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Note

The pneumococcal polysaccharide S4: a structural re-assessment

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During an investigation of the effect of non-carbohydrate substituents on the conformation, dynamics, and immunology of bacterial polysaccharides used as vaccines, n.m.r. studies of the depyruvylated capsular polysaccharide from *Streptococcus pneumoniae* type 4 (depyruvyl S4) showed a discrepancy between our results and those expected from the published structure (**1**) of the repeat unit[1]. On the basis of these results, we propose a revised structure (**2**) which differs in the linkage to the ManNAc residue. Jansson *et al.*[1] assigned this linkage on the results of methylation analysis of the pyruvylated polysaccharide. The pyruvate moiety is linked at O-2 and O-3 of the α-Gal residue. A full analysis of our conformational data will be published separately.

$$\rightarrow 4)\text{-}\beta\text{-ManNAc-}(1\rightarrow 3)\text{-}\alpha\text{-FucNAc-}(1\rightarrow 3)\text{-}\alpha\text{-GalNAc-}(1\rightarrow 4)\text{-}\alpha\text{-Gal-}(1\rightarrow$$

1

$$\rightarrow 3)\text{-}\beta\text{-ManNAc-}(1\rightarrow 3)\text{-}\alpha\text{-FucNAc-}(1\rightarrow 3)\text{-}\alpha\text{-GalNAc-}(1\rightarrow 4)\text{-}\alpha\text{-Gal-}(1\rightarrow$$

2

The native pyruvylated polysaccharide was too viscous to allow full assignment of the n.m.r. spectrum, but was in close agreement with the data reported by Jansson *et al.*[1]. Depyruvylation with dilute acid (10mM HCl, 100°, 1 h) and desalting by gel filtration (BioGel P4 in aqueous 2% ammonium hydrogencarbonate) gave a sample (17 mg) that was suitable for full n.m.r. analysis. The ^{13}C-n.m.r. spectrum of this sample at 85° was in agreement with data reported by Jansson *et al.*[1]. The one-dimensional ^{1}H-n.m.r. spectrum is shown in Fig. 1a with assignments. Analysis of the COSY-45 spectrum (Fig. 2) allowed a full assignment, apart from one ambiguity. The GalNAc H-4 and Gal H-4 signals are coincident, and the *galacto* H-4 to H-5 connectivities could not be traced. The two *galacto* H-5/H-6/H-6′ systems were easily traced, with the H-6 signals coincident in each case and H-5 resonances at 4.14 and 4.40 p.p.m. Due to the small H-4/H-5 coupling constant and

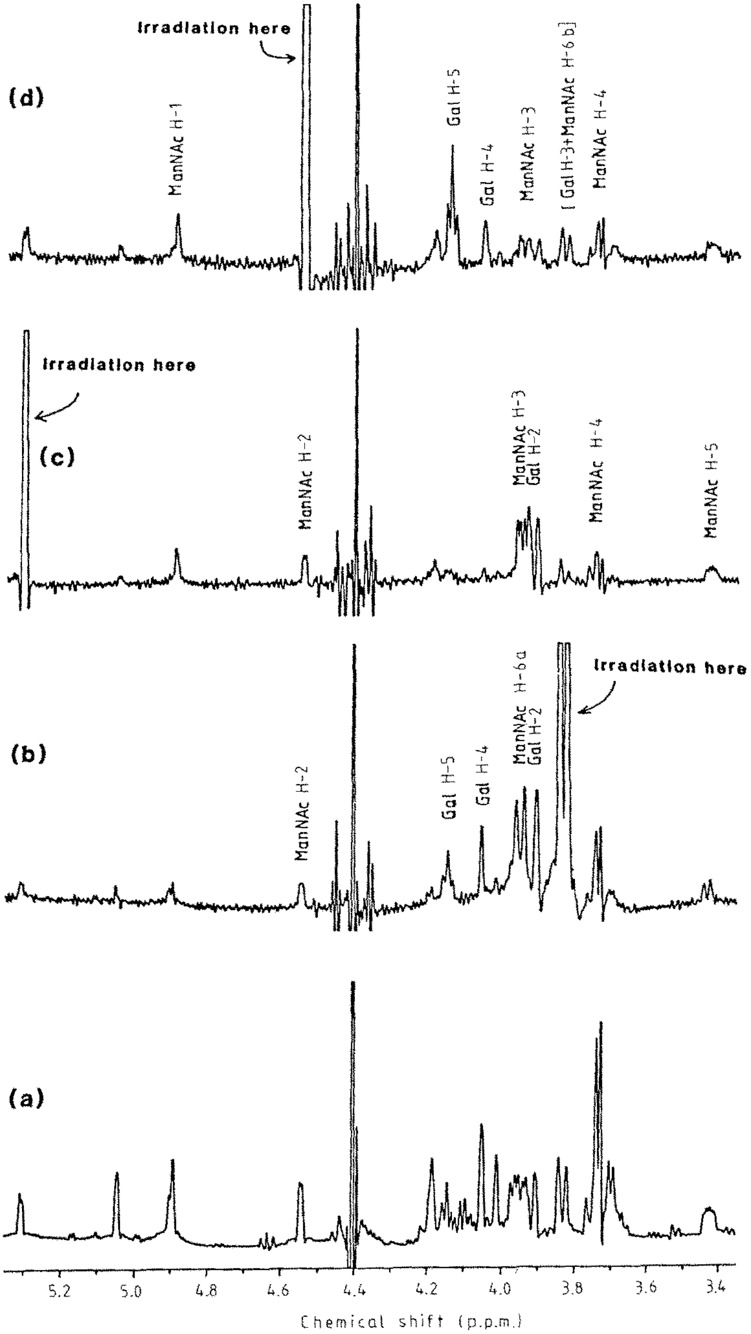

Fig. 1. (a) ^{1}H-N.m.r. spectrum of depyruvyl S4: the off-resonance reference spectrum for (b)–(d). (b) N.O.e. difference spectrum, irradiation of [Gal H-3 + ManNAc H-6b]. (c) N.O.e. difference spectrum, irradiation of Gal H-1. (d) N.O.e. difference spectrum, irradiation of ManNAc H-2.

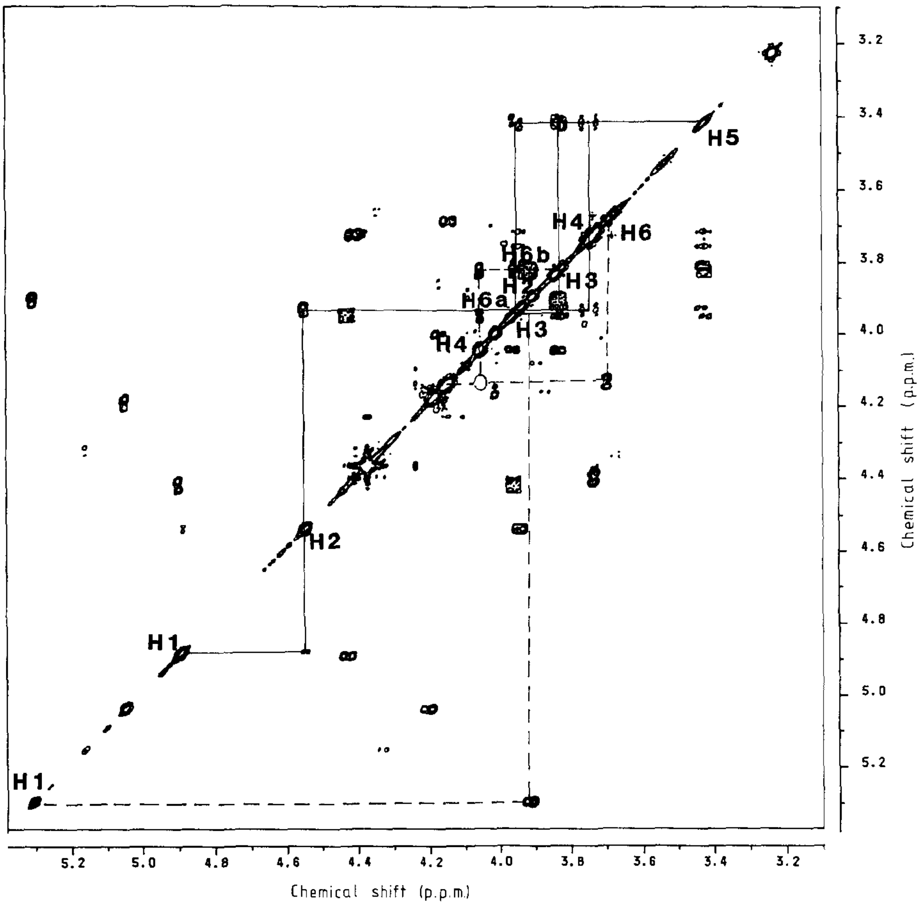

Fig. 2. COSY-45 spectrum of depyruvyl S4. The connectivity of the α-Gal (------) and β-ManNAc (——) residues are traced.

the rapid T_2 relaxation rate of the polymer, a relayed COSY was not expected to resolve this ambiguity and so results from n.O.e. experiments were used to assign these spin systems to specific residues.

Initial n.O.e. time-course experiments were carried out using increasing periods of pre-irradiation, from 0.05 to 2 s. The use of a 0.2-s pre-irradiation resulted in good n.O.e. difference spectra that were free of significant spin-diffusion artefacts. Subsequent n.O.e. spectra, and those illustrated, were collected under these conditions. Pre-irradiation of the [Gal H-3 + ManNAc H-6b] signals at 3.83 p.p.m. caused an enhancement of a triplet at 4.14 p.p.m. (which was therefore assigned as α-Gal H-5), and an apparent doublet at 3.95 (assigned from the COSY-45 as the ManNAc H-6a) (Fig. 1b). The GalNAc H-5/H-6/H-6′ spin system was assigned by default. The full assignment is given in Table I, with assignments for the β-ManNAc residue in comparable systems[2,3]. All coupling constants measured

TABLE I

^{1}H-N.M.R. ASSIGNMENTS FOR THE DEPYRUVYLATED POLYSACCHARIDE[a]

Unit	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6a*	*H-6b*
→3)-β-ManNAc-(1→	4.89	4.55	3.94	3.75	3.42	3.95	3.83
→3)-α-FucNAc-(1→	5.04	4.20	4.17	4.01	4.10	1.25	
→3)-α-GalNAc-(1→	4.90	4.43	3.96	4.05	4.40	3.74	3.74
→4)-α-Gal-(1→	5.30	3.91	3.85	4.05	4.14	3.70	3.70
→3)-β-ManNAc-(1→[b]	4.89	4.64	3.95	3.78	3.49	3.94	3.82
→3)-β-ManNAc-(1→[c]	4.83	4.67	3.99	3.56	3.44		
→4)-β-ManNAc-(1→[c]	4.87	4.39	3.92	3.70	3.49		

[a] 500 MHz, 343 K, referenced to external TSP-d_4 at 0 p.p.m. [b] Found[2] in the sequence →4)-α-Glc-(1→3)-β-ManNAc-(1→, 500 MHz, 343 K. [c] Found[3] in the sequence →3)-β-ManNAc-(1→4)-β-ManNAc-(1→.

from the one-dimensional spectrum or estimated from the COSY-45 were in agreement with the assignment, and showed the usual conformations for the monosaccharide units (*i.e.*, $^4C_1$ for the D sugars, and $^1C_4$ for the L-FucNAc residue).

N.O.e. experiments with irradiation of the Gal H-1 (Fig. 1c, 5.30 p.p.m.) led to strong enhancements of a doublet at 3.91 p.p.m. (Gal H-2) and a double doublet at 3.94 p.p.m. (assigned as ManNAc H-3), and weak enhancements of the ManNAc H-4 (t, 3.75 p.p.m.) and ManNAc H-2 (d, 4.55 p.p.m.) signals. This pattern of n.O.e. enhancements suggested a Gal-(1→3)-ManNAc linkage rather than a (1→4) linkage. Irradiation of the ManNAc H-2 resonance (4.55 p.p.m., Fig. 1d) led to a strong enhancement of the Gal H-5 signal, and weaker enhancements at ManNAc H-3, ManNAc H-4, [Gal H-3 + ManNAc H-6b], and [Gal H-4 + GalNAc H-4]. Observation of this relatively large n.O.e. indicates the close proximity of the Gal H-5 and ManNAc H-2, which was further investigated by computer modelling.

Previous work[4] on an analogous disaccharide, α-D-Fuc*p*-(1→3)-α-D-Man-OMe estimated the Fuc H-5/Man H-2 distance at 2.43 Å, from n.m.r. and computer modelling studies. In the present work, computer modelling of the disaccharide

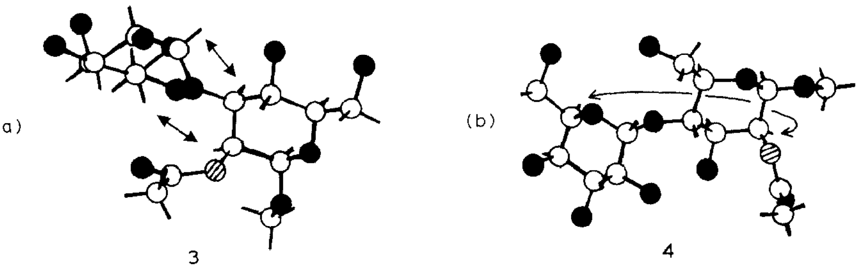

Fig. 3. Computer-generated, lowest energy conformations of the disaccharides (a) α-Gal-(1→3)-β-ManNAc-OMe (**3**) and (b) α-Gal-(1→4)-β-ManNAc-OMe (**4**). The arrows in (a) indicate the observed strong inter-residue n.O.e.s. The arrows in (b) indicate the ManNAc H-2 to Gal H-5 distance.

TABLE II

OBSERVED INTER-RESIDUE N.O.E.S IN THE DEPYRUVYLATED S4 POLYMER, AND ESTIMATED INTER-PROTON DISTANCES IN THE MODEL DISACCHARIDES **3** AND **4**

Proton irradiated	*Observed inter-residue n.O.e.*	*Estimated inter-proton distances (Å)*	
		3	**4**
α-Gal H-1	s[a] β-ManNAc H-3	2.47	4.47
	w β-ManNAc H-1	5.05	5.65
	w β-ManNAc H-2	4.31	5.94
	w β-ManNAc H-4	3.59	2.26
	w β-ManNAc H-5	4.33	3.87
β-ManNAc H-2	s α-Gal H-5	2.44	6.44
	w [α-Gal H-3 + β-ManNAc H-6]	4.26	5.79
	w [α-Gal H-4 + α-GalNAc H-4]	4.89	8.02

[a] Key: s = strong n.O.e.; w = weak n.O.e.

TABLE III

ESTIMATED GLYCOSIDIC BOND ANGLES FOR THE LOWEST ENERGY CONFORMATIONS OF DISACCHARIDES **3**, **4**, AND α-D-Fuc*p*-(1→3)-β-D-Man*p*-OMe (FROM REF. 4)

α-Gal-(1→3)-ManNAc (**3**)	H-1-C-1-O-3-C-3	−52	C-1-O-3-C-3-H-3	−22
α-Fuc-(1→3)-ManOMe[4]	H-1-C-1-O-3-C-3	−50	C-1-O-3-C-3-H-3	0
α-Gal-(1→4)-ManNAc (**4**)	H-1-C-1-O-4-C-4	27	C-1-O-4-C-4-H-4	22

α-D-Gal*p*-(1→3)-β-D-Man*p*NAc-OMe (**3**), using the MM2CARB program[5] for energy minimisation, resulted in a lowest energy conformation (Fig. 3) in which the Gal H-5/ManNAc H-2 distance is 2.44 Å (Table II), and the angles about the glycosidic bond are similar to those found[4] for α-D-Fuc*p*-(1→3)-α-D-Man*p*-OMe (Table III). This conformation corresponds closely to that found in the computer model of the depyruvylated polymer[6]. On the other hand, computer modelling of the (1→4)-linked disaccharide α-D-Gal-(1→4)-β-D-ManNAc-OMe (**4**) indicated a Gal H-5 to ManNAc H-2 distance of >5 Å, and an n.O.e. would not be expected. No low-energy conformations with this distance less then 3 Å were found, and the minimum energy conformation gave a distance of more than 5 Å.

EXPERIMENTAL

N.m.r. spectra were recorded on a Bruker AM500 spectrometer operating in the Fourier-transform mode under the control of an Aspect 3000 computer, and running standard Bruker software as previously described[2]. The COSY-45 spectrum was collected with a digital resolution of ~1.2 Hz/point. N.O.e. difference spectra were obtained using the NOEDIFF program, with interleaved accumulation of 8 scans at each irradiation frequency, up to a total of 640, with a pre-irradiation time of 0.2 s. Irradiation power was "40L", and a 3-s relaxation delay was allowed after each accumulation. Spectra were recorded at an indicated probe temperature of 343 K in deuteriated water. Computer modelling was carried out using the ChemX system (developed and distributed by Chemical Design, Oxford, U.K.), using the modified MM2 program for energy minimisation (Quantum Chemistry Program Exchange No. 501, Indiana University, Bloomington, IN).

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