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Carbohydrate Research 296 (1996) 23–37

CARBOHYDRATE RESEARCH

Cyclolaminarinose. A new biologically active β-(1 → 3) cyclic glucan [1]

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Received 21 June 1996; accepted in revised form 12 September 1996

Abstract

A unique glucan has been isolated from a recombinant strain of a *Rhizobium meliloti* TY7, a cyclic β-(1 → 2) glucan mutant carrying a locus specifying β-(1 → 3; 1 → 6) glucan synthesis from *Bradyrhizobium japonicum* USDA110. This compound, which appears to have considerable hydrophobic affinity, was separated from a perchloric acid cell extract by adsorption to a C-18 silica column. Unlike those cyclic glucans previously isolated from *Rhizobium meliloti* or *Bradyrhizobium japonicum*, this molecule contains neither phosphoglycerol nor phosphocholine substituents, respectively. 2D NMR, FAB mass spectrometric analysis and high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) confirmed that this glucan is a single, cyclic decasaccharide (cyclolaminarinose) in which one of the residues is substituted in its 6-position with β-laminarabiose. This structural assignment was confirmed by mass spectral and NMR analyses of the product obtained from two consecutive Smith degradations. Unlike the complex ^{13}C spectrum of the unoxidized material, the spectrum of this product consisted of only six resonances due to rapid time averaging of its symmetrical structure on the relatively slow NMR timescale. Synthesis of this newly described cyclic β-glucan in the *R. meliloti ndv*B mutant restored the symbiotic and hypoosmotic adaptation characteristics of the *R. meliloti* wild type strain. © 1996 Elsevier Science Ltd.

Keywords: Cyclolaminarinose; Cyclic decasaccharide; *Rhizobium meliloti*; Glucan

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PII S0008-6215(96)00238-8

1. Introduction

It is common to find a mixture of substituted and unsubstituted β-(1 → 2) cyclic glucans associated with different species of *Agrobacterium* and *Rhizobium* species in which ring sizes can vary from 17–40 glucose residues [1]. Substitution of these glucans is often in the form of anionic groups such as phosphoglycerol, succinate, or methylmalonate [1]. In contrast, the slower growing members of the *Bradyrhizobium japonicum* species synthesize a mixture of branched β-(1 → 3; 1 → 6) cyclic glucans with dps of 10–13 [2,3]. Approximately half of this mixture is found to be a branched β-(1 → 3; 1 → 6) cyclic glucan with a dp of 13, carrying a C-6 substituted phosphocholine [3]. It was established that this cyclic glucan structure was composed of alternating blocks of three β-(1 → 3) and three β-(1 → 6) glucose units with a single branched glucose at a C-6 position [3].

Both the β-(1 → 2) and β-(1 → 3; 1 → 6) cyclic glucans and their substituted derivatives have been implicated as factors in the recognition/colonization process [4–6] and osmotic regulation [7–10] of the organisms from which they are derived. It has been proposed that β-(1 → 3; 1 → 6) cyclic glucans may be functional analogs of the β-(1 → 2) cyclic glucans [9]. However, there is no DNA homology between the β-(1 → 2) glucan synthesis genes (*ndv*B or *chv*B from *Rhizobium* or *Agrobacterium*) and genomic DNA of *B. japonicum* [4,5]. In an attempt to establish whether the function of either of these respective glucans is interchangeable in terms of host/organism responses, we mobilized a locus specifying synthesis of β-(1 → 3; 1 → 6) cyclic glucans from *B. japonicum* USDA 110 to an *ndv*B mutant of *Rhizobium meliloti*, TY7 [11]. In this study we report that the resulting recombinant strain of *R. meliloti*, TY7 (p5D3), produced a new cyclic glucan that is composed only of β-(1 → 3) linkages in the ring and a C-6 linked branch composed of a β-(1 → 3) linear glucan chain. In addition, this recombinant strain, unlike its precursor TY7, produced effective nodules and adapted readily to growth in hypoosmotic medium as does the wild-type *R. meliloti*.

2. Experimental

Growth of Rhizobial strains.—*R. meliloti* 102F34 and TY7, an *ndv*B mutant, was obtained from S. Stanfield [4]. For glucan isolation, the strains were grown in GYM medium [12] until late log phase. Antibiotics were added to the media when appropriate. *R. meliloti* TY7 (p5D3) is a recombinant strain carrying a genomic DNA clone from *B. japonicum* USDA 110, obtained after triparental mating [11,13].

Isolation and purification of cyclic glucan.—The cells from GYM growth medium were harvested at late log phase (A_{600} = 0.7–0.9) and washed with GYM salts before extracting. Bacteria from 12 L of growth medium were frozen with liquid nitrogen and extracted in the cold with 0.8 M perchloric acid [3]. The paste was centrifuged at 20,000 × *g* and the clear straw colored liquid was carefully neutralized to pH 6.9 with potassium hydroxide while cooling in an ice bath. The resulting precipitate was separated from the clear extract by centrifugation at 20,000 × *g* for 20 min. The bulk water was removed by rotary film evaporation and the final crude extract was lyophilized

to a white powder. This material was dissolved in 40 mL of distilled water and placed in a dialysis bag (1500 molecular weight cutoff) and dialyzed against 12 L of distilled water for 3 days, changing the water every 24 h. The contents of the dialysis bag was evaporated on a rotary film evaporator to a 3 mL volume and lyophilized to give approximately 30 mg of white powder. This material was dissolved in 80 mL of distilled water and placed on 0.6 g (1 g/50 mg of sample) of preconditioned [3] C-18 silica support (Worldwide Monitoring, Horsham, PA) and eluted with 160 mL of water followed by 160 mL of 30% methanol–water. The methanol–water fraction containing the cyclic glucan was concentrated on a rotary film evaporator to remove the methanol and was subsequently lyophilized to yield 14 mg of the cyclic glucan as a white amorphous powder.

Oligosaccharide analysis by HPAEC-PAD.—Conditions for high performance anion-exchange chromatography and pulsed amperometric detection (HPAEC-PAD) were similar to those reported previously [14]. HPAEC-PAD was performed with a Dionex 4500 system, which included a Pulsed Electrochemical Detector (PED; gold working electrode) operated in the integrated amperometry mode ($E_1 = 0.35$ V, 0.48 s; $E_2 = 0.95$ V, 0.12 s; $E_3 = -0.25$ V, 0.07 s; integration: 0.28–0.48 s), and a pressurized bottle post-column delivery system that added 500 mM sodium hydroxide prior to the detector. A CarboPac PA1 column (4×250 mm^2; Dionex) and a CarboPac PA Guard column (3×25 mm^2; Dionex) were utilized. The mobile phase (0.8 mL/min) consisted of a linear gradient of 5–375 mM CH_3COONa in 100 mM NaOH for 90 min (obtained by mixing eluants consisting of 100 mM NaOH and 500 mM CH_3COONa–100 mM NaOH) and then up to 500 mM CH_3COONa in 100 mM NaOH at 100 min. Chromatograms were collected and analyzed with a Chrom Perfect Direct (Justice Innovations) chromatography data system that included a DT2804 A-D board. Maltrin M-040 maltodextrin was generously provided by Grain Processing (Muscatine, IA) and α- and β-cyclodextrins were generously provided by American Maize-Products (Hammond, IN).

Permethylation of TY7(p5D3).—The glucan (2 mg) was dissolved in 1 mL of dimethylsulfoxide (Me_2SO), 1 mL of 2 M dimsyl sodium in Me_2SO was added and the solution stirred for 3 h. The solution was then cooled in an ice bath, 1 mL of methyl iodide was added and the solution was allowed to warm to room temperature and stirred for an additional 1 h. The solution was chilled in an ice bath and 1 mL of H_2O was added slowly. The excess methyl iodide was then removed under a stream of N_2 and the solution was extracted with an equal volume of chloroform; the chloroform extract was washed twice with an equal volume of H_2O and then concentrated to dryness. The residue was dissolved in 1 mL of 0.25 M H_2SO_4 and hydrolyzed overnight at 100 °C. The solution was then cooled and neutralized with barium carbonate and concentrated to dryness. The residue was reduced with $NaBH_4$ and the products were acetylated with acetic anhydride in pyridine. The methylated alditol acetates were analyzed by GC–MS using a Hewlett–Packard 5890 GC fitted with a 15 m SP 2330 capillary column (Supelco, Bellefont, PA) interfaced to a Hewlett–Packard 5989A MS engine. The column was programmed from 125 to 250 °C at 4 °C/min.

Smith degradation of TY7(p5D3) glucan.—A 9.7 mg sample of glucan was dissolved in 5 mL of 50 mM $NaIO_4$ and the solution was stored in the cold (refrigerator) for 72 h.

The excess periodate was then decomposed with ethylene glycol (100 μl). $NaBH_4$ (15 mg) was added to the solution and after 5 h at room temperature the solution was neutralized with 50% acetic acid. After concentration overnight under a stream of N_2, the residue was taken up in a solution of methanol–cetic acid (90/10) and concentrated under N_2; this process was repeated 3 times. The oxidized product was treated with N TFA at room temperature for 40 h and concentrated to dryness. The final residue was taken up in H_2O and fractionated on a C-18 column (see above). The column (pretreated with methanol) was eluted with 15 mL H_2O followed by 30% aq methanol. The methanol fraction was concentrated to dryness to yield 7.8 mg of product. This material after HPLC, NMR, and mass spectral analysis was treated to a second Smith degradation sequence.

End group analysis.—One mg of glucan was reduced with $NaBH_4$, then hydrolyzed and the aldononitrile derivative was prepared in the usual manner [3]. GLC showed only the aldononitrile acetate of glucose, indicating that no reducing glucose unit was present in the sample.

FAB mass spectral analysis.—Mass spectra were obtained with a VGZAB-2SE/FPD magnetic sector mass spectrometer (VG Analytical, Manchester, UK) by fast atom bombardment ionization at 8 keV using a thioglycerol–*m*-nitrobezoic acid (MNBA) matrix. An exponential downscan from 4000–100 amu at a scan rate of 15 s/decade with a 2 s reset time was used.

Nuclear magnetic resonance spectroscopy.—^1H and ^{13}C NMR spectroscopy was performed using Bruker DMX 500 and 750 and Varian UnityPlus 400 MHz spectrometers at 35 °C. The samples were exchanged at least twice in D_2O before obtaining the spectra in D_2O. The 1D ^{13}C spectra were obtained with a 70° pulse and 10,000–50,000 scans, depending on the quantity of sample available, and a repetition time of 1.8 s. Spectral widths were 25,000 Hz with 32 K data points. Linebroadening was 3–5 Hz depending on the level of noise in the spectrum. Chemical shifts were measured relative to external *p*-dioxane (67.4 ppm)

The HSQC experiments were run in the phase-sensitive mode using the TPPI method with a GARP sequence for ^{13}C decoupling [15]. Low power presaturation was applied to the residual HDO signal. The proton spectral width was 5009 Hz (10 ppm) and the carbon spectral width was 50 ppm with a total 256 data points zero filled to 2048 and processed with a sine bell function and 0.0 Hz linebroadening. The ^1H homonuclear 2D COSY experiments were performed on a Bruker DMX 750 MHz spectrometer with low power presaturation of the water signal.

The two-dimensional gradient heteronuclear multiple bond correlation (HMBC) [16] spectrum of the TY7(p5D3) cyclic glucan was recorded on a Bruker DMX 500 MHz spectrometer. The spectrum was collected with 272 transients of 4 K complex points in the ^1H direct dimension with the ^{13}C correlations evolved in 128 increments in the indirect dimension. The ^1H sweep width was 10 ppm and the ^{13}C sweep width was 80 ppm. The HMBC low-pass filter delay was set to 3.3 ms to remove one-bond ^{13}C–^1H correlations, with the long-range couplings optimized using a 60 ms delay. Data were processed as a 4 K by 512 real matrix. The ^1H dimension was multiplied by a 12 Hz exponential and the indirect ^{13}C dimension was multiplied by a 90° shifted sine function. The resulting spectrum was obtained in the absolute value mode.

3. Results and discussion

Isolation and structure analysis of I.—Extraction of the recombinant strain *R. meliloti* TY7(p5D3) with 0.8 M perchloric acid yielded a material which was easily separated from a water solution of a complex mixture of oligosaccharides by adsorption on a C-18 silica open tube chromatography column followed by elution with 30% methanol–water. This method yielded compound **I** which eluted as one predominant peak at approximately 66 min from the CarboPac PA1 column used for HPAEC-PAD (Fig. 2A). The proposed structure of this compound is shown in Fig. 1. From the carbohydrate and methylation analysis we determined that **I** contains only glucose and that the structure consists of ten residues with 1,3 linkages, one with a 1,3,6 linkage and a single non-reducing terminal unit. The per-*O*-acetylated D-gluconononitrile derivatives obtained from the per-*O*-methylated glucan (**I**) extracted from TY7(p5D3), namely 2,3,4,6-tetra-*O*-methyl, 2,4,6-tri-*O*-methyl and 2,4-di-*O*-methyl, were in the ratio 1.0:10.6:1.2. The FAB mass spectrum of **I** displayed an M + H ion at 1945 and an M + Na at 1967 (data not shown) demonstrating that the glucan contains twelve

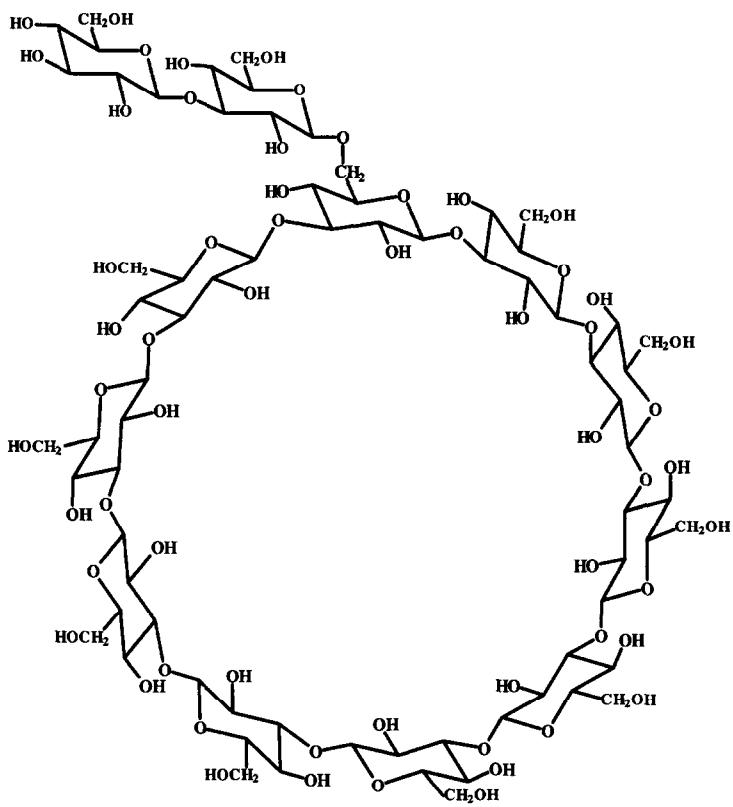

Fig. 1. Structure of β-(1 → 3) cyclic glucan **I** isolated from *R. meliloti* TY7(p5D3).

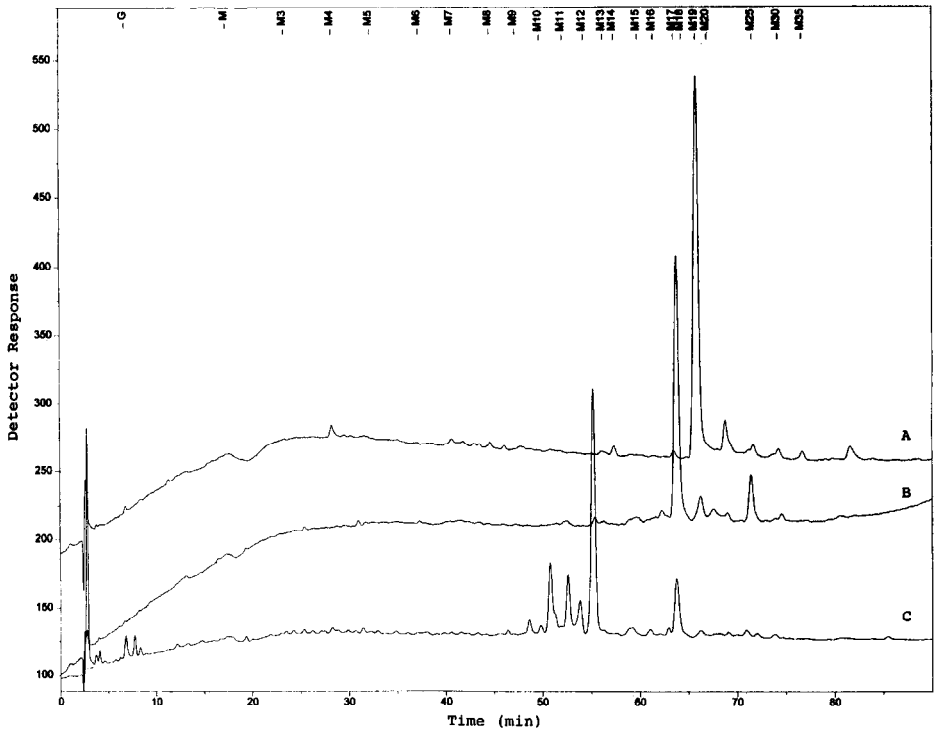

Fig. 2. HPAEC-PAD separation of gluco-oligosaccharides. The elution of the *R. meliloti* TY7(p5D3) β-(1 → 3) cyclic glucan (A) before Smith degradation, (B) after one Smith degradation sequence, and (C) after two Smith degradation sequences. The elution order of a series of malto-oligosaccharides present in Maltrin M040 maltodextrin is indicated by the labels above the chromatograms (G = glucose, M = maltose, M3 = maltotriose, etc.). See the Experimental section for chromatographic conditions.

glucosidically linked hexoses units with no reducing end. Relatively minor amounts of other glucans were evident but their structures have not yet been elucidated.

Following the Smith degradation, the HPAEC-PAD retention time of **I** shifted approximately one glucose unit (66 to 64 min) to the lower molecular weight side of the original peak (Fig. 2B). While elution of **I** corresponded to a dp 19 malto-oligosaccharide (see reference positions of malto-oligosaccharides in Fig. 2), Koizumi et al. [14] demonstrated that β-(1 → 3) gluco-oligosaccharides eluted later than α-(1 → 4) gluco-oligosaccharides during HPAEC-PAD analysis (the HPIC-AS6 and CarboPac PA1 columns are equivalent). We also confirm the HPAEC-PAD elution behavior of cyclic gluco-oligosaccharides reported by Koizumi et al. [14]; α-cyclodextrin (dp 6 cyclic α-(1 → 4) gluco-oligosaccharide) eluted between dp 4 and dp 5 linear malto-oligosaccharides whereas β-cyclodextrin (dp 7) eluted between dp 9 and dp 10 linear malto-oligosaccharides. Therefore, both the substitution/anomeric configuration and the cyclic nature of **I** contribute to its elution compared to dp 12 malto-oligosaccharide. The FAB mass spectrum showed that the molecular weight of the oxidation product diminished by one glucose unit [M + 1 (1783), M + Na (1805) dp 11 glucose units]

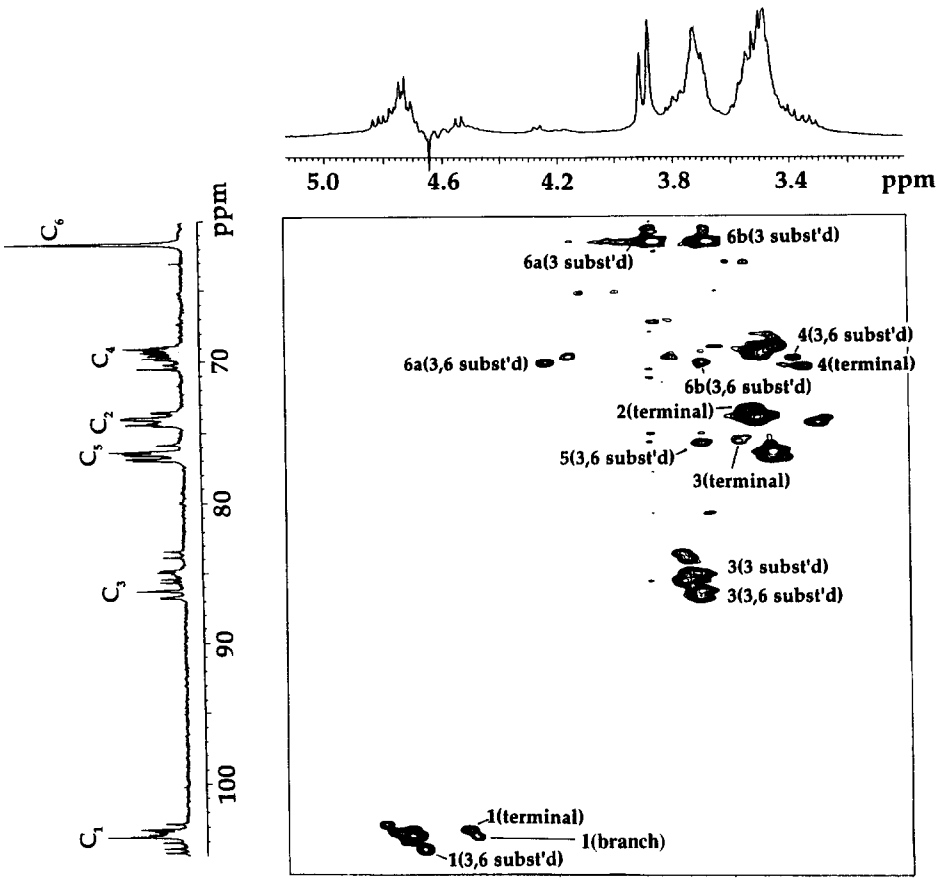

Fig. 3. Two dimensional HSQC spectrum of compound **I**. Parameters used to obtain these spectra are given in the experimental section. Additional shift assignments are given in Results and discussion and in Table 2.

(data not shown) indicating that no (1 → 6)-linked glucose residues (excluding trisubstituted 1,3,6 glucopyranoses) were present in the cyclic or linear branch portion of **I** and that the cyclic portion of the product remained intact [dp of 11 glucose units minus 18 mass units (elements of water)]. Also, since the ^{13}C NMR spectrum of the product from the oxidized, reduced and hydrolyzed **I** remained complex as one might expect if the branch were still present in the structure, we conclude that this cyclic glucan must have at least two glucose units in its single branch and that only a terminal non-reducing glucose was removed in the degradation process.

The HSQC NMR spectrum of **I** and the corresponding 1D spectra shown in Fig. 3 show that there is a significant amount of asymmetry in **I**. Although the 1D ^{1}H spectrum is extremely crowded, even at 500 MHz, the 100 MHz ^{13}C spectrum shows a significant dispersion of each carbon (C-1 to C-6) in the molecule. The C-1 resonances are observed in the range from δ 102.8 to 104.8 for the β-C-1 resonances of the (1 → 3)-linked glucose units corresponding to the proton range δ 4.70 to 4.82, $J_{1,2}$ 8 Hz

Table 1
^{1}H and ^{13}C chemical shift compilation for **I** [a]

Carbon number	^{13}C (ppm)	^{1}H (ppm) [b]
Terminal glucose		
1	103.3(1)	4.58(1)
2	73.4	3.54
3	75.8(1)	3.58
4	70.5	3.38
5	77.0	3.58
6a	61.6	3.72
6b		3.90 [b]
3,6-Substituted glucose		
1	104.2(1)	4.68(1)
2	74.2	3.55
3	73.6(1)	3.54
4	70.2(1)	3.42
5	77.0	3.70
6a	70.5(2)	3.70(1)
6b		4.26(1)
3-Substituted glucose (linear and cyclic structure)		
1	102.8(1); 103.2(2); 103.5(1); 103.6(1)	4.50(branch); 4.70(1); 4.73(1); 4.77
	103.8(branch,1),(3); 104.5(1); 104.8(1)	4.79(1); 4.82
2	73.5; 73.6; 74.0; 74.3; 74.4	3.55; 3.60
3	83.4(1); 83.8(1); 84.8(1); 84.9(1); 85.4(1)	3.76; 3.77; 3.78(branch); 3.81
	85.6(1); 86.1(1); 86.2(2); 86.7(1); 86.8(1)	3.82; 3.82
4	68.8; 69.0; 69.2; 69.3; 69.5; 69.7; 70.2	3.53; 3.55
5	76.1; 76.5; 76.6; 76.8; 76.9; 77.0	3.53; 3.53; 3.75
6a	61.6(1)	3.72(11)
6b		3.90(11)

[a] Numbers in parenthesis indicate the relative area of each resolvable carbon/proton resonance.
[b] Resolved in the carbon dimension.

[17]. The C-1 of the first branch glucose unit off the ring (glycosylated to a C-6-OH) is observed at δ 103.8 and its corresponding proton is seen at δ 4.50. That all linkages are of the β-configuration is demonstrated by the fact that we do not observe any ^{13}C anomeric resonances at a field higher than δ 102.8. Typically anomeric carbon resonances of α-(1 → 3) glucose units are observed at approximately δ 100.0 in acyclic [18] and cyclic glucans [19]. No evidence of NMR peaks attributable to reducing-end C-1 or H-1 [17,18] were detected, indicating that this structure is cyclic with a non-reducing terminal glucose which was confirmed by chemical analyses (see experimental section). At δ 103.4 we observe the β-C-1 of the 3,6-substituted glucose with its corresponding proton at δ 4.68 ($J_{1,2}$ 8.0 Hz) [3,20]. The C-1 of the terminal non-reducing glucose resonance is observed at δ 103.8 and its proton is observed at δ 4.58 [17]. In contrast to the broad, ill-defined proton spectrum representing all H-3 resonances centered at approximately δ 3.78, the ^{13}C spectrum shows a wide dispersion of eleven of the twelve C-3 resonances. Table 1 summarizes the NMR data. Fig. 4 gives an expanded view of the 1D and HSQC contour plot of eleven of the C-3 glycosylated

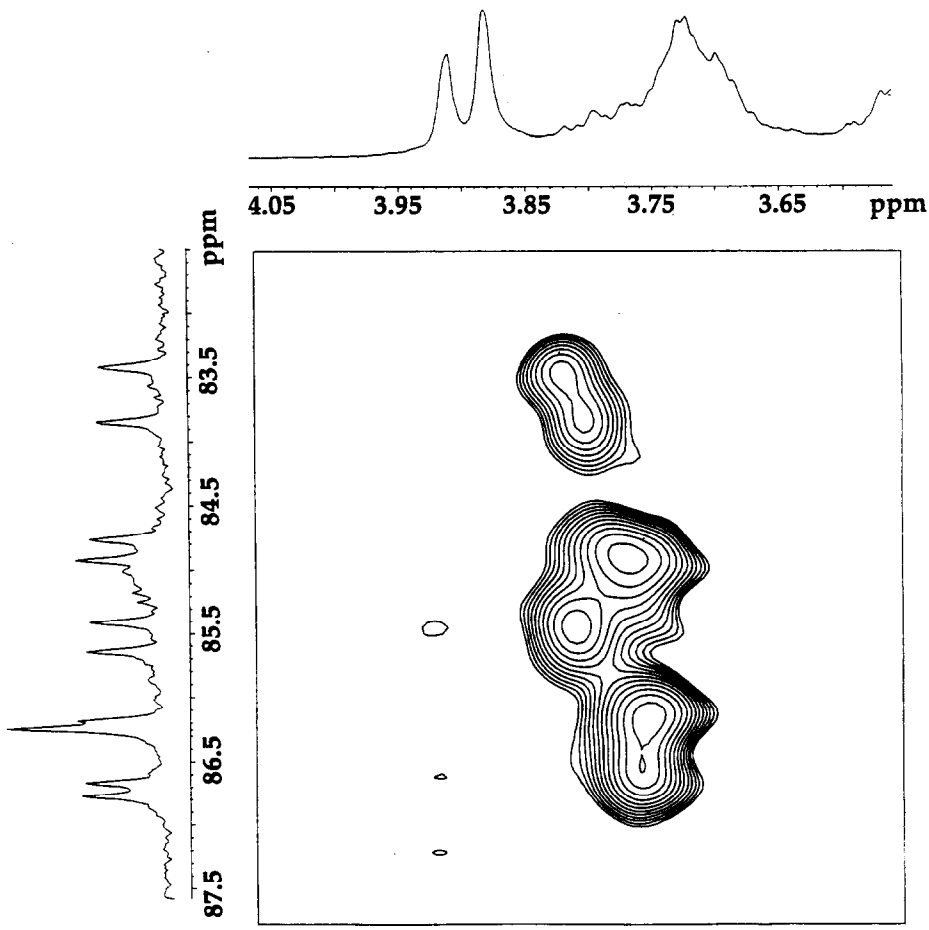

Fig. 4. Expanded C-3 region of the 125 MHz two-dimensional ^{13}C–^{1}H HSQC spectrum of compound **I**, showing resolution of some of the C-3 protons in the contour plot. Other projections of the 1D spectrum at δ 3.90 and δ 3.70–3.75 represent protons not observed in this section of the contour plot (see Table 1).

carbons. The resonance at δ 86.2 contains two degenerate shifts for two equivalent C-3 carbons and a poorly resolved shoulder is also observed at δ 86.1 (total area = 3 carbons). An additional resonance assigned to a C-3 in the non-glycosylated terminal glucose is observed at δ 75.8 [18], with its corresponding ^{1}H shift seen at δ 3.58. A more detailed analysis of the ^{1}H NMR spectrum of compound **I** was accomplished with a 2D ^{1}H COSY experiment (spectra not shown) at 750 MHz (see Table 1).

Although the ^{13}C and ^{1}H spectra of the isolated product following the Smith degradation remained almost identical to **I** and equally complex, two obvious differences emerge. One resonance at δ 86.1 in the C-3 region was not present (total relative area of the C-3 region = 10) and the C-3 of the terminal glucose unit was shifted slightly downfield to δ 76.0 (see Fig. 5b). However, the ^{13}C resonance corresponding to a glycosylated C-6 of a ring glucose was observed in the spectrum at δ 70.5 (with its

resolved proton cross peaks seen at δ 3.70 and 4.26) [15,20] confirming that a glucan branch is still attached to the ring.

In an attempt to establish the size of the cyclic glucan ring we treated **I** with a specific endolytic β-(1 → 6) glucanase [21] to remove the branch glucan from the ring. The progress of this enzymatic hydrolysis was followed by ^{13}C NMR. Unfortunately this enzyme is not active in the hydrolysis of a β-(1 → 6) glucan linkage in this chemical environment. Likewise, treatment of **I** with both laminarinase and controlled acetolysis gave no selective removal of the branched portion of this molecule. However, the 2D HMBC spectrum of **I** gave direct evidence for the existence of only two glucose units in the linear branch chain. The HMBC spectrum (data not shown) gave the following $J_{1,3}$ C–H coupling connectivities: H-6 (3,6 substituted) δ 3.70 → C-1 (branch glucose attached to the ring C-6–OH) δ 103.8 → H3 (branch glucose attached to the ring C-6–OH) δ 3.78 → C-1 (terminal glucose) δ 103.3 → H-3(terminal glucose) δ 3.58. Thus, starting with the H-3 resonance of the terminal ring we have observed that there is only one additional (1 → 3)-linked glucose unit between it and the C-6 linkage to the cyclolaminarinose ring.

Additional proof of structure **I** comes from chemical degradation whereby two or three consecutive Smith degradation reactions gave only one major product that was retained on the C-18 silica column. The HPAEC-PAD profile of this product (Fig. 2C) shows that it has a retention time substantially shorter (55 min versus 66 and 64 min) than the parent compound and singly oxidized product, respectively. Thus, loss of the glucose branch appears to significantly influence the overall interaction of the molecule with the stationary phase. Retention of mono- and oligo-saccharides on the pellicular anion-exchange CarboPac PA1 column is due to both the relative acidities of the hydroxyl groups [22] and the accessibility of the oxyanions to the stationary phase [23]. The bulk of the pyranose oligosaccharide retention behavior has been attributed to the interaction of the highly acidic (relative to all other OH groups except the anomeric OH) 2-OH groups and the positively charged pellicular resin [24]. Apparently, the orientation of these hydroxyl groups in cyclic oligosaccharides changes as ring size increases so that stationary phase interaction is either more (i.e., β-cyclodextrin) or less (i.e., α-cyclodextrin) favorable relative to their linear equivalents. Alternatively, Koizumi et al. [14] suggested that hydrophobic interactions might explain the different elution pattern of cyclic glucans from the CarboPac PA1 column relative to their linear equivalents. Therefore, the shift in retention following the first Smith degradation is due to the decreased dp of the linear side chain which contains 2-OH groups that are readily accessible to interact with the stationary phase. The dramatic decrease in retention time following the second Smith degradation can be explained by a relatively less accessible distribution of 2-OH groups or decreased hydrophobicity in the completely cyclic structure compared to **I**.

The FAB mass spectrum corresponded to a molecular weight of a cyclic glucan of dp 10 (M + 5Na, 1735). At the present time it is unclear why this particular structure has such a strong propensity to complex with sodium, since the other two derivatives of this molecule appear to complex with only a single sodium. The ^{13}C NMR spectra of **I** and its oxidation products (dp 11, dp 10) clearly demonstrate that, following the two degradation reactions, the asymmetric branch point on the cyclic structure has been

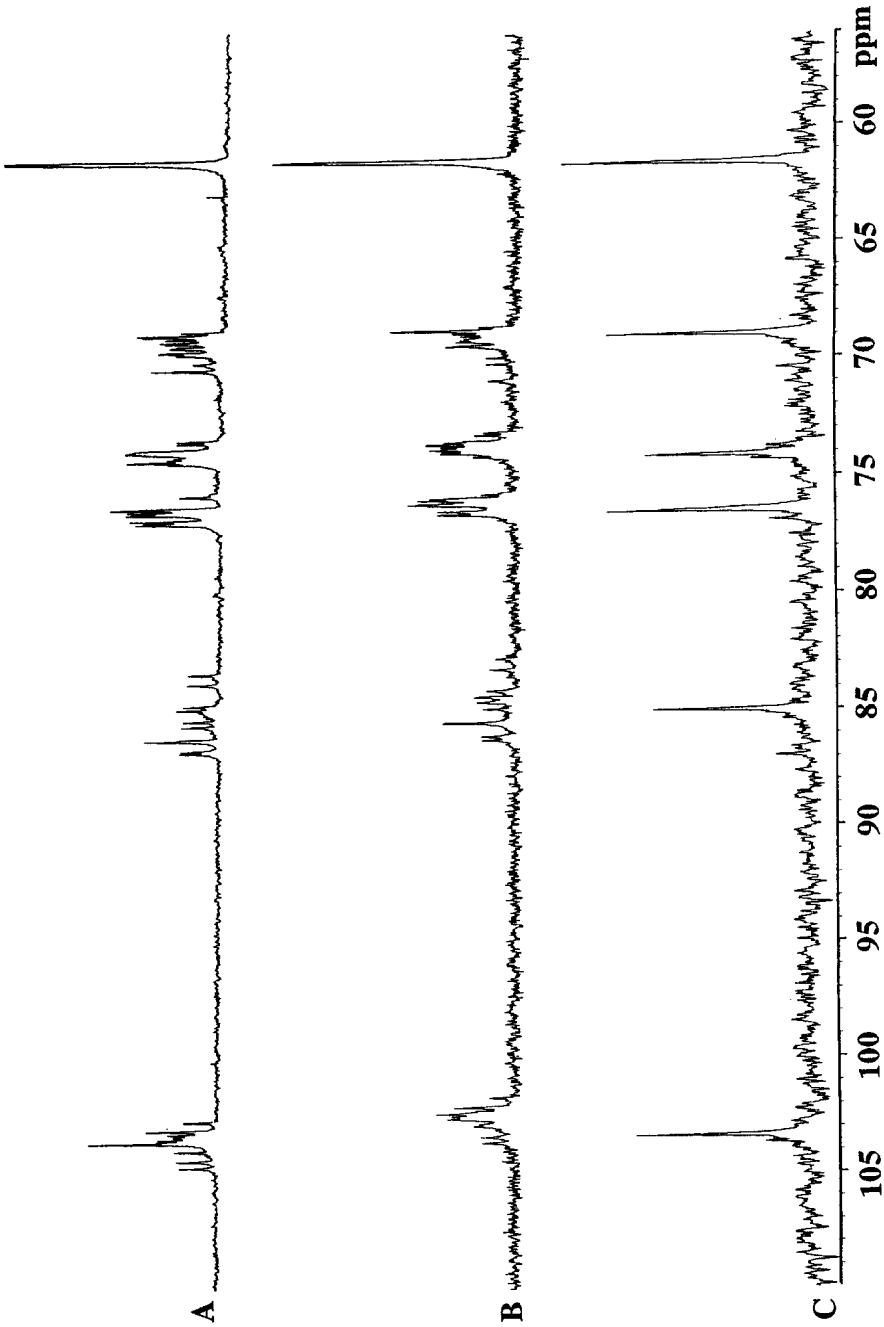

Fig. 5. 100 MHz ^{13}C spectra of (A) **I**, dp 12; (B) product (dp 11) obtained from a single Smith degradation sequence carried out on **I**; (C) product (dp 10) obtained from two consecutive Smith degradation reactions carried out on **I**.

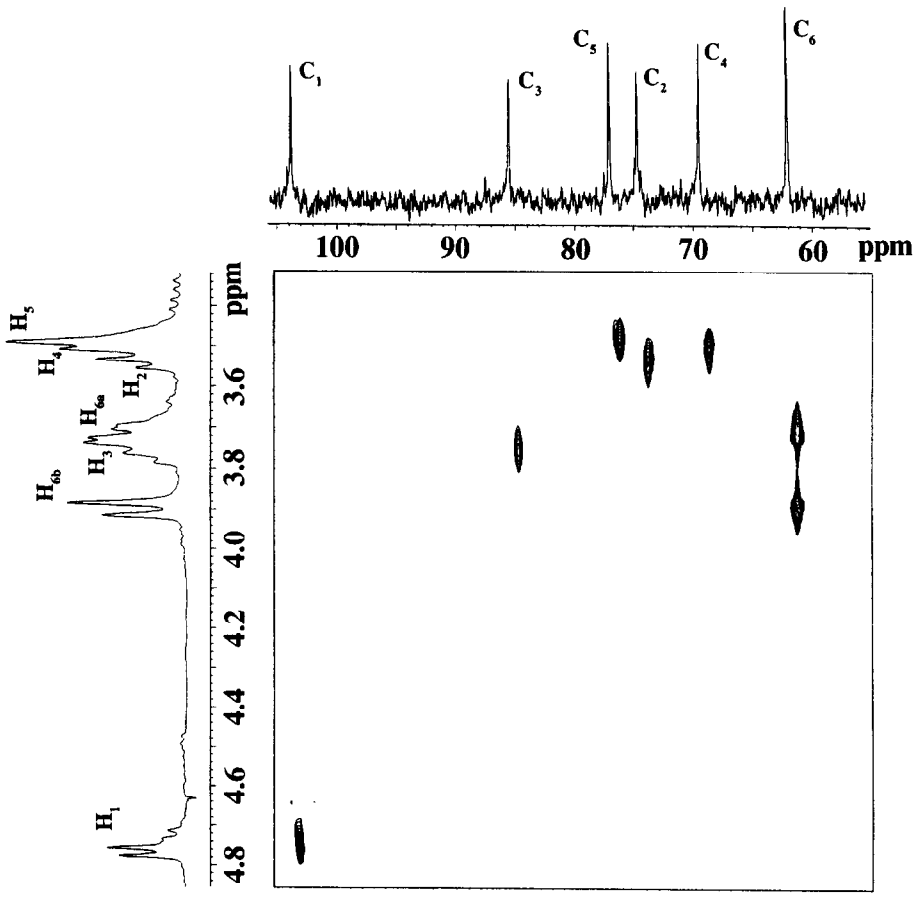

Fig. 6. 100 MHz two-dimensional $^{13}C-^{1}H$ HMQC spectrum of unbranched cyclolaminarinose isolated after two consecutive Smith degradation sequences on compound **I**.

removed, leaving a molecule with a ten-fold axis of symmetry (it appears averaged out on the relatively slow NMR time scale; compare Fig. 5A–C). In assessing the spectra of these compounds, it is particularly surprising to note the far reaching effect a single branch has on inducing so much asymmetry within what would appear to be a relatively symmetrical molecule. That is, one would not have anticipated that almost all of the ring C-3 resonances would be non-degenerate (see Fig. 4). Fig. 6 shows the $^{13}C-^{1}H$ HMQC NMR spectrum of unbranched cyclolaminarinose. All of the ^{13}C shifts obtained for cyclolaminarinose are essentially the same as those of laminarin with the exception of C-3 which is 0.14 ppm upfield in the former. Shifts are as follows: C-1, δ 103.37; C-2, δ 74.12; C-3, δ 84.91; C-4, δ 68.93; C-5, δ 76.44; C-6, δ 61.54.

Although earlier reports [25] suggest that a mixture of various branched β-(1 → 3) cyclic glucans with dp 11 were formed by the *B. japonicum* membrane enzyme and UDP-glucose, no conclusive evidence for the proposed structures was presented. Likewise, a reported attempt to synthesize a six-membered β-(1 → 3) cyclic glucan has only

Table 2
Comparison of nodulation of alfalfa by mutant and wild-type *Rhizobium meliloti*

Inoculum	% Plants nodulating [a]	Number of nodules/plant
R. meliloti 102F34	100	5.2±2.8
R. meliloti TY7	0 [b]	0.0
R. meliloti TY7 (p5D3)	94	4.1±2.3

[a] Alfalfa (*Medicago sativa* cv. sarnac) seeds were surface sterilized and inoculated with a stationary phase culture of *R. meliloti*, plants were grown in modified Leonards jar assemblies [5], and nodules were examined six weeks after inoculation.
[b] Plants had several pseudonodules devoid of any bacteria.

been reported to the stage before deprotection of the corresponding 4,6-ethylidene derivative which has not yet been thoroughly characterized [26].

Properties and biological activity of **I**.—The hydrophobic properties of **I** and other cyclic glucans derived from various plant interacting microorganisms [1,3] are interesting in light of the fact that these compounds may function as carriers and/or solubilizing agents for hydrophobic signal molecules [1,27]. Recent studies have demonstrated, for example, that β-(1 → 2) cyclic glucans when added to a *Rhizobium*–legume nodulation system enhance the nodulation process [5,12]. They have also been shown to enhance the solubility of naringenin (a legume-derived flavonoid) which induces the transcription of nodulation genes in *Rhizobium* species [28]. However, when the cyclic glucan synthesis was not active, as reported in a *R. meliloti* mutant, the organism elicited the formation of inactive nodules in alfalfa [11,29] and did not produce flagella [29]. Table 2 gives the percentage of plants nodulated and the levels of nodule

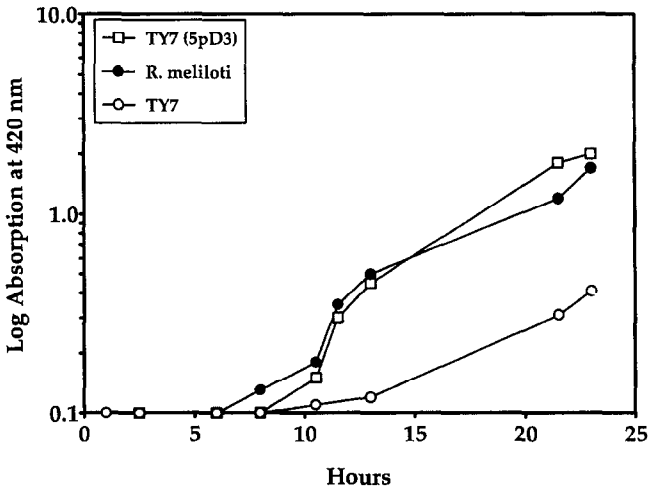

Fig. 7. Hypoosmotic adaptation of wild type and mutant *R. meliloti* strains. Each cell type was grown until stationary phase in GYM medium (low osmolarity) supplemented with 10 mM NaCl. The cells were then diluted 1:100 with fresh GYM medium without NaCl. Growth was monitored spectrophotometrically at 420 nm.

production in the wild type *R. meliloti* 102F34 as compared with TY7 and the recombinant strain TY7(p5D3). Fig. 7 illustrates that in the absence of cyclic glucan production, the *ndv*B mutant TY7 is slow to adapt to growth in low osmolarity GYM medium [12] whereas the growth rate of the recombinant strain is similar to the wild-type.

The β-(1 → 3) cyclic glucan reported in the present study is very different in structure, cavity size and substitution characteristics from those reported for both wild-type precursors strains *B. japonicum* USDA 110 and *R. meliloti* 102F34. Its presence in the recombinant strain TY7(p5D3) appears to restore the wild-type characteristics to the *ndv*B mutant with regard to hypoosmotic adaptation and symbiotic competence on alfalfa. A similar glucan produced by an *ndv*C mutant of *B. japonicum* USDA 110 (strain AB-1) [30] restores hypoosmotic competence but not symbiotic effectiveness on soybean. This is interesting in that it supports the hypothesis that there is a specific function for β-glucans in addition to their role as osmolytes. More details concerning the biological functions and physical properties of compound **I** and related compounds will be the subject of future studies.

Acknowledgements

We thank Dr. Yair Shachar-Hill for helpful discussions, Ms. Dina Grimes for preparing the figures for this publication, and Professor Stanley Opella for his generosity in letting us have access to his NMR facility and instrumentation at the University of Pennsylvania. This work was supported in part by USDA CSRS NRI Competitive Research Grant No. 93-37305-9233 to DLK and AAB.

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