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Structural investigation of a heteropolysaccharide isolated from the green fruits of *Capsicum annuum*

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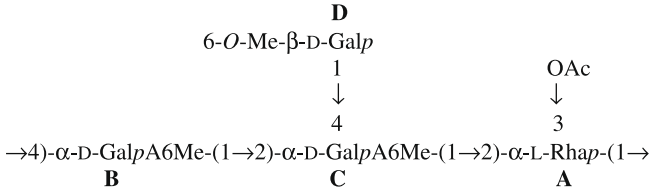
ABSTRACT

A water-soluble polysaccharide isolated from the hot water extract of the green fruits of *Capsicum annuum* was found to consist of 3-*O*-acyl-L-rhamnose, D-methyl galacturonate, 6-*O*-methyl-D-galactose in a molar proportion of nearly 1:2:1. Structural investigation of the polysaccharide was carried out using total hydrolysis, methylation analysis, periodate oxidation followed by GLC–MS, and NMR experiments. On the basis of the above-mentioned experiments it is concluded that the following repeating unit is present in the polysaccharide



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Several *Capsicums* with 20–27 species are reported[1] of which fruits of five important species (*Capsicum annuum, Capsicum baccatum, Capsicum chinense, Capsicum frutescens,* and *Capsicum pubescens*) of this genus are commonly available and cultivated worldwide including India.[2] Green fruits of *C. annuum* are available in the local market of Midnapore city throughout the whole season and used as a vegetable. The chemical constituent of this fruit was found to consist of a volatile alkaloid, capsaicin[3] that is reported to concentrate in the inner part of the fruit from which pungency arises. Capsaicin plays an important role in the physiological and pharmacological effects on the sensory and cardiovascular systems.[4] Fruits are also a good source of Vitamins A, C, and E, which are present in high concentration in various types[5] of peppers. Flavonoid and phenolic components in *Capsicum* are reported as antioxidants[6] which play an important role in cancer chemoprevention.[7] The extract of the whole fruit *Capsicum* is used by the local people as a tonic for the heart and stomach stimulant.[8] The fresh fruit with mustard seeds is used as a drug in India as a counter-irritant.[9] The hot water extract of crude polysaccharide of fruits of *C. annuum* showed potent anti-complementary activity[10] but no work relating to the structure of the polysaccharide was carried out. In the present investigation a polysaccharide was isolated using hot water extraction and repeated purifications and was characterized with a view to study its structural as well as the immunological properties. Here, only the structural characterization of the polysaccharide is reported.

The pure polysaccharide has a specific rotation of $[\alpha]_D^{25} + 36.0$ (*c* 0.6, water). The molecular weight[11] of the polysaccharide was found to be $\sim 1.65 \times 10^5$ Da. Paper chromatographic analysis of the hydrolyzed polysaccharide showed the presence of galacturonic acid, rhamnose, and a slow moving spot nearer to galactose. The absolute configuration of the sugar units was determined by the method of Gerwig et al.[12] and confirmed by NMR spectroscopy. GLC analysis of the alditol acetates of the sugars showed the presence of rhamnose and 6-*O*-methyl-galactose. The carboxy-methyl-reduced[13] polysaccharide on hydrolysis, followed by GLC analysis of the corresponding alditol acetates showed the presence of galactose, 6-*O*-methyl-galactose, and rhamnose. From the above GLC analysis it is clear that the uronic acids are methyl esterified. Methylation studies,[14] followed by GLC–MS analysis revealed the presence of 1,2,3,5-tetra-*O*-acetyl-4-mono-*O*-methyl-rhamnitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-galactitol in a molar ratio of 1:1. From the above-mentioned analysis we can conclude that the methoxy-galactose is present at the non-reducing end. The methylated reduced[15] polysaccharide showed peaks corresponding

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to 1,2,3,5-tetra-*O*-acetyl-4-mono-*O*-methyl-rhamnitol, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-galactitol, 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-galactitol, and 1,2,4,5,6-penta-*O*-acetyl-3-mono-*O*-methyl-galactitiol in a molar ratio of nearly 1:1:1:1. These results indicate the presence of non-reducing-end methoxy-Gal*p*, (1→2,3)-linked-Rha*p*, (1→4)-linked-Gal*p*A6Me, and (1→2,4)-linked-Gal*p*A6Me. Thereafter, a periodate oxidation experiment was carried out with the polysaccharide. The periodate oxidized, reduced material upon hydrolysis with trifluoro acetic acid followed by GLC analysis showed the presence of rhamnose, which indicates that methoxy-galactose was consumed during oxidation. The GLC–MS analysis of the periodate-oxidized followed by methylated reduced[15] polysaccharide showed that 1,2,3,5-tetra-*O*-acetyl-4-mono-*O*-methyl-rhamnitol and 1,2,4,5,6-penta-*O*-acetyl-3-mono-*O*-methyl-galactitol were retained. This result indicates that the non-reducing-end methoxy-Gal*p*, (1→4)-linked-Gal*p*A6Me was consumed by the diol oxidizing reagent.

The ^{1}H NMR spectrum (500 MHz) (Fig. 1) of this polysaccharide at 27 °C showed four anomeric proton signals at δ 5.12, 5.07, 4.94, and 4.62 ppm in a molar ratio of nearly 1:1:1:1. The signal has an unusually down field chemical-shift of one of the ring protons in the anomeric region, indicated by 5.08 ppm. The singlet at δ 2.05 is indicative of the CH_3 proton of an acetic ester. The doublet at δ 1.24 ppm may be for the CH_3 proton of deoxy sugar, rhamnose. The sugar residues were designated as **A**–**D** according to their decreasing anomeric proton chemical shifts (Table 1). In the ^{13}C NMR spectrum (125 MHz, Fig. 2) at 27 °C four anomeric carbon signals appeared at δ 99.6, 99.98, 100.8, and 104.78 ppm in a ratio of nearly 1:1:1:1. Furthermore δ 17.05, 20.56, 53.29, and 61.17 ppm were assigned for CH_3 of rhamnose, CH_3 of acetic ester, carbomethoxy carbon, and *O*-methyl carbon, respectively.

Residue **A** has an anomeric proton chemical shift at 5.12 ppm. From the 2D homocorrelation spectrum, the identity of each proton signal was assigned. Residue **A** was determined as Rha*p* due to signals for an exocyclic CH_3 group and the weak coupling between H-1, H-2, and H-3. $J_{H\text{-}1,H\text{-}2} \sim 1.9$ Hz, $J_{H\text{-}1,C\text{-}1} \sim 170$ Hz, indicate that it is an α-linked residue. In addition to this, the C-5 signal of rhamnose appeared at δ 68.88 ppm, instead of 75 ppm, characteristic signal for β-rhamnosyl residue, hence it is further supported that rhamnose is present in α-configuration. The resonance for H-3 of rhamnose is shifted an additional 1.27 ppm downfield, to 5.08 ppm with respect to the standard value of methyl glycosides[16,17] strongly suggesting that *O*-3 of the rhamnose is the site of the acetylation. Each carbon signal was identified by using the ^{13}C–^{1}H heterocorrelation spectrum. The signal for C-3 of the residue **A** was shifted 0.74 ppm downfield with respect to the standard value of methyl glycosides. Moreover, the appended group exhibits a three-bond ^{1}H–^{13}C coupling with the sugar, **A** H-3, **A** OAc (carbonyl carbon) proving the site of acetylation in the HMBC experiment (Fig. 3). The downfield shift of C-2 (75.58) signal with respect to the standard value of methyl glycosides[16,17] and methylation analysis indicates that residue **A** is 1,2-linked 3-*O*-acetyl L-rhamnose. The spin system of residue **B**, which has only five protons with a relatively high chemical shift of the H-5 signal (δ 4.43) and weak coupling between H-3, H-4, and H-5, indicated that residue **B** is of D-Gal*p*A. The anomeric signal of moiety **B** at δ 5.07 and $J_{H\text{-}1,C\text{-}1} \sim 171$ Hz, indicated that D-galacturonosyl residue is α-linked. The C-4 peak of residue **B** at δ 78.08 showed a downfield shift compared to that of standard methyl glycosides[16,17] due to the α-glycosylation effect. The carbon signals of residue **B** were observed at δ 68.27, 70.98, 72.14, and 171.08 or 170.55 (not assigned) corresponding to C-2, C-3, C-5, and C-6 (carbonyl carbon), respectively. The residue **C** has an anomeric proton chemical shift at δ 4.94 ppm and this spin system also consisting of only five protons with a weak coupling between H-3, H-4, and H-5, indicate that it is a D-Gal*p*A. The singlet of H-1 and the characteristic $J_{H\text{-}1,H\text{-}2}$ coupling constant value of <3 Hz, the H-1/H-2 intra-residual correlation in the NOESY spectrum (Fig. 4), and $J_{H\text{-}1,C\text{-}1} \sim 170$ Hz, showed that the residue **C** is α-D-Gal*p*A. The downfield shift of C-2 (79.41 ppm), C-4 (78.79 ppm) signals, with respect to the standard value of methyl glycosides indicates that residue **C** is 1,2,4-linked α-D-Gal*p*A. The carbon signals of residue **C** were observed at δ 70.98, 72.14, and 170.55 or 171.08 (not assigned) corresponding to C-3, C-5, and C-6 (carbonyl carbon), respectively. The carbonyl groups of both galacturonic acids are present as methyl ester. The presence of a carboxymethyl group in residues **B** and **C** is confirmed by the appearance of intra-residual coupling between the ester carbonyl carbon (δ 171.08 or δ 170.55) and the carboxymethyl proton (δ 3.78) in the HMBC experiment (Fig. 3, Table 2). These indicate that residue **B** is the methyl ester of a 1,4-linked α-D-Gal*p*A and residue **C** is methyl ester of a 1,2,4-linked α-D-Gal*p*A. The residue **D** has an anomeric proton chemical shift at δ 4.62 ppm. A large coupling constant $J_{H\text{-}1,H\text{-}2}$ value (~8.5 Hz) and $J_{H\text{-}1,C\text{-}1}$ value (162 Hz) indicate that it is a β-linked residue. The $J_{H\text{-}2,H\text{-}3}$ value (~9.1 Hz) and $J_{H\text{-}3,H\text{-}4}$ value (~3.4 Hz) indicate that it

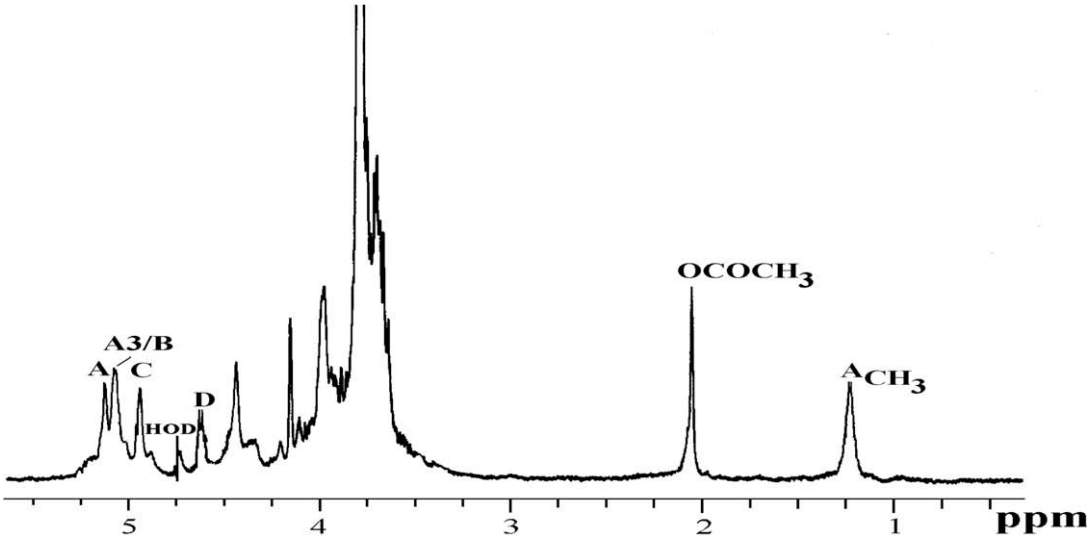


Figure 1. ^{1}H NMR spectrum (500 MHz, D_2O, 27 °C) of the polysaccharide isolated from *C. annuum*.

Table 1
^{1}H and ^{13}C NMR chemical shifts of polysaccharide recorded in D_2O at 27 °C for *C. annuum*[a,b] (δ, ppm)

Glycosyl residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a,H-6b/C-6	COOMe	3-*O*-COMe	6-*O*-Me
A →2)-α-L-Rha*p*-(1→ 3↑ OAc	5.12 99.6	3.96 75.58	5.08 72.24	3.61 71.84	4.01 68.88	1.24 17.05		2.05[f] 20.5[g] 171.23[h]	
B →4)-α-D-Gal*p*A6Me-(1→	5.07 99.98	3.68 68.27	3.81 70.98	3.98 78.08	4.43 72.14	171.08[e] or 170.55[e]	3.78[c] 53.29[d]		
C →2,4)-α-D-Gal*p*A6Me-(1→	4.94 100.8	3.71 79.41	3.76 70.98	4 78.79	4.43 72.14	170.55[e] or 171.08[e]	3.78[c] 53.29[d]		
D 6-*O*-Me-β-D-Gal*p*-(1→	4.62 104.78	3.65 71.8	3.75 73.73	4.15 70.98	3.81 74.93	3.72, 3.66 68.21			3.8 61.17

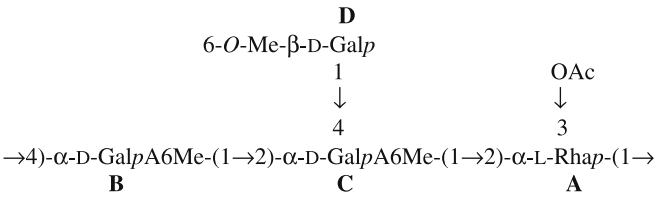
[a] Values of the ^{13}C chemical shifts were recorded with reference to using acetone as internal standard and fixed at 31.05 ppm at 27 °C.
[b] Values of the ^{1}H chemical shifts were recorded and assigned with respect to the HOD signal fixed at 4.74 ppm at 27 °C.
[c] Proton value of the ester group.
[d] ^{13}C chemical shift value of the methyl carbon in ester group.
[e] ^{13}C chemical shift value of the carbonyl group of the carboxyl group.
[f] Methyl proton value of OAc group.
[g] Methyl carbon value of OAc group.
[h] Carbonyl carbon value of OAc group.

is a β-D-galactosyl residue. Chemical shifts from H-1 to H-6 were assigned from 2D-COSY and TOCSY spectra. On the basis of the proton assignments, the chemical shifts of C-1 to C-6 were obtained from the ^{1}H–^{13}C HMQC spectrum (Fig. not shown). The methoxy group at C-6 of residue **D** was confirmed by the appearance of cross coupling between the methoxy proton (δ 3.8 ppm) and the C-6 atom of D-galactose [**D** OCH_3 (H), **D** C-6] and between methoxy carbon δ 61.17 and its H-6a, H-6b atoms [**D** OCH_3 (C), **D** H-6a] and [**D** OCH_3 (C), **D** H-6b] in the HMBC experiment (Fig. 3, Table 2). Thus considering the result of methylation analysis and NMR experiment, it may be concluded that **D** is a β-linked, nonreducing-end 6-*O*-Me D-galactopyranosyl moiety.

The sequence of the glycosyl residues of the polysaccharide was determined from NOESY (Fig. 4, Table 3) as well as ROESY (Fig. not shown) experiments followed by confirmation HMBC experiment. Residue **A** has interresidue NOE contacts from H-1 to H-4 of residue **B**, residue **B** has NOE contacts from H-1 to H-1, and H-2 of residue **C.** Similarly, residue **C** has an interresidue NOE contact from H-1 to H-1, and H-2 of residue **A**, and finally cross-peak between **D** H-1 and **C** H-4 was observed along with other intra-residue contacts. Thus, from the NOESY experiment the following sequences are established:

A (1→4) **B**; **B** (1→2) **C**; **C** (1→2) **A**; **D** (1→4) **C**

The sequences of glycosyl residues were determined using long-range ^{13}C–^{1}H correlation in the HMBC spectrum (Fig. 3). The acetyl group at C-3 of residue **A** was confirmed the results of the NOESY experiment (Table 3). Cross-peaks were found between H-1 of residue **A** and C-4 of residue **B** (**A** H-1, **B** C-4); and between C-1 of residue **A** and H-4 of residue **B** (**A** C-1, **B** H-4). The 3-position of residue **A** attached with acetyl group was confirmed by the presence of cross-peak **A** H-3, **A** OAc (carbonyl carbon). Cross-peaks were observed between H-1 (δ 5.07) of residue **B** and C-2 (δ 79.41) of residue **C** (**B** H-1, **C** C-2), C-1 of residue **B** (δ 99.98), and H-2 of residue **C** (**B** C-1, **C** H-2). The cross-peaks between H-1 (δ 4.94) of residue **C** and C-2 (δ 75.58) of residue **A** (**C** H-1, **A** C-2), C-1 (δ 100.8) of residue **C** and H-2 of residue **A** (**C** C-1, **A** H-2) were observed. Similarly, the cross-peaks between H-1 (δ 4.62) of residue **D** and C-4 (δ 78.79) of residue **C** (**D** H-1, **C** C-4), C-1 (δ 104.78) of residue **D**, and H-4 (δ 4.00) of residue **C** (**D** C-1, **C** H-4) were observed. Therefore, the tetrasaccharide repeating unit for the polysaccharide is assigned as follows:



1. Experimental

1.1. Isolation, fractionation, and purification of the crude polysaccharide

The fruits of C. annuum (1.0 Kg) were collected from the local market, washed with water, and then cut into small pieces and boiled in 250 mL of distilled water for 6 h. The whole mixture was kept overnight at 4 °C and then filtered through linen cloth. The filtrate was centrifuged at 10,000 rpm (using a Heraeus Biofuge Stratos Centrifuge) for 1 h at 4 °C. The supernatant was collected and precipitated in ethanol (1:5 v/v). It was kept overnight at 4 °C and centrifuged as above. The precipitated material was washed with ethanol five times and then dialyzed through dialysis tubing of cellulose membrane (Sigma–Aldrich, retaining MW >12,400) against distilled water for 12 h to remove low-molecular weight materials. The aqueous solution was then collected from the dialysis tubing and freeze-dried, yielding 1.1 g of crude polysaccharide.

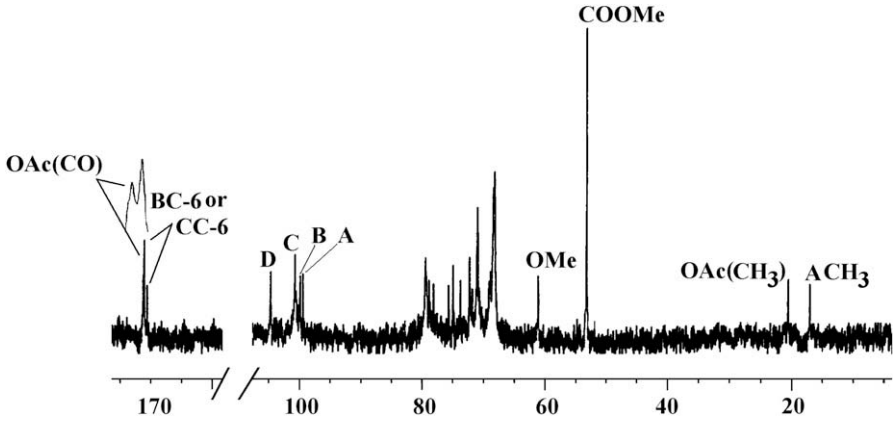


Figure 2. ^{13}C NMR spectrum (125 MHz, D_2O, 27 °C) of the polysaccharide isolated from *C. annuum*.

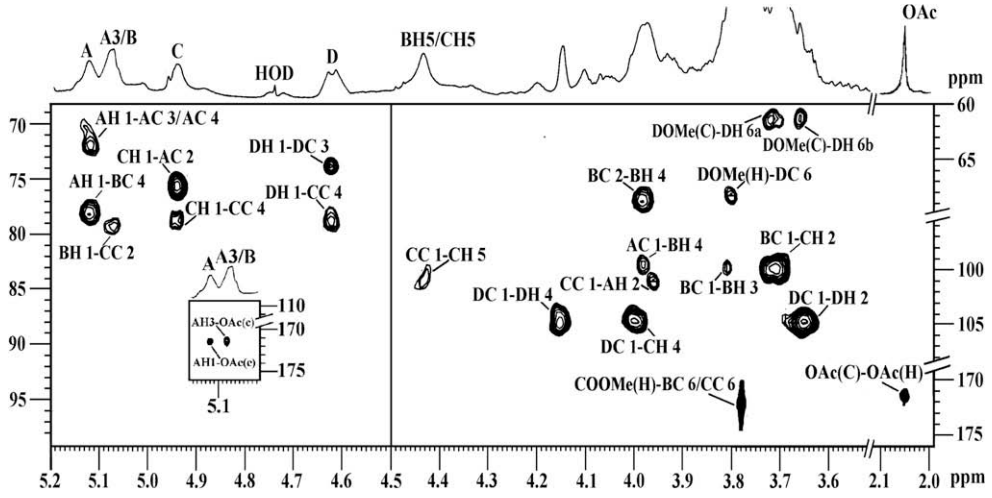


Figure 3. HMBC spectrum of polysaccharide, isolated from *C. annuum*. The delay time in the HMBC experiment was 80 ms.

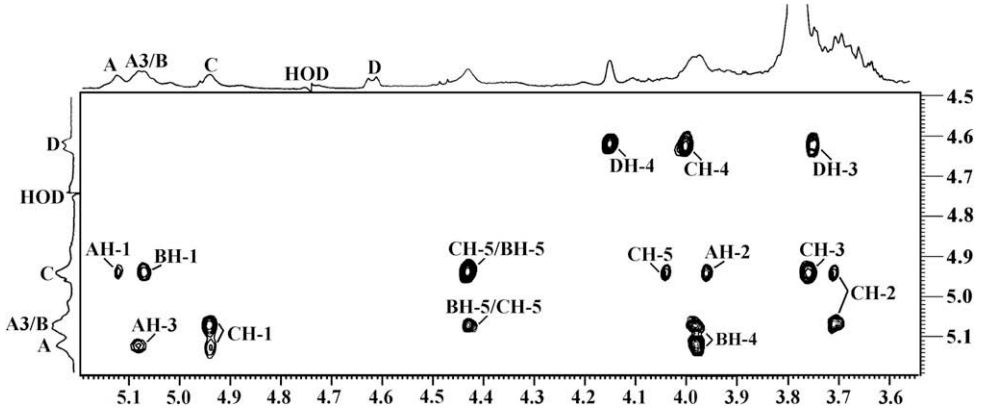


Figure 4. NOESY spectra of the polysaccharide isolated from *C. annuum*. The NOESY mixing time was 300 ms.

The crude polysaccharide (30 mg) was purified by gel permeation chromatography on column (90 × 2.1 cm) of Sepharose 6B in water as eluant with a flow rate of 0.5 mL min^{-1} using Redifrac fraction collector. Eighty-five test tubes (2 mL each) were collected, and monitored spectrophotometrically at 490 nm with phenol–sulfuric acid reagent[18] using a Shimadzu UV–vis spectrophotometer, model-1601. One fraction (test tubes 22–45) was collected and freeze-dried, yielding 21 mg of material. The purification process was carried out in seven lots, yield 144 mg.

1.2. Monosaccharide analysis

1.2.1. Alditol acetate analysis

The polysaccharide sample (4.0 mg) was hydrolyzed with 2 M CF_3CO_2H (2 mL) in a round-bottomed flask at 100 °C for 16 h in a boiling water bath. The excess acid was removed by co-distillation with water. Then, the hydrolyzed product was divided into two parts. One part was examined by paper chromatography in solvent systems X and Y. Another part was reduced with $NaBH_4$, followed

Table 2
The significant $^{3}J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide of *C. annuum*

Residue	Sugar linkage	Observed connectivities	
		Inter	Intra
A	→2)-α-L-Rha*p*-(1→ 3↑ OAc	**A**H-1/**B**C-4 **A**C-1/**B**H-4	**A**H-1/**A**C-3 or **A**H-1/**A**C-4
B	→4)-α-D-Gal*p*A6Me-(1→	**B**H-1/**C**C-2 **B**C-1/**C**H-2	**B**C-1/**B**H-3 **B**C-2/**B**H-4
C	→2,4)-α-D-Gal*p*A6Me-(1→	**C**H-1/**A**C-2 **C**C-1/**A**H-2	**C**H-1/**C**C-4 **C**C-1/**C**H-5
D	6-*O*-Me-β-D-Gal*p*-(1→	**D**H-1/**C**C-4 **D**C-1/**C**H-4	**D**H-1/**D**C-3 **D**C-1/**D**H-2 **D**C-1/**D**H-4
A	→2)-α-L-Rha*p*-(1→ 3↑ OAc		**A**OAc(H)/**A**OAc(CO) **A**OAc(CO)/**A**H-1 **A**OAc(CO)/**A**H-3
B	→4)-α-D-Gal*p*A6Me-(1→		**B**COOMe(H)/**B**C-6
C	→2,4)-α-D-Gal*p*A6Me-(1→		**C**COOMe(H)/**C**C-6
D	6-*O*-Me-β-D-Gal*p*-(1→		**D**OMe(H)/**D**C-6 **D**OMe(C)/**D**H-6a, **D**H-6b

Table 3
NOE data for the polysaccharide isolated from *C. annuum*

Anomeric proton	δ_H	NOE contact protons		
Glycosyl residue		δ_H	Residue	Atom
→2)-α-L-Rha*p*-(1→ 3↑ **A** OAc	5.12	3.98 5.08 4.94	**B** **A** **C**	H-4 H-3 H-1
→4)-α-D-Gal*p*A6Me-(1→ **B**	5.07	4.94 3.71 3.98 4.43	**C** **C** **B** **B**/**C**	H-1 H-2 H-4 H-5
→2,4)-α-D-Gal*p*A6Me-(1→ **C**	4.94	5.12 3.96 5.07 3.71 3.76 4.43	**A** **A** **B** **C** **C** **C**/**B**	H-1 H-2 H-1 H-2 H-3 H-5
6-*O*-Me-β-D-Gal*p*-(1→ **D**	4.62	4.00 3.75 4.15	**C** **D** **D**	H-4 H-3 H-4

by acidification with dilute CH_3CO_2H. It was then co-distilled with pure CH_3OH to remove excess boric acid. The reduced sugars were acetylated with 1:1 pyridine–Ac_2O in a boiling water bath for 1 h to give the alditol acetates. These were then analyzed by GLC using column (A) 3% ECNSS-M on Gas Chrom Q (100–120 mesh) and column (B) 1% OV-225 on Gas Chrom Q (100–120 mesh) at 170 °C. Gas–liquid chromatography-mass spectrometric (GLC–MS) analysis was also performed on a Hewlett–Packard 5970A automatic GLC–MS system, using an HP-5 capillary column (25 m × 25 mm). The program was isothermal at 150 °C; hold time 2 min, with a temperature gradient of 4 °C min^{-1} up to a final temperature of 200 °C. Quantification was carried out from the peak area, using response factors from standard monosaccharide.

1.2.2. Preparation of carboxy methyl reduced polysaccharide[13]

The polysaccharide (1.0 mg) was dissolved in 1 M imidazole-hydrochloric acid buffer, pH 7.0 (200 μL/mg) and cooled on ice. $NaBH_4$ (40 mg) was then added, and the reaction mixture was maintained on ice for at least 1 h. The excess $NaBH_4$ was decomposed by adding HOAc (100 μL/40 mg $NaBH_4$) slowly to the cooled sample. An equal volume of redistilled water was then added, and the reduced polysaccharide was precipitated by adding 3–4 vols of 95% (v/v) EtOH (2 mL). The sample was reprecipitated two more times with 95% EtOH and freeze-dried. The carboxymethyl-reduced polysaccharide was hydrolyzed with 2 M CF_3CO_2H for 18 h at 100 °C, and after the usual treatment, the sugars were analyzed by GLC.

1.3. Methylation analysis

The polysaccharide (4.0 mg) was methylated using the Ciucanu and Kerek method.[14] The methylated products were isolated by partition between $CHCl_3$ and water (5:2, v/v). The organic layer containing products was washed with 3 mL water three times and dried. The methylated products were then hydrolyzed with 90% HCOOH (1 mL) at 100 °C for 1 h, reduced with $NaBH_4$, acetylated with 1:1 Ac_2O–pyridine, and analyzed by GLC (using columns A and B) and GLC–MS (using HP-5 fused silica capillary column) using the same temperature program indicated as above. A portion of the methylated polysaccharide (2.0 mg) was dissolved in dry

THF (2 mL), refluxed with lithium aluminium hydride[15] (40 mg) for 5 h, and kept overnight at room temperature. The excess of the reducing agent was decomposed by adding EtOAc and aq THF. The inorganic materials were filtered off. The carboxyl-reduced methylated product was hydrolyzed with formic acid as before, and the alditol acetate of the reduced methylated sugars was prepared in the usual way and analyzed by GLC and GLC–MS experiments.

1.4. Periodate oxidation

The polysaccharide (5 mg) was oxidized with 0.1 M $NaIO_4$ (2 mL) at 27 °C in the dark during 48 h. The excess periodate was destroyed by adding 1,2-ethanediol, and the solution was dialyzed against distilled water. The dialyzed material was reduced by $NaBH_4$ for 16 h and neutralized with AcOH. The resulting material was co-distillation with MeOH to remove excess boric acid. The periodate-reduced material was divided into two portions. One portion was hydrolyzed with 2 M CF_3CO_2H for 18 h, and alditol acetates were prepared as usual for the GLC analysis. Another portion was methylated by the Ciucanu and Kerek method followed by LAH reduction and alditol acetate preparation. These alditol acetates were analyzed by GLC–MS.

1.5. Paper chromatographic studies

Paper partition chromatographic studies were performed on Whatmann Nos. 1 and 3 MM sheets. Solvent systems used were (X) BuOH–HOAc–H_2O (v/v/v, 4:1:5, upper phase), and (Y) EtOAc–pyridine–H_2O (v/v/v, 8:2:1). The spray reagent used was alkaline silver nitrate solution.[19]

1.6. Absolute configuration of the monosaccharides

The method used by Gerwig et al.[12] is as follows. After the hydrolysis of the polysaccharide (1 mg) by trifluoroacetic acid, the acid was removed by co-distillation with water. A solution of 250 μL of 0.625 M HCl in R-(+)-2-butanol was added to it, and the mixture was heated at 80 °C for 16 h. The reactants were than evaporated, and per-*O*-TMS-derivatives were prepared with *N*,*O*-bis(trimethylsilyl) trifluroacetamide (BSTFA). The products were analyzed by GLC using a capillary column (SPB-1, 30 m × 0.26 mm) with a temperature program (3 °C/min) from 150 to 210 °C. The (+)-2-butyl 2,3,4,6-tetra-*O*-TMS-glycosides obtained were identified by comparing with those prepared from the D- and L-enantiomers of the monosaccharide.

1.7. Optical rotation

Optical rotation was measured by a Perkin–Elmer model 241 MC spectropolarimeter at 25 °C.

1.8. Determination of molecular weight

The molecular weight of the polysaccharide fraction was determined by a gel-chromatographic technique. Standard dextrans[11] T-200, T-70, and T-40 were passed through a Sepharose 6B column, and then the elution volumes were plotted against the logarithms of their respective molecular weights. The elution volume of the polysaccharide was then plotted in the same graph, and the molecular weight of the polysaccharide was determined.

1.9. NMR studies

Prior to NMR-spectroscopic analysis, sample was kept over P_2O_5 in vacuum for several days, and then deuterium-exchanged[20] by lyophilizing with D_2O (99.96% atom ^2H, Aldrich) four times. With a Bruker Avance DPX-500 spectrometer, ^1H, TOCSY, DQF-COSY, NOESY, ROESY, and HMQC, HMBC NMR spectra were recorded at 27 °C. The ^1H and ^{13}C (both ^1H coupled and decoupled) NMR spectra were recorded at 27 °C. Proton chemical shifts refer to residual HOD at δ 4.74 ppm using the WEFT pulse sequence.[21] Carbon chemical shifts refer to internal acetone at δ 31.05 ppm. The 2D-DQF-COSY experiment was carried out using standard Bruker software at 27 °C. The 2D-TOCSY experiment was recorded at a mixing time of 150 ms, and complete assignment required several TOCSY experiments having mixing times ranging from 60 to 300 ms. Both NOESY and ROESY spectra were recorded with a mixing time 300 ms. The HMBC spectrum was recorded using a delay time of 80 ms.

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