl, and pyruvate acetal groups) and carbohydrate (D-xylose[10] and 4-*O*-methyl-L-galactose[11]) substituents for the agarose disaccharide repeating-units, and also because of the presence of the labile 3,6-anhydrogalactosyl residue, which affords dimethyl acetal derivatives[1] under controlled conditions.

The present paper is a first approach to obtain useful information on the main

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constituents in agar-type polysaccharides by a combination of chromatographic techniques.

EXPERIMENTAL

Material.—The red seaweed *Laurencia gemmifera* was collected in Cuba in a zone at the north coast of the province of Ciego de Avila, known as Dogs's Bay. Sulfated galactan (SG) was extracted from dry, ground seaweed (10 g) with distilled water (200 mL) for 6 h at 100°C. After centrifugation, the residue was reextracted twice, the supernatants were combined, evaporated (in vacuo, < 40°C) to 300 mL and precipitated with 5% Cetavlon according to the Scott procedure[12]. The solid was further dissolved in 4 M NaCl, precipitated with EtOH (4 vol), centrifuged, washed with EtOH (3 × 50 mL), dissolved in distilled water, dialyzed, and lyophilized. The yield of SG was 2.1 g (21%). The ^{13}C NMR spectrum of SG confirmed the agar nature of the polysaccharide and that it was free of floridean starch (unpublished results).

Analytical procedures.—Qualitative paper chromatography (descending), was performed on Filtrak FN 11 paper (Germany) and preparative PC on Filtrak FN 18 using 6:4:3 (v/v) 1-butanol–pyridine–water as eluent in both cases. Spots corresponding to non-reducing monosaccharides were detected with $AgNO_3$ and KOH after periodic acid oxidation[13], neutral monosaccharides with aniline hydrogenphthalate reagent, and 3,6-anhydrogalactose derivatives with *o*-aminophenol reagent[14].

Fast-flow liquid chromatography was performed using a glass column (40 × 3 cm) filled with TSK gel Toyopearl HW-40F (Merck). The eluent was distilled water at a flow rate of 1 mL · min^{-1}. The column was coupled to a system composed of a 2132 Microperpex peristaltic pump (LKB, Sweden), an LKB 2142 refractive-index detector, and an LKB 2210 recorder. Fractions of 3 mL were collected and pooled according to the elution profile.

An LKB HPLC system composed of two 2150 HPLC pumps, a 2150 LC controller, and a Rheodyne 7125 injector coupled to a C-R6A data processor (Shimadzu, Japan) was used. The column was a Lichrosorb RP 18 5 μm (250 × 4 mm, LKB). Water (AnalaR, BDH) at 2 mL · min^{-1} was used for elution and the eluted material was monitored with an LKB 2142 refractive-index detector.

The samples of agarobiose dimethyl acetal, 2-*O*-methylagarobiose dimethyl acetal, methyl α- and β-galactopyranosides, methyl galactofuranosides, 3,6-anhydro-L-galactose dimethyl acetal and 2-*O*-methyl-3,6-anhydro-L-galactose dimethyl acetal were obtained and purified from the methanolyzates of the red seaweed, *Rhodomela larix*[5] and were kindly supplied, along with standard methyl 3,6-anhydro-α-L-galactose, by Dr. A.I. Usov of the Institute of Organic Chemistry of Moscow. 6-*O*-Methyl-D-galactose was a gift from Dr. Vicente Verez from the University of Havana. Methyl glycosides of xylose and 6-*O*-methyl-D-galactose were prepared in sealed ampoules using 1% HCl in dry MeOH for 24 h at 80°C.

All other reagents were reagent grade and used as received, unless otherwise stated.

Methanolysis procedures.—Partial methanolysis of SG was performed by dispersing 200 mg of the dried polysaccharide in 25 mL of 0.5% HCl in dry MeOH and refluxing the mixture for 2 h. After cooling to room temperature, $PbCO_3$ was added. The solid was separated by centrifugation and washed twice with MeOH. The supernatants were mixed and evaporated in vacuo (< 40°C). The residue was treated with 25 mL of 0.1 M $Ba(OH)_2$ and heated for 2 h at 60°C, made neutral with CO_2. The mixture was filtered and the filtrate evaporated to dryness. The solid was dissolved in water for PC, FFLC, and HPLC experiments. For HPLC determinations, the samples (20 μL) were pre-filtered (0.22 μm, Millipore).

RESULTS AND DISCUSSION

Partial methanolysis of the sulfated galactan (SG) afforded seven fractions as detected in PC. Five could be identified by comparison with standards, and two having lower R_f values could not be identified by PC (Table I, Fig 1). Fractions A and B reacted with the *o*-aminophenol reagent, indicating the presence of 3,6-anhydrogalactopyranosyl derivatives. Treatment of both fractions with lactate dehydrogenase[15] indicated the presence of pyruvic acid or a derivative thereof. It is known that, whereas ester sulfate groups are released during methanolysis, pyruvic acid acetal groups remain stable[17]. Thus, although further structural analysis could not be performed, we suggest that fraction A may be the pyruvic acid acetal of agarobiose dimethyl acetal (**15**) first reported by Hirase [3] and fraction B its 2-*O*-methyl derivative (**16**).

An attempt to fractionate the products of methanolysis of SG was made on TSK HW-40F gel. Fig. 2 shows the elution profile obtained. Pooled fractions I, II, and III were analyzed by RP-HPLC together with the total extract and a mixture of standards (Fig. 3).

As may be seen (Fig. 3, chromatogram *a*), the mixture of standards was completely resolved. The methyl glycosides of galactose (peaks 3–5) and xylose

TABLE I

Preparative paper chromatography of the products of partial methanolysis of SG

Fraction	R_f	Compound [a]	Yield (mg)
A	0.18	**15** [b]	8
B	0.25	**16** [b]	5
C	0.41	**8, 9**	31
D	0.48	**2**	39
E	0.53	**3**	15
F	0.62	**10, 11**	12
G	0.80	**4, 5**	37

[a] For identification of compounds see Fig. 1. [b] Proposed structure.

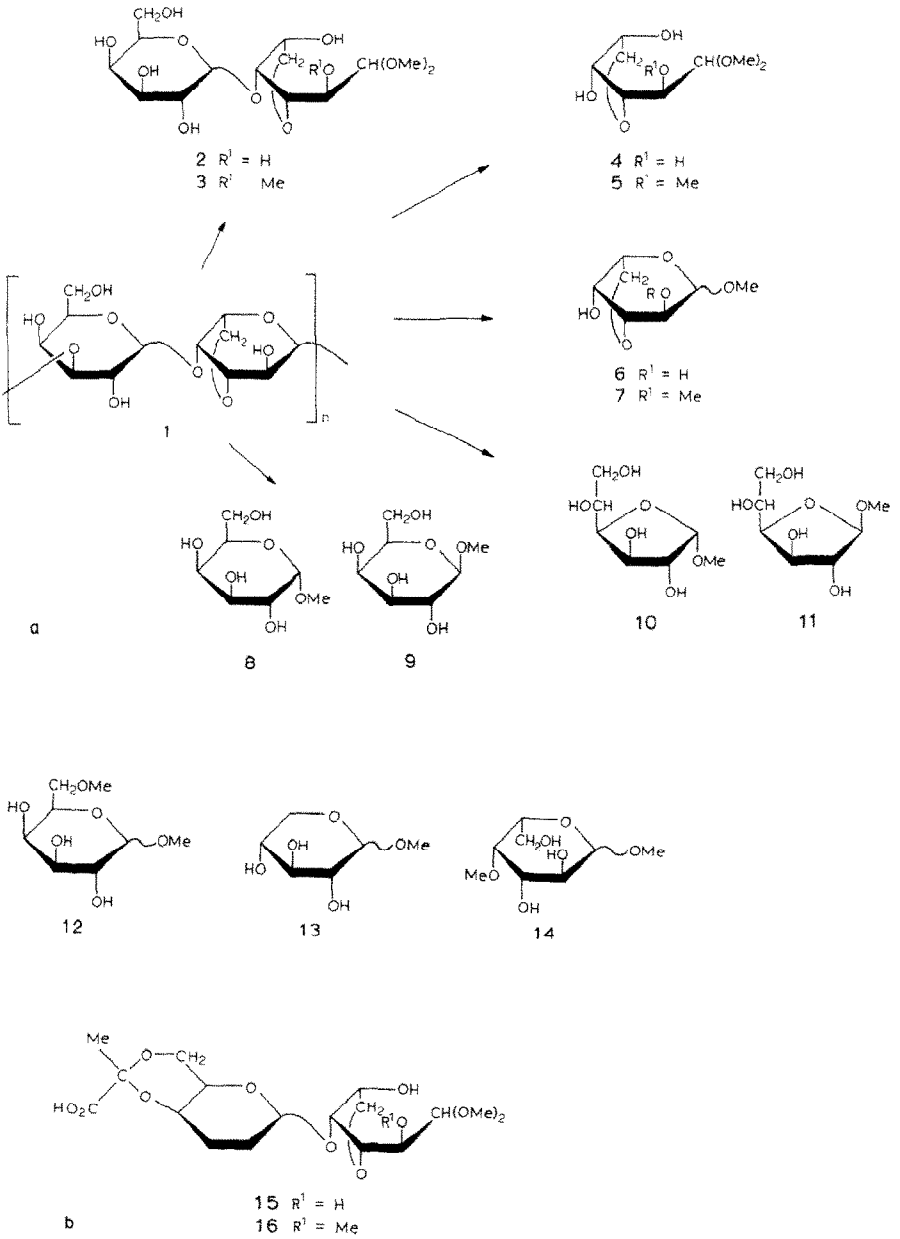

Fig. 1. (a) Main structures obtained during partial methanolysis of an agar-type polysaccharide. (b) Minor structures that could be present during the same process as a result of the presence of substituents in agarobiose repeat units. **1**, Agarobiose repeat unit; **2**, agarobiose dimethyl acetal; **3**, 2^1-*O*-methylagarobiose dimethyl acetal; **4**, 3,6-anhydro-L-galactose dimethyl acetal; **5**, 2^1-*O*-methyl-3,6-anhydro-L-galactose dimethyl acetal; **6**, methyl 3,6-anhydro-α-L-galactopyranoside; **7**, methyl 2-*O*-methyl-3,6-anhydro-α-L-galactopyranoside; **8** and **9**, methyl α- and β-D-galactopyranosides; **10** and **11**, methyl α- and β-D-galactofuranosides; **12**, methyl 6-*O*-methylgalactopyranosides; **13**, methyl D-xylopyranosides; **14**, methyl 4-*O*-methyl-L-galactopyranosides; **15**, pyruvated agarobiose dimethyl acetal; **16**, pyruvated 2^1-*O*-methyl agarobiose dimethyl acetal.

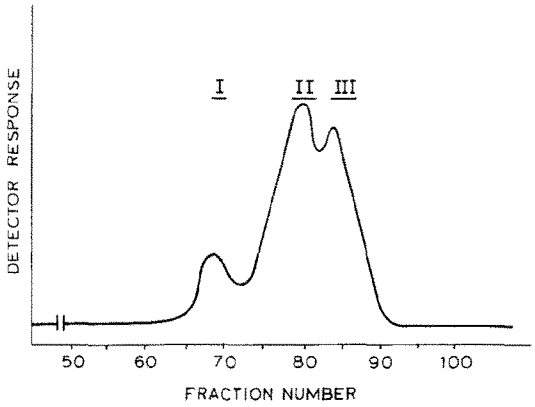

Fig. 2. Elution profile in TSK HW-40F gel of the products of partial methanolysis of SG.

(peaks 6 and 7) were eluted from the reversed phase column in ~6 min. The less-polar 3,6-anhydrogalactose dimethyl acetal (compound **4**, peak 8) and its 2^1-*O*-methyl derivative (compound **5**, peak 9) required ~11 min for elution. Methyl 6-*O*-methylgalactosides (compound **12**, peaks 10 and 11) were eluted after 12.5 min, methyl 3,6-anhydro-α-L-galactopyranoside (compound **6**, peak 12) required 16 min for elution, and agarobiose dimethyl acetal (compound **2**, peak 13) and its 2^1-O-methyl derivative (compound **3**, peak 14) were the most strongly retained of the standards and required 18 min for total elution.

Peak 1 was present in all chromatograms and it was determined to be free, galactose, released during incomplete methanolysis, as indicated by coinjection of a galactose standard.

Methyl xylosides and pyruvic acid acetals of agarobiose dimethyl acetal were not identified as minor constituents in the total extract of SG (chromatogram *b*, Fig. 3). Only when the total extract was fractioned on TSK gel, could methyl xylosides be detected in fraction II (chromatogram *c*, peaks 6 and 7). Traces of xylose were observed by GLC of aldononitrile derivatives of the products of total acid hydrolysis of SG (unpublished results).

The same result was obtained for pyruvic acid acetals of agarobiose dimethyl acetal when fraction I (Fig. 2) was recorded (Fig. 3, chromatogram *c*, peaks 15 and 16).

The correspondence between compounds **15** and **16** (Fig. 1) and peaks 15 and 16 (Fig. 3) was demonstrated when fractions A and B obtained from preparative PC (Table I) were injected onto the column and the chromatogram recorded (experiments not shown).

The absence in all chromatograms of peaks corresponding to methyl 3,6-anhydrogalactosides (compounds **6** and **7**, peak 12) proves that even mild methanolysis converts all of these residues into the respective dimethyl acetal derivatives.

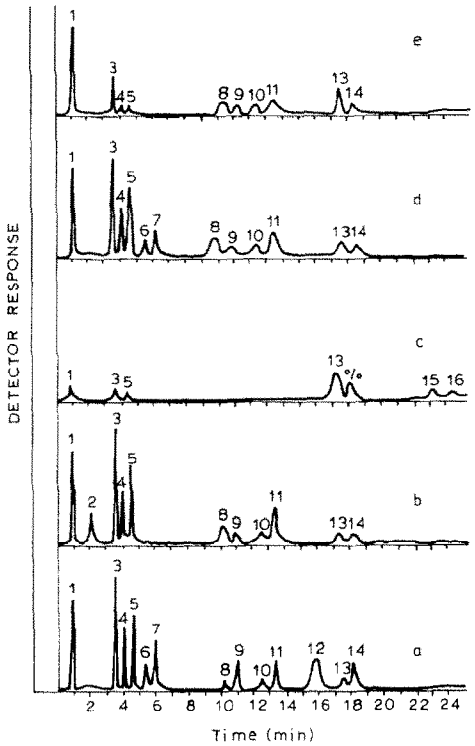

Fig. 3. HPLC chromatograms of (a) mixture of standards; (b) methanolysis products of SG; (c) fraction I of TSK gel separation; (d) fraction II of TSK gel separation; (e) fraction III of TSK gel separation. 1, galactose; 2, unidentified peak; 3, methyl α-D-galactopyranoside (**10**); 4, methyl β-D-galactopyranoside (**11**); 5, methyl α- and β-D-galactofuranosides; 6, 7, methyl α- and β-xylopyranosides; 8, 3,6-anhydro-L-galactose dimethyl acetal (**4**); 9, 2[1]-*O*-methyl 3,6-anhydro-L-galactose dimethyl acetal (**3**); 10, 11 methyl 6-*O*-methyl-D-galactopyranosides (**12**); 12, methyl 3,6-anhydro-α-L-galactopyranoside (**6**); 13, agarobiose dimethyl acetal (**2**); 14, 2[1]-*O*-methylagarobiose dimethyl acetal (**15**); 16, pyruvated 2[1]-*O*-methyl-agarobiose dimethyl acetal (**16**).

The presence of the methyl glycosides of 6-*O*-methyl-D-galactose (compound 2) was also confirmed in the total extract (Fig. 3, chromatogram *b*, peaks 10 and 11) and in fractions II and III from the TSK-gel separation (Fig. 3, chromatograms *d* and *e*, peaks 10 and 11). Although methyl glycosides of 6-*O*-methyl-D-galactose could not be obtained by preparative PC, the presence of 6-*O*-methyl-D-galactose as a constituent of SG was proved by GLC of aldononitriles derivatives of the products of acid hydrolysis of SG and also by ^{13}C NMR of the polysaccharide (results to be reported later).

Thus, partial methanolysis followed by a combination of TSK-gel separation and RP-HPLC of the products gives a total picture of the major and minor constituents of agar-type polysaccharides. This contrasts with the classical procedure (total acid hydrolysis followed by GLC analysis) for qualitative and quantitative determination of monosaccharides, in which information concerning 3,6-anhydrogalactosyl residues is lost because of conversion in the acid medium of these residues into

5-(hydroxymethyl)furfural. Furthermore, RP-HPLC analysis of the products of partial methanolysis of agar-type polysaccharides only requires 25 min for completion, whereas, for instance, GLC of alditol acetates of neutral monosaccharrides requires[16] more than 1 h.

No attempts to quantitate these analyses was made.

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