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Note

Structural analysis of a second acidic exopolysaccharide of *Rhizobium meliloti* that can function in alfalfa root nodule invasion

Steven B. Levery*†, Hangjun Zhan‡, Chi Chang Lee‡, John A. Leigh‡, and Sen-itiroh Hakomori*‡**

* *The Biomembrane Institute, 201 Elliott Ave W, Seattle, WA 98119 (U.S.A.) and Departments of Chemistry†, Microbiology‡, and Pathobiology**, University of Washington, Seattle, WA 98 195 (U.S.A.)*

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Symbiotic bacteria of the genus *Rhizobium* secrete a variety of acidic polysaccharides that appear to be essential for root nodulation[1–7]. Normal strains of *R. meliloti* produce a calcofluor-binding exopolysaccharide (EPS), succinoglycan, a polymer having an octasaccharide repeating unit with the sequence [4,6-*O*-(1-carboxyethylidene)-β-Glc-(1→3)-β-Glc-(1→3)-β-Glc-(1→6)-β-Glc-(1→6)]→4)-β-Glc-(1→4)-β-Glc-(1→4)-β-Glc-(1→3)-β-Glc-(1→. Each repeating unit is further substituted with approximately one acetyl and one succinyl group at undetermined sites[8,9]. *Rhizobium meliloti exo* mutants, which either produce no succinoglycan or a non-succinylated form of the glycan, form "empty" nodules on alfalfa that are ineffective in nitrogen fixation, since they contain no intracellular bacteroids[2,10,11]. Recently, we reported the isolation and characterization of *R. meliloti* mutants, secreting a structurally distinct exopolysaccharide (EPSb), wich are also capable of forming nitrogen-fixing nodules on alfalfa, although a number of them are incapable of producing succinoglycan[12]. Virtually identical results have been reported by Glazebrook and Walker[13]. In this paper, we describe the structural characterization of EPSb by ^{1}H-n.m.r. spectroscopy, along with monosaccharide and methylation analysis by g.l.c.–m.s.

Analysis of monosaccharide composition. When native EPSb was subjected to anhydrous acidic methanolysis, followed by per-*O*-trimethylsilylation of the resultant monosaccharide methyl glycosides, g.l.c.–e.i.m.s. analysis gave the pattern shown in Fig. 1. Peaks whose retention times and e.i. mass spectra were readily assignable as derivatives of galactose and glucose were followed by two major peaks not immediately identifiable. The e.i. mass spectra were found to match closely those observed by Dudman and Lacey[14] for the anomeric methyl 4,6-*O*-(1-carboxyethylidene)-D-galacto-pyranoside methyl esters, while the g.l.c. peak ratios appeared similar. This result was consistent with the earlier identification of a resonance for the methyl group of pyruvic acetal in the 1-D ^{1}H-n.m.r. spectrum of EPSb[12].

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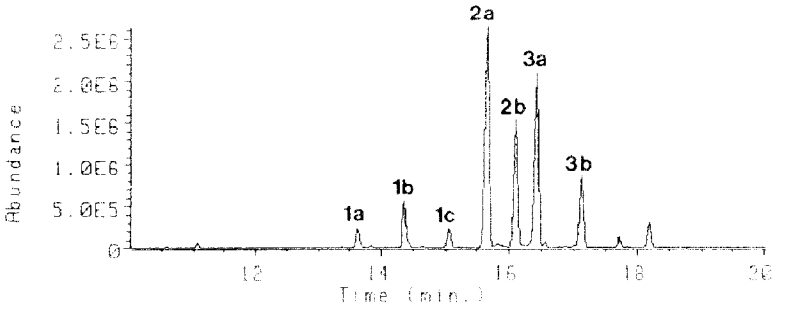


Fig. 1. Gas chromatography electron impact-mass spectrometry of per-*O*-trimethylsilyl monosaccharide methyl glycosides produced from native EPSb following methanolysis. Derivatives were identified as those derived from galactose (1a,b,c); glucose (2a,b); and the methyl ester of 4,6-*O*-(1-carboxyethylidene)galactose (3a,b).

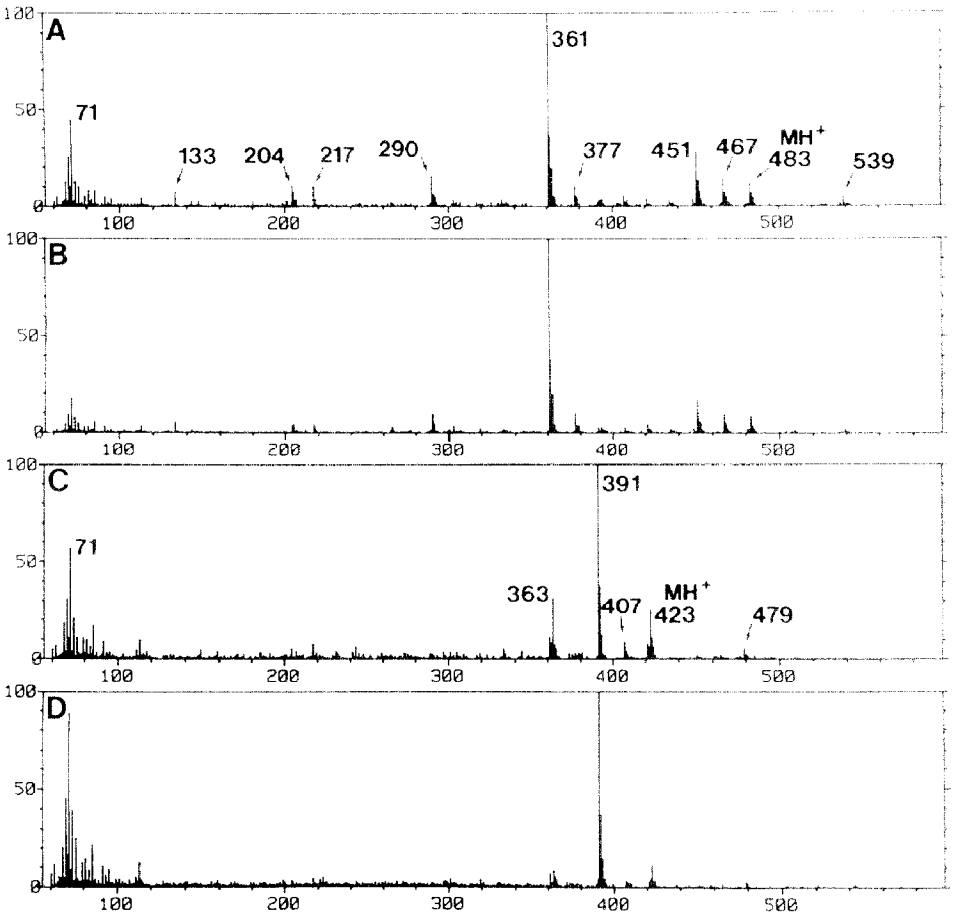
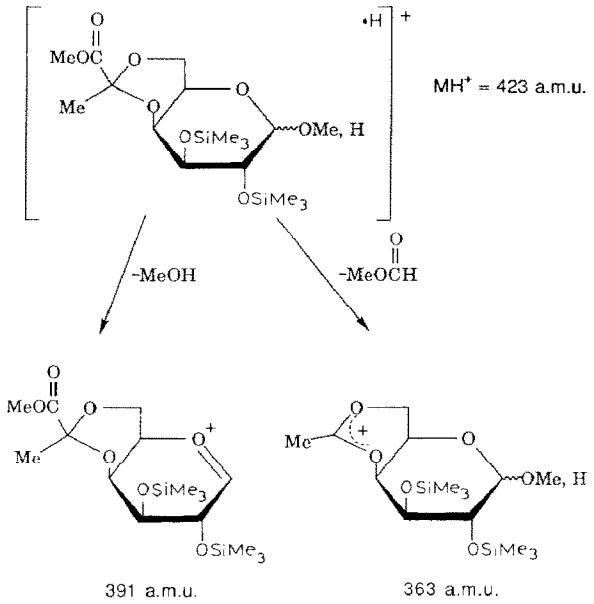


Fig. 2. Chemical-ionization mass spectra (isobutane) of derivatives 2a, 2b, 3a, and 3b (panels A-C respectively) identified in Fig. 1. Spectra of derivatives 1a–c were qualitatively similar to those in panels A and B, but with m/z 451 (MH^+) as base peak.

The identification of the two peaks as arising from 1-carboxyethylidene derivatives was confirmed by analysis using g.l.c.–c.i.m.s. with isobutane as reagent gas. The c.i. mass spectra of both derivatives were characterized by quasimolecular ions at m/z 423 ($M{\cdot}H^+$) and 479 ($M{\cdot}C_4H_9^+$), corresponding to the correct calculated nominal mass of 422 a.m.u. (see Fig. 2C). These peaks were accompanied by abundant fragments from loss of neutral MeOH (m/z 391) and methyl formate (m/z 363) (Scheme 1). This pattern can be contrasted with that typical for methyl per-*O*-trimethylsilyl hexopyranosides (see Fig. 2A), with quasimolecular ions at m/z 483 ($M{\cdot}H^+$) and 539 ($M{\cdot}C_4H_9^+$), accompanied by fragments for neutral losses of CH_4 (m/z 467), MeOH (m/z 451), and, in most cases, an additional loss of $(CH_3)_2HSiOCH_3$) (m/z 361).



Scheme 1

TABLE I

Sugar composition of EPSb from *Rhizobium meliloti* strain Rm7011 exoA (pMuc)

	Component sugar (%)		
	Glc	*Gal*	*4,6-O-1-carboxyethylidene-Gal*
(1) Native	51.6	12.1	36.3
(2) Aq. HOAc treated	54.8	45.2	not detected

Further confirmation of the nature of the substituted monosaccharide was obtained by sugar analysis following treatment of EPSb with mild aqueous acid, a procedure known to remove the extremely labile pyruvic acetal group. In this case, galactose and glucose were detected as their per-*O*-trimethylsilyl methyl glycosides in a ratio of ~1:1, with no trace of the 1-carboxyethylidene methyl ester derivatives. The quantitative results are summarized in Table I. Similar results were obtained from native EPSb following degradation by 0.25M H_2SO_4–90% HOAc and analysis of monosaccharides as alditol acetates (data not shown).

^{1}H-N.m.r. spectroscopy. — In order to obtain better resolution, ^{1}H-n.m.r. spectroscopy was performed at an elevated temperature (350 ± 2K), which was chosen to be close to that used in previous studies of acidic *Rhizobium* exopolysaccharides[14,15]. One

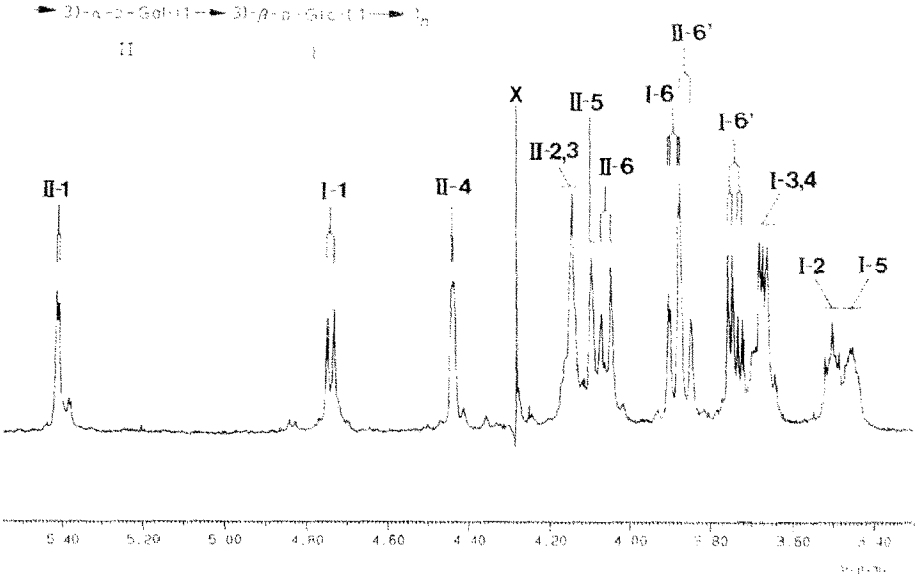


Fig. 3. 1-D ^{1}H-n.m.r. spectra of depyruvated EPSb in D_2O at 350 ± 2K.

TABLE II

^{1}H-N.m.r. chemical shifts (p.p.m. from sodium 3-(trimethylsilyl) propionate-2,2,3,3-d_4) and coupling constants (Hz) for depyruvated EPSb in D_2O at 350 ± 2K

Sugar residue	*H-1* $(^3J_{1,2})$	*H-2* $(^3J_{2,3})$	*H-3* $(^3J_{3,4})$	*H-4* $(^3J_{4,5})$	*H-5* $(^3J_{5,6})$	*H-6* $(^2J_{6,6'})$	*H-6'* $(^2J_{6,6'})$
β-Glc→3 (I)	4.75 (7.9)	3.51 (8.4)	3.68 (n.d.)[a]	3.68 (n.d.)[a]	3.46 (2.6)	3.90 (5.7)	3.75 (−12.6)
α-Gal→3 (II)	5.42 (3.2)	4.16 (n.d.)[a]	4.16 (2.9)	4.45 (<1.5)	4.10 (n.d.)[a]	4.07 (n.d.)[a]	3.87 (−13.3)

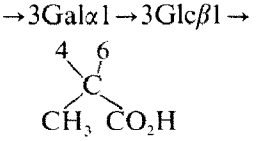
[a] (n.d.) = Not determined.

each of 3-proton singlets at typical chemical shifts for acetate (2.152 p.p.m.) and pyruvate (1.459 p.p.m.) were initially observed in the spectrum of intact EPSb, as reported previously[12]. On heating, the latter peak was seen to decay, being replaced by a singlet at 1.464 p.p.m. (free pyruvate), while the former peak was replaced by a singlet at 1.907 p.p.m. (free acetate). In a short time, the pyruvate group was completely lost, even when 100mM phosphate buffer at pD 9.8 was used as solvent. Therefore, all spectra for this study were acquired after complete hydrolysis of the pyruvic acetal. The downfield portion of a resolution-enhanced 1-D n.m.r. spectrum of depyruvated EPSb is reproduced in Fig. 3.

Unambiguous assignments for all proton resonances in the ^{1}H-n.m.r. spectrum of depyruvated EPSb were obtained by successive applications of the 2-D DQF-PS-COSY, RELAY, TOCSY, and TQF-COSY sequences. Results from the latter two experiments are reproduced in Fig. 4A, and the assignments are summarized in Table II. Only two spin-systems were observed, which could be followed from anomeric resonances at 5.42 and 4.75 p.p.m.; analysis of splitting constants made it clear that these were from α-Gal and β-Glc residues, respectively. It was hoped that PS-NOESY data would give indications of linkage sites by displaying clear interglycosidic enhancements. The expected off-diagonal peaks were indeed observed, one interglycosidic cross-relaxation for each anomeric proton (Fig. 4B). However, in each case the n.O.e. cross peak corresponded to a pair of degenerate resonances, to the α-Gal H-2/3 from β-Glc H-1, and to the β-Glc H-3/4 from α-Gal H-1. Thus, it was impossible to specify more precisely the inter-residue linkage sites. This was left to methylation analysis, as described next.

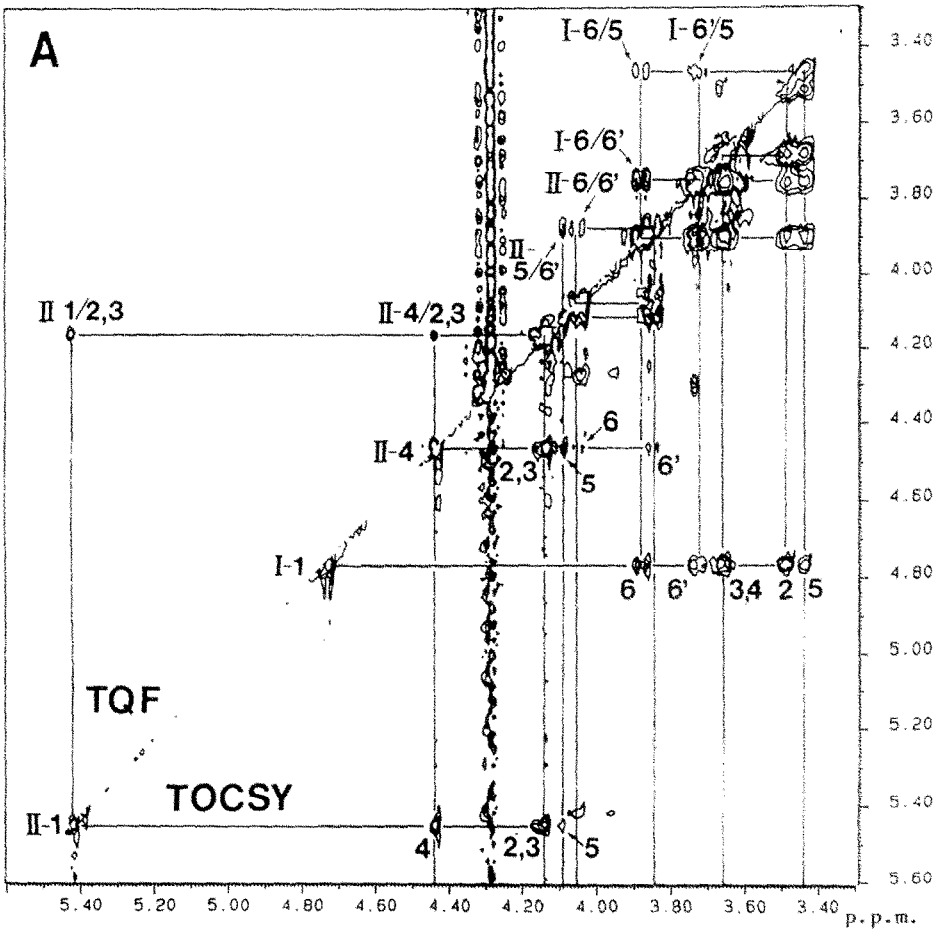
Methylation analysis. — Following successive permethylation, hydrolysis, reduction, and acetylation, intact EPSb yielded a simple pattern of partially methylated alditol acetates (PMAAs): mainly 2,4,6-tri-*O*-Me-Glc and 2-mono-*O*-Me-Gal, with a small amount of 2,4,6-tri-*O*-Me-Gal. When EPSb was depyruvated by acid treatment prior to permethylation, only 2,4,6-tri-*O*-Me-Glc and α-Gal were obtained. This clearly indicates →3Glc and →3Gal linkages exclusively, while confirming the 4,6-location of the 1-carboxyethylidene groups.

These results show that EPSb has a simple repeating-unit structure,



confirming our previous proposal[12]. An acetate group is present at an as yet undetermined site. The same structure was also proposed by Glazebrook and Walker[13], who further specified the site of acetylation as the 6-position of β-Glc. Details of that structure elucidation appeared just as this paper was being submitted[16].

It is interesting to consider how EPSb can perform the same function as the *R. meliloti* succinoglycan, which has a considerably more complex octasaccharide repeat-


A
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4
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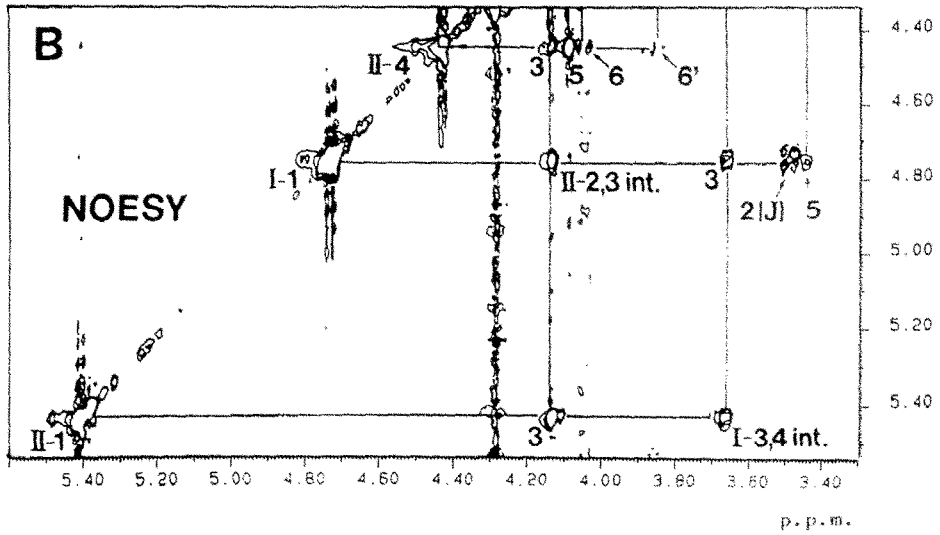
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5.40 5.20 5.00 4.80 4.60 4.40 4.20 4.00 3.80 3.60 3.40
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ing-unit. As pointed out by Glazebrook and Walker[13], the two structures have one feature in common, the β-Glc-(1→3)-Gal disaccharide linkage, which they suggest might represent a common recognition site in the nodule invasion process, particularly if the corresponding OAc groups occupy the same position in both polysaccharides. However, the corresponding galactose residue in *R. meliloti* succinoglycan is not pyruvated as in EPSb. Thus, the proposed determinant would have to occupy a rather small area of molecular topology, including only the unmodified part of the galactose ring (around HO-2), and a contiguous portion of the glucosyl residue. Whether this is sufficient to confer the required specificity might be questionable. It is worth noting that, even within the family of related succinoglycan structures produced by different species and strains of *Rhizobia*, the extent to which host-related specificity is conferred by shared *vs.* unique features is an issue still open to debate[15,17]. Further biological experiments, along with a careful analysis of the three-dimensional structural features of the polysaccharides, will be required for clarification of this question.

EXPERIMENTAL

Isolation of EPSb. — EPSb was isolated from culture supernatants of *R. meliloti* strain Rm7011 exoA (pMuc) as previously described[11].

Depyruvation. — Samples of EPSb were treated with 10% v/v aq. HOAc (1 mL), heated in a sealed tube for 20 h at 100°. Following removal of HOAc by flushing with N_2 at 37°, using EtOH as co-distallant, samples were dried *in vacuo* over NaOH for 3 h.

Monosaccharide analysis. — To samples of EPSb, before and after depyruvation (100 μg each), in 13 × 100 test tubes with Teflon-lined screw caps, were added M HCl in anhydrous MeOH (1.0 mL). These were sealed and heated for 16 h at 80°. The solutions were made neutral with Ag_2CO_3, and Ac_2O (50 μL) was added. After 6 h at 4°, the tubes were centrifuged and the MeOH supernatant removed, washing the precipitate once with MeOH (1.0 mL). The combined MeOH solutions were evaporated in clean tubes under N_2, and then dried *in vacuo* over P_2O_5 for 2 h. The monosaccharide methyl glycosides were converted into pertrimethylsilyl ethers[18,19] and analyzed by g.l.c.–e.i.m.s. on a Hewlett–Packard 5890A gas chromatograph interfaced to a 5970B mass-selective detector (g.l.c.-MSD), using a 30-m DB-5 bonded phase fused silica capillary column (splitless injection, temperature program 150–250° at 4°/min). Monosaccharide derivatives were identified by characteristic retention times and mass spectra compared either with those of standard compounds, or with spectra published for pyruvated compounds[14]. Alternatively, the analysis was performed on a system consisting of a Hewlett–Packard 5890J gas chromatograph interfaced to a Jeol HX-110/DA-5000 mass spectrometer/data system operating in the chemical-ionization (isobutane) mode.

Fig. 4. Sections of 2-D ^{1}H-n.m.r. spectra of depyruvated EPSb: A, upper left-hand sector, TQF-COSY; lower right-hand sector, TOCSY; B, NOESY. J, in panel B, refers to a cross-peak corresponding mainly to *J* coupling between β-Glc H-1 and H-2 which was not suppressed; int. refers to interglycosidic dipolar correlations.

Methylation analysis. Samples of EPSb, before and after depyruvation (200 μg each), were peracetylated using 1:1 Ac_2O–pyridine (1.0 mL) in a sealed tube at room temperature for 4 days. Following removal of reagents by evaporation under N_2 at 37°, using toluene as co-distillant, the samples were dried *in vacuo* over P_2O_5 and permethylated[20]. The permethylated samples were hydrolyzed in 0.25M H_2SO_4 90% HOAc, reduced with $NaBD_4$, and acetylated according to published procedures[21], and the resultant partially methylated alditol acetates analyzed by g.l.c.–m.s. under conditions previously described[22], with identification of derivatives made by comparison of retention times with authentic standards and by characteristic electron-impact spectra[23,24].

Proton nuclear magnetic resonance spectroscopy. — Samples (~3 mg) were deuterium exchanged by repeated lyophilization from D_2O (Aldrich, 99.96 atom %), then dissolved in 0.4 mL of this solvent for ^{1}H-n.m.r. All spectra were recorded at 350 ± 2K on a Bruker AM-500 Fourier transform spectrometer/Aspect 3000 data system. For 1-D spectra, the sweep width was 4000 Hz, collected over 16K data points. Sodium 3-(trimethylsilyl)propionate-*2,2,3,3,-d*$_4$ was used as the internal chemical-shift standard.

Pure absorption phase-sensitive correlated spectroscopy (PS-COSY) was performed using the method of time-proportional phase increments (TPPI)[25] with double quantum filtering (DQF)[26–27]. Time domain spectra were acquired with a sweep width of 4000 Hz over 4K data points in t_2, and 1K experiments in t_1. One-step relayed coherence-transfer spectroscopy (RELAY)[28] was performed using a mixing time (τ) of 52 msec. A total of 512 t_1 experiments were collected. An increment to t_2 of 250 μsec per experiment gave a t_{2max} = 128 msec. The data were transformed as a 2K × 1K matrix, with a non-phase-shifted sine-bell window function applied in both dimensions, as recommended[29].

Total correlation spectroscopy (TOCSY)[30] was performed using the MLEV-17 pulse scheme described by Bax and Davis[31], with a total mixing time of 230 msec. A total of 590 TPPI experiments were collected with 96 transients per t_1, and a PD of 2.0 sec. The data were transformed as a 2K × 1K matrix, with phase-shifted sine-bell apodization applied in both dimensions (π/4 in t_2 and t_1). Triple quantum filtered 2-D correlation spectroscopy (TQF-COSY) was performed as described by Piantini *et al.*[32], and Shaka and Freeman[33], 512 t_1 experiments were collected, and the data transformed as a 2K × 2K matrix, with sine-bell apodization applied in both dimensions.

Phase-sensitive homonuclear dipolar-correlated 2-D n.m.r. spectroscopy (NOESY) was performed in the TPPI mode[34] with a mixing time of 300 msec, and a PD of 2.0 sec. A random variation of ±20% of the mixing time was introduced to suppress zero-quantum (scalar) correlations; 512 t_1 experiments were collected, and the data transformed as a 2K × 2K matrix with phase-shifted sine-bell apodization applied in both dimensions (π/2 in t_2 and t_1).

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