Carbohydrate Research, 88 (1981) 139–145
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

Note

Structural studies on the porphyran from *Porphyra columbina* (Montagne)*

LUIS H. VILLARROEL AND ALBERTO B. ZANLUNGO
Departamento de Química, Facultad de Ciencia, Universidad Técnica del Estado, Casilla 5659, Santiago 2 (Chile)
(Received July 16th, 1980; accepted for publication, August 4th, 1980)

Porphyran is a soluble polysaccharide isolated from seaweeds of the order Bangiales, and especially from the genera *Porphyra*[2–10]. *Porphyra columbina* is a seaweed found in the central and southern part of Chile, as well as in New Zealand and Australia[11].

The seaweed was exhaustively extracted with water, affording a polysaccharide shown to be inhomogeneous in electrophoresis. Separation of the porphyran into two homogeneous fractions was achieved by precipitation with cetyltrimethylammonium bromide, giving fractions F-1 and F-2. Analytical data for these fractions and for the whole polysaccharide are summarized in Table I.

The chemical composition (percentual or molar) of the porphyran from *Porphyra columbina* is in good agreement with data for similar polysaccharides. The specific optical rotation of this porphyran compares well with that reported for the polysaccharide from *Porphyra capensis*[2] (−60°), whereas that of fraction F-2 is in good agreement with the specific rotation (−79°) of a fraction of the phycocolloid of *Porphyra umbilicalis*[3]. The molar composition of the latter fraction (Gal:Gal-6-*O*-Me:Gal-3,6-An:sulfate 1.00:0.57:0.35:0.65) also compares well with the result given in Table I for F-2. The low, negative value of the specific rotation of F-1 is reasonable, taking into consideration the low proportion of 3,6-anhydro-α-L-galactosyl residues.

The ^{1}H-n.m.r. spectra (see Table I) show several proton-signals in the anomeric region. Some of them (δ 5.30–5.05) have been assigned to H-1 of the 3,6-anhydro-α-L-galactosyl units, whereas others (δ 4.68 and 4.52) should correspond to H-1 of the β-D-galactopyranosyl and 6-*O*-methyl-β-D-galactopyranosyl residues[12]. The singlet at δ 3.36–3.41 can be assigned to the methoxyl group of the 6-*O*-methyl-D-galactosyl residues[13].

*Work (by L. H. V.) done in partial fulfilment of the degree of Master of Science, Universidad Técnica del Estado. Part X of the series "Polysaccharides from Chilean Seaweeds"; for Part IX, see ref. 1.

TABLE I

ANALYTICAL DATA FOR WHOLE POLYSACCHARIDE AND FRACTIONS FROM PORPHYRAN OF *Porphyra columbina*

Property	*Whole polysaccharide (1)*	*Fraction F-1*	*Fraction F-2*
Yield (%)	15.6	66 (from 1)	2.9 (from 1)
$[\alpha]_D$ (degrees)	−52 (*c* 0.2)	−14 (*c* 0.5)	−75 (*c* 0.6)
SO_3 (%)	15.5	11.9	10.3
OCH_3 (%)	3.6	4.3	4.1
3,6-anhydrogalactose (%)	11.6	9.6	15.5
N (%)	0.6	0.5	0.3
Molar ratios of galactose:6-*O*-methyl-galactose:3,6-anhydro-galactose:sulfate	1.00:0.44:0.30:0.73	1.00:0.49:0.24:0.53	1.00:0.48:0.39:0.47
^{1}H-n.m.r. (D_2O), δ	[a]5.30, 5.26, 5.15, 5.06, 4.68, 4.52, 3.41, 3.39	[a]5.30–5.26, 5.16, 5.07, 4.68, 4.52, 3.40	[b]5.25, 5.06, 4.52, 3.36
^{13}C-n.m.r. (D_2O), p.p.m.		104.11 103.81 103.62 103.34 102.73 101.68 98.63	103.98 102.69 101.64 98.85

[a]Acetone as internal reference standard. [b]DSS as internal reference standard.

In the ^{13}C-n.m.r. spectra (see Table I), the ratio of the respective integration for β- and α-linked, anomeric carbon atoms[14] is at least 3:1 for fraction F-2; for F-1, this ratio is much greater, in qualitative agreement with the percentages of 3,6-anhydro-L-galactose in the two fractions.

The lower content of sulfate groups in fraction F-2 (one per 4 sugar units) in relation to that of F-1 (one per 3.2 sugar residues) may afford a partial explanation of the quick destaining of the first-mentioned fraction, after its detection with Toluidine Blue in glass-fiber and poly(acrylamide)-gel electrophoreses.

A methylation study of fraction F-1 allowed the isolation of 2,4,6-tri-*O*-methyl-D-galactose as the main product; 2,3-di-*O*-methyl-D-galactose and 2,3,4,6-tetra-*O*-methyl-D-galactose were also present in significant amounts in the hydrolyzate. The isolation, in high proportion, of the 2,4,6-trimethyl ether indicates that a great part of the D-galactose or 6-*O*-methyl-D-galactose, or both, is linked through O-3.

The identification of the 2,3-di- and 2,3,4,6-tetra-*O*-methyl derivatives suggests that there are branching points in fraction F-1; a similar conclusion was reached by Bowker and Turvey[10] in the methylation analysis of the galactan sulfate from *Laurencia pinnatifida*.

On the other hand, undermethylation could not be precluded, as the percentage

of methoxyl groups, although constant through three sets of methylations, was lower than the calculated value (>27.6%). Moreover, 2,3-di-*O*-methyl-L-galactose could also have been formed from the L-galactose 6-sulfate residue, as demonstrated by Bowker and Turvey[10].

EXPERIMENTAL

General. — Melting points were determined with an Electrothermal melting-point apparatus and are uncorrected. Optical rotations were measured with a Hilger–Watts polarimeter. Evaporations were performed *in vacuo*, the bath temperature being kept below 50°. Analytical, paper chromatography was performed by the descending technique on Whatman No. 1 paper; spots of methylated sugars were made visible by spraying with *p*-anisidine hydrochloride in aqueous 1-butanol, and heating for 5 min. Analytical and preparative t.l.c. were performed on glass plates coated with silica gel HF_{254} (Merck); zones were detected by u.v. light, or by charring with sulfuric acid. The solvent systems (v/v) used were: *A*, 2-butanone–water azeotrope; *B*, 4:1:5 1-butanol–ethanol–water; *C*, 8:2:1 ethyl acetate–pyridine–water; *D*, 40:11:9 1-butanol–ethanol–water, *E*, 1-butanol saturated with water; *F*, 20:3 benzene–ethanol, *G*, 99:1 ethyl acetate–ethanol; *H*, 1:1 ethyl acetate–petroleum ether; and *I*, 4:1 ethanol–petroleum ether. Glass-fiber electrophoresis and poly(acrylamide)-gel electrophoresis were conducted according to techniques reported elsewhere[15]; staining was performed with Toluidine Blue, and destaining, with 5% acetic acid in water. Reduction of sugars was achieved with sodium borohydride[16]. The g.l.c. analysis was performed in a Varian 3700 gas chromatograph equipped with a flame-ionization detector, and having stainless-steel columns (2.0 m × 2.1 mm i.d.) packed with Chromosorb W and the liquid phases *J* (3% of OV-17), *K* (3% of OV-225), *L* (3% of ECNSS-M), *M* (20% of Apiezon L), and *N* (3% of SP-2340). I.r. spectra were recorded with a Perkin–Elmer Model 621 spectrometer. Proton-n.m.r. spectra (100- and 270-MHz) were performed and integrated at 100 and 270 MHz, respectively, with a Varian HA-100 spectrometer and with a 270-MHz spectrometer built at the University of British Columbia with Nicolet parts. Tetramethylsilane was used as the solvent, sodium 2,2-dimethyl-2-silapentane-5-sulfonate or acetone being the internal, reference standard for deuterium oxide solutions. The concentration of samples was 1–2% for polysaccharides, and 6–8% for the other samples. Coupling constants were measured on 250- and 500-Hz sweep-width spectra. ^{13}C-N.m.r. spectra were recorded at 20 MHz with a Varian CFT-20 spectrometer, for solutions (1.5–3.0%) in deuterium oxide; the chemical shifts were determined relative to tetramethylsilane, through the use of an internal, 1,4-dioxane reference taken at 67.40 p.p.m. relative to Me_4Si. The spectra were recorded with complete proton-decoupling.

Analytical samples were dried over P_2O_5 at 100° and 0.1 torr. The percentage of 3,6-anhydrogalactose was determined according to the procedure reported by Yaphe[17]. Microanalyses for sulfate, methoxyl, and nitrogen were performed by Dr. B. B. de Deferrari (University of Buenos Aires). The content of galactose was assumed

to be the difference from 100%, after taking into consideration all of the aforementioned analyses, as well as the content of the counterion (Na^+).

Extraction and hydrolysis of the polysaccharide. — The ground, dried seaweed (100 g) was extracted thrice with water (1.0-L portions) at room temperature, and the same procedure was repeated twice with hot water (80°). The two extracts were separately filtered through muslin, clarified in a centrifuge (15,000 r.p.m.), dialyzed against tap water, and concentrated to a thin syrup which was poured into acetone (5 vol.). Both precipitates were collected, washed with acetone, and dried *in vacuo*; their analyses (chemical and spectroscopic) showed almost identical values. These fractions were combined, giving 15.6 g (15.6%) of a product that was dissolved in hot water and poured into ethanol (10 vol.), to afford fibrous polysaccharide, $[\alpha]_D^{20}$ −52° (*c* 0.2, water): SO_3, 15.5%; OCH_3 3.6%; 3,6-anhydrogalactose, 11.6%; ν_{max}^{KBr} 3,500–3,200, 1,350, 1,220, 930, and 810 cm^{-1}; ^{1}H-n.m.r. data (D_2O, 100 MHz, 95°; 270 MHz; internal acetone): δ 5.30, 5.26, 5.15, 5.06, 4.68, 4.52, 3.41, and 3.39.

On staining, the glass-fiber electrophoretogram of this polysaccharide showed two diffuse bands, one of which remained on the sheet for a few seconds when it was introduced into the solvent used for destaining.

Complete acid hydrolysis of this polysaccharide (15 mg) with 0.5M sulfuric acid, followed by paper chromatography (systems *C* and *D*) of the hydrolyzate, showed the presence of galactose and 6-*O*-methylgalactose as the only sugars. A portion of the hydrolyzate was reduced and the alditols acetylated, giving a product that showed, in g.l.c. (column *L*), two peaks that could be superposed on those of authentic samples of hexa-*O*-acetylgalactitol and penta-*O*-acetyl-6-*O*-methyl-D-galactitol.

Mercaptolysis. — This was performed on 2.0 g of the polysaccharide, according to the technique reported by O'Neill[18]. After processing, the resulting, neutral, aqueous solution was concentrated to 40 mL; on cooling, this afforded crystals (0.20 g) of D-galactose diethyl dithioacetal, m.p. 140–142°, $[\alpha]_D^{20}$ −4.7° (*c* 0.5, water); lit.[18] m.p. 141–142°, $[\alpha]_D^{25}$ −4.7° (water). This compound was acetylated as usual, giving penta-*O*-acetyl-D-galactose diethyl dithioacetal, m.p. 76–77°; lit.[19] m.p. 77.5–78.5°; ^{1}H-n.m.r. data ($CDCl_3$): δ 1.24 (t, 3 H, *J* 7.5, -S-CH_2-CH_3), 2.04 (s, 3 H, acetyl), 2.12 (s, 9 H, 3 acetyl), 2.14 (s, 3 H, acetyl), 2.70 (m, 4 H, -S-CH_2-CH_3), 3.84 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 3.86 (dd, 1 H, $J_{6',5}$ 6.0 Hz, H-6'), 4.30 (dd, 1 H, $J_{6,5}$ 5.0, $J_{6,6'}$ 12.0 Hz, H-6), 5.18 (dd, 1 H, $J_{2,3}$ 1.7, $J_{2,1}$ 8.0 Hz, H-2), 5.20 (m, 1 H, H-5), 5.32 (dd, 1 H, $J_{4,3}$ 9.2, $J_{4,5}$ 2.0 Hz, H-4), and 5.80 (dd, 1 H, $J_{3,2}$ 1.7, $J_{3,4}$ 9.2 Hz, H-3).

The mother liquor from the crystallization of D-galactose diethyl dithioacetal was extracted continuously with ethyl ether for 18 h, and the extract was evaporated to a syrup; its t.l.c. (silica gel, system *F*) showed three components, which were separated on preparative plates.

The compounds obtained by extraction of the band having the smallest R_F value crystallized from 1 : 1 ethanol–ether, giving galactose diethyl dithioacetal (70 mg),

m.p. 140–142°, which, in t.l.c. (systems *F* and *G*), had the same R_F value as an authentic sample of D-galactose diethyl dithioacetal.

The compound extracted from the band having the intermediate R_F value crystallized from ethanol–ether, giving 6-*O*-methyl-D-galactose diethyl dithioacetal (20 mg), m.p. 141–142°, $[\alpha]_D^{23}$ −38° (*c* 0.1, water); lit.[9] m.p. 141.5–142°, $[\alpha]_D^{18}$ −30° (*c* 0.1, water); ^{1}H-n.m.r. data (D_2O): δ 1.25 (t, 6 H, *J* 6.0 Hz, -S-CH_2-CH_3), 2.75 (d, 4 H, *J* 6.0 Hz, -S-CH_2-CH_3), and 3.41 (s, 3 H, OCH_3).

The fastest-moving compound in t.l.c. was isolated as a powder (8 mg), $[\alpha]_D^{18}$ +9° (*c* 0.18, water); lit.[9] for 3,6-anhydro-D-galactose diethyl dithioacetal, m.p. 111–112°, $[\alpha]_D$ −9.69° (water). This compound, in t.l.c. (silica gel), and after multiple developments (systems *F* and *G*), traveled the same distance from the origin as a standard sample of 3,6-anhydro-L-galactose diethyl dithioacetal. Acetylation of the 3,6-anhydro-L-galactose diethyl dithioacetal isolated gave a syrup which, in t.l.c. (silica gel, systems *H* and *I*), showed several spots; their attempted separation did not allow the isolation of 2,4,5-tri-*O*-acetyl-3,6-anhydro-L-galactose diethyl dithioacetal. The same behavior was observed on acetylation of an authentic sample (30 mg) of 3,6-anhydro-L-galactose diethyl dithioacetal.

Fractionation of the polysaccharide. — To a solution of the polysaccharide (3.5 g) in water (350 mL) was added a solution of cetyltrimethylammonium bromide according to the technique reported by Scott[20]; the resulting precipitate was filtered off, and purified by the aforementioned technique, giving fraction F-1 (2.3 g, 66%), $[\alpha]_D^{20}$ −14° (*c* 0.5, water): SO_3, 11.9%; OCH_3, 4.27%; 3,6-anhydrogalactose, 9.6%; N, 0.5%; ν_{max}^{KBr} 930 (sh) and 810 cm^{-1}; ^{1}H-n.m.r. data (D_2O, 100 MHz, 95°, 270 MHz; internal acetone): δ 5.30–5.26, 5.16, 5.07, 4.68, 4.52, and 3.40; ^{13}C-n.m.r. data (D_2O): 104.11, 103.81, 103.62, 103.34, 102.73, 101.68, and 98.63 p.p.m.

From the solution remaining after the filtration of the F-1-Cetavlon complex, and after the usual processing, fraction F-2 (0.1 g, 2.9%) was obtained, $[\alpha]_D^{19}$ −75° (*c* 0.6, water); SO_3, 10.3%; OCH_3, 4.14%; 3,6-anhydrogalactose, 15.5%; N, 0.3%; ν_{max}^{KBr} 930 and 820–810 cm^{-1}; ^{1}H-n.m.r. data (D_2O, 100 MHz, 95°, 270 MHz; internal DSS): δ 5.25, 5.06, 4.52, and 3.36; ^{13}C-n.m.r. data (D_2O): 103.98, 102.69, 101.64, and 98.85 p.p.m.

Electrophoresis [fiber-glass and poly(acrylamide) gel] of F-1 showed only one band (detection with Toluidine Blue). Fiber-glass electrophoresis of F-2 showed only one band on staining, but it vanished quickly in the solution used for the removal of the excess of stain. The homogeneity of F-2 was verified by gel chromatography on Sepharose 6-B, after the fraction had been stained[21] with Procion Blue 3 GS.

Methylation of fraction F-1. — Methylation of F-1 (10 g) was performed with dimethyl sulfate and sodium hydroxide according to the procedure reported by Anderson *et al.*[22]. Three sets of methylations, each one lasting 5 days, were performed. Chemical analysis showed that the methoxyl content (23%) was practically constant after the first set, and a similar conclusion was reached through i.r. spectroscopy (no absorption at 3500–3200 cm^{-1}). A portion of the crude, methylated polysaccharide (0.10 g) was purified by chromatography on a column (15 × 1 cm) of silica gel

60 (Merck) with 3:2 chloroform–methanol, affording a polysaccharide that had methoxyl 25.2%.

A portion of the methylated polysaccharide (1.9 g) was refluxed with 45% formic acid (150 mL) for 16 h, and the hydrolyzate was chromatographed on a column (70 × 3.5 cm) of cellulose with system *E*. Fractions (10 mL) were collected, and, after performing paper chromatography (systems *A* and *E*), they were combined into three main fractions A, B, and C.

A syrup (70 mg) was obtained after evaporating the solvent from fraction A. It was identified as 2,3,4,6-tetra-*O*-methylgalactose by: (*a*) paper chromatography in solvent system *A*, with an authentic sample as reference, (*b*) g.l.c. (after reduction and acetylation) in columns *K*, *L*, and *M*, where it co-chromatographed with an authentic sample of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol.

By evaporation of the solvent, fraction B gave a solid (0.27 g), which crystallized from ethyl ether, affording 2,4,6-tri-*O*-methyl-D-galactose as needles, m.p. 101–103°, $[\alpha]_D^{23}$ +128→+93° (*c* 0.5, water); lit.[23] m.p. 102–105°, $[\alpha]_D^{23}$ +124→+90.4° (water). The identification of this compound was corroborated by: (*a*) its reaction with aniline, which gave 2,4,6-tri-*O*-methyl-*N*-phenyl-D-galactopyranosylamine, m.p. 178–179°, $[\alpha]_D^{17}$ +36° (*c* 0.5, acetone); lit.[24] m.p. 178–179°, $[\alpha]_D^{20}$ +38° (acetone); and (*b*) g.l.c. (after reduction and acetylation) in columns *J*, *K*, and *L*, where it co-chromatographed with an authentic sample of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-galactitol.

After evaporation of the solvent, fraction C gave a syrup (0.23 g) which, in paper chromatography (system *A*), showed a mixture of dimethylated sugars. A portion of this syrup was reduced, and the product acetylated, giving a sample that, in g.l.c. (columns *K*, *L*, *M*, and *N*), showed two main peaks. One of them had the same retention time as that of an authentic sample of 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-D-galactitol; the other did not coincide with any of the peracetates of the following standard compounds: 2,4-di-*O*-methyl-D-galactitol, 2,6-di-*O*-methyl-D-galactitol, 3,4-di-*O*-methylgalactitol, and 4,6-di-*O*-methyl-D-galactitol.

ACKNOWLEDGMENTS

This research was supported by the Programa de Desarrollo Científico y Tecnológico (PRDCYT) of the Organization of the American States, and by the Dirección de Investigaciones Científicas y Tecnológicas of the UTE. The authors thank Drs. G. G. S. Dutton (Univ. of British Columbia), A. M. Stephen (Univ. of Cape Town), C. T. Bishop (NRCCanada), and W. Yaphe (McGill Univ.) for the gift of standard compounds. They also thank Dr. L. D. Hall and the Chemistry Department of the University of British Columbia for facilities provided to one of them (A.Z.) for recording ^{1}H-n.m.r. spectra (270 MHz) while he was working under a Fellowship of a joint program of NRCCanada–CIDA.

REFERENCES

1 A. B. ZANLUNGO, *Bot. Mar.*, in press.
2 J. R. NUNN AND M. M. VON HOLDT, *J. Chem. Soc.*, (1957) 1094–1097.
3 S. PEAT, J. R. TURVEY, AND D. A. REES, *J. Chem. Soc.*, (1961) 1590–1595.
4 J. R. TURVEY AND D. A. REES, *Nature*, 189 (1961) 831–832.
5 D. A. REES AND E. CONWAY, *Biochem. J.*, 84 (1962) 270–275.
6 D. A. REES AND E. CONWAY, *Nature*, 195 (1962) 398–399.
7 J. C. SU AND W. Z. HASSID, *Biochemistry*, 1 (1962) 468–474.
8 N. S. ANDERSON AND D. A. REES, *J. Chem. Soc.*, (1965) 5880–5887.
9 D. M. BOWKER AND J. R. TURVEY, *J. Chem. Soc., C*, (1968) 983–988.
10 D. M. BOWKER AND J. R. TURVEY, *J. Chem. Soc., C*, (1968) 989–992.
11 T. LEVRING, H. A. HOPPE, AND O. J. SCHMID, *Marine Algae*, Cram, De Gruyter, and Co., Hamburg, 1969.
12 D. WELTI, *J. Chem. Res.*, (1977) 312–313.
13 E. G. GROS, I. O. MASTRONARDI, AND A. R. FRASCA, *Carbohydr. Res.*, 16 (1971) 232–234.
14 G. K. HAMER, S. S. BHATTARCHARJEE, AND W. YAPHE, *Carbohydr. Res.*, 54 (1977) c7–c10; S. S. BHATTARCHARJEE AND W. YAPHE, *ibid.*, 60 (1978) c1–c3.
15 B. J. DAVIS, *Ann. N. Y. Acad. Sci.*, 121 (1964) 404–427; C. A. PASTERNAK AND P. W. KENT, *Research*, 5 (1952) 486–487.
16 M. L. WOLFROM AND A. THOMPSON, *Methods Carbohydr. Chem.*, 2 (1963) 67.
17 W. YAPHE, *Anal. Chem.*, 32 (1960) 1327–1330.
18 A. N. O'NEILL, *J. Am. Chem. Soc.*, 77 (1955) 6324–6326.
19 M. L. WOLFROM, *J. Am. Chem. Soc.*, 52 (1930) 2464–2473.
20 J. E. SCOTT, *Methods Carbohydr. Chem.*, 5 (1965) 38–44.
21 W. F. DUDMAN AND C. T. BISHOP, *Can. J. Chem.*, 46 (1968) 3079–3084.
22 N. S. ANDERSON, T. C. S. DOLAN, AND D. A. REES, *J. Chem. Soc., C*, (1968) 596–601.
23 D. J. BELL AND S. WILLIAMSON, *J. Chem. Soc.*, (1938) 1196–2000.
24 E. L. HIRST AND J. K. N. JONES, *J. Chem. Soc.*, (1946) 506–512.