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Note

N.m.r. structure determination of 3-*O*-(α-D-glucopyranosyluronic acid)-L-galactopyranose, an aldobiuronic acid isolated from the unicellular red alga *Rhodella reticulata*

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When cultivated in a medium of brackish water the unicellular red alga *Rhodella reticulata* produces a sulfated polysaccharide. Total hydrolysis of the polysaccharide adhering to the cell, as well as that dissolved in the medium, showed[1] it to be composed mainly of xylose, glucose, galactose, glucuronic acid, and rhamnose. In the present study, the polysaccharide was isolated from the medium during the stationary-growth phase of the micro-algae, and a disaccharide was obtained from it *via* hydrolysis under mildly acidic conditions[2]. Herein is described the determination of the structure of the disaccharide by the combined application of several 2D-n.m.r. spectroscopic methods. It is shown that the isolated disaccharide fraction consists of a single aldobiuronic acid, and that this is a 3-*O*-(α-glucopyranosyluronic acid)-galactopyranose that exists in aqueous medium as an ~1:2 mixture of the α and β anomers (**1α,β**), respectively.

N.m.r.-spectral parameters (^{1}H- and ^{13}C-) for **1α** and **1β** are listed in Tables I and II; position numbers for the glycosyluronic moiety are primed. Two major anomeric proton resonances were found at δ 4.65 and δ 5.24 ($^3J_{H\text{-}1,H\text{-}2}$ 7.8 and $^3J_{H\text{-}1',H\text{-}2'}$ 3.7 Hz, respectively), and one minor anomeric-proton signal at δ 5.27 ($^3J_{H\text{-}1,H\text{-}2}$ 3.7 Hz). The ratio of major to minor protons was ~2:1. Analogously, two low-field carbonyl ^{13}C{^{1}H}-n.m.r. signals in the ratio of ~2:1 were observed, at δ 177.11 and 177.02, respectively. The anomeric-carbon signals for the major species

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TABLE I

^{1}H-N.M.R.-SPECTRAL PARAMETERS FOR 3-*O*-(α-D-GLUCOPYRANOSYLURONIC ACID)-L-GALACTOPYRANOSE (**1α,β**) IN D_2O

Hydrogen atom	*δ*[a]			$^3J_{H,H}$ *in Hz (s.d.)*	
	1β	**1α**		**1β**	**1α**
H-1	4.65	5.27	H-1,H-2	7.8(1)	3.7(1)
H-2	3.61[b]	3.98[b]	H-2,H-3	10.3(1)	10.6(2)
H-3	3.78[b]	3.88[b]	H-3,H-4	3.4(1)	
H-4	4.01[b]	4.09	H-4,H-5	0.9(1)	
H-5	3.70[c]		H-1′,H-2′	3.7(1)	
H-6,6a	3.63[c]		H-2′,H-3′	10.1(2)	
H-1′	5.24		H-3′,H-4′	9.1(2)	
H-2′	3.63[b]		H-4′,H-5′	10.1(1)	
H-3′	3.79[b]				
H-4′	3.47[b]				
H-5′	4.06[b]				

[a]P.p.m. downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, at 300 MHz, and sample pH ~7. [b]Assignment from COSY, RELAY-COSY, and 2 D J-resolved spectra. [c]Assignment from HETCOR spectrum.

TABLE II

$^{13}C\{^1H\}$-N.M.R.-SPECTRAL PARAMETERS FOR 3-*O*-(α-D-GLUCOPYRANOSYLURONIC ACID)-L-GALACTOPYRANOSE (**1α,β**) IN D_2O

Carbon atom	*δ*[a]	
	1β	**1α**
C-1	97.18	93.17
C-2	72.49, 72.23[b]	68.59[c]
C-3	81.73	78.63
C-4	69.46	70.09[c]
C-5	76.01	71.48[c]
C-6	61.86[c]	62.01[d]
C-1′	101.29	101.23
C-2′	72.49, 72.23[b]	
C-3′	73.65	
C-4′	72.81	
C-5′	73.40	
C-6′	177.11	177.02

[a]P.p.m. downfield from tetramethylsilane, 75 MHz; sample pH ~7. Unassigned **1α** absorbance: δ 72.61 (HETCOR correlated to δ 3.98 proton). Spectrum measured in presence of trifluoroacetic salt: $\delta(CF_3CO^-)$ 117.21, $^1J_{^{13}C-^{19}F}$ 292.0(3) Hz; $\delta(CF_3CO^-)$ 163.42, $^2J_{^{13}C-^{19}F}$ 35.3(2) Hz. [b]C(2,2′) not differentiated in assignment (one high-intensity HETCOR correlation cross-peak between δ 72.3 and 3.62 absorbance). [c]Assigned by analogy to corresponding peaks[12] for α-D-galactopyranose residue of 3,6-anhydro-α-L-Gal-(1→3)-α-D-Gal. [d]Assigned from the DEPT spectrum; pulse-width 135°.

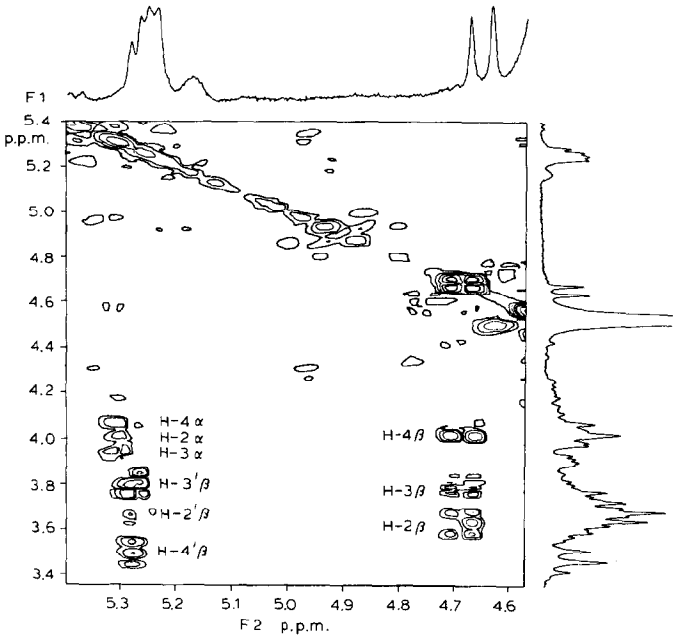


Fig. 1. Double-step RELAY-COSY (partial) spectrum of aldobiuronic acid **1** in D_2O at 25°. Signals appearing on the diagonal for H-1α, H-1'α, and H-1β are at δ 5.27, 5.24, and 4.65, respectively (see Table I).

were found at δ 101.29 and 97.18, whereas those for the minor species were at δ 101.23 and 93.17. As there were no other substantial ^{13}C signals in the anomeric region, the ^{13}C spectrum served to demonstrate that only a single kind of aldobiuronic acid was contained in the syrupy specimen isolated. Overall, these findings indicated that the aldobiuronic acid consists of an α-anomeric nonreducing end-group linked to β- and α-glycose residues in the ratio of ~2:1.

COSY, RELAY-COSY, and J-resolved 2D-n.m.r. experiments. — Connectivity networks of coupled protons were identified by using a double quantum filtered COSY 2D-n.m.r. spectrum[3] (200 MHz). Chemical-shift ambiguity and degeneracy were noted for signals from proton pairs H-2,2', and also for H-3,3', which was resolved *via* a relayed coherence transfer (RELAY-COSY) 2D-n.m.r. experiment[4]. As shown in Fig. 1, this experiment allowed for an extension of these correlations to the respective signals for H-4 and H-4', which were clearly resolved. The observed splittings, given as vicinal coupling-constants, $^3J_{H,H}$, were measured by means of a homonuclear 2D-n.m.r. J-resolved spectrum[5]. Using a semiquantitative Karplus-type[6] relationship, the magnitudes of these vicinal coupling-constants permitted the assignment of configurations at C-1 to C-5 and at C-1' to C-5'. The $^3J_{H,H}$ values listed in Table I point to axial orientations for H-1,-2,-3, and -5, and an equatorial one for H-4 of the major species, corresponding to the *β-galacto-pyranosyl* configuration in the $^4C_1$(D) or $^1C_4$(L) conformation. The proton chemical-

shift values for this major species (see Table I) are each ~0.13(8) p.p.m. downfield *vis-a-vis* those of the protons of β-D-galactopyranose[7], and also the four corresponding vicinal coupling-constants are all very similar to those for β-D-galactopyranose [root-mean-square (r.m.s.) difference[7], 0.3 Hz].

Similarly, the $^3J_{H,H}$ values listed in Table I point to axial orientations for H-2′,-3′,-4′, and -5′, and to an equatorial disposition for H-1′; and all of these values are closely similar to those of α-glucopyranose (r.m.s. difference[7] 0.5 Hz). Hence, the nonreducing-end group must have the *α-glucopyranosyl* configuration in the $^4C_1$(D) or $^1C_4$(L) conformation, and the D configuration was assigned to it on the basis of optical rotation measurements[2]. The proton chemical-shift values of the α-glucopyranosyluronic acid moiety in **1β** are each ~0.21(7) p.p.m. downfield of those for corresponding protons in α-D-glucopyranose[7], and the four vicinal coupling-constants measured are very similar to corresponding values for α-D-glucopyranose (r.m.s. difference[7] 0.5 Hz).

For a glycopyranosyluronic acid group, the H-1 or -1′ and H-5 or -5′ terminal signals of the coupling networks must each reflect only one vicinal proton, whereas the splitting patterns of internal nuclei H-2, -3, -4, or of -2′, -3′, or -4′ should be affected by two vicinal neighbors. Significantly, it was found that the H-5′ signal, at δ 4.06, is only a doublet [10.1 Hz ($^3J_{H\text{-}4',H\text{-}5'}$)]. Additional coupling was not found between it and methylene-type protons potentially present on C-6′. Thus, the H-5′

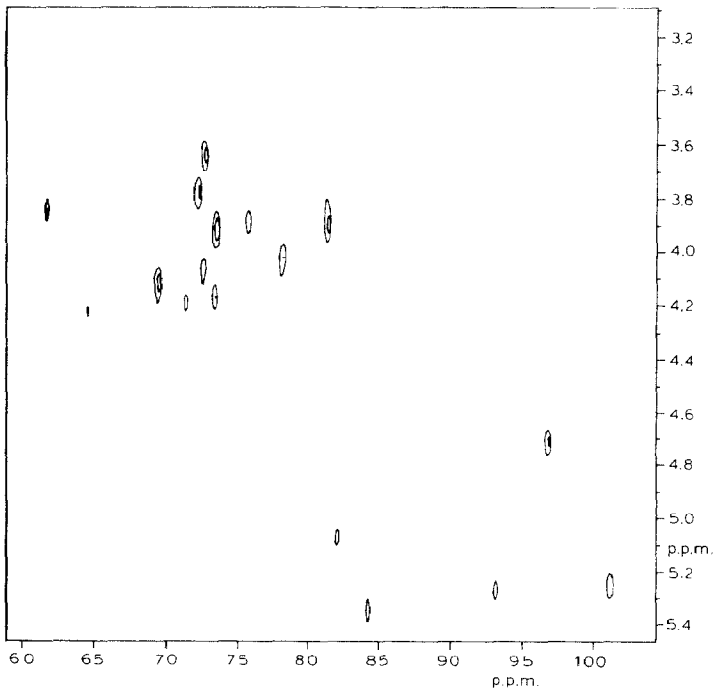


Fig. 2. ^1H–^{13}C Heterocorrelation spectrum (300 MHz) for aldobiuronic acid **1**.

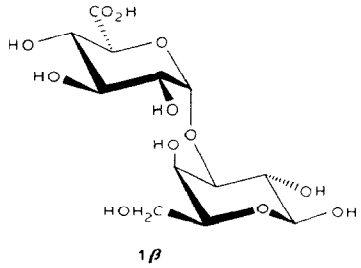
nucleus is the coupling network terminus of the glycopyranosyluronic acid group.

An α-galactopyranosyl configuration for the minor species was ascertained by analogy. The relevant $^3J_{H,H}$ values listed in Table I demonstrate axial orientation for H-2 and -3, whereas H-1 is equatorial. Similarly, the two vicinal coupling-constants measured for this α anomer are both very similar to those in α-D-galactopyranose[7] (r.m.s. difference 0.1 Hz).

^{13}C-N.m.r., DEPT, and HETCOR spectra. — The DEPT[8] (75 MHz, pulse angle 135°) ^{13}C-n.m.r. spectrum showed methylene carbon resonances for the major and minor species at δ 61.86 and 62.01, respectively, in the ratio of ~2:1. The major methylene H-6, -6a assignments, at δ ~3.63, were obtained from a homonuclear decoupled 2D-HETCOR[9] experiment[10]. Conversely, nine of the ten methine carbon assignments were made with the aid of the HETCOR correlation cross-peaks (see Fig. 2) relating previously assigned methine proton resonances. The one unassigned methine carbon signal (δ 76.01) for the major species was designated that of the C-5 nucleus (found to be HETCOR-correlated with a δ-3.70 proton signal, which was then assigned as H-5). Assignment of the latter was not readily forthcoming from the COSY spectrum, most likely because of the small value, 0.9(1) Hz, for $^3J_{H\text{-}4,H\text{-}5}$. Methine carbons associated with glycosidic linkages of the reducing moieties of the disaccharide diastereomers (**1β** and **1α**) were found to resonate characteristically downfield, at δ 81.73 and 78.63, respectively. As correlations of the major C-3 (δ 81.7) with H-3 (δ 3.78), and the minor C-3 (δ 78.6) with H-3 (δ 3.88), were evident in the HETCOR[9] spectrum, the glycosidic bond from the α-glucopyranosyluronic acid group to the β- or α-galactopyranose residue was clearly characterized as being (1→3).

With the exception of the C-1 signal, ^{13}C-n.m.r. chemical-shift values for the α-glucopyranosyluronic acid group in **1β** are more closely similar to those found[10] for corresponding carbons in α-D-glucopyranosyluronate (pH 7.8) (r.m.s. difference, 1.57 p.p.m.) *vis-a-vis* those for the corresponding uronic acid[10] (pH 1.8) (r.m.s. difference, 2.10 p.p.m.). This is consistent with the pH value (~7) of the aldobiuronic acid sample. The chemical shifts of C-1 to C-6 in **1β** are also closely similar to those reported[11] for corresponding nuclei in the β-D-galactopyranose residue of neoagarobiose [*O*-(3,6-anhydro-α-L-galactopyranosyl)-(1→3)-β-D-galactose (**2β**)], the repeating unit of agarose (**1β** *vs.* **2β** r.m.s. difference, 0.54 p.p.m.). Analogously, the ^{13}C signals of the minor component (**1α**) at δ 68.59, 70.09, and 71.48 were assigned to C-2, C-4, and C-5, respectively: the corresponding values for **2α** are[11] δ 68.38, 69.84, and 70.88, respectively (r.m.s. difference, 0.49 p.p.m.). As expected, the chemical-shift values of carbons α and γ to the axial OH-1 of **1α** were characteristically shifted upfield *vis-a-vis* those from the corresponding carbons of the **1β** anomer (Δδ 4.02, 3.12, and 4.53 for C-1, C-3, and C-5, respectively).

Derivatives of 3-*O*-(D-glucopranosyluronic acid)-D-galactopyranose have been isolated from other polysaccharide sources, including mesquite gum *(Prosopis juliflora)*[12] and Keta gum *(Feronia elephantum)*[13]. Hydrolysis of the heparin–protein



1β

linkage region afforded 3-*O*-(β-D-glucopyranosyluronic acid)-D-galactopyranose[14], which was subsequently synthesized by Flowers[15]. Kieras *et al.*[16] obtained 3-*O*-(α-D-glucopyranosyluronic acid)-L-galactopyranose from the unicellular marine red alga *Porphyridium cruentum*. On studying *Porphyridium cruentum* and the fresh-water alga *Porphyridium aerugineum*, Percival and Foyle[17] reported a diastereoisomeric aldobiuronic acid, 3-*O*-(β-D-glucopyranosyluronic acid)-D-galactopyranose, from both algae; the β linkage was deduced from the optical rotation of the disaccharide[17]. A direct correlation in structure between the *Rhodella* product and these aldobiuronic acids was not feasible on the basis of the information earlier available. The present study, which reports the first n.m.r. evidence for structure in the series, should facilitate correlations in the future.

Dubinsky *et al.*[2] recently showed that the acid-catalyzed hydrolyzate of **1** consists of glucuronic acid and galactose and, using the enzyme D-galactose oxidase, found the L configuration for the galactose. Thus, the aldobiuronic acid isolated from *Rhodella reticulata* is presumably the same as that obtained by Kieras *et al.*[16] from *Porphyridium cruentum* polysaccharide. As the analysis of the ^{13}C-n.m.r. spectrum can account for all of the lines of significant intensity, it shows that no other aldobiuronic acid had been obtained by the procedure employed in this study.

EXPERIMENTAL

3-O-(α-D-Glucopyranosyluronic acid)-L-galactopyranose (**1α,β**). — This compound was isolated from the unicellular red alga *Rhodella reticulata* by acid hydrolysis and column chromatographic techniques[2]. Although the aldobiuronic acid was isolated in the presence of trifluoroacetic salts, these did not interfere with, or detract from, the high quality of the ^{1}H- and ^{13}C-n.m.r. spectra recorded.

N.m.r. spectroscopy. — (^{1}H- and ^{13}C)-N.m.r. spectra (7.0 T) were recorded for solutions in deuterium oxide at 293 K, and at 299.9 and 75.4 MHz, respectively, with a Varian XL-300 Fourier-transform n.m.r. spectrometer. The WALTZ[18] broad-band decoupling technique was utilized for the ^{13}C-n.m.r. spectra (including the DEPT-135 spectrum[8], as well as HETCOR[9]), and the deuterium oxide solvent was used as an internal lock. 1,4-Dioxane was used in ^{13}C-n.m.r. spectra as an internal secondary standard (δ 67.4) referenced to tetramethylsilane. Sodium 4,4-

dimethyl-4-silapentane-1-sulfonate was used as an internal standard for ^{1}H-n.m.r. spectra. The homonuclear 2D experiments were recorded at ambient temperatures using a standard Varian microprogram. Pseudo-echo shaping was used for processing with zero-filling in both dimensions. The spectral width was 640 Hz. For COSY-90 experiments[3], 256 increments with 64 transients for each f.i.d. were acquired; the corresponding parameters for RELAY-COSY[4] were 128 increments with 128 transients, and, for 2D J-RESOLVED[5] spectra, 64 increments with 64 transients, all recorded at 200.1 MHz with a Varian XL-200 F.t.-n.m.r. spectrometer.

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