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Note

NMR spectroscopic investigation of oligoglucuronates prepared by enzymic hydrolysis of a (1 → 4)-β-D-glucuronan

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An exopolysaccharide (EPS), excreted in the culture medium of the mutant strain M5N1 CS (NCIMB 40472) of *Rhizobium meliloti*, has been characterized as a partially acetylated (1 → 4)-β-D-glucuronan [1–3]. A similar structure, known as mucoric acid, has thus far only been detected in cell walls of fungi [4] and in extracellular material of Mucorales [5].

The native and the deacetylated polymer were hydrolysed by treatment with cellulase to give a series of homologous oligomers (Fig. 1).

Here we report ^{13}C and ^{1}H NMR spectroscopic analysis of sodium deacetylated (1 → 4)-β-D-oligoglucuronates.

The ^{1}H and ^{13}C NMR spectra of deacetylated glucuronan [2] and monomers such as glucuronic acid or sodium glucuronate were used as references for the chemical-shift assignments. Some discrepancies are noted in the literature values for the carbon chemical shifts of D-glucuronic acid and D-glucuronate monomers [6–8].

The ^{1}H chemical-shift values of sodium glucuronate and glucuronic acid were determined in D_2O solutions at 50°C (Table 1). Values for glucuronate agree with the literature data [9].

The downfield region of the ^{1}H NMR spectrum of oligosaccharides of dp 3 (Fig. 2) and dp > 15 (Fig. 3) shows signals corresponding to H-1 of the different residues and to

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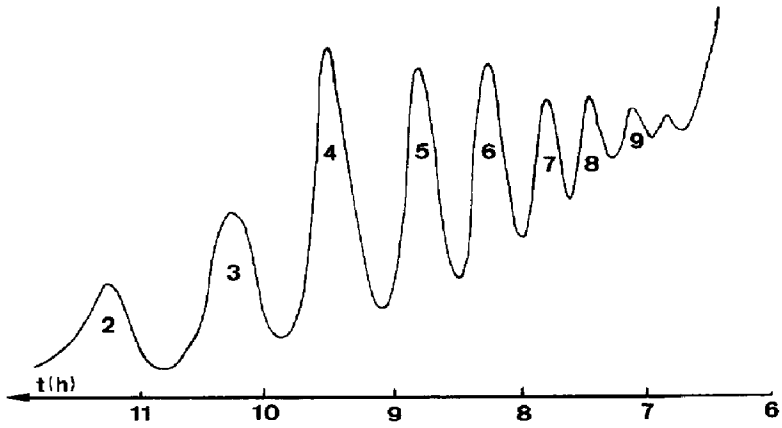

Fig. 1. Fractionation of oligosaccharides obtained by enzymic hydrolysis of a (1 → 4)-β-D-glucuronan on a Bio-Gel P6 column (column 100×2.5 cm; flow-rate, 60 mL h^{-1}; detector, R.I.). The degree of polymerization is indicated by numbers under the peaks.

H-4 of the unsaturated residue. The doublet at 5.83 ppm (J 4 Hz) was attributed to H-4 of the unsaturated unit, in accord with the literature data [10,11]. In the ^1H NMR spectrum of the oligomer having dp > 15 (Fig. 3), signals at 5.21 and 4.64 ppm, with coupling constants of 3.3 and 8.0 Hz, respectively, were attributed to H-1α and H-1β of the reducing unit by comparison with chemical-shift values for α- and β-glucuronates. As the H-1 chemical shift of deacetylated EPS is 4.53 ppm [2,3], resonances at 5.10 and 4.53 ppm were attributed to H-1 of an unsaturated residue and to H-1 of the repeating unit. Similar signals were observed for the oligomer of dp 3. A simple determination of the degree of polymerization of the oligomer was obtained by comparison of the integral of the H-1 or H-4 signals of the unsaturated unit to the integral of the H-1 signal of the central unit.

In Fig. 3, the signals at 3.37, 3.62, 3.69, and 3.86 were attributed respectively to H-2, H-3, H-4, and H-5 of the repeating unit by comparison with the chemical shifts of various protons in glucuronate and deacetylated EPS [2,3]. For oligomers of low dp, the signals of the different units were assigned by selective irradiations of H-4// and H-1α and β signals. The results reported in Table 1 were confirmed by 2D proton–proton correlation experiments and 1D TOCSY experiments performed on a UNITY 400 Varian instrument equipped with a reverse 5-mm ^1H–X probe [12].

The ^{13}C spectra of glucuronic acid and sodium glucuronate were reexamined, and values in D_2O are reported in Table 2. Variations of concentration from 50 to 200 g L^{-1} did not modify the signal resonances. A reversed sequence of chemical shifts for β-D-glucuronate in solution (δC-5 > δC-3 > δC-2) and for β-D-glucuronic acid in solution (δC-3 > δC-5 > δC-2) was confirmed by the study of homo H–H and hetero H–C nuclear chemical-shift correlations (not reported here). A similar phenomenon was observed for the anomeric forms of α-D-glucuronate and α-D-glucuronic acid with δC-3 > δC-5 > δC-2 and δC-3 > δC-2 > δC-5 sequences, respectively.

In the ^{13}C NMR spectra of oligomers dp > 15 (Fig. 4), we observed six major signals at 176.25, 104.43, 83.05, 77.49, 76.44, and 74.90 ppm which correspond to carbons of

Table 1
^{1}H chemical shifts and coupling constants of glucuronic acid, sodium glucuronate, oligoglucuronan, and (1 → 4)-β-D-deacetylated glucuronan

	δ (ppm) H-1	H-2	H-3	H-4	H-5
Glucoronic acid					
α	5.19	3.52	3.68	3.52	4.25
β	4.69	3.31	3.54	3.63	4.01
Sodium glucuronate					
α	5.24	3.58	3.72	3.52	4.06
β	4.63	3.27	3.51	3.53	3.71
Oligoglucuronan, dp 2					
α	5.15 [a]	3.53	3.66	3.66	4.08
β	4.55	3.24	3.47	3.69	3.75
(//)	5.04	3.76	4.03	5.79	
Oligoglucuronan, dp 3					
α	5.23 (3.8)	3.58	3.78	3.69	4.19 (9.8)
β	4.66 (7.9)	3.33 (8.4)	3.62	3.69	3.86 (6.8)
(')	4.53 (7.8)	3.40	3.62	3.69 (9.6)	3.86 (6.8)
(//)	5.16 (5.1)	3.81	4.15 (4.1)	5.83 (4.0)	
Oligoglucuronan, dp 4					
α	5.17	3.57	3.76	3.67	4.22
β	4.59	3.25	3.57	3.69	3.90
	4.49	3.31	3.58	3.73	3.83
"	4.52	3.33	3.62	3.73	3.83
(//)	5.11	3.79	4.08	5.94	
Oligoglucuronan, dp > 15					
α	5.21 (3.3)	n.d.	3.79	n.d.	n.d.
β	4.64 (8.0)	3.32	n.d.	n.d.	n.d.
(n)	4.53 (7.8)	3.37 (8.2)	3.62 (8.6)	3.69 (9.1)	3.86 (9.2)
(//)	5.10 (4.8)	3.82	4.15 (4.2)	5.81 (3.7)	
(1 → 4)-β-D-Deacetylated glucuronan					
(n)	4.53 (7.7)	3.36 (8.0)	3.61 (8.8)	3.69 (9.1)	3.85 (9.0)

[a] Values in parentheses (expressed in Hz) are coupling constants. The prime (') refers to the unit close to the reducing unit, (") refers to β-D-glucosyluronic group attached to the unit ('), (//) refers to the nonreducing unit, and symbol (n) corresponds to the chemical shifts of the repeating unit.

the repeating unit (n). They are unambiguously attributed to C-6, C-1, C-4, C-5, C-3, and C-2, respectively, by comparison with carbon chemical-shift values [2,3] of glucuronate and deacetylated glucuronan. In Figs. 4 and 5, signals at ~ 147 and ~ 109 ppm are attributed, respectively, to C-5 and C-4 of a 4,5-unsaturated glucuronic unit; these results are in good agreement with literature data [13].

The signals corresponding to both central units of the oligomer having dp 4 (Fig. 5) are readily characterized as the chemical shifts are close to those of the repeating unit in the oligomer having dp > 15 and to those of the homopolymer. The other major signals (102.24, 68.21, and 71.85 ppm) are attributed to carbons of the unsaturated unit (noted C//). The signal at 102.24 ppm corresponds to C-1// and the signals at 71.85 and 68.21 ppm are assigned respectively to C-2 and C-3 of an unsaturated residue by

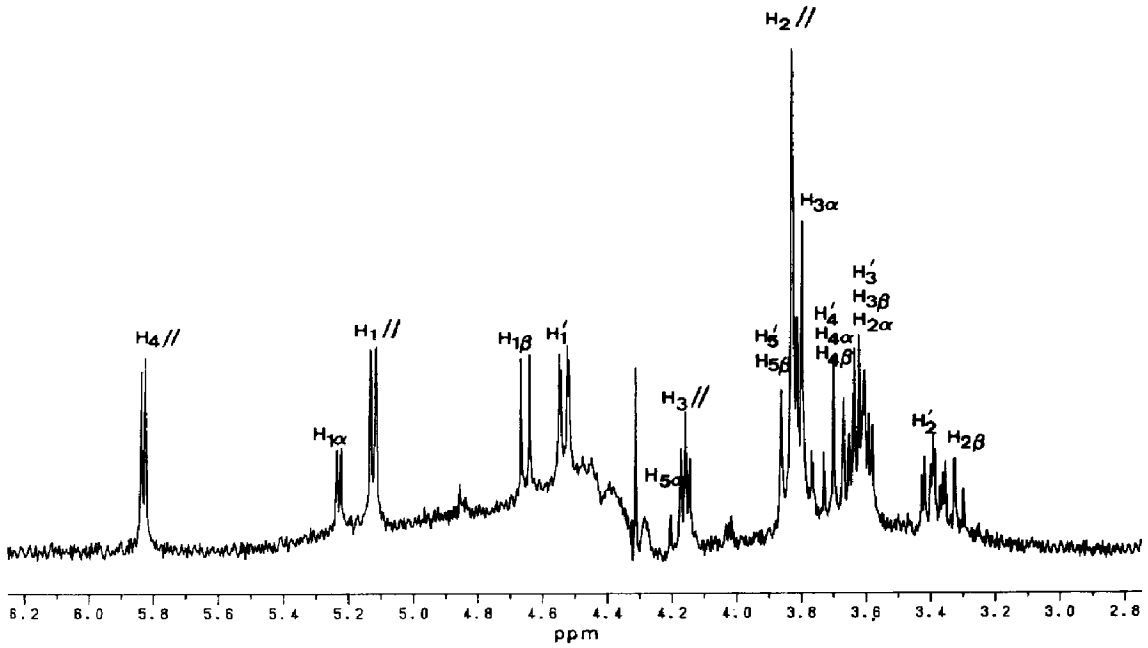

Fig. 2. ^{1}H NMR spectrum of oligoglucuronan, dp 3 in D_2O (at 300 MHz; T, 50°C).

comparison with analysis of the heteronuclear H–C chemical-shift correlation of the dp 2 oligomer.

Because of the anomeric equilibrium, each carbon atom of the reducing unit gives two signals; based on the chemical shifts of the α and β monomers, signals at 97.57 and 93.62 ppm are assigned to C-1β and C-1α respectively and signals at 82.84 and 83.13 ppm to C-4β and C-4α. Chemical shifts of C-2α 72.92, C-2β 75.64, C-3α 73.08, C-3β 76.31 and C-5α 73.35 C-5β 77.56 were assigned on the basis of the results obtained on the oligomer of dp 2. Because of the low stability of the low dp oligosaccharides (dp 3 and 4), we chose to confirm the ^{13}C chemical shifts by the fastest method. Thus we avoided the time-consuming HETCOR method, and employed an ^{1}H selective inversion experiment, which gives only the corresponding ^{13}C signal [14].

By comparison of ^{13}C NMR spectra obtained with oligomers of different dp, we observed a decrease of signals of the two terminal units: signals of C-1 and C-2 of the unit noted (′) and signals of C-3, C-4, and C-5 of the unit close to the unsaturated residue. The other signals were assumed to correspond to the signals of the repeating unit of the β-(1 → 4)-glucuronan [2]. Slight variations in chemical shifts may be related to an increase in rigidity associated with a change in conformation when the degree of polymerization increases.

In conclusion, the results show a random split of the β-(1 → 4)-glucuronan, with the enzyme acting as a lyase and causing β-elimination of 4-*O*-linked glycosidic bonds. An homologous series of oligomers, whose structure is schematically presented in Fig. 6 results. Because of low enzyme activity, it is not obvious that cellulase would be efficient in cleavage of a β-(1 → 4) linkage between two glucuronic units.

1. Experimental

Production of EPS. — The mutant strain *R. meliloti* M5N1 CS (NCIMB 40472) was cultivated at 30°C in a 20-L reactor on RC medium [15] supplemented with sucrose (1%). EPS was isolated from the broth by microfiltration (pore size 0.2 μm), purified by ultrafiltration (100 000 NMWCO membrane) and dried under vacuum at ambient temperature.

Deacetylation of EPS. — Because of the low activity of the enzyme on the native acetylated polysaccharide, the polymer was deacetylated. A complete deacetylation of a 1 g L^{-1} solution of polymer was achieved by treatment with NaOH (pH 10) during 4 h at 80°C (during this drastic treatment a major decrease in molecular weight was observed). The solution was then neutralized with HCl. The deacetylated polysaccharide was precipitated by 2-propanol (50%, v/v) in the presence of M NaCl and dried under vacuum at room temperature.

Procedure for the enzyme assay. — To 500 mL of deacetylated poly (glucuronate) solution (1 mg mL^{-1}) in distilled water heated at 35°C, was added 1 mL of Celluclast 1.5 L (Novo). The enzyme activity was followed by monitoring the decrease of viscosity

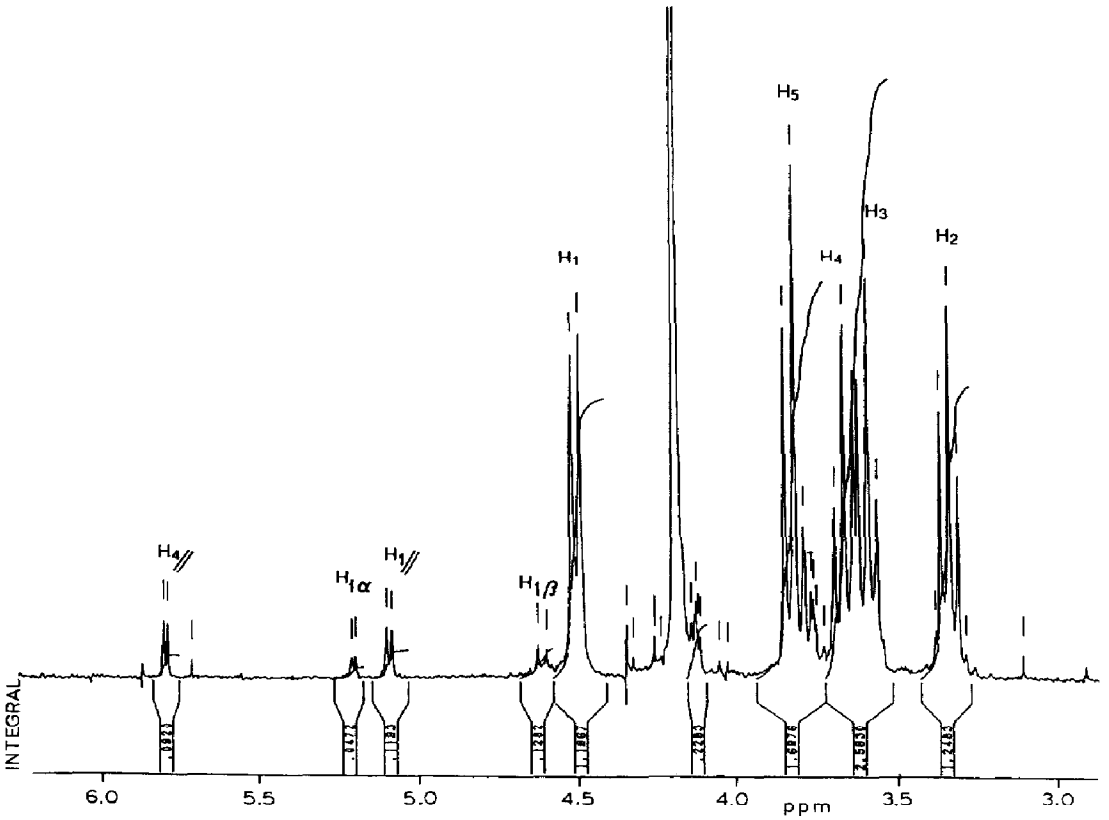

Fig. 3. ^{1}H NMR spectrum of oligoglucuronans, dp > 15 in D_2O (at 300 MHz; T, 70°C).

Table 2
^{13}C chemical shifts of glucuronic acid, sodium glucuronate, oligoglucuronates and deacetylated glucuronan (D_2O; 353 K)

	δ (ppm) C-1	C-2	C-3	C-4	C-5	C-6
Glucuronic acid						
α	93.59	72.33	73.69	72.62	71.77	173.18
β	97.41	75.08	76.63	72.45	75.74	174.07
Sodium glucuronate						
α	93.36	72.65	73.92	73.31	72.87	177.68
β	97.18	75.40	76.94	73.08	77.13	176.76
Oligoglucuronan, dp 2						
α	93.72 [a]	73.00	73.27	82.63	73.51	n.d.
β	97.77	75.82	76.21	82.44	77.93	n.d.
(//)	102.11	71.73	68.00	108.85	146.95	n.d.
Oligoglucuronan, dp 3						
α	93.69	72.88	73.08	83.26	73.36	n.d.
β	97.68	75.58	76.30	82.97	77.67	n.d.
(′)	104.23	74.84	76.02	82.20	77.45	n.d.
(//)	102.16	71.72	68.07	108.76	147.00	n.d.
Oligoglucuronan, dp 4						
α	93.62	72.12	73.08	83.13	73.25	n.d.
β	97.57	75.64	76.31	82.84	77.56	n.d.
(′)	104.03 [b]	74.74 [c]	76.05	82.62	77.19	n.d.
(″)	104.13 [b]	74.62 [c]	75.64	82.23	77.46	n.d.
(//)	102.24	71.85	68.21	108.91	147.15	n.d.
Oligoglucuronan, dp > 15						
α	93.93	73.25	73.57	83.35	73.57	n.d.
β	97.92	75.91	n.d.		77.72	n.d.
(*n*)	104.43	74.90	76.44	83.05	77.49	176.25
(//)	102.58	72.16	68.52	109.13	146.97	n.d.
(1-4)-β-D-Deacetylated glucuronan						
	104.45	74.90	76.46	83.09	77.51	176.47

[a] The prime (′) refers to the unit close to the reducing unit, (″) refers to β-D-glucosyluronic group attached to the unit (′), (//) refers to the nonreducing unit, and symbol (*n*) corresponds to the chemical shifts of the repeating unit.
[b,c] ^{13}C Assignments may be interchanged.

in the mixture incubated at 35°C. The reaction was stopped by heating for 10 min at 100°C.

Fractionation of oligoglucuronates. — The separation of oligoglucuronates was performed on a glass chromatographic column (100 × 2.5 cm) packed with Bio-Gel P6 (Biorad). Samples (300 mg in 4 mL) were injected through a Rheodyne injector with a 5-mL loop. A MR minipump from Touzart et Matignon (France) was used to propel the eluent (a 5×10^{-2} M $NaNO_3$ solution), at a flow rate of 60 mL h^{-1}. Detection was achieved with an Iota refractometer (Jobin Yvon, France). A 201-202 model Gilson Collector was used to collect fractions of 10 mL. Fractions corresponding to the same peak were combined, concentrated, desalted on a column (210 × 1.5 cm) of Bio-Gel P2 and lyophilized.

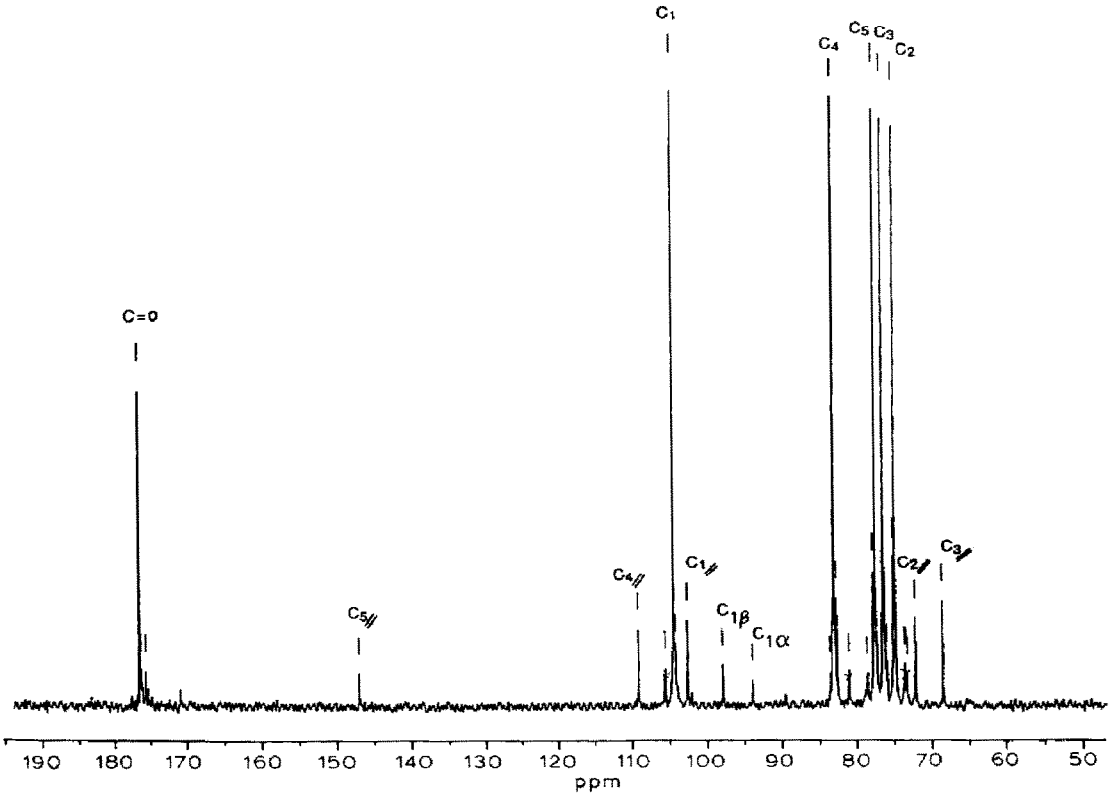

Fig. 4. ^{13}C NMR spectrum of oligoglucuronan, dp > 15 in D_2O (at 75 MHz; T, 70°C).

NMR studies. — NMR analyses were performed with an AC-300 Bruker Fourier transform spectrometer with a 5-mm $^{1}H,^{13}C$ dual probe. To equilibrate exchangeable protons, the different glucuronan oligomers were twice dissolved in D_2O (1 mL) and lyophilized, and then dispersed in 500 μL of 99.96% D_2O.

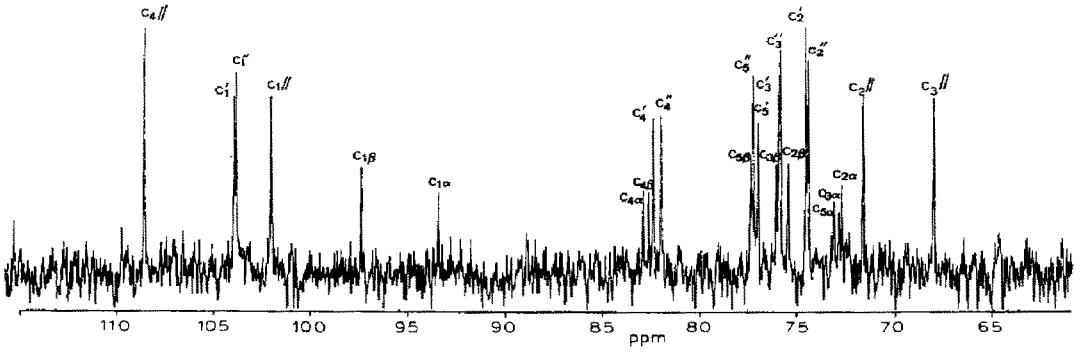

Fig. 5. ^{13}C NMR spectrum of oligoglucuronans, dp 4 in D2O (at 75MHz; T, 50°C).

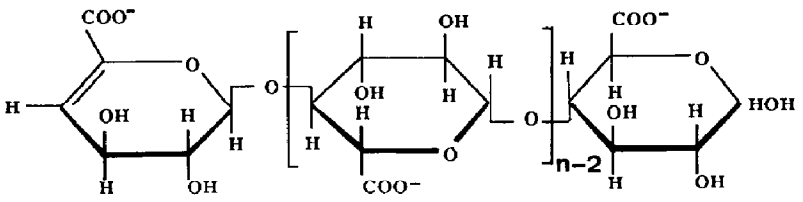

Fig. 6. Schematic representation of the structure of the oligosaccharides released by the action of enzyme on a (1 → 4)-β-D-glucuronan.

^{1}H NMR spectra were obtained using a spectral width of 3000 Hz, a 16K data-block size and a pulse duration of 8 μs; 16 scans with AQ = 2.73 s were accumulated. An H_2O presaturated signal experiment with a delay of 3 s and decoupler power of 20 dB at low range, using the standard Bruker PRESAT sequence, was used; selective ^{1}H signal irradiations were performed using the PRESATHD Bruker sequence under the same conditions for water signal presaturation and 25 dB at low range power for selective irradiation.

^{13}C NMR spectra were recorded using a spectral width of 15000 Hz, a 16K data-block size and a pulse duration of 6 μs; the number of scans was ~ 30 000 with standard C.P.D. decoupling conditions. Acetone was used as the external standard (δ_H 2.10 ppm; δ_C 31.45 ppm).

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