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Application of 1D and 2D NMR techniques to the structure elucidation of the O-polysaccharide from *Proteus mirabilis* O:57

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SUMMARY

The LPS O-polysaccharide (O-PS) produced by *Proteus mirabilis* serotype O:57 (ATCC 49995) was shown by NMR spectroscopy and chemical analysis to be a high-molecular-weight acidic branched polymer of pentasaccharide repeating units, composed of D-glucose, D-galactose, 2-acetamido-2-deoxy-D-galactose and glycerophosphate residues (1:2:2:2:1). Application of one- and two-dimensional NMR methods allowed the complete assignment of notoriously crowded ^{1}H and ^{13}C spectra of the O-PS, leading to the determination of its structure. Several of the NMR techniques used were applied for the first time to the structure elucidation of polysaccharides. The resonances of galactose H5, H6 and H6' were identified by a 1D analog of 3D NOESY-TOCSY and 2D $\{^{1}H,^{1}H\}$ triple-quantum experiments. The position and the nature of the phosphate group were determined from 2D ^{31}P (ω_1)-half-filtered COSY and 2D ^{31}P-relayed COSY spectra. 2D HMQC-TOCSY and 2D single-quantum proton–carbon long-range correlation techniques were used to overcome the difficulties of severe overlap and higher order effects in the ^{1}H NMR spectrum of the O-PS. The latter technique, together with 2D NOESY, enabled us to identify the substitution positions, the anomeric configurations and the sequence of the component glycose residues in the O-PS.

INTRODUCTION

Strains of *Proteus mirabilis* and *Proteus vulgaris* belong to a gram-negative bacterial species particularly implicated in urinary tract infections and the production of urinary stones, which generally contain struvite ($MgNH_4PO_4.6H_2O$) or carbonate-apatite ($Ca_{10}(PO_4)_6CO_3$) crystals (McLean et al., 1985,1991). While urease production is a common virulence determinant for these

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pathogens (Griffith et al., 1976), this alone is not cause enough to account for stone formation. Capsular polysaccharides (CPS) and lipopolysaccharides (LPS) of pathogenic strains of *Proteus* have been hypothesized to be potential virulence factors which possibly accelerate crystal growth (Clapman et al., 1990; McLean et al., 1991) and cause aggregation of urine-precipitated components to form stones in an alkaline environment, induced from associated urease activity.

The structures of many O-polysaccharides (O-PS) of *Proteus* have been reviewed (Knirel et al., 1993). We have undertaken investigations of the structures of CPS and LPS from clinically isolated *Proteus* strains exhibiting consistent experimental pathogenicity. There is accumulating evidence that a common characteristic of these polysaccharides is their acidic nature. The acidic characteristics have been shown to originate in the presence of component hexuronic acid (Beynon et al., 1992), substituent pyruvate ketals (Perry and McLean, 1994) or phosphate groups. This paper describes the application of novel NMR techniques to the elucidation of the fine structure of the LPS O-chain of *P. mirabilis* O:57, in which the acidic component is a phosphoglycerol substituent. Concurrent work is being undertaken to investigate the role of the characterized isolated polysaccharides in urinary stone formation.

MATERIALS AND METHODS

LPS and O-PS production

Cells of *P. mirabilis* serotype O:57 (ATCC 49995) were grown in brain–heart infusion (Difco) at 37 °C in a fermenter (28 L, New Brunswick) and were extracted by a modified enzyme aqueous phenol method (Johnson and Perry, 1976). Isolation procedures involving ultracentrifugation (105 000 × g, 4 °C, 12 h) to precipitate LPS were the same as those previously described (Beynon et al., 1992). Treatment of LPS with 2% acetic acid (100 °C, 2 h) afforded insoluble lipid A, which was removed by low-speed centrifugation. The lyophilized water-soluble hydrolysis products were separated by Sephadex G-50 chromatography; fractions containing O-PS, core oligosaccharide and released Kdo were collected and lyophilized.

Chemical analysis methods

Glycose analyses, gas–liquid chromatography (GLC), mass spectrometry (MS), methylations, dephosphorylations, periodate oxidations and chromatographic separations were done as previously described (Moreau et al., 1988).

Nuclear magnetic resonance spectroscopy

Spectra were recorded on Bruker AMX-500 (500.13 MHz ^{1}H) and AMX-600 (600.13 MHz ^{1}H) spectrometers. The observed ^{1}H chemical shifts are reported relative to internal acetone (2.225 ppm). The carbon chemical shifts are quoted relative to the methyl group of internal acetone (31.07 ppm). The phosphorus chemical shifts are reported relative to the external reference signal of 85% H_3PO_4, contained in a sealed capillary tube. The O-PS sample (30 mg) was exchanged in D_2O and, following lyophilization, the NMR sample was prepared by redissolving it in 0.5 ml D_2O (99.96% deuterated). All NMR data sets were recorded at 42 °C, without sample spinning, except for the ^{31}P-relayed $\{^1H,^1H\}$ correlation experiment which was acquired at 25 °C.

500 MHz 1D TOCSY spectra (Kessler et al., 1989), of 16 scans each, were obtained using a half-Gaussian pulse (Friedrich et al., 1987) of 106 or 67 ms, depending on the position of the

neighbouring signal. The duration of the spin-lock period, realized via the MLEV-17 sequence (Bax and Davis, 1985), was 95 ms for β-glycoside and 135 ms for α-glycoside residues. Trim pulses of 2.5 ms were applied before and after the spin-lock.

600 MHz 1D NOESY spectra (Kessler et al., 1989), of 128 scans each, were acquired using a half-Gaussian pulse of 78 ms and a mixing time of 200 ms. H5 and H6 resonances in β-D-Gal*p* residues were assigned applying a 1D analog of the 3D NOESY-TOCSY experiment (Uhrín et al., 1994). Both selective pulses were 78 ms half-Gaussian pulses. Mixing times of 200 and 23 ms (including two 2.5 ms trim pulses) were used for the NOESY step and for the TOCSY transfer, respectively. For each spectrum 512 scans were accumulated.

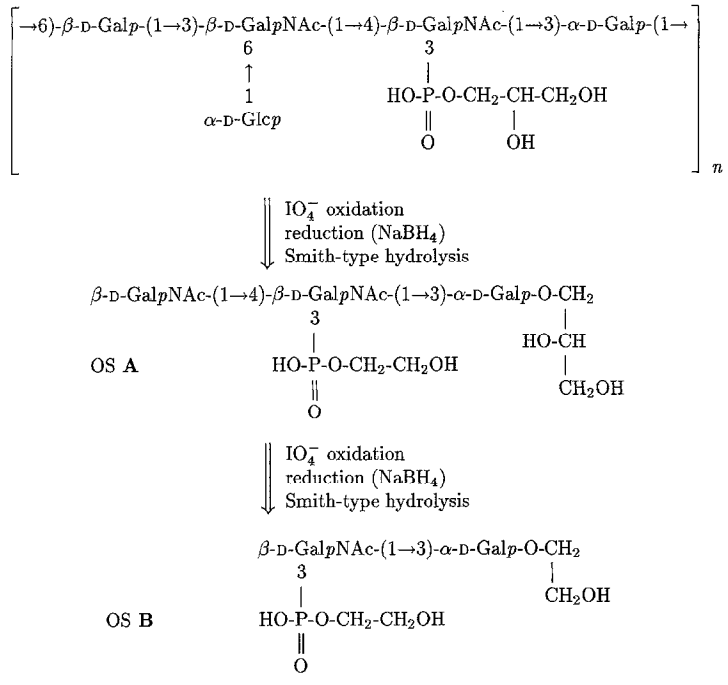
A 2D $\{^1H,^1H\}$ triple-quantum experiment (Brownstein and Bornais, 1990) was acquired at 600 MHz with the rf carrier placed at 4.0 ppm. The spin-echo period for the creation of triple-quantum coherences was set to 60 ms and the relaxation time was 2 s. A data matrix of 128×2048 points was acquired with 48 scans per t_1 increment and acquisition times of 66.7 ms and 0.74 s in the t_1 and t_2 domains, respectively. The data matrix was zero-filled to 512×2048 points and processed in the magnitude mode with a squared sine function in both dimensions.

A 2D ^{31}P (ω_1)-half-filtered $\{^1H,^1H\}$ COSY experiment (Bolton, 1985; Otting and Wüthrich, 1990), acquired at 500 MHz, was optimized for a $J_{P,H}$ of 10 Hz, i.e., the spin-echo filter period was set to 50 ms. The (t_1,t_2) matrix consisted of 128×2048 data points. Acquisition times of 141.8 ms and 1.13 s were used in t_1 and t_2, respectively, together with 32 scans per t_1 increment. The data matrix was zero-filled to 512×2048 points and processed in the magnitude mode with a sine function in both domains.

A 500 MHz 2D ^{31}P-relayed $\{^1H,^1H\}$ correlated spectrum (Delsuc et al., 1984; Neuhaus et al., 1984) was acquired and processed using the same conditions as the previously described ^{31}P (ω_1)-half-filtered COSY experiment, except that 128 scans were acquired for each FID. Since only one resonance line appeared in the ^{31}P spectrum of the O-PS, both 180° pulses were omitted from the original pulse sequence and the decoupler rf carrier was set to the chemical shift of the ^{31}P signal. The fixed evolution interval was set to 16 ms. This value corresponds to the spin–spin relaxation time of the ^{31}P nucleus in the O-PS, estimated from the line width at half height of the phosphorus line at 25 °C ($T_2=1/\pi\Delta_{1/2}$). The spin–spin relaxation at this temperature was considerably slower ($T_2^{25\,°C}$ = 16 ms) than that at 42 °C ($T_2^{42\,°C}$ = 10 ms), the temperature at which all the other experiments were performed. This observation led us to decrease the temperature for this particular experiment. The phase-sensitive spectrum was acquired using the TPPI procedure (Marion and Wüthrich, 1983). The first increment, $t_1(0)$, was calculated according to $t_1(0) = DW(t_1) - 4/\pi t_{90(1H)}$ (Archer et al., 1992), where $DW(t_1)$ and $t_{90(1H)}$ are the sampling delay in t_1 and the duration of a 90° ^1H pulse. A zero-order phase correction of 90° and a first-order correction of −180° were applied in F_1.

A 500 MHz 2D NOESY spectrum was acquired using the TPPI procedure, with a mixing time of 180 ms. The first increment of the 2D matrix was calculated according to the procedure described for the ^{31}P-relayed $\{^1H,^1H\}$ correlated spectrum and the corresponding phasing was applied. Acquisition times of 127 ms and 0.51 s were used in t_1 and t_2, respectively, and 32 scans were acquired in each of 512 experiments into 2048 data points. The 2D matrix was zero-filled to 1024×2048 data points and multiplied by a squared sine function, shifted by $\pi/3$.

A DEPT (Doddrell et al., 1982) spectrum was acquired using a 135° editing pulse, yielding opposite phases for signals of methine and methylene carbons.



Scheme 1. Stepwise periodate oxidation of *Proteus mirabilis* O:57 LPS O-polysaccharide.

All the carbon–proton correlation spectra were acquired at 600 MHz. HMQC (Bax et al., 1983) and HMQC-TOCSY (Lerner and Bax, 1986; Gronenborn et al., 1989) were acquired using WALTZ-16 (Shaka et al., 1983) for the ^{13}C decoupling during the acquisition. A spin-lock of 22 ms was applied in the proton frequency for the HMQC-TOCSY experiment. The long-range proton correlation via single-quantum coherences (LR-HSQC) (Uhrín et al., 1992) was optimized for $J_{C,H}$=12.5 Hz by using an evolution delay of 40 ms.

No BIRD pulse was applied before the start of any of the heteronuclear sequences used. In the LR-HSQC experiment, resonances of protons from ^{12}C isotopomers were suppressed by a 2.5 ms spin-lock pulse, applied along the x-axis immediately before the acquisition. All the other pulses were cycled as described previously (Uhrín et al., 1992). The ^{13}C rf carrier frequency was set to 80 ppm with a sweep width of 62 ppm. Acquisition times were 27.3 ms in t_1 and 0.27 s in t_2, except for the long-range correlation experiment where the acquisition time in t_2 was set to 0.55 s. In each t_1 increment, 48 scans were acquired for HMQC, 192 for HMQC-TOCSY and 256 for LR-HSQC experiments. Phase-sensitive acquisition of the data in the t_1 dimension was done by TPPI. Data matrices of 512 × 1024 points were acquired (512 × 2048 points for the LR-HSQC experiment), zero-filled to 1024 × 2048 points and multiplied prior to Fourier transformation by a squared sine function, shifted by $\pi/3$ in both domains. The first increment, $t_1(0)$, was calculated according to $t_1(0) = DW(t_1) - 4/\pi t_{90(13C)} - t_{180(1H)}$ (Archer et al., 1992), where $DW(t_1)$ is the sampling delay in t_1 and $t_{90(13C)}$ and $t_{180(1H)}$ are the duration of a 90° ^{13}C pulse and a 180° ^{1}H pulse, respectively. Zero- and first-order phase corrections of 90° and −180°, respectively, were applied in F_1.

RESULTS

The LPS produced by *P. mirabilis* serotype O:57 was obtained in 8% yield by extraction of fermenter-grown cells with hot aqueous phenol, followed by ultracentrifugation of the dialysed and concentrated separated aqueous phase. Mild hydrolysis of the LPS released an insoluble lipid A and Sephadex G-50 gel filtration chromatography of the concentrated water-soluble products afforded the O-polysaccharide (O-PS, K_{av} 0.03, 22%), a core oligosaccharide (K_{av} 0.34, 24%) and a fraction (K_{av} 0.95, 11%) containing 3-deoxy-D-manno-octulosonic acid and phosphate.

The O-PS had $[\alpha]_D$ +72° (c 1.0, water), chemical analysis yielded C, 38.34; H, 5.58; N, 2.82; and ash, 8.0%, and colorimetric analysis gave phosphate 7.2%. Analysis of the dephosphorylated (48% HF) O-PS gave a composition of D-galactose (D-Gal), 2-amino-2-deoxy-D-galactose (D-GalN), and D-glucose (D-Glc) (2:2:1); the configuration of the glycoses was determined by capillary GLC of their trimethylsilylated-derived 2-(*S*)-butyl glycosides (Gerwig et al., 1978, 1979). The native O-PS liberated glycerol (ca. 3%) on alkaline hydrolysis (1 M NaOH, 95 °C, 2 h). The compositional analysis indicates that the O-PS is a polymer of repeating pentasaccharide units, containing a phosphoglycerol substituent.

Methylation analysis of the O-PS as well as the dephosphorylated O-PS gave unusually and inexplicably low yields of methylated aminodeoxyglycose products, allowing for only nonstoichiometric tentative identification of their linkages. GLC-MS analysis of reduced ($NaBD_4$) and acetylated hydrolysis products of methylated O-PS afforded 2,3,4,6-tetra-*O*-methyl-D-Glc, 2,4,6-

TABLE 1
NMR CHEMICAL SHIFTS (PPM) OF THE O-POLYSACCHARIDE COMPONENT OF THE LPS PRODUCED BY *PROTEUS MIRABILIS* O:57 LPS

Assignment		Residue					
		α-Glc (**a**)	α-Gal (**b**)	β-GalNAc (**c**)	β-GalNAc (**d**)	β-Gal (**e**)	Glycerol (**f**)
^1H	H1	4.96	4.93	4.72	4.68	4.48	3.88–3.90
	H2	3.56	3.89	4.12	3.97	3.53	3.89
	H3	3.71	3.89	3.89	4.23	3.64	3.67, 3.61
	H4	3.42	4.15	4.14	4.26	4.03	–
	H5	3.67	3.91	3.90	3.70	3.83	–
	H6	3.76	3.74	3.93	3.81	3.82	–
	H6'	3.87	3.74	3.69	3.81	3.76	–
	NAc	–	–	2.03	2.06	–	–
^{13}C	C1	99.4	100.2	103.2	103.7	105.7	67.3
	C2	72.2	68.3	51.9	52.8	71.5	71.6
	C3	73.9	79.6	80.6	75.5	73.3	63.1
	C4	70.5	70.1	69.1	75.5	69.0	–
	C5	72.8	71.7	73.3	74.8	73.2	–
	C6	61.5	62.1	67.8	62.1	66.9	–
NAc	CH_3	–	–	23.3	23.3	–	–
	CO	–	–	175.3[a]	175.9[a]	–	–

[a] These signals can be interchanged.

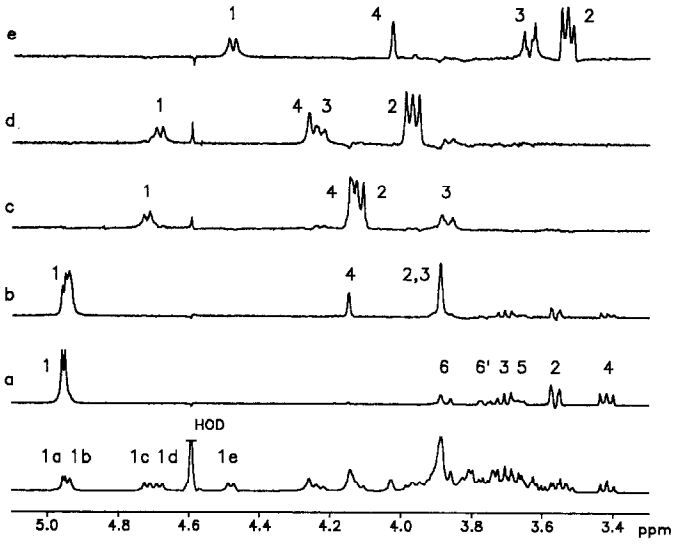


Fig. 1. A set of 500 MHz 1D TOCSY spectra of the O-PS. A half-Gaussian pulse was applied for selective excitation of anomeric protons in residues **a**–**e**.

tri-*O*-methyl-D-Gal, 2,3,4-tri-*O*-methyl-D-Gal, 6-*O*-methyl-D-GalNMe and 4-*O*-methyl-D- GalNMe (trace), suggesting that the repeating unit contains a nonreducing D-Glc*p* residue, 3-*O*- and 6-*O*-monosubstituted D-Gal*p* residues and 3,6-di-*O*- and 3,4-di-*O*-substituted D-Gal*p*NAc residues.

Smith-type hydrolysis of the periodate oxidized and reduced ($NaBH_4$) O-PS (Scheme 1) resulted in oxidation of the 6-*O*-linked D-Gal*p* and terminal nonreducing D-Glc*p* residues. On Biogel P2 gel filtration chromatography, this resulted in a major fraction (**A**, K_{av} 0.36) which had $[\alpha]_D$ +64° (c 2.3, water) and was composed of D-Gal, D-GalN, phosphate, glycol and glycerol (1.2:0.9:0.6:0.5). The formation of the trisaccharide **A** indicates that the D-Glc*p* residue was present in the O-PS as a single unsubstituted nonreducing side group and that the 6-*O*-linked D-Gal*p* residue was present as an unsubstituted residue in a linear backbone chain.

Periodate oxidation of **A**, followed by sequential reduction ($NaBH_4$), Smith-type hydrolysis and Biogel P2 chromatography, gave an oligosaccharide (**B**, K_{av} 0.40) which had $[\alpha]_D$ +61° (c 0.3, water) and was composed of D-GalN, D-Gal, phosphate and glycol (1.0:1.0:0.8:1.2). The disaccharide **B** was not further oxidized by periodate, indicating that the D-GalNAc residue is protected by phosphate, substituted at either positions O3 or O4, and that it forms the nonreducing terminal end of **B**. Methylation and ^1H NMR data were consistent with the characterization of **A** and **B** depicted in Scheme 1. The results of the periodate oxidations are consistent with the position linkages and configuration subsequently obtained from a detailed NMR study of the native O-PS.

The ^1H NMR spectrum of the O-PS showed, inter alia, signals for *N*-acetyl groups at 2.03 (3H) and 2.06 (3H) ppm, together with five anomeric proton signals at 4.96, 4.93, 4.72, 4.68 and 4.48 ppm (Table 1), consistent with the proposed pentasaccharide repeating unit. In agreement with this, the ^{13}C NMR spectrum of the O-PS showed signals for the five anomeric carbons at 105.7,

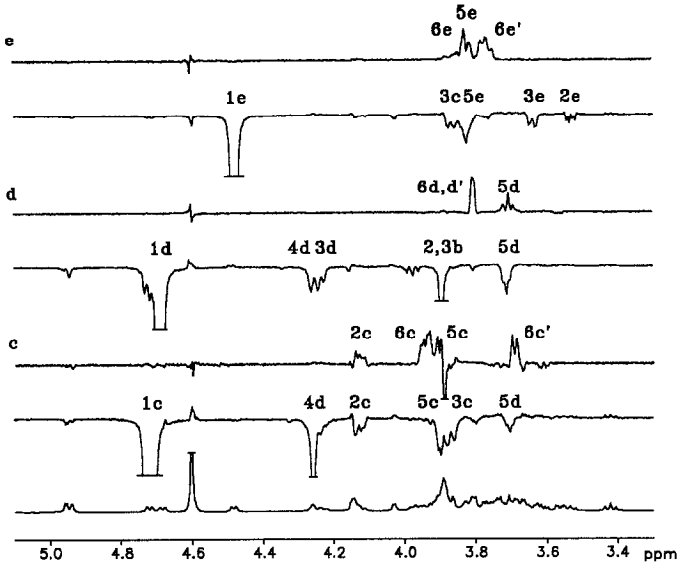


Fig. 2. A set of 600 MHz 1D NOESY and 1D NOESY-TOCSY spectra of the O-PS. Corresponding 1D NOESY (negative phase) and 1D NOESY-TOCSY (positive phase) spectra are placed together and labelled **c**, **d** and **e**, according to the anomeric proton to which the initial selective pulse was applied. A second selective pulse in the 1D NOESY-TOCSY experiments was always applied to the H5 proton of the respective residue.

103.7, 103.2, 100.20 and 99.4 ppm, acetamido signals at 175.9 and 175.3 ($NHCOCH_3$) and 23.3 ppm (2C) ($NHCOCH_3$), as well as C2 signals at 52.8 and 51.9 ppm from two D-GalNAc residues. The 1D ^{31}P NMR spectrum (pH 2.9) showed a singlet at −1.6 ppm.

^1H resonances of the O-PS were partially assigned by a series of 1D TOCSY experiments (Fig. 1), from which the configurations of the individual glycoside ring systems were determined, one being an α-D-Glc*p* (**a**), and three (**c**, **d** and **e**) having β-D-Gal*p* configurations. In addition, a glycose having the α-D-Gal*p* configuration was tentatively assigned to the remaining residue **b**. Due to strong coupling between H2 and H3 resonances of this residue, the H1b to H4b signals appeared as broad singlets. Significant differences in the chemical shifts for the corresponding signals of H1 to H4 in β-D-Gal*p* residues **c**, **d** and **e** suggested different linkage and/or substitution patterns (NAc, PO_4^-) in these residues.

In 1D TOCSY experiments, the transfer of magnetization was relayed as far as H4 for all the D-Gal*p* residues. In principle, the assignment of the galactopyranosyl resonances could be completed by using a 3D NOE-HOHAHA experiment (Oschkinat et al., 1988; Vuister et al., 1988). In this experiment the NOEs between the H1 and H5 in β- and H4 and H5 in both α- and β-D-Gal*p* residues, followed by a short HOHAHA mixing time, would produce cross peaks H1/H5/H6, H1/H5/H6' and H4/H5/H6, H4/H5/H6', respectively. A 1D analog of this 3D experiment, a 1D NOESY-TOCSY, was employed for the assignment of H5, H6 and H6' resonances in β-D-Gal*p* residues. First the H5 resonances were located by 1D NOESY experiments, using a selective NOE transfer from H1; then the selective NOE transfer from H1 was followed by a selective TOCSY transfer from H5 in the 1D NOESY-TOCSY experiment (Fig. 2). Since the $J_{4,5}$ is very small, a short mixing time (~20 ms) used for the TOCSY transfer caused the proton magnetization to

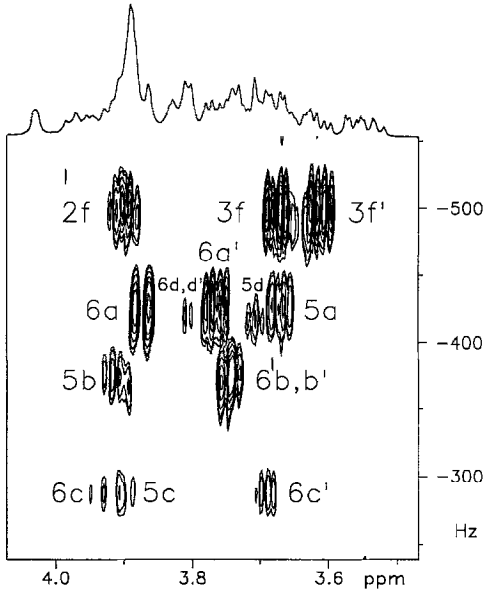


Fig. 3. A partial 600 MHz 2D {^{1}H,^{1}H} triple-quantum spectrum of the O-PS, with the relevant part of the 1D spectrum at the top.

oscillate between the H5, H6 and H6' protons only. Despite severe overlap between H5, H6 and H6' in residues **c**, **d** and **e**, all the assignments could be made using this procedure.

The benefits of this approach are evident from a comparison of 1D NOESY-TOCSY spectra (Fig. 2) and a 2D {^{1}H,^{1}H} triple-quantum spectrum of the O-PS (Fig. 3). In the latter spectrum, signals of residues **c** and **d** appeared as weak cross peaks, while resonances of residue **e** were not observed. This can be attributed to higher order effects, resulting from very close chemical shifts of the H6c/H5c, H6d/H6d' and H6e/H5e resonance pairs. The most intense signals observed in the {^{1}H,^{1}H} triple-quantum spectrum (Fig. 3) belong to the well-separated resonances of residue **a**. Two more sets of signals were found in this spectrum, at F_1 frequencies of −374 and −497 Hz. One of them arises from residue **b**, while the other is associated with residue **f**, and was subsequently proved to belong to a phosphate-substituted glycosyl moiety.

A discrimination between the spin systems of residues **b** and **f** was obtained from a 2D ^{31}P(ω_1)-half-filtered COSY. In this experiment only resonances of protons having a heteronuclear J-coupling with phosphorus appear on a diagonal, while the off-diagonal signals belong to protons coupled via homonuclear J-coupling with protons experiencing the coupling with phosphorus. The spectrum has an asymmetric character, since the magnetization pathway from protons not having a coupling with phosphorus to their homonuclearly coupled neighbours is eliminated. A set of resonances, consisting of a diagonal signal at ~3.9 ppm and corresponding off-diagonal signals at 3.67 and 3.61 ppm, was observed in the 2D ^{31}P(ω_1)-half-filtered COSY spectrum (Fig. 4a). These signals, identical with those identified in the {^{1}H,^{1}H} triple-quantum spectrum (Fig. 3) at the F_1 frequency of −497 Hz, were therefore assigned to residue **f**. Hence, by elimination, signals in the {^{1}H,^{1}H} triple-quantum spectrum at the F_1 frequency of −374 Hz were assigned to H5,6,6' of residue **b**. Another diagonal signal observed in the 2D ^{31}P(ω_1)-half-filtered

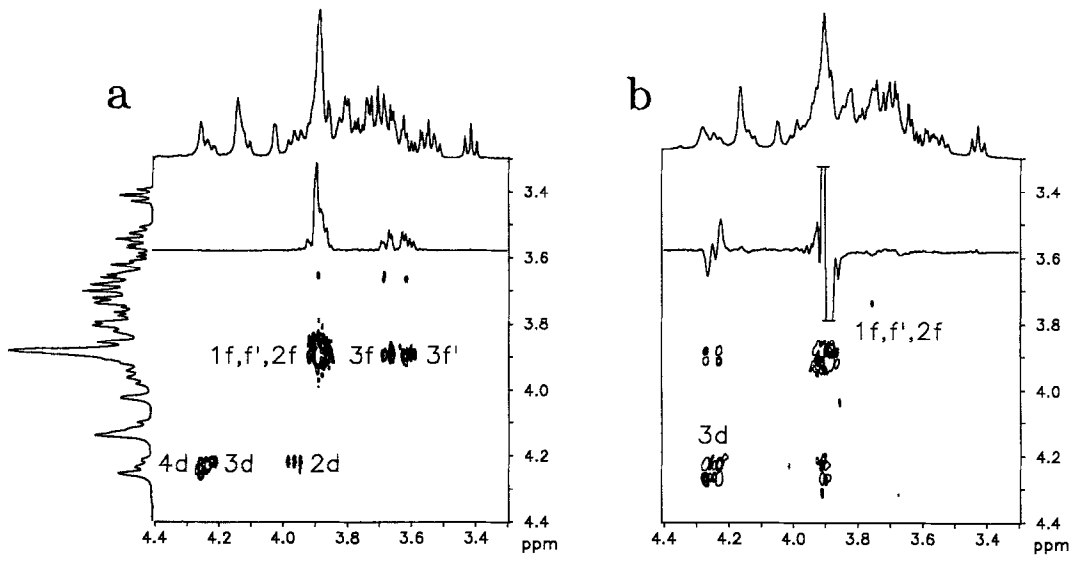


Fig. 4. (a) A 500 MHz 2D $^{31}P(\omega_1)$-half-filtered $\{^1H,^1H\}$ COSY spectrum of the O-PS. Only protons coupled with a ^{31}P nucleus appear on the diagonal; off-diagonal signals represent spins coupled to protons having a $J_{P,H}$ coupling. (b) A 500 MHz 2D ^{31}P-relayed $\{^1H,^1H\}$ correlated spectrum of the O-PS. A cross peak between the 3d and 1f,1f' protons appears because both H3d and H1f,1f' are coupled to the same ^{31}P nucleus, although their mutual proton–proton coupling is zero. Spectra (a) and (b) are magnitude- and phase-sensitive, respectively. F_2 traces at approximately 3.9 ppm are shown in both spectra.

COSY spectrum of the O-PS belonged to H3d and showed cross peaks to H4d and H2d. Thus, the presence of a phosphoryl group could be established at C3d. This was not evident from the 1D TOCSY spectrum of the **d** ring (Fig. 1d), because of partial overlap between the H3d and H4d resonances, which obscured identification of the additional splitting of H3d caused by $^3J_{P,H3d}$.

The chemical shifts and splitting pattern of two signals for H3f (dd, couplings of 11.7 and 3.7 Hz) and H3f' (dd, couplings of 11.7 and 5.6 Hz) observed in the 2D $^{31}P(\omega_1)$-half-filtered COSY spectrum were indicative of the CH_2 protons of a -$C^{2f}H$.OH-$C^{3f}H_2$OH moiety. Due to the nature of this experiment, the corresponding diagonal signal at 3.90 ppm would normally originate from a proton coupled to the phosphorus (i.e. H2f). This interpretation of the data would point to the presence of 1,2-ethanediol in the O-PS, which is not in agreement with the observed liberation of glycerol on hydrolysis of the native O-LPS. Results from a 2D ^{31}P-relayed $\{^1H,^1H\}$ correlated experiment (Fig. 4b) also suggested that the proton resonating at 3.90 ppm is coupled to the phosphorus. Both these spectral results could, however, also be explained by the presence of a glycerol moiety, in which the CH_2 (1f,f') and CH (2f) signals at 3.90 ppm are overlapping. As described below, the latter alternative was confirmed by proton–carbon correlated experiments.

For the repeating unit of the O-PS, the following NOE contacts were observed in the 2D NOESY spectrum (Fig. 5), as well as in a series of 1D selective NOE spectra (Fig. 2): 1a→6c'; 1b→6e,6e',4e; 1c→4d,3d,5d; 1d→2b or 3b; 1e→3c,4c. Given the intensity ratio between the 1c→4d and 1c→3d,5d as well as the 1e→3c,4c NOEs, the linkages 1c→4d and 1e→3c could be established. The position of the linkage between the **d** and **b** residue, however, remained uncertain since the resonances H2b and H3b overlapped. Also, the cross peak between H1e and H3c,

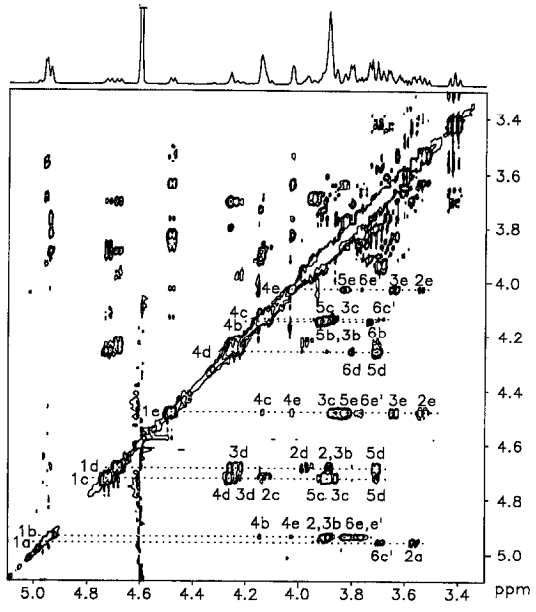


Fig. 5. A 500 MHz 2D NOESY spectrum of the O-PS. Cross peaks of all anomeric and H4 protons of galactopyranosyl residues are labelled. An F_2 projection is shown at the top of the 2D spectrum.

located in the highly crowded spectral area around 3.9 ppm, needed further verification of its assignment.

^{13}C NMR spectroscopy was employed to remove the linkage ambiguities, as well as to establish the character of the **f** substituent. Assignment of carbon resonances was complicated by severe overlap in the proton spectrum of the O-PS. An HMQC experiment indicated at least eight resonances around 3.9 ppm. An HMQC-TOCSY experiment, however, enabled assignment of almost all the carbon resonances in the ^{13}C spectrum of the O-PS. Using a short mixing time (~20 ms), magnetization transfer in the proton dimension occurred in this experiment primarily to

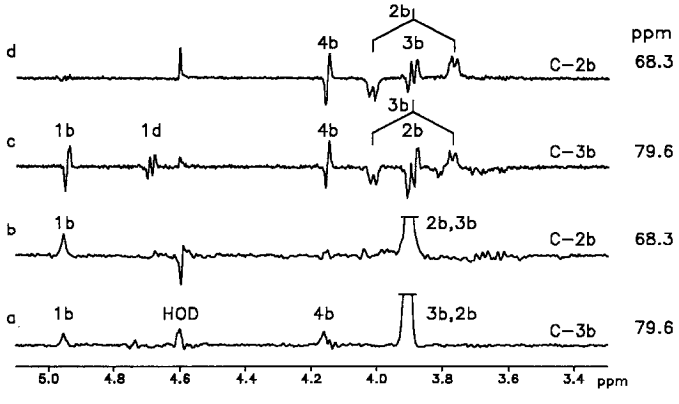


Fig. 6. F_2 traces through the HMQC-TOCSY (a,b) and LR-HSQC spectra (c,d) of the O-PS. These correlations were used to establish the position of the glycosidic linkage between the **d** and **b** rings.

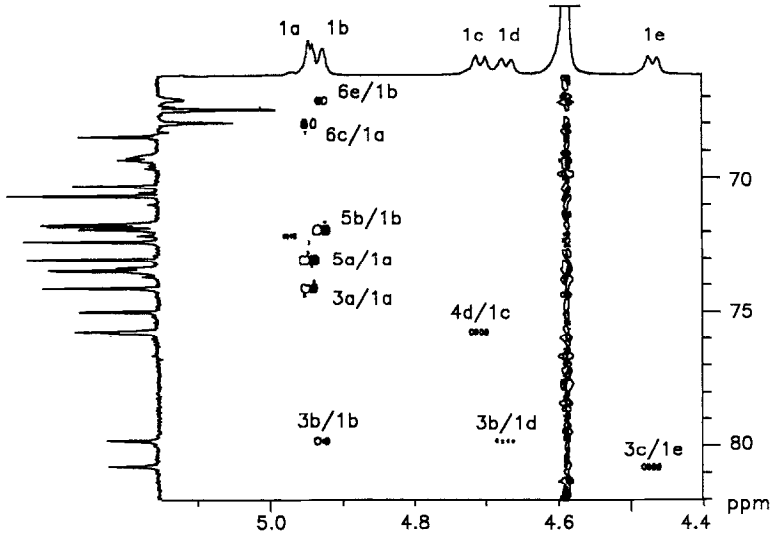


Fig. 7. A partial 2D LR-HSQC spectrum of the O-PS. Only the long-range correlation region of the anomeric protons is shown. Corresponding parts of the proton and DEPT spectra with opposite phases for CH and CH_2 resonances are plotted along the F_2 and F_1 axes, respectively. Only one contour was drawn and positive signals were filled with black. For an explanation of the opposite phase of CH and CH_2 cross peaks, see the Discussion.

protons adjacent to those attached to ^{13}C nuclei. Large coupling constants mediated significant transfer of magnetization, yielding intense cross peaks for diaxial proton–proton arrangements, while less intense cross peaks were observed for axial–equatorial pairs. By analogy to the 1D TOCSY experiments, no transfer occurred between H4 and H5 protons in D-Gal*p* residues. The linkage between the **d** and **b** rings was established by comparing the F_2 traces through the HMQC-TOCSY spectrum at 79.6 and 68.3 ppm (Figs. 6a,b). In the first trace, except for an intense signal of H2b,3b at 3.9 ppm, relay signals of comparable intensity to H1b and H4b were observed, while in the second trace, in addition to the H2b,3b signal, an intense relay signal to H1b and a very weak signal to H4b appeared. The lower field signal (79.6 ppm) was therefore assigned to C3 and the higher field signal (68.3 ppm) to C2, indicating the linkage between the **d** and **b** rings to be 1d→3b. This was further confirmed in a long-range proton–carbon correlation experiment. F_2 traces for C3b and C2b, taken from the LR-HSQC spectrum, are seen in Figs. 6c and d. In accord with the long-range proton–carbon coupling constants for α-D-Gal*p* residues (Morat et al., 1988), both C2b and C3b showed a coupling to H4b, but only C3b gave rise to an observable cross peak to H1b. In addition to these signals, cross peaks for C3b/H2b and C2b/H3b, as well as for C3b/H3b and C2b/H2b pairs were observed. Significantly, the carbon resonance at lower field (79.6 ppm) showed an interresidue coupling to H1d. All the other glycosidic connectivities, as suggested from the 2D NOESY experiment, were confirmed in a long-range correlation spectrum (Fig. 7), including the problematic 1e→3c connectivity.

The identity of the **f** substituent was established from heterocorrelated spectra. Three carbon resonances belonging to this substituent were identified in the HMQC, HMQC-TOCSY and LR-HSQC spectra, confirming the presence of a glycerol spin system in which three protons (H1f, 2f, 2f') resonate at 3.90 ppm. Although protons 1f, 1f' and 2f overlapped in the ^{13}C-decoupled

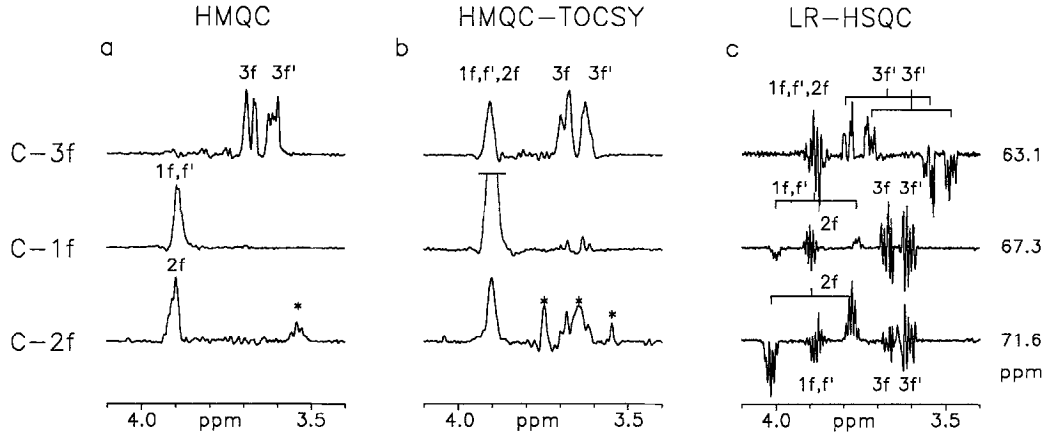


Fig. 8. F_2 traces through the ^{13}C resonances of the glycerol residue **f** (-C^1H_2-C^2H.OH-C^3H_2OH), extracted from different heterocorrelated experiments: (a) HMQC; (b) HMQC-TOCSY; and (c) LR-HSQC. Signals due to overlap of ^{13}C resonances with C2f are labelled with an asterisk.

HMQC and HMQC-TOCSY spectra, the LR-HSQC experiment acquired without ^{13}C decoupling displayed the long-range coupling pattern expected for a glycerol residue (Fig. 8).

These results explain the appearance of a diagonal peak at 3.90 ppm and corresponding cross peaks of 3f and 3f' protons in the 2D $^{31}P(\omega_1)$-half-filtered COSY spectrum (Fig. 4a). Because of overlapping energy levels between H1f, f' and 2f, magnetization originating at H1f,f' was transferred by a mixing pulse of a $^{31}P(\omega_1)$-half-filtered COSY up to H3f,f', the terminal -CH_2OH group of the glycerol moiety. The cross peak observed in the ^{31}P-relayed $\{^1H,^1H\}$ correlated spectrum (Fig. 4b) was primarily due to an H3d/H1f,f' connectivity and not to H3d/H2f. Finally, the signals at −497 Hz observed in the $\{^1H,^1H\}$ triple-quantum spectrum (Fig. 3) were those of H2f, H3f and H3f'. Because of higher order effects, no signals were observed in this spectrum for 1f, 1f' and 2f protons.

Based on the results of the chemical analysis and combined use of homo- and heteronuclear correlated NMR spectroscopy, the structure given in Fig. 9 is ascribed to the polysaccharide O-antigen from *Proteus mirabilis* O:57 LPS.

DISCUSSION

One of the obstacles encountered in applying ^1H NMR spectroscopy to carbohydrate structural analysis is the assignment of glycopyranosyl signals of residues containing axial hydroxyl substituents (e.g. *manno* and *galacto* hexoses). Several techniques for the complete assignment of proton resonances in D-Gal*p* residues have been presented so far. They include identification of resonances for the three-spin system H5,H6,H6' in each residue, followed by establishment of connectivities to the previously identified signals of H1 to H4. Tracing the H5,H6,6' signals from ordinary phase-sensitive double-quantum-filtered COSY spectra (Rance et al., 1983) often fails, due to severe overlap in the spectral region where these resonances usually appear. Various methods have been applied so far to enable this analysis, including that of partially relaxed 2D COSY (Saunders and Stevens, 1986), triple-quantum-filtered 2D COSY (Piantini et al., 1982;

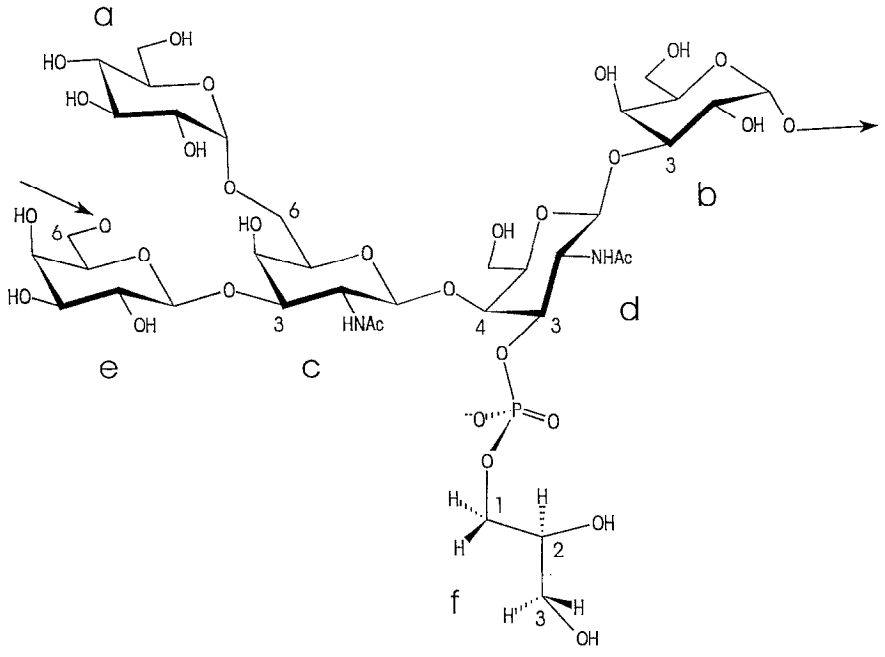


Fig. 9. Structure of the repeating unit of the O-PS from *Proteus mirabilis* O:57.

Homans et al., 1986), {^{1}H,^{1}H} triple-quantum spectroscopy (Brownstein and Bornais, 1990; Van Halbeek and Poppe, 1992), E.COSY (Griesinger et al., 1985; Abeygunawardana et al., 1991a), and 1D chemical-shift selective COSY (Hall and Norwood, 1988; Dabrowski, 1989). However, none of these techniques appears to be universally applicable. Partially relaxed COSY suffers from the occurrence of different relaxation times of methine protons and gives better results for suppression of methylene resonances. Signals from some three-spin systems can be missing in triple-quantum-filtered COSY and {^{1}H,^{1}H} triple-quantum spectra (Abeygunawardana et al., 1990; Reddy et al., 1993) because of possible degeneracy between nuclei involved and/or the presence of very small couplings. Although the E.COSY technique simplifies cross peaks to a certain extent, depending on the character of the spin system and the values of the coupling constants, the level of simplification could still be insufficient, especially if higher order effects are present (Abeygunawardana et al., 1991a).

There are few NMR techniques which can be used for linking the resonances of the three mutually coupled protons H5, H6 and H6' to the rest of the spin system. Since the $J_{4,5}$ coupling in glycoses having the D-Gal*p* configuration is very small ($J_{4,5}$<1 Hz), the H4/H5 cross peak is only observed in DQF-COSY spectra of small oligosaccharides, but not in polysaccharides. The relayed HOHAHA experiment (Inagaki et al., 1989), which has been successfully applied for the assignment of the D-Gal*p* resonances in the relatively small Forssman antigen, is also likely to fail for larger molecules. A more general approach, especially useful for polysaccharides, is based on the detection of H1→H5 intraresidue NOEs in β-D-Gal*p* residues or H4→H5 NOEs in both α-and β-D-Gal*p* residues (Abeygunawardana et al., 1991a,b).

Combination of 1D selective NOESY and 1D NOESY-TOCSY experiments, applied for the

assignment of H5, H6 and H6' resonances in β-D-Gal*p* residues in this study, was shown not to be limited to first-order spectra and/or spin systems with relatively small coupling constants and therefore to complement previously developed methods.

Introduction of ^{31}P-filtered correlation techniques, originally proposed in a one-dimensional form (Cohen et al., 1984; De Waard and Vliegenthart, 1989), gives information not only about the protons directly experiencing a phosphorus coupling, but also about their homonuclearly coupled partners. This provides a substantial advantage over simple proton–phosphorus correlation experiments (Pardi et al., 1983; Byrd et al., 1986; Sklenář et al., 1986). By optimizing the filter interval in the X(ω_1)-half-filtered experiment for the three-bond ^{31}P-^{1}H coupling constants, a 2D ^{31}P(ω_1)-half-filtered COSY experiment was introduced in this study. Two proton spin systems, separated by a phosphoryl group, could be clearly distinguished due to the introduction of the second domain. The 2D experiment is also a method of choice when more than one phosphoryl group occurs within the polysaccharide repeating unit.

A complementary ^{31}P-relayed {^{1}H,^{1}H} correlated experiment (Delsuc et al., 1984; Neuhaus et al., 1984) can be applied for bridging proton spin systems separated by a phosphoryl group. In general, this experiment is less sensitive in comparison with the ^{31}P(ω_1)-half-filtered COSY, because of significantly shorter T_2 than T_1 ^{31}P relaxation times, which determine the relaxation of coherences evolving in the respective experiments and also because of competitive evolution of passive ^{31}P-^{1}H coupling constants during the fixed evolution interval of the ^{31}P-relayed {^{1}H,^{1}H} correlated experiment.

^{13}C chemical shifts are less likely to coincide than their ^{1}H counterparts, which provides a means for removing ambiguities in ^{1}H assignments. Proton-detected heteronuclear relay techniques (Lerner and Bax, 1986; Groncnborn et al., 1989) have proven to be especially useful, since they are able to overcome ambiguities associated with severe overlap and higher order effects which occur in ^{1}H spectra. This feature has been extensively used in the form of an HMQC-COSY experiment by Bush and co-workers (Abeygunawardana et al., 1991a,b; Reddy et al., 1993). It is our experience that the TOCSY transfer can also be used for effective proton magnetization transfer, provided that the mixing time is kept rather short (≃20 ms), thus limiting transfer of magnetization beyond the immediate neighbours. An in-phase character of relay signals promises a higher sensitivity of the HMQC-TOCSY experiment compared to the HMQC-COSY technique.

Long-range proton–carbon coupling constants also provide useful information on polysaccharide structures. Interresidue couplings across the glycosidic bond indicate the glycosylation pattern of a polysaccharide, while intraresidue couplings are helpful for assignment and for elimination of higher order effects in ^{1}H NMR spectra (Abeygunawardana et al., 1990,1991a,b; Reddy et al., 1993). The LR-HSQC experiment (Uhrín et al., 1992), which makes use of single-quantum carbon magnetization, was applied in this study. This technique yields pure phase signals in antiphase with respect to the heteronuclear coupling, with additional in-phase splitting from proton–proton couplings. It is therefore suitable also for determining the values of the coupling constants. A BIRD^{r,x} pulse (Uhrín et al., 1993) applied during the long-range evolution interval of this experiment causes methylene carbon cross peaks to appear with an opposite phase to those of methine carbons (Uhrínová et al., 1991; Mele et al., 1992). Multiplicity differentiation of the long-range correlation peaks is a unique feature of the LR-HSQC experiment and provides additional information, which might be useful for the assignment of ^{13}C signals in crowded regions.

CONCLUSIONS

A direct analysis of a native intact LPS by NMR spectroscopy is feasible, although the procedure is not always straightforward and a number of problems may be encountered, even at higher magnetic fields (≥500 MHz). The source of these difficulties lies in the severe overlap of resonances, which occurs mostly in ^{1}H NMR spectra. If pairs of coupled spins resonate very closely, further complications arise from higher order effects. Standard techniques of 2D NMR spectroscopy might be insufficient and special methods, tailored for saccharides, need to be applied as illustrated in the present structural analysis of the O-PS of *Proteus mirabilis* O:57.

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