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Full assignment of the ^{1}H and ^{13}C spectra and revision of the O-acetylation site of the capsular polysaccharide of *Streptococcus pneumoniae* Type 33F, a component of the current pneumococcal polysaccharide vaccine

Xavier Lemercinier and Christopher Jones*

Laboratory for Molecular Structure, National Institute for Biological Standards and Control Blanche Lane, South Mimms, Herts EN6 3QG, UK

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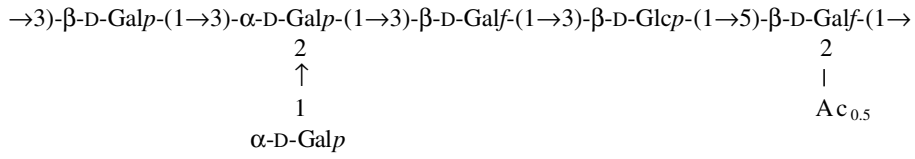
Abstract—The structure of the capsular polysaccharide from *Streptococcus pneumoniae* Type 33F was originally determined by a combination of chemical methods and limited use of NMR spectroscopy [*Can. J. Biochem. Cell Biol.* **1984**, *62*, 666–677]. We report full ^{1}H and ^{13}C assignments and confirm the structure of the saccharide repeat unit, but find that the site of O-acetylation is *O*-2 of the →5)-β-D-Gal*f*, rather than the →3)-β-D-Gal*f* residue. We find that a slightly higher percentage of the repeat units are O-acetylated:



Keywords: Capsular polysaccharide; Pneumococcal; Vaccine

1. Introduction

Streptococcus pneumoniae remains an important bacterial pathogen causing both invasive and non-invasive diseases, mainly in children and the elderly.[1] The World Health Organisation has estimated that pneumococcal infections cause in excess of 1 million deaths per year in the under-fives, mainly due to acute respiratory infections and in developing countries.[2] Ninety pneumococcal serotypes have been defined, differing in the structure of their capsular polysaccharide (CPS). The presence of an appropriate level of anti-CPS antibodies is generally considered protective against disease caused by that serotype, and immunological cross-protection between serotypes with CPSs of similar structure occurs. Immunisation of adults has relied on the use of multivalent vaccine comprising capsular polysaccharides (CPS) from the 23 most important serotypes of *S. pneumoniae*.[3] Such vaccines, prepared with plain CPS, induce

Abbreviations: 1D, one-dimensional; 2D, two-dimensional; COSY, correlation spectroscopy; CPS, capsular polysaccharide; Hib, *Haemophilus influenzae* type b; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; ROESY, rotating frame Overhauser effect spectroscopy; HSQC, heteronuclear single quantum correlation.

* Corresponding author. Tel.: +44 (0)1707 641211; fax: +44 (0)1707 646730; e-mail: cjones@nibsc.ac.uk

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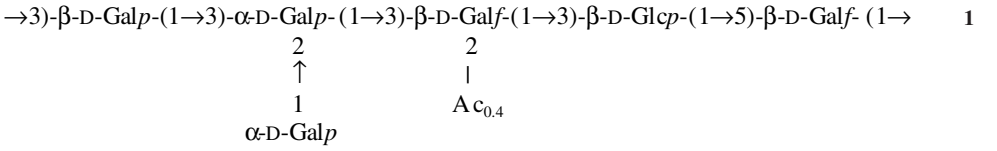
only relatively short-term protection and are ineffective in children less than two years of age.[4,5] Recently, glycoconjugate vaccines against *Haemophilus influenzae* type b (Hib) and *Neisseria meningitidis* Group C, prepared by covalent conjugation of the relevant CPS (or oligosaccharides derived from the CPS) to a carrier protein have been proved to induce higher avidity, complement-activating antibody isotypes which improve protection, and to elicit immunological memory. These vaccines are effective in infants.[6] The success of these vaccines prompted the development of conjugate vaccines against *S. pneumoniae*.[4]

The CPS from pneumococcal Type 33F is a component of both 23-valent polysaccharide vaccines currently available.[2] Although the Type 33F is not included in the 7-, 9- or 11-valent pneumococcal conjugate vaccines currently in development or in clinical trials,[7–10] it remains a potential candidate for a future conjugate vaccine containing more or different serotypes. A high level of antibiotic resistance was recently observed in Type 33F strains isolated from otitis media patients in southern Israel.[11] Historically, control of the identity and purity of CPSs material intended for vaccine production has relied on a combination of immunological and compositional tests. The most common serotyping method, counter-immunoelectrophoresis, does not work for uncharged CPSs, although modern techniques such as those based on specific-antibody coated fluorescent microspheres can be used.[12] In the case of *S. pneumoniae* Type 33F, the British and European Pharmacopoeia specify only that an immunologic identity test and quantification of the levels of protein and nucleic acids contaminants, and total nitrogen and phosphorus assays be performed. No component of the CPS itself has to be quantified.[13]

In previous papers, we have shown that NMR can be used as an alternative to the combination of immunological and compositional tests to confirm the identity of bacterial CPSs. In the case of the Hib CPS ^{1}H NMR can be used as an identity test,[14] and for the *N. meningitidis* and *Salmonella enterica* serovar Typhi CPS as both an identity test and as a method to quantify the level of O-acetylation.[15,16] Validation of the method is easier if the structure is known and full spectral assignments are available. We report here ^{1}H and ^{13}C assignments for *S. pneumoniae* Type 33F CPS undertaken to support implementation of the NMR method for the 23-valent vaccines. These assignments were incompatible with the published structure, and we report the results of that investigation.

A structure, **1**, of the Type 33F pneumococcal CPS was first proposed by Richards and Perry,[17] derived principally by degradative chemical methods and with limited use of ^{1}H and ^{13}C NMR spectroscopy. The authors proposed the following structure for the repeat unit:



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Partial O-acetylation on the *O*-2 of →3)-β-D-Gal*f*-(1→ was proposed, and ^{13}C and anomeric ^{1}H assignments for the de-O-acetylated CPS and a small number of ^{1}H and ^{13}C assignments for the native CPSs reported, based on analyses of the 1D spectra. Using two-dimensional (2D-) homo- and heteronuclear techniques, we obtained full ^{1}H and ^{13}C assignments for both the native and de-O-acetylated CPS, which differ slightly from those published.[17] Our data also show that the single site of O-acetylation is *O*-2 of the →5)-β-D-Gal*f*-(1→ residue.

2. Results and discussion

2.1. Assignments of the ^{1}H and ^{13}C one- and two-dimensional spectra

The ^{1}H spectrum of the native CPS contains several resonances of unequal intensities in the anomeric area, revealing heterogeneity in the chain. Other key features include the absence of major signals at chemical shifts characteristic of the methyl groups of 6-deoxy sugars (around 1.3 ppm) and a single peak in the area of *N*- and *O*-acetyl methyls. A resonance at 3.2 ppm arises from the choline groups of the pneumococcal C-polysaccharide teichoic acid, a common contaminant of the pneumococcal CPS.[18] The anomeric region of the HSQC spectrum is presented in Figure 1 with the corresponding $^{1}J_{C,H}$ obtained from another HSQC spectrum recorded without ^{13}C decoupling. The cross-peaks are labelled from A to F in the same order as the corresponding residue will appear in the final structure. The six anomeric cross-peaks can be classified from their ^{1}H and ^{13}C shifts and the values of the $^{1}J_{H1,C1}$ as originating from two β-furanose, two α- and two β-pyranose residues. Based on peak shapes and intensities, the O-acetylation is incomplete and probably occurs at a single site. ^{1}H and ^{13}C assignments of the spin systems of the 6 sugar rings were obtained from the DQFCOSY, TOCSY, HSQC or HSQC-TOCSY spectra (Table 1).

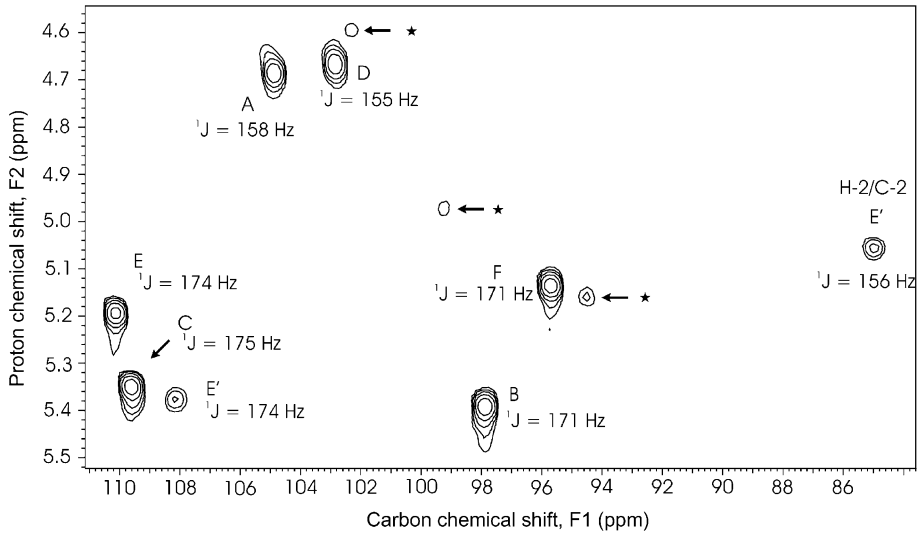


Figure 1. Part of the 500 MHz HSQC spectrum, obtained at 30 °C, showing the cross-peaks from the anomeric positions and from the O-acetylated position. Peaks arising from the Type 33F CPS residues are labelled with letters A–F as explained in the text, and the O-acetylated residue is labelled E′. The values of $^1J_{C\text{-}1,H\text{-}1}$, obtained from a spectrum obtained without ^{13}C decoupling, are shown for each cross-peak from the CPS. Cross-peaks arising from anomeric resonances of the teichoic acid pneumococcal C-polysaccharide are labelled with an asterisk.

Table 1. Pneumococcal Type 33F CPS ^1H and ^{13}C NMR assignments

		$^1J_{H1,C1}$ (Hz)	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6 C-6	H-6′	–OCOMe
→3)-β-D-Gal*p*-(1→	**A**	158	4.69 104.93	3.70 71.08	3.72 82.21	4.09 69.76	3.73 75.85	3.82 62.16	3.84	
→2,3)-α-D-Gal*p*-(1→	**B**	171	5.40 97.92	4.20 69.93	4.21 77.54	4.30 70.53	4.17 72.02	3.77 62.02	3.80	
→3)-β-D-Gal*f*-(1→	**C**	155	5.36 109.74	4.44 80.53	4.15 86.41	4.33 82.98	4.00 71.16	3.70 64.33	3.69	
→3)-β-D-Glc*p*-(1→	**D**	175	4.68 102.90	3.50 74.58	3.69 83.29	3.50 69.30	3.50 76.74	3.96 61.99	3.96	
→5)-β-D-Gal*f*-(1→	**E**	174	5.20 110.21	4.22 82.38	4.32 76.92	4.20 82.37	4.10 78.01	3.80 62.15	3.82	
→5)-β-D-Gal*f*(2OAc)-(1→	**E′**	n.d.	5.39 108.20	5.06 85.01	4.51 76.14	4.29 83.85	n.d.	n.d.	n.d.	2.17 21.54
α-D-Gal*p*-(1→	**F**	171	5.14 95.75	3.86 69.14	3.96 70.40	3.97 70.55	4.35 71.94	3.78 62.45	3.78	

Data were collected at 500 MHz and at a nominal probe temperature of 30 °C.

To minimise spontaneous de-O-acetylation, the spectra were first obtained at 30 °C: at this temperature, the ^1H spectral lines were sometimes too broad to extract coupling constants less than 5 Hz. When higher resolution was needed or to take advantage of minor chemical shift changes induced by a temperature change, additional spectra were run at 70 °C.

For the β-pyranose residues, severe overlap prevented the values of $^3J_{H\text{-}2,H\text{-}3}$ and $^3J_{H\text{-}3,H\text{-}4}$ being obtained from the DQFCOSY spectrum. These couplings were instead obtained from an HSQC spectrum recorded with an acquisition time of 820 ms and at 70 °C. The chemical shifts and couplings indicate the presence of one β-Glc*p* residue (spin system D $\delta_{H\text{-}3}$ 3.69 t, $J_{2,3}$–$J_{3,4} = 7.5$ Hz) and one β-Gal*p* residue (spin system A $\delta_{H\text{-}3}$ 3.72 d, $J_{2,3} = 8.5$ Hz). This is confirmed by the observation of a strong H-3 to H-4 TOCSY cross-peak for spin system D and its absence for spin system A. The observed ^{13}C chemical shift of the C-3s (which are 8.5 ppm and 6.55 ppm downfield of the value observed for unsubstituted Gal and Glc[19,20]) indicate glycosylation at *O*-3 for both residues.

^1H and ^{13}C chemical shifts of the α-pyranose residues (spin systems B and F) correspond to Gal residues. In the case of the spin system B, a ^{13}C downfield shift of ~7 ppm relative to the expected value of 70.13 ppm

Table 2. Inter-residue correlations defining the sequence of sugar residues in the repeating unit of pneumococcal Type 33F CPS

Sugar residue and atom				NOESY cross-peaks			Long-range HSQC		HSQC-NOESY spectrum		HSQC-ROESY spectrum	
				δ (ppm)	Intensity[a]	Assign	δ (ppm)	Assign	δ (ppm)	Assign	δ (ppm)	Assign
→3)-β-D-Gal*p*-(1→	**A**	4.70	H-1	4.21	n.r.	H-3 (B)			77.54	C-3 (B)		
		104.93	C-1				4.21	H-3 (B)	4.21	H-3 (B)	4.21	H-3 (B)
→2,3)-α-D-Gal*p*-(1→	**B**	5.40	H-1	5.14	Strong	H-1 (F)	86.50	C-3 (C)	86.41	C-3 (C)	86.41	C-3 (C)
		5.40	H-1	3.86	Weak	H-2 (F)			95.75	C-1 (F)	95.75	C-1 (F)
		5.40	H-1	3.96	Weak	H-3 (F)						
		5.40	H-1	4.15	Strong	H-3 (C)						
		97.92	C-1						4.15	H-3 (C)		
		97.92	C-1						5.14	H-1 (F)		
→3)-β-D-Gal*f*-(1→	**C**	5.37	H-1	3.70	Strong	H-3 (D)					83.29	C-3 (D)
		109.74	C-1				3.70	H-3 (D)				
→3)-β-D-Glc*p*-(1→	**D**	4.68	H-1	4.10	Medium	H-5 (E)						
		4.68	H-1	3.82	Medium	H-6 (E)						
		102.90	C-1								4.10	H-5 (E)
→5)-β-D-Gal*f*-(1→	**E**	5.20	H-1	3.72	n.r.	H-3 (A)			82.22	C-3 (A)	82.21	C-3 (A)
		110.21	C-1						3.69	H-3 (A)	3.69	H-3 (A)
→5)-β-D-Gal*f*(2OAc)-(1→	**E′**	5.39	H-1	3.75	n.r.	H-3 (A′)						
→α-D-Gal*p*-(1→	**F**	5.14	H-1	4.20	n.r.	H-2 (B)			97.92	C-1 (B)	97.92	C-1 (B)
		5.14	H-1	5.40	Strong	H-1 (F)			69.92	C-2 (B)		
		95.75	C-1						5.40	H-1 (B)		
		95.75	C-1						4.20	H-2 (B)		

[a] n.r. = not resolved, because overlap of resonances from adjacent protons prevents discrimination of possible contributions.

for C-3 indicates a glycosylation at this position. Whilst the chemical shift of C-2 (69.93 ppm) corresponds to that of an unsubstituted position, data from NOESY, ROESY and long-range HSQC spectra confirm that this position is glycosylated (Table 2). Vicinal 2,3-disubstitution imposes strong steric hindrance that can restrict motion or alter bond angles compared to monosubstituted sugars, resulting in different glycosylation shift values.[21] For spin system B, H2 and H3 have identical chemical shifts and same multiplicities at 30 °C, and an HSQC-TOCSY spectrum at 70 °C was necessary to distinguish them. The ^{13}C chemical shifts of the other α-pyranose (spin system F) are all within 0.8 ppm of the expected values for an unsubstituted sugar,[19,20] indicating a terminal side-chain residue. The ^{1}H and ^{13}C chemical shifts of the two furanose residues correspond to galactose. Comparing the ^{13}C chemical shifts of these two β-D-Gal*f* clearly show different substitution patterns. In residue C, the C-3 resonance is ~10 ppm downfield and C-2 1.15 ppm upfield of the corresponding carbons in residue E, indicating a C-3 linkage. On the other hand, in residue E linkage at C-5 is indicated by a downfield shift of 6.8 ppm of the C-5 resonance and an upfield shift of 2.4 ppm for the C-6 resonance.

2.2. Inter-residue linkage positions

The sequence of linkages between the sugar residues were determined by combining data from a NOESY spectrum, HSQC-NOESY and HSQC-ROESY (Fig. 2) spectra and long-range ^{1}H–^{13}C correlations observed in an HSQC experiment tuned to observe small ^{1}H–^{13}C coupling constants. These data are summarized in Table 2. These correlations establish unequivocally the connectivities between the six sugars residues and confirm the structure determined previously.[17]

2.3. Comparison with results previously published

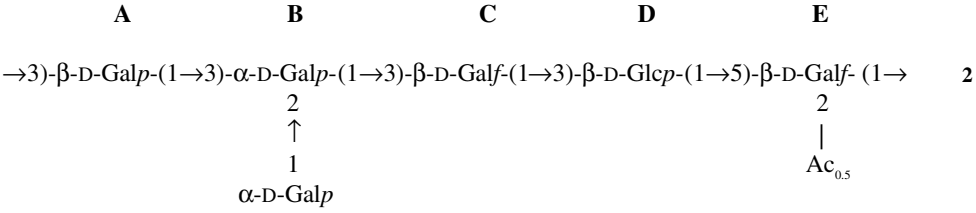
The structure obtained on the basis of our results is identical to that proposed previously for the de-O-acetylated CPS.[17] In that paper, the authors give ^{13}C and ^{1}H assignments (anomeric protons only) based on the 1D ^{1}H and ^{13}C spectra of the native CPS and of the various oligomers obtained by degradation. Two-dimensional spectra, such as HSQC (see Fig. 1) highlight inconsistencies in their assignments; for example, the lowest field proton anomeric, arising from α-D-Gal*p*(**B**), is assigned to one of the two β-D-Gal*f* residues by Richards et al. Our ^{13}C assignments agree with those of Richards et al.[17] for both β-pyranosyl residues and the side-chain α-Gal*p* residue. The most important differences arise for the ^{13}C resonances **B** C-2, **C** C-2, **D** C-2 and **E** C-2.

2.4. O-Acetylation site

Richards et al. noted[17] that a signal present at 85.1 ppm in the ^{13}C spectra of native O-acetylated material is absent in spectra of de-O-acetylated material. The C-3 signal of non-O-acetylated →3)-β-D-Gal*f*-(1→ residue,

at 86.5 ppm, is present in both cases but is more intense for the de-O-acetylated material. On the basis of peak intensities and because the chemical shift difference of 1.4 ppm between these signals corresponds to the upfield shift normally experienced by carbons β to an O-acetylated position,[22] the authors tentatively assigned the peak at 85.1 ppm as C-3 of a putative C-2 O-acetylated →3)-β-D-Gal*f*-(1→ residue. In our study, the HSQC spectrum shows, at a chemical shift typical of an anomeric signals of a β furanose residue, a third low intensity cross-peak (δ_H 5.39 ppm, δ_C 108.20 ppm, labelled E′ in Fig. 1) which correlates in COSY and TOCSY spectra to a weak resonance at 5.06 ppm (δ_C 85.01 ppm in the HSQC spectrum). This low field proton chemical shift confirms that one of the furanose residues is O-acetylated at C-2. Correlations in DQFCOSY and HSQC spectra locate the H-3 and C-3 resonances of this residue at δ_H 4.51 and δ_C 76.14 ppm, and the H-4 at δ_H 4.29 ppm. These values are compatible only with the →5)-β-D-Gal*f*-(1→ residue. The H-2 and C-2 resonances of the O-acetylated →5)-β-D-Gal*f*-(1→ residue are 0.8 ppm and 2.4 ppm downfield of those of the non-O-acetylated residue, with a downfield shift of 0.2 ppm for H-3 and an upfield shift of approximately 0.8 ppm for C-3. These values are similar to those found in the literature for chemical shift modifications induced by O-acetylation.[22] The NOESY spectrum contains a strong correlation between H-1 of the non-O-acetylated →5)-β-D-Gal*f*-(1→ (**E**) and H-3 of →3)-β-D-Gal*p*-(1→ (**A**), whilst the H-1 of the 2-O-acetylated →5)-β-D-Gal*f*-(1→ correlates to a resonance with a slightly different chemical shift value, 0.04 ppm downfield, consistent with a secondary chemical shift on H-3 of →3)-β-D-Gal*p*-(1→ adjacent residue. Such a situation has been previously observed, in the case of the meningococcal CPS.[23] Other shifted resonances of the →3)-β-D-Gal*p*-(1→ aglycone of the 2-O-acetylated →5)-β-D-Gal*f*-(1→ were not identified.

The following revised structure, **2**, for the repeating unit of the pneumococcal Type 33F CPS is therefore proposed:



F

Four samples of pneumococcal Type 33F CPS from different sources were analysed and all showed initial degrees of O-acetylation of approximately 50%. During our study, we noted that the spectra of material in solution at room temperature changed with time, suggesting either migration or a loss of the *O*-acetyl group. *O*-Acetyl group migration has been observed in other CPSs,[23] and is known to happen easily for furanose residues.[24] As no evidence for *O*-acetyl migration was detected for the Type 33F CPS, and as the decrease in the intensities of the resonances from the C-2 O-acetylated →5)-β-D-Gal*f*-(1→ matched increases in the intensities of the free acetate and non-O-acetylated →5)-β-D-Gal*f*-(1→, facile loss of the *O*-acetyl group rather than migration seems likely (Fig. 2).

2.5. Identification of minor resonances

Pneumococcal teichoic acid (called pneumococcal C-polysaccharide) is an ubiquitous contaminant of pneumoccocal CPS samples.[18,25] Minor anomeric signals, identified by an asterisk in Figure 1, correspond to data published earlier for C-polysaccharide.[18] Observation of a phosphocholine resonance at 3.2 ppm and the methyl group on the 6-deoxysugar at 1.25 ppm confirm this, whilst ^{31}P resonances indicate the presence of only one phosphocholine substituent per repeat unit. The proportion of teichoic acid present in the samples was estimated from the relative intensities of the phosphocholine and four lowest field anomeric resonances of the CPS as approximately 7% (molar ratio of repeat units).

3. Experimental

Three CPS samples were material destined for vaccine production and the fourth was from the ATCC (American Type Culture Collection, Manassas, USA). Polysaccharide (~5 mg for ^{1}H spectra, ~20 mg for heteronuclear studies) was lyophilised twice from 400 μL of deuterated water (>99.5% ^{2}H, Apollo…) and redissolved in 700 μL of deuterated water for analysis. NMR spectra were obtained on Varian Unity 500 or Inova 500 spectrometers using 5 mm indirect detection probes and running VNMR versions 6.1A, 6.1B or 6.1C. Spectral widths were 6000 Hz (^{1}H) or 20,000 Hz (^{13}C). The pulse programs used were those in the Varian library, except for the introduction of spin-echo sequences into the TOCSY and ROESY programs. The mixing times for the TOCSY[26] experiments

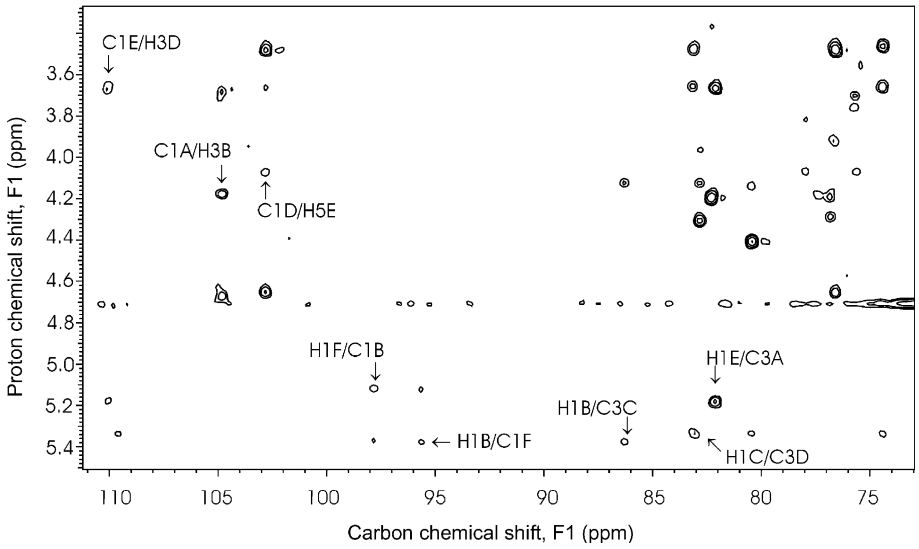


Figure 2. Part of the 500 MHz HSQC-ROESY spectrum, obtained at 30 °C with a 100 ms mixing time, showing trans-glycosidic correlations which were used to establish the sequence of and connectivities between the sugar residues. Residue labeling A–F is as explained in the text.

were 25 and 60 ms, 75 ms for the NOESY experiment,[27] and 100 ms for the ROESY experiment.[28] The HSQC sequence was that of Wider and Wüthrich,[29] with and without ^{13}C decoupling. The HSQC-TOCSY and HSQC-ROESY sequences were variants of those of Crouch et al.,[30] modified to use the Wider and Wüthrich HSQC sequence. The mixing times in the HSQC-ROESY experiments were 25, 60 and 100 ms. Inter-residue ^{1}H–^{13}C correlations were observed in 'long range' HSQC experiments with delays of 20 and 40 ms. Spectra were collected at an indicated probe temperature of 30 °C, apart from a limited set of 2D spectra which were obtained at 70 °C. ^{1}H and ^{13}C chemical shifts are referenced to internal TSP-d_4 at zero (^{1}H) and −1.80 ppm (^{13}C), respectively, and ^{31}P to external 85% phosphoric acid at zero ppm.

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