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THE CYCLIC STRUCTURE OF β-D-(1→2)-LINKED D-GLUCANS SECRETED BY *Rhizobia* AND *Agrobacteria*[*,†]

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ABSTRACT

The β-D-(1→2)-linked D-glucans secreted by *Rhizobia* and *Agrobacteria* have been characterized by using fast-atom bombardment–mass spectrometry and ^{13}C-nuclear magnetic resonance spectroscopy. The D-glucans are shown to be unbranched, cyclic molecules varying in size from 17 to at least 24 residues. Some of these molecules are fragmented during permethylation procedures.

INTRODUCTION

Rhizobia and *Agrobacteria*, the nodule- and crown gall-forming bacteria, synthesize and secrete an interesting family of low-molecular-weight β-D-(1→2)-linked D-glucans[2–7]. The observation that these D-glucans lack reducing-terminal D-glucose residues led us[5] and others[6,7] to suggest that they may have a cyclic structure. However, cyclic molecules could not be distinguished from linear molecules containing unidentified functional groups at the reducing termini of the D-glucosyl chains. We now present data, obtained by fast-atom bombardment–mass spectrometry (f.a.b.–m.s.) and ^{13}C-n.m.r. spectroscopy, that unambiguously demonstrate that the β-D-(1→2)-linked D-glucans are unbranched, cyclic molecules. We also show that the D-glucans are partially degraded under the conditions routinely used to permethylate polysaccharides, and that this degradation can result in misleading data when the methylation products are analyzed.

Two recent technological innovations have made the m.s. part of this work possible. These are the development of high-field-magnet m.s.[8], which allows the study of large molecules at high sensitivity, and the introduction of the novel ioni-

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zation technique of f.a.b.–m.s.[9]. F.a.b.–m.s. is eminently suited to the molecular-weight determination of high-mass, polar molecules[10,11], and recent reports have demonstrated[12,13] its applicability to the determination of oligosaccharide structures. The studies reported herein constitute a further illustration of the valuable role that f.a.b.–m.s. can play in the characterization of complex, biological samples.

EXPERIMENTAL

Mass spectrometry. — Fast-atom-bombardment–mass spectra were recorded with a VG Analytical High Field ZAB-1F mass spectrometer operated at accelerating voltages of 8 kV (mass range 3300), 7 kV (3770), or 5 kV (5280 u). Xenon was used as the bombarding gas, and the atom gun was operated at 10 kV.

Spectra, which were recorded on u.v.-sensitive chart-paper, were acquired by using a 500-s, mass-controlled, linear scan over the full mass-range defined by the accelerating voltage used. Improved signal-to-noise ratio of the high-mass peaks was in some cases achieved by operating the instrument in the "magnet-integrating scan" mode. In these experiments, scans of ~400-u span were recorded at a scan rate of 1 u/s. Peak masses were assigned either by manual counting or by comparison with the Hall probe-controlled, mass marker, which was calibrated between *m/z* 3000 and 5500 by using the f.a.b. spectrum of cesium iodide. In all numbers presented in the text, mass assignments correspond to either the counted or the calibrated mass (details given in the Figure legends) of the most-abundant ion in each cluster. At high mass, the most intense peak is the ^{13}C-isotope peak. A distinction must be made between counted and calibrated spectra, because values assigned by manual counting correspond to the nominal masses of the ions, whereas those assigned by calibration correspond to the accurate masses of the ions. For a permethylated carbohydrate with a molecular weight of 5000, the difference between the exact and the nominal values is ~2 u.

F.a.b.-mass spectra were obtained by dissolving native D-glucans in 5% aqueous acetic acid (10–20 μg/μL), and loading 1 μL of the solution into a drop of glycerol on the stainless-steel target. In salt-dosing experiments, a 1-μL aliquot of the salt solution (M NaCl or M KCl) was also added. Permethylated D-glucans were dissolved in methanol (5–10 μg/μL), and 1 μL was loaded into the glycerol on the target, followed by additions of M aqueous ammonium thiocyanate (1 μL) and 1-thioglycerol (0.2 μL). In the Results and Discussion section, the term "matrix" is used to describe the mixture of glycerol and additives in which the sample is dissolved during the f.a.b. experiments.

Nuclear magnetic resonance — ^{13}C-N.m.r. spectra were recorded with a Bruker 250-MHz, Fourier-transform, n.m.r. spectrometer. The samples were dissolved in D_2O at a concentration of ~50 mg/mL in a 5-mm, n.m.r. tube. Chemical shifts were assigned relative to 1,4-dioxane, whose chemical shift was taken to be 67.4 p.p.m. downfield from Me_4Si. The machine parameters included a spectrum

frequency of 62.9 MHz, broad-band decoupling, 32 k of memory, 31° pulse, 1.8 s between pulses, and a sweep width of 13.5 kHz. The spectra were recorded at ambient temperature, and a typical experiment consisted of 28,000 pulses.

Bacterial cultures and isolation of β-D-(1→2)-linked D-glucans. — The *Rhizobium* and *Agrobacterium* strains were those previously described[5], except for *Agrobacterium tumefaciens* C58-C1, which was a gift from M. Van Montagu (State University of Ghent, Ghent, Belgium). Growth of cultures, and purification of β-D-(1→2)-linked D-glucans, were performed as previously described[5].

Methylation analysis. — Freeze-dried, β-D-(1→2)-linked D-glucan (0.5 mg) was dissolved in dry Me_2SO (0.5 mL). Sodium methylsulfinyl carbanion (0.18 mmol) was added, and the solution was stirred for 2 h. Methyl iodide or perdeuteriomethyl iodide (0.18 mmol) was added, and the solution was stirred for 1 h. Sodium methylsulfinyl carbanion (0.18 mmol) was again added, and the solution was stirred for 1 h. Methyl iodide or perdeuteriomethyl iodide (1.8 mmol) was added, and the mixture was stirred overnight. Other methylations were performed by using milder or harsher conditions, as described in the text. *O*-Methylated products were isolated by modifying the procedure of T. Waeghe of this laboratory (unpublished procedure), as follows. Water (0.5 mL) was added to the reaction mixture. The resulting suspension was applied to a "Baker 10" (1 mL) C18 silica cartridge (J. T. Baker Chemical Co.) The reaction vessel was rinsed with 1:1 Me_2SO–H_2O (500 μL), and rinse was also applied to the C18 silica cartridge. Me_2SO and salts were eluted with H_2O (1.2 mL), followed by 15% MeCN (0.6 mL) and then by 20% MeCN (0.6 mL). *O*-Methylated products were eluted with 100% MeCN (0.6 mL), and finally with abs. ethanol (0.6 mL). The MeCN and ethanol eluates were pooled. Glycosyl-linkage compositions were determined as previously described[5].

Assay for reducible glucose. — Reducing-terminal D-glucose residues were analyzed as previously described[5], except that gas–liquid chromatography was performed in a Supelco SP-2330 glass capillary-column, with an oven temperature of 200°. D-Glucose pentaacetate was eluted as three peaks, and D-glucitol hexaacetate as a single peak.

Partial hydrolysis of the glucan. — The β-D-(1→2)-linked D-glucan was dissolved (2 mg/mL) in 2M trifluoroacetic acid (TFA), and the solution heated for 70 min at 68°, resulting in hydrolysis of 4% of the glycosidic linkages. Heating for an additional 90 min resulted in hydrolysis of 9% of the linkages. The 4%- and 9%-hydrolyzed samples were analyzed by ^{13}C-n.m.r. spectroscopy. The 9%-hydrolyzed sample was separated into several fractions by liquid chromatography on Bio-Gel P-2, BioRad, as previously described[5], and the fractions were analyzed by f.a.b.–m.s.

RESULTS AND DISCUSSION

Fast-atom bombardment–mass spectrometry of the β-D-(1→2)-linked D-glu-

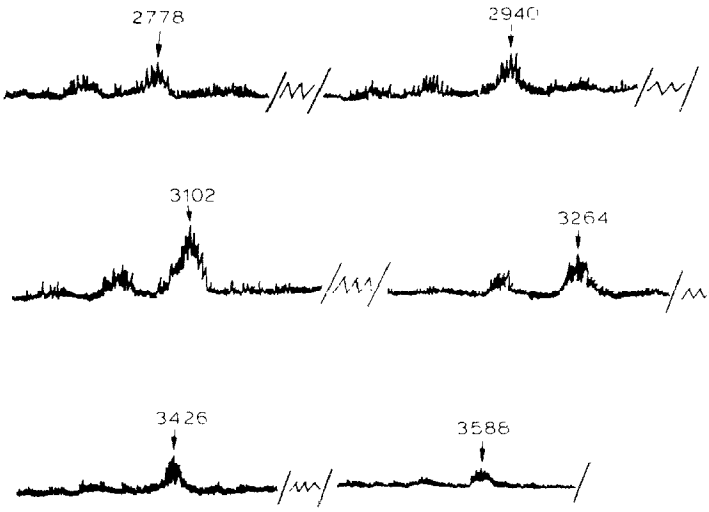


Fig. 1. Positive f.a.b.–mass spectrum (m/z >2700) of a sample of *R. leguminosarum* β-D-(1→2)-linked D-glucan. [The mass spectrometer was operated at 7 kV accelerating voltage and a mass-controlled, linear scan of 500-s duration, from m/z 3700 to 10, was acquired. The counted mass of the most abundant ion in each cluster is given.]

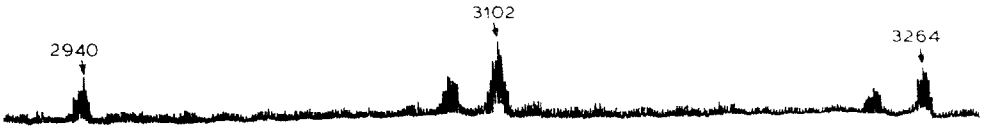


Fig. 2. Positive f.a.b.–mass spectrum of a sample of *R. leguminosarum* β-D-(1→2)-linked D-glucan, spectrum obtained by scanning from m/z 3300 to 2900 at a scan rate of 1 μ/s. [The mass spectrometer was operated at 8 kV accelerating voltage. The spectrum was counted manually from the m/z 2940 signal, which was assigned by comparison with the spectrum shown in Fig. 1.]

cans. — Samples of β-D-(1→2)-linked D-glucan from *Rhizobium leguminosarum* 128c53, *R. phaseoli* 127 K14, and *Agrobacterium tumefaciens* 11156 (ATCC) and C58-C1 were analyzed by f.a.b.–m.s. Comparable data were obtained for all three samples. Full positive f.a.b.–m.s. spectra were acquired by scanning the mass range 3700–10 u. These spectra were characterized by the presence of molecular-ion clusters in the high-mass region (m/z >2700), and a second ion-cluster for each molecular-ion cluster equivalent to the loss of a water molecule. No significant fragment-ions were present at m/z <2700.

The major signals present in the spectrum obtained for a sample of D-glucan from *R. leguminosarum* are illustrated in Fig. 1. This spectrum was manually counted to mass 3000, but a poor signal-to-noise ratio of the background ions at m/z >3000 prevented further counting. To overcome this problem, slow mass-scans over a narrow mass-range were conducted, yielding readily countable spectra of the quality illustrated in Fig. 2.

The data contained in Figs. 1 and 2 are interpreted as follows. Sodium-

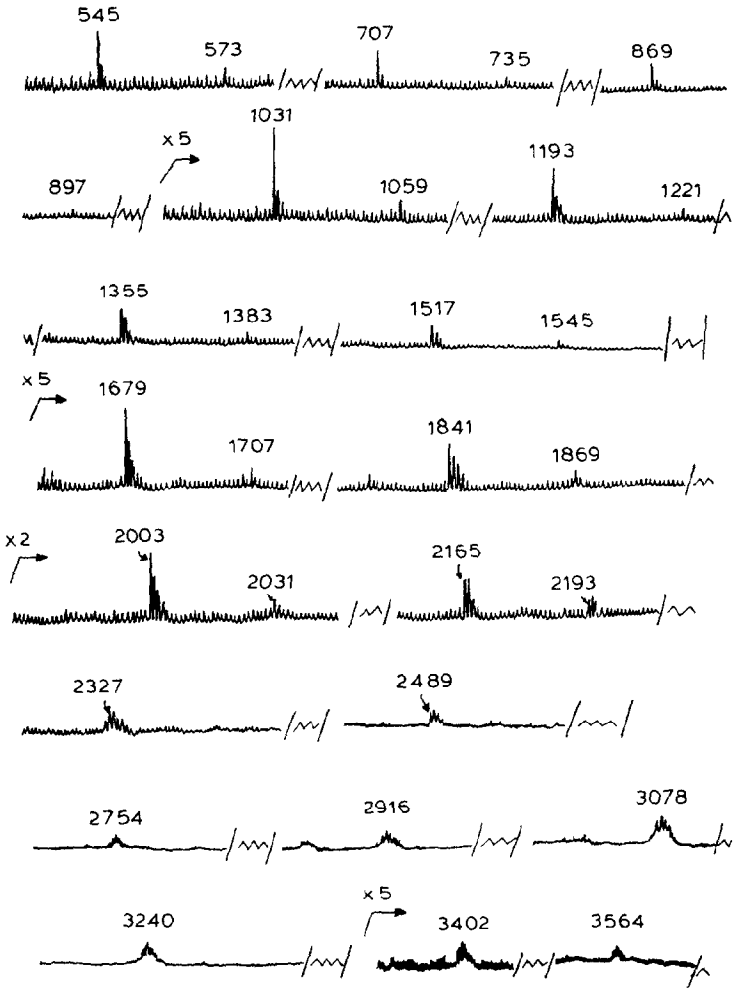


Fig. 3. Negative f.a.b.–mass spectrum (m/z >500) of a sample of *R leguminosarum* D-glucan. [The mass spectrometer was operated at 7 kV accelerating voltage and a mass-controlled, linear scan of 500-s duration from m/z 3700 to 10 was acquired. The spectrum was counted manually.]

cationized, molecular-ion clusters are present 162 u apart (the mass of a glucosyl residue) at m/z 2778, 2940, 3102, 3264, 3426, and 3588 (see the Experimental section for an explanation of the mass assignments used for clusters of high-mass ions). Loss of water from each molecular ion yields the weaker clusters 18 u below the major signals. The presence of sodium was established by salt-dosing experiments. Addition of sodium chloride to the sample did not alter the spectra, whereas addition of potassium chloride shifted all signals 16 u to higher mass, consistent with the replacement of sodium by potassium. The molecular weights (given as the nominal, ^{12}C-isotopic values) of the major D-glucans present in the sample

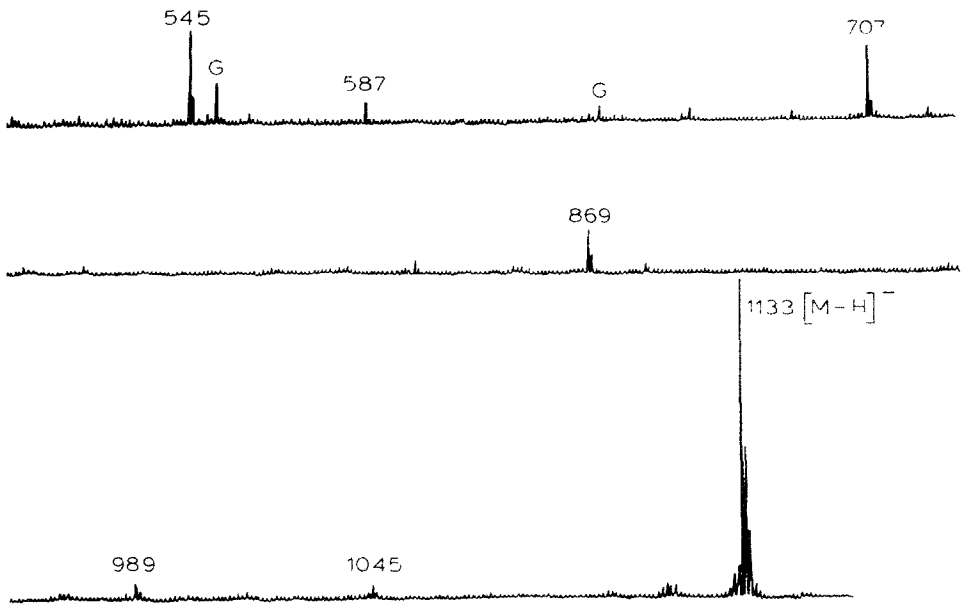
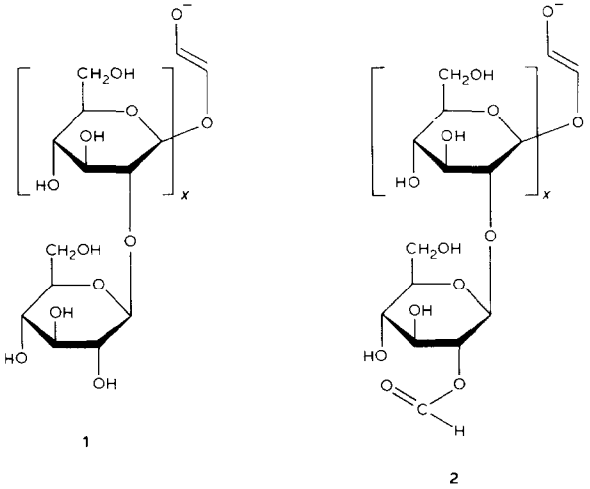


Fig. 4. Negative f.a.b.–mass spectrum (m/z >500) of cyclomaltoheptaose (β-cyclodextrin). [Glycerol cluster ions are labelled G.]

are, therefore, 2754, 2916, 3078, 3240, 3402, and 3564, which correspond precisely to the molecular weights of cyclic polymers varying in size from 17 to 22 D-glucosyl residues.

Corroborative evidence for the existence of cyclic D-glucans was afforded by negative f.a.b.–m.s. The negative, f.a.b.–mass spectrum of the *R. leguminosarum* glucan mixture is shown in Fig. 3. Molecular-ion clusters are present at m/z 2754, 2916, 3078, 3240, 3402, and 3564, *i.e.*, at the masses calculated for the ^{13}C peak in the $(M - H)^-$ cluster of cyclic D-glucans containing 17 to 22 D-glucosyl residues.

Extensive fragmentation occurs in the negative-ion mode (in contrast to the behavior of these molecules in positive f.a.b.–m.s.), giving rise to the series of intense signals commencing at m/z 545 and continuing at intervals of 162 u up to m/z 2489 (see Fig. 3). Fragment-ions that may occur at m/z <500 are difficult to distinguish from signals arising from the matrix (see the Experimental section for a definition of this term). The fragment-ions that occur at m/z >500 are, unfortunately, structurally uninformative, because it is known from other work that the same ions are present in the spectra of a variety of D-glucans, including linear straight-chain and linear branched molecules[14]. To confirm that cyclic D-glucans can be cleaved to yield fragment-ions identical in mass to those produced by their linear counterparts, cyclomaltohexa-, -hepta-, and -octa-ose- (α-, β-, and γ-cyclodextrin) standards (Sigma) were analyzed in a manner similar to that for the β-D-glucans. Typical data obtained for β-cyclodextrin are reproduced in Fig. 4; the signals at m/z 545, 707, and 869 should be noted. The $(545 + 162_n)$ u series of ions observed in all of the D-glucan and cyclodextrin spectra is analogous to the Series A ions previ-

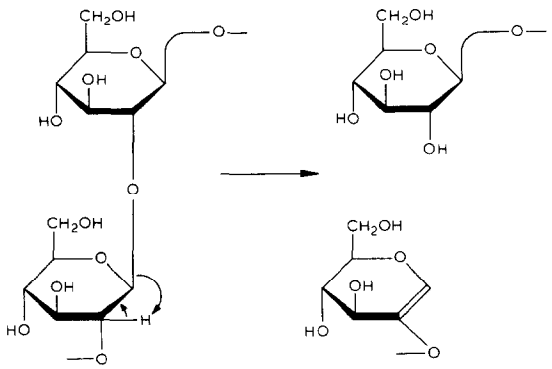


1 2

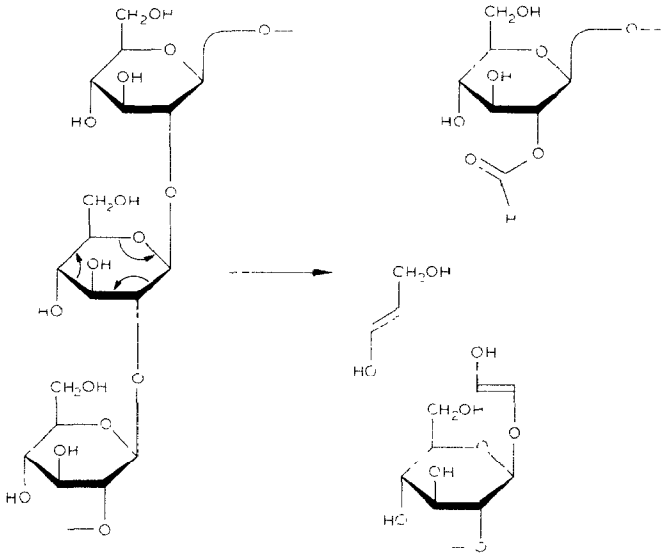
Fig. 5. Structure assigned to the major fragment-ions in the negative f.a.b.–mass spectra of the β-D-(1→2)-linked D-glucans. [The negative charge was assigned to the position indicated, because this structure is resonance-stabilized, but the charge possibly resides elsewhere in the ion.]

ously described in a report of the f.a.b.–mass spectra of a 6-*O*-methylglucose polysaccharide[15]. Structure **1** (see Fig. 5) is assigned to this $(545 + 162_n)$ u ion series present in the β-D-(1→2)-linked D-glucan spectra. Comparable structures may be drawn for D-glucans linked at O-3 and O-4.

To generate ions of structure **1** from a cyclic polymer, two cleavages must occur. One cleavage affords the reducing end of the ion, and another cleavage, the nonreducing end of the ion. A mechanism such as that illustrated in Scheme 1 could produce the nonreducing end of the ion (see upper product, Scheme 1). A mechanism such as that illustrated in Scheme 2 could produce the reducing end of the ion (see lower product, Scheme 2). These two cleavages could occur simultaneously, or consecutively, in either order, in conjunction with the loss of a proton, to yield an ion of type **1** (see Fig. 5).



Scheme 1. Proposed mechanism for glycosidic cleavage in negative f.a.b.–m.s.



Scheme 2. Proposed mechanism for cleavage of a D-glucosyl residue to produce either type **1** or type **2** fragment-ions. [Type **1** ions are formed if the cleavage shown is accompanied by a glycosidic cleavage (see Scheme 1); type **2** ions are formed if the cleavage shown is repeated at another position in the chain.]

Linear D-glucans should yield type **1** ions (see Fig. 5) if only the cleavage illustrated in Scheme 2 occurs. These ions are, in fact, observed in the spectra of linear D-glucans[14]. Linear D-glucans should also yield a different series of ions if only the cleavage illustrated in Scheme 1 occurs. This series corresponds in mass to $[M - H]^-$ ions of intact, linear D-glucans having fewer D-glucosyl residues than the parent D-glucan. This series, which is not observed in the spectra of circular D-glucans, is usually observed in the spectra of linear D-glucans.

A detailed examination of Fig. 3 reveals a second, considerably weaker, series of fragment-ions lying 28 u above each of the members of the *m/z* 545 series. Structure **2** is here suggested for this ion series (see Fig. 5). This fragment-ion series may arise from two occurrences of the fragmentation process shown in Scheme 2, once at each end of the fragment (see Scheme 2, upper and lower products).

In summary, the major, high-mass signals present in both the positive and negative spectra of the β-D-(1→2)-linked D-glucan samples correspond to molecular ions ($[M + Na]^+$ and $[M - H]^-$, respectively) of cyclic polymers varying in size from 17 to 22 residues. The fragmentation pattern exhibited by these molecules in the negative-ion mode is consistent with the assignment of cyclic structures. It is, however, recognized that a possible, alternative explanation of the data is that linear β-D-(1→2)-linked D-glucans are unstable in the glycerol matrix, and completely eliminate one molecule of water during the f.a.b. experiment. This possibility was discounted by examining the behavior in f.a.b.–m.s. of linear β-D-(1→2)-linked D-glucans formed by hydrolysis of cyclic β-D-(1→2)-linked D-glucan. After

TABLE I

^{13}C-N M R -SPECTRAL CHEMICAL-SHIFT[a] DATA

Sample	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-1'*	*C-2'*	*C-3'*	*C-4'*	*C-5'*	*C-6'*
α-Sophorose	92.4	81.2	72.3	70.1[b]	71.7	61.3[b]	104.4	73.9	76.5[b]	70.1[b]	76,5[b]	61.3[b]
β-Sophorose	95.2	82.2	76.5[b]	70.1[b]	76.5[b]	61.3[b]	103.2	74.1	76.5[b]	70.1[b]	76.5[b]	61.3[b]
β-D-Glucan	102.6[b]	82.8[b]	76.8[c]	69.5[b]	76.1[c]	61.4						

[a]Assigned by using the values of Usui *et al* [16] [b]Signal is in a group of signals centered at the chemical shift indicated. [c]Unambiguous assignment not possible with present data

separation by exclusion chromatography on Bio-Gel P-2, the hydrolysis products were analyzed by negative f.a.b.–m.s. A number of large fragments were isolated from the hydrolysis products, in addition to intact starting-material. The signals present in the f.a.b.–m.s. spectra of these compounds were of the exact mass calculated for the $(M - H)^-$ ions of fully hydrated, linear molecules. For example, one of the column fractions had a spectrum containing signals at *m/z* 1709, 1961, and 2123 that correspond to the pseudomolecular ions of linear polymers that are 11,

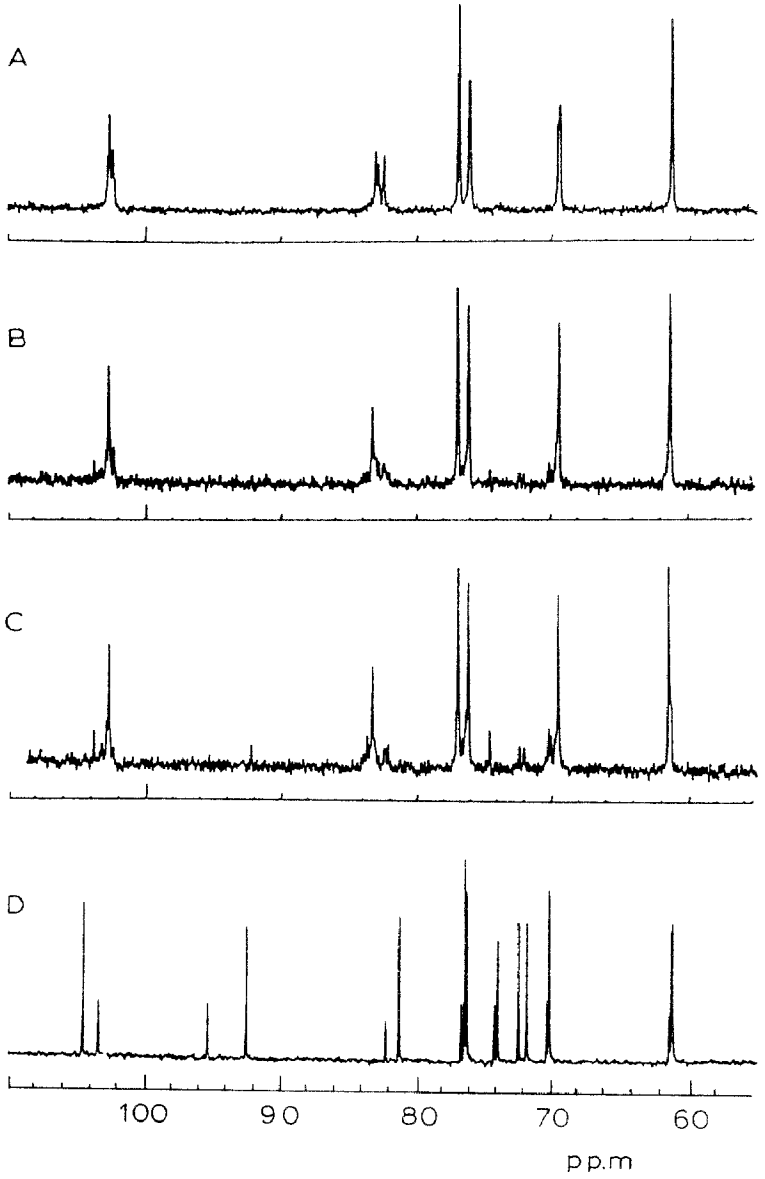


Fig. 6. ^{13}C-N.m.r. spectra of (A) native, (B) 4% *c*-hydrolyzed, and (C) 9% *c*-hydrolyzed β-D-(1→2)-linked D-glucan isolated from *A. tumefaciens* 11156 (ATCC), and (D) sophorose.

12, and 13 residues in length. Only very minor signals were present for loss of water from each molecular ion. These results provide evidence that the linear β-D-(1→2)-linked D-glucans behave analogously to their 3-, 4-, and 6-linked relatives, and are not more likely to undergo dehydration reactions when subjected to the conditions of f.a.b.–m.s. We conclude, therefore, that the β-D-(1→2)-linked D-glucans isolated from *Rhizobia* and *Agrobacteria* are cyclic molecules.

Two structures are possible for a cyclic D-glucan polymer: (*1*) a cyclic polysaccharide having a side chain(s) extending from a branch point(s), or (*2*) an circular polysaccharide having no terminal or branched residues. Methylation analysis is normally the method chosen for determining the number of branching residues and nonreducing termini in a polysaccharide. However, this procedure could not be used to distinguish between possibilities (*1*) and (*2*), because the β-D-(1→2)-linked D-glucans are partially degraded during methylation. Instead, we used ^{13}C-n.m.r. spectroscopy in order to establish that these cyclic D-glucans are unbranched.

^{13}C-N.m.r. spectroscopy of the β-D-(1→2)-linked D-glucans. — ^{13}C-N.m.r.-spectral studies were conducted on the β-D-glucan samples by using sophorose (2-*O*-β-D-glucosyl-α,β-D-glucose) as a model compound. Assignment of signals for sophorose and the native D-glucan are given in Table I. These assignments are based on the results of Usui *et al.*[16] (see also, Fig. 6). However, Usui *et al.* did not distinguish between the two signals from C-2 of the nonreducing D-glucosyl group of α-sophorose and β-sophorose. These two signals are resolved in the ^{13}C-n.m.r. spectrum in Fig. 6; the weaker signal, at 74.1 p.p.m., is assigned to β-sophorose, and the stronger, at 73.9 p.p.m., to α-sophorose, because α-sophorose is the more abundant of the two species[16]. The chemical-shift data uniformly differ from those of Usui *et al.*[16] by ~1 p.p.m., which is not unusual for spectra recorded with different instruments. The carbon atoms of the reducing D-glucose residue are referred to as C-1–C-6, and those of the D-glucosyl group as C-1′–C-6′.

Signals in the spectrum of the β-D-(1→2)-linked D-glucan may be unambiguously assigned, except for those of C-3 and C-5, which could not be distinguished, by comparison to the sophorose standard. The carbon atoms of β-D-(1→2)-linked D-glucosyl residues have chemical shifts similar to those of the corresponding carbon atoms of the (nonreducing) D-glucosyl group of β-sophorose, except for that of C-2. The chemical shift of C-2 of β-D-(1→2)-linked D-glucosyl residues is similar to that of C-2 of the reducing D-glucose residue of β-sophorose; this is because a substituent on the oxygen atom attached to any carbon atom of a sugar affects[16] the chemical shift of the signal of that carbon atom, moving it downfield by 8 to 11 p.p.m. The β-D-(1→2)-linked D-glucosyl residues have such a substituent attached to O-2 (*i.e.*, the next D-glucosyl residue), so that the chemical shift of C-2 of a β-D-(1→2)-linked D-glucosyl residue is ~82 p.p.m., not 74 p.p.m.

The absence of a signal having a chemical shift of ~74 p.p.m. (see Fig. 6) in the ^{13}C-n.m.r. spectrum of the β-D-(1→2)-linked D-glucan indicates that the native polymer contains no D-glucosyl residues that are nonsubstituted at O-2, and there

TABLE II

ADDITIONAL ^{13}C-N.M.R.-SPECTRAL SIGNALS RESULTING FROM PARTIAL HYDROLYSIS OF β-D-(1→2)-LINKED D-GLUCAN WITH ACID

Carbon atom number	*Position of carbon atom*	*Predicted chemical shift*	*Observed chemical shift*	
			4% Hydrolysis	*9% Hydrolysis*
2	nonreducing terminus	74.1	74.5	74.6
1	nonreducing terminus	103.2	103.7	103.8
1	reducing terminus (α)	92.4	n.d.[a]	92.3
1	reducing terminus (β)	95.2	n.d.	n.d.
3	reducing terminus (α)	72.3	72.2	72.3
5	reducing terminus (α)	71.7	71.9	72.0

[a]Not detected

are, therefore, no (nonreducing) terminal D-glucosyl groups in the molecule. The similar absence of signals at 92–96 p.p.m. (C-1 of reducing glucose) is consistent with the absence of a reducing D-glucose residue.

The validity of the foregoing conclusion was tested by determining the limits of detection of the n.m.r. spectrometer with a sample containing terminal D-glucose residues and D-glucosyl groups at a concentration equivalent to that expected if they are present at one residue per molecule in the cyclic β-D-(1→2)-linked D-glucan analyzed. The ^{13}C-n.m.r. spectra of two samples of partially hydrolyzed D-glucan were compared to that of native, cyclic D-glucan, using similar concentrations of D-glucan and the same data-acquisition parameters (see Fig. 6). The results of this experiment (see Fig. 6) indicated that (*1*) the limit of detection for several carbon nuclei of interest is below that required to detect 1 unique residue in 20, and (2) when terminal residues are generated by partial hydrolysis of the D-glucan with acid, signals having appropriate values of chemical shift arise in the ^{13}C-n.m.r. spectrum of the hydrolysis product(s). It was, therefore, concluded that, if a terminal residue (1 out of 20) were present in the molecule it would be detectable by ^{13}C-n.m.r. spectroscopy.

Terminal (nonreducing) D-glucosyl groups and (reducing) D-glucose residues are generated by partial hydrolysis of the D-glucan with acid. Signals in the ^{13}C-n.m.r. spectrum of the hydrolyzate may be assigned to certain carbon nuclei of these new sugar types. Although some of the relevant signals are obscured by larger signals, others have distinct chemical-shifts, and are readily detected. The predicted chemical-shifts of these distinct signals are given in Table II, as well as ^{13}C-chemical-shift data obtained from D-glucan samples that had been subjected to 4 and 9% hydrolysis of its glycosidic linkages (as determined by the reducing D-glucose assay). However, in the ^{13}C-n.m.r. spectrum of 4%-hydrolyzed D-glucan, all of the expected signals, except that for C-1 of the newly produced reducing terminus, are visible above the noise. In the ^{13}C-n.m.r. spectrum of the 9%-hydro-

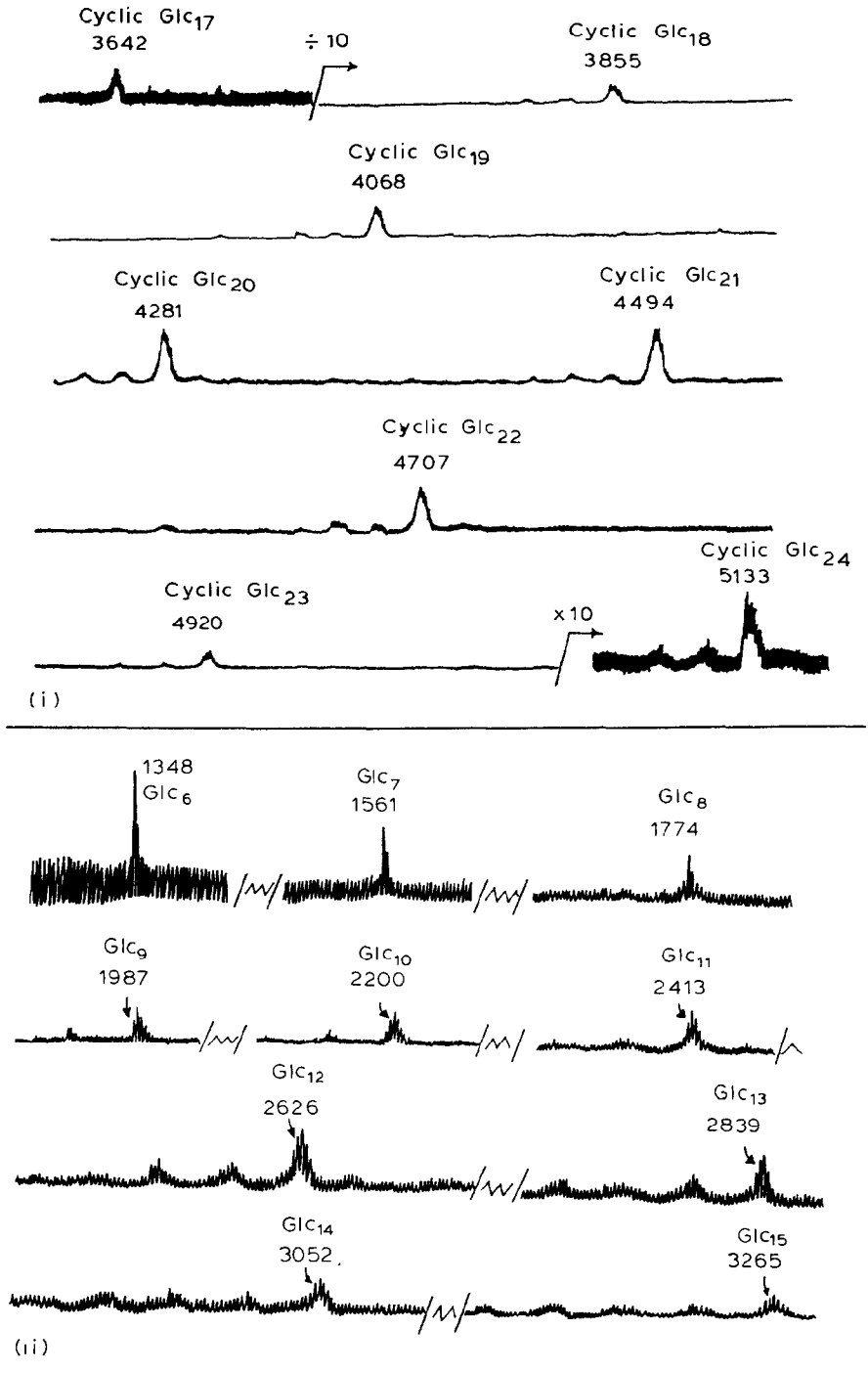


Fig 7. F.a.b.–mass spectra recorded for a sample of per(deuteriomethyl)ated β-D-glucan prepared as described in the Experimental section. [Scan (i) was obtained at an accelerating voltage of 5 kV from *m/z* 5280 to 3300, and peaks were assigned by calibration against the mass marker. Scan (ii) was obtained at an accelerating voltage of 8 kV from *m/z* 3300 to 10, and peaks were assigned by counting the spectrum. Ammonium-ion dosing was used, and all signals correspond to ammonium-cationized species.]

lyzed D-glucan, a signal at 92 p.p.m., corresponding to C-1 of the reducing D-glucose residue in the α-anomeric form, is *also* clearly visible (see Fig. 6).

From these data, it was concluded that the sensitivity of the n.m.r. technique is sufficient to permit observation of both the C-2 signal of a (nonreducing) terminal D-glucosyl group and several of the signals of a (reducing) D-glucose residue should either of these be present in a β-D-glucan. The absence of the relevant signals is evidence that the D-glucans are unbranched, circular molecules.

Interestingly, in the ^{13}C-n.m.r. spectrum of the native D-glucan, signals from C-1, C-2, and C-4 appear as multiplets, whereas these signals begin to coalesce in the spectra of samples of partially hydrolyzed D-glucan, and this presumably constitutes further evidence that the native D-glucan has a circular structure. Variation in ring size, corresponding to the number of D-glucosyl residues per molecule, is likely to result in slightly different angles between adjacent D-glucosyl residues, and this might result in slightly altered chemical-shifts for corresponding carbon nuclei within the variously sized, D-glucan rings.

Methylation analysis of the β-D-(1→2)-linked D-glucans. — Significant levels of 2,3,4,6-tetra-*O*-methylglucose had been detected in the methylation products of the β-D-(1→2)-linked D-glucans[5]. The formation of this derivative of terminal D-glucosyl groups is inconsistent with the unbranched, cyclic structure of the aforedescribed D-glucan. The proportion of this derivative detected varies, depending on the conditions used to methylate the D-glucan and the method used to isolate the methylation products. When the D-glucan is methylated under relatively mild conditions (*e.g.*, a single addition of sodium methylsulfinyl carbanion followed by addition of methyl iodide), the products contain less than 2% of the tetra-*O*-methylglucose, but they also contain significant proportions of di- and mono-*O*-methylglucose derivatives as a result of undermethylation. When the D-glucan is subjected to harsher conditions during methylation (*e.g.*, up to four alternate additions of potassium methylsulfinyl carbanion and methyl iodide), the products may contain >10% of the tetra-*O*-methyl derivative but greatly lessened proportions of di- and mono-*O*-methyl derivatives[5].

The origin of the "anomalous" tetra-*O*-methyl derivative was investigated by using f.a.b.–m.s. to monitor the products of the permethylation reaction before hydrolysis and acetylation. The f.a.b.–mass spectra revealed the presence of linear degradation products varying in size from disaccharides to oligomers containing 20 D-glucosyl residues, in addition to the methylated, cyclic molecules. Samples subjected to the two-step alkylation procedure described in the Experimental section yielded spectra of the type shown in Fig. 7 (i and ii). These spectra were obtained by using ammonium-ion dosing (see Experimental), a procedure that had been shown[17] to afford f.a.b.–mass spectra of very high quality from per-*O*-methylated carbohydrates. All of the signals observed in these spectra are those of ammonium-cationized molecular-ions. Two separate scans were needed, with reloading between scans, in order to achieve optimal sensitivity. These were a high-mass scan from *m/z* 5300 to 3300 and a low-mass scan from *m/z* 3300 to 100. A resolution of

2500 was chosen for both mass ranges, in order to preserve sensitivity at high mass. Consequently, the high-mass peaks appear as unresolved clusters. The masses assigned to these signals correspond to the calibrated mass of the most-intense part of the peak.

The major signals shown in Fig. 7 (i) occur at the values calculated for $(M + NH_4)^+$ of per(deuteriomethyl)ated, cyclic D-glucans containing 17 to 24 residues. The sensitivity achievable by ammonium-f.a.b.–m.s.of permethylated samples is greater than that normally obtained in f.a.b.–m.s. of underivatized D-glucans, which explains why components greater than 22 residues in length are observed in the spectra of the permethylated D-glucans. The major signals shown in Fig. 7 (ii) arising from linear molecules, *i.e.*, *m/z* 1348, 1561, 1774, 1987, 2200, 2413, 2626, 2839, 3052, and 3265, are the ammonium-cationized, molecular ions of per(deuteriomethyl)ated linear D-glucans (methyl glycoside at the originally reducing terminus) containing 6 to 15 D-glucosyl residues. In this sample, larger linear molecules are of very low abundance [see Fig. 7 (i)], and smaller molecules are absent (data not shown). In other experiments, if the methylation conditions used were particularly harsh, large proportions of short, linear molecules (2–5 residues) were observed, whereas, if milder conditions were used, larger linear molecules (up to 20 residues) were present, together with significant proportions of undermethylated, cyclic and linear derivatives.

In conclusion, the f.a.b.–m.s. data indicate that the formation, during methylation, of tetra-*O*-methyl derivatives results from cleavage of circular β-D-(1→2)-linked D-glucan. The resulting linear molecules are not detected in significant proportions before methylation, either by f.a.b.–m.s. or by the reducing glucose assay. Furthermore, these molecules are detected in varying proportions after methylation, depending upon the methylation conditions used.

CONCLUSION

High-field-magnet, f.a.b.–m.s. and high-field, ^{13}C-n.m.r. spectroscopy constitute a very powerful combination of techniques for structural determination of biological molecules. These methods have now been used to establish that the β-D-(1→2)-linked D-glucans secreted by *Rhizobia* and *Agrobacteria* are unbranched, cyclic molecules containing a chain of 17 to, at least, 24 D-glucosyl residues. By use of f.a.b.–m.s., it has also been shown that the D-glucans are subject to partial degradation during methylation.

NOTE ADDED IN PROOF

P. M. Hisumatsu, A. Amemura, T. Matsuo, H. Matsuda, and T. Harada have published results[18] that are similar to those presented in this paper.

REFERENCES

1 W F DUDMAN, L.-E. FRANZEN, M. MCNEIL, A. G. DARVILL, AND P ALBERSHEIM, *Carbohydr Res*, 117 (1983) 169–183.
2 R A. DEDONDER AND W Z. HASSID, *Biochim Biophys Acta*, 90 (1964) 239–248
3 P. A. J. GORIN, J. F T SPENCER, AND D. W. S. WESTLAKE, *Can J. Chem*, 39 (1961) 1067–1073
4 J. A. LIPPINCOTT AND B. B LIPPINCOTT, *Annu Rev Microbiol*, 29 (1975) 377–405
5 W. S. YORK, M. MCNEIL, A. G DARVILL, AND P ALBERSHEIM, *J Bacteriol*, 142 (1980) 243–248
6 L. P T M ZEVENHUIZEN AND H. J SCHOLTEN KOERSELMAN, *Antonie van Leeuwenhoek J Microbiol Serol*, 45 (1979) 165–175
7 M. ABE, A. AMEMURA AND S HIGASHI, *Plant Soil*, 64 (1982) 315–324
8 H R. MORRIS, A. DELL, AND R A. MCDOWELL, *Biomed. Mass Spectrom.*, 8 (1981) 463–473.
9 M. BARBER, R. S BORDOLI, R. D SEDGWICK, AND A N TYLER, *Nature (London)*, 293 (1981) 270–275.
10 H R MORRIS, A. DELL, A T. ETIENNE, M JUDKINS, R A MCDOWELL, M PANICO AND G W TAYLOR, *Pure Appl. Chem*, 54 (1982) 267–279
11 A. DELL AND H. R MORRIS, *Biochem Biophys Res Commun*, 106 (1982) 1456–1462
12 L. S. FORSBERG, A. DELL, D. J. WALTON AND C E BALLOU, *J Biol Chem*, 257 (1982) 3555–3563
13 A. DELL, H R. MORRIS, H EGGE, H. VON NICOLAI AND G STRECKER, *Carbohydr Res*, 115 (1983) 41–52.
14 A DELL AND J. E OATES, unpublished results
15 A DELL AND C E BALLOU, *Biomed Mass Spectrom*, in press
16 T USUI, N YAMAOKA, K MATSUDA, K. TUZIMURA, H. SUGIYAMA, AND S SETO, *J Chem Soc, Perkin Trans 1*, (1973) 2425–2432
17 A. DELL, J. E OATES, H. R. MORRIS, AND H. EGGE, *Int. J. Mass Spectrom. Ion Phys*, in press.
18 M HISUMATSU, A. AMEMURA, T MATSUO, H. MATSUDA AND T HARADA, *J Gen Microbiol*, 128 (1982) 1873–1879.