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Note

Structure of 3-*O*-(α-D-glucopyranosyluronic acid)-L-galactopyranose, an aldobiouronic acid isolated from the polysaccharides of various unicellular red algae

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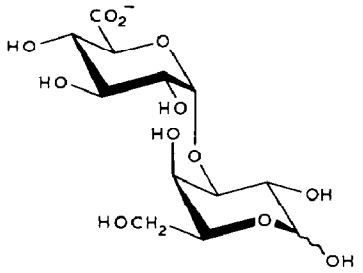
In preliminary accounts of the composition of the extracellular polysaccharide produced by the brackish water, unicellular, red alga *Rhodella reticulata*, total hydrolysis revealed mainly xylose, glucose, galactose, glucuronic acid, rhamnose, arabinose, and 3- and 4-*O*-methylpentose[1,2]. An aldobiouronic acid, isolated after mild acid-catalysed hydrolysis of the polysaccharide, was shown[3,4] by 1D- and 2D-n.m.r. spectroscopy to be 3-*O*-(α-glucopyranosyluronic acid)-α,β-galactopyranose (**1**). However, biochemical data and criteria for the absolute configuration were not presented.



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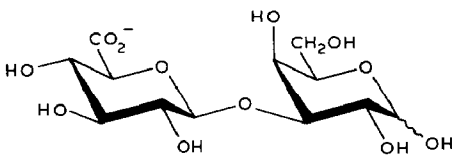

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Kieras *et al.*[5] reported the isolation of **1** from the unicellular, marine, red alga *Porphyridium cruentum*. Percival *et al.*[6], in studying *P. cruentum* and the fresh-water alga *P. aerugineum*, mentioned the isolation of 3-*O*-(β-D-glucopyranosyluronic acid)-D-

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galactopyranose (**2**). We now report the isolation of **1** from *P. sp.*, *P. cruentum*, *P. aerugineum*, and *R. reticulata*, and that the galactose moiety is L.

The extracellular polysaccharides from *R. reticulata*, *P. sp.*, *P. cruentum*, and *P. aerugineum* were isolated during the stationary phase of growth of the algae. Mild acid-catalysed hydrolysis of each polysaccharide yielded (t.l.c. and h.p.l.c.) a mixture of monosaccharides, a disaccharide (R_F 0.29), and higher oligosaccharides. Each total hydrolysate was subjected to ion-exchange chromatography (DE-52) and fractions eluted with aqueous sodium chloride were subjected to gel-filtration chromatography on Sephadex G-10 to give the aldobiouronic acid fraction with R_F 0.29.

The procedure for characterising the aldobiouronic acid **1** (R_F 0.29) isolated from the *R. reticulata* polysaccharide is typical. The peak at *m/z* 355 in the f.a.b.-mass spectrum of **1** was characteristic of $(M-1)^+$ of an aldobiouronic acid $C_{12}H_{19}O_{12}$. The ^{13}C-n.m.r. resonances at 177.11 and 177.02 p.p.m. (ratio ~2:1) were characteristic[3,4] of a COOH group.

Total hydrolysis of **1** afforded (t.l.c. and h.p.l.c.) a mixture of galactose, glucuronic acid, and glucuronolactone. Delactonisation, borohydride reduction, and acetylation followed by g.l.c. revealed galactitol hexa-acetate[7]. Lactonisation of the carboxylic acid in a hydrolysate of **1**, followed by borohydride reduction, and acetylation gave (g.l.c.) the hexa-acetates of glucitol and galactitol. The ratio of galactose to glucuronic acid + glucuronolactone in the total hydrolysate determined by h.p.l.c. was the same as that for the hexa-acetates of galactitol to glucitol. Thus, **1** was a glucuronosyl-galactose.

Delactonisation, borohydride reduction, and acetylation of the products of hydrolysis of the purified disaccharide from *P. sp.* gave[8] (g.l.c.) galactitol and glucitol in the ratio ~4:1. The inhomogeneity of the disaccharide sample was also seen in the ^{13}C-n.m.r. spectrum. G.l.c.–e.i.-m.s.[8] analysis of the derived methylated disaccharide indicated the major component to be *O*-methylated 3-*O*-(methyl glucopyranosyluronate)galactitol (derived from **1**), but the minor component could not be characterised[8].

^{13}C-N.m.r. (D_2O) spectra of the disaccharides isolated from *R. reticulata* and from *P. aerugineum* were identical, and contained resonances for 3-*O*-(α-glucopyranosyluronic acid)galactopyranose[3,4] (**1**) with an α,β-ratio of ~1:2. Details of the n.m.r. data have been reported elsewhere[3,4]. The ^{13}C-n.m.r. spectra for the aldobiouronic acid isolated from *P. sp.* and from *P. cruentum* were similar, but major and minor species were present (ratio ~4:1). The major species had an α,β-ratio of ~1:2 and its resonances were identical with those for **1**.

Heany-Kieras and Chapman[5a] noted the presence of two aldobiouronic acids in addition to **1** in the acid hydrolysate of the *P. cruentum* polysaccharide, namely, 3-*O*-(2-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactopyranose and -D-glucopyranose. Although the ^{13}C-n.m.r. data for the aldobiouronic acid fraction obtained from the *P. cruentum* polysaccharide indicated heterogeneity, signals of OMe groups were not detected.

The $J_{1,2}$ value of 3.7 Hz for the glucopyranosyl moiety is indicative of the α-$^4C_1$(D) or $^1C_4$(L) conformation[4]. The D configuration was assigned on the basis of optical

rotation data (see below). The galactose obtained after hydrolysis of the aldobiouronic acid from each of the four sources was not oxidised by D-galactose dehydrogenase (EC 1.1.1.48) or D-galactose oxidase (EC 1.1.3.9), but was oxidised by L-fucose dehydrogenase (E.C. 1.1.1.122) which also accepts L-galactose as a substrate. The sample of **1** from *R. reticulata* had $[\alpha]_D^{25}$ +48° (*c* 0.5, water). As a first approximation, it is assumed that the $[\alpha]_D$ value of **1** may be *estimated* from the sum of those of its components. From the $[\alpha]_D^{24}$ values[9] for β-D-glucuronic acid [+11.7° (water)] and a 1:2 α,β-mixture (+36.3°), the value for α-D-glucuronic acid is estimated to be +85°. The $[\alpha]_D$ value[10] for mutarotated D-galactose is +80.2°. Thus, the value calculated for **1** based on α-D-glucuronic acid and mutarotated L-galactose is +5° [+85° + (−80.2°)], *cf.* −165° based on α-L-glucuronic acid.

Changes in ^{13}C-n.m.r. chemical shifts have recently been correlated[11,12] with the absolute stereochemistry of sugars in diastereomeric disaccharide units. For example, substituent effects in methyl 3-*O*-[α-(D or L)-fucopyranosyl]-α-D-galactopyranosides{[D- or L-(1→3)-D]-**3**} were estimated[11] using appropriate chemical shifts for [D- or L-(1→3)-D]-**3**, α-L-fucopyranose, and methyl α-D-galactopyranoside. Comparison of values for externally diastereotopic pairs of nuclei shows that fucosyl anomeric carbons (C-1′), galactosyl-substituted carbons (C-3), and adjacent carbons (C-2 and C-4) differ significantly in magnitude[11] and may be useful for stereochemical diagnosis. Substituent shifts for C-1′, C-2, C-3, and C-4 were, respectively, +8.40, −0.73, +8.16, and +0.07 p.p.m. for [L-(1→3)-D]-**3**, and +3.15, −1.58, +5.16, and −3.53 p.p.m. for [D-(1→3)-D]-**3** [(+)-value = downfield shift][11]. Substituent shifts of +8.33, −0.81, +8.43, and −0.21 p.p.m. for the corresponding carbons in α-**1**, and +8.39, −0.6(2), +7.93, and −0.24 p.p.m. for β-**1** were based on ^{13}C chemical shifts for (α or β)-**1** (ref. 4), α-D-glucopyranosyluronic acid (pH 7.8) (ref. 13), and (α or β)-D-galactopyranose[13]. Clearly, the sets of values for (α or β)-**1** and [L-(1→3)-D]-**3** are very similar, whereas they differ significantly from those for [D-(1→3)-D]-**3**. Therefore, the D-(1→3)-L absolute stereochemistry ascertained for aldobiouronic acid **1** is completely consistent with these chemical shift changes.

EXPERIMENTAL

The unicellular red algae *R. reticulata* (UTEX LB 2320), *P. sp.* (UTEX 637), and *P. cruentum* (UTEX 161) were obtained from the culture collection of the University of Texas. *R. reticulata* was cultivated in a brackish water medium according to Schlosser[14]. *P. sp.* and *P. cruentum* were cultivated in an artificial sea-water medium according to Jones *et al.*[15]. *P. aerugineum* was cultivated in a fresh-water medium according to Savins[16]. The algae were grown in a controlled room (24 ± 1°, continuous illumination supplying 155 $\mu E.m.^{-2}.s^{-1}$), in conical tubes aerated with sterile air containing 2–3% of CO_2. Cultures at the stationary phase of growth were centrifuged (27500*g*, 30–40 min, 0–4°), and each supernatant solution was dialysed in 2.3-cm diameter dialysis tubing (8000 mol. wt. cut-off) against double-distilled water at 4° until the conductivity of the water reached 1 μS (El-Hamma Instruments model TH-250 conductometer), and then lyophilised.

Each polysaccharide (500 mg) was hydrolysed in 2M trifluoroacetic acid at 100° for 2 h in a glass tube fitted with a Teflon-lined screw cap. The hydrolysate was filtered through a Whatman GF/C filter, then concentrated to dryness at 40°. Excess of acid was removed by repeated addition and evaporation of ethanol. A solution of the resulting residue in distilled water (20 mL) was filtered through a membrane filter (Schleicher & Schull, 0.25 μm, 25 mm), and applied to a column (2.0 × 10 cm) of DE-52 ion-exchanger (Whatman). Neutral components were eluted with distilled water (fractions monitored by the phenol–sulfuric acid method[17]). Elution with 0.5M ammonium hydrogencarbonate afforded anionic fractions, and the ammonium hydrogencarbonate was removed by repeated concentration *in vacuo* with the addition of water. A solution of the residue in distilled water (20 mL) was chromatographed on a column (2.5 × 150 cm) of Sephadex G10 (Pharmacia) pre-equilibrated with distilled water. Fractions of 2.5 mL were eluted with distilled water, and analysed (phenol–sulfuric acid method).

Positive fractions were subjected to t.l.c. on Silica Gel 60 (Merck), using ethanol–1-butanol–water–acetic acid–pyridine (100:10:30:3:10), and detected with a 0.1% solution of orcinol in aqueous 20% H_2SO_4 at 100–110°. The component (**1**) with R_F ~0.29 was isolated, and corresponded to ~7.5% of the polysaccharide.

Total hydrolysis of **1** was performed with 4M HCl for 4 h at 100° in a sealed tube. The monosaccharides were analysed by t.l.c. and h.p.l.c. (Varian Instruments Model LC-5000) using a CHO-620 carbohydrate column and a Varian RI-3 refractive index detector. Sugars were eluted with distilled water at 1 mL/min. Galactose, glucuronic acid, and glucuronolactone were revealed by t.l.c. (R_F 0.69, 0.31, and 0.60, respectively) and h.p.l.c. (T 9.35, 5.60, and 18.25 min, respectively).

The total hydrolysate of **1** was delactonised (1 drop of conc. aqueous NaOH), treated with $NaBH_4$, and acetylated with acetic anhydride–pyridine (1:1). Another separate sample was lactonised in acidic medium and treated with $NaBD_4$, followed by acetylation. The resulting alditol acetates were analysed by g.l.c. [Girdel gas chromatograph series-300, FID detector, Chrompack CP-Sil 5CB methyl silicone column, nitrogen carrier gas, 130° initial oven temperature (15 min), 2°/min to 230°, then 230° for 10 min]. The hexa-acetates of galactitol and glucitol have T 40.48 and 40.08 min, respectively.

F.a.b.-m.s. was performed on a Kratos Model MS50RF instrument (negative-ion mode, 6 kV, primary particle flux produced by Xenon, and a glycerol–water–NaOH matrix). Optical rotations were measured with a Perkin–Elmer MC-141 polarimeter. Each isolated disaccharide fraction was analysed by ^{13}C-n.m.r. spectroscopy (50.3 MHz) with a Bruker WP-200-SP F.t. spectrometer operating in the broad-band proton decoupling mode. The D_2O solvent was used as an internal lock, and 1,4-dioxane as an internal secondary standard (δ 67.4) referenced to Me_4Si.

Oxidation with D-*galactose dehydrogenase.* — D- or L-Galactose (200 μL, 70 mg/mL), or the total hydrolysate of **1**, was mixed with NAD^+ (100 μL, 20 mg/mL) in glycine–sodium pyrophosphate buffer (2.68 mL, 0.1M, pH 9). After the addition of the enzyme solution (20 μL, ~0.5 U), the formation of NADH was followed by measurement of the absorbance at 340 nm.

Oxidation with D-*galactose oxidase.* — D- or L-Galactose, or the hydrolysate of **1** (with and without Dowex-1 anion-exchange treatment), was incubated at 37° with D-galactose oxidase (0.5 U), peroxidase (4 U), and *o*-tolidine (0.1 mg) in sodium acetate buffer (0.5 mL, 0.05M, pH 7.0). The absorbance was measured periodically at 425 nm on a Spectronic spectrophotometer.

Oxidation with L-*fucose dehydrogenase.* — A standard aqueous solution of D- or L-galactose (100 μL, 0.2 mg/mL), or the total hydrolysate of **1** (after chromatography on Dowex-1), was mixed with NAD^+ (100 μL, 20 mg/mL) in glycine–sodium pyrophosphate buffer (4.0 mL, 0.1M, pH 9). After the addition of the enzyme solution (100 μL, ~0.5 U), the formation of NADH was followed by measurement of the absorbance at 340 nm.

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