Carbohydrate Research, 198 (1990) 353–357
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

Note

A novel method for the determination of the stereochemistry of pyruvate acetal substituents applied to the capsular polysaccharide from *Streptococcus pneumoniae* Type 4

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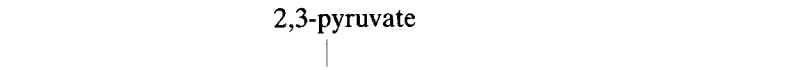
(Received November 3rd, 1988; accepted for publication, August 16th, 1989)

Bacterial polysaccharides often carry non-carbohydrate substituents that affect their chemistry, conformation, and immunology. Pyruvate acetals are amongst the most common substituents and their formation creates a new chiral centre at C-2. Pyruvate acetals have been found 4,6-linked to Gal, Glc, and Man, and 3,4-linked to Gal. The chemical shifts of the ^{13}C and ^{1}H resonances of the methyl group of the pyruvate acetal, or the carboxyl-reduced group, have been correlated with the stereochemistry at C-2, using data from model systems[1,2].

Less common locations of the pyruvate acetal group occur in the capsular polysaccharides from *Streptococcus pneumoniae* Type 4 (S4, positions 2 and 3 of α-Gal)[3,4] and *Klebsiella* K1 and K58 (positions 2 and 3 of β-GlcA)[5–7]. The structure of the repeating unit of the S4 polysaccharide was shown to be **1**, but no methods for the determination of the stereochemistry at the pyruvate acetal C-2 were available. A method based on n.O.e. is applicable to these systems and is illustrated by application to the polysaccharide S4.



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The ^{1}H-n.m.r. spectrum of the deuterium-exchanged polysaccharide was fully assigned from the COSY-45[8], phase-sensitive NOESY[9], and 1D n.O.e. spectra at 343 K. The assignments for the α-Gal and β-ManNAc residues are given in Table I, and Fig. 1 illustrates how they were obtained from the spectra. Of particular note is the coincidence of the α-Gal H-2 and β-ManNAc H-3 resonances at 4.016 p.p.m. An initial phase-sensitive NOESY experiment, obtained (400 MHz, 343 K) with a mixing time of 100 ±20 ms, showed extensive spin diffusion (illustrating the rapid proton T_2 relaxation), and was repeated with a mixing time of 50 ±20 ms. The

0008-6215/90/$ 03.50

rapid relaxation also prevented resolution of the smaller (3–4 Hz) couplings between, for example, the α-Gal H-1 and H-2. A 1D n.O.e. time-course experiment, with pre-irradiation of the pyruvate methyl for 0.05, 0.10, and 0.15 s, showed a steady build-up in the intensity of the n.O.e. between 0.05 and 0.10 s, but extensive spin diffusion was apparent with a pre-irradiation time of 0.15 s. Fig. 2 shows the 500-MHz 1D ^{1}H-n.m.r. spectrum of the polysaccharide together with the n.O.e. difference spectra obtained after pre-irradiation of the α-Gal H-1 and the pyruvate methyl group. The same Lorentzian-to-Gaussian resolution-enhancement functions were used for the normal and n.O.e. difference spectra. The spectrum with irradiation of the α-Gal H-1 showed an intense intra-residue n.O.e. and the expected inter-residue n.O.e. across the glycosidic bond to the [α-Gal H-2 + β-ManNAc H-3] resonance at 4.016 p.p.m.[4], together with weak enhancements of the β-ManNAc H-1 (4.893 p.p.m.) and H-4 (t, 3.750 p.p.m.) signals, probably due to spin diffusion. The n.O.e. difference spectrum with pre-irradiation of the pyruvate methyl resonance showed an enhancement of the α-Gal H-3 signal. The small (3–4 Hz) α-Gal H-1,2, α-Gal H-3,4, and β-ManNAc H-2,3 couplings are not visible in the normal or n.O.e. difference spectra, and the [α-Gal H-2 + β-ManNAc H-3] and α-Gal H-3 resonances appear as doublets with 10–11 Hz ($J_{2,3}$ for Gal, $J_{3,4}$ for β-ManNAc). The peaks show the "lean-to" effect expected for second-order effects on tightly coupled systems, as $\Delta\delta/J_{2,3} = \sim 2.5$ at 500 MHz. Fig. 2 (*d* and *e*) also shows the data obtained from the phase-sensitive NOESY spectrum with a mixing time of 50 ms (400 MHz, 343 K), with a cross-peak between the α-Gal H-1 and the [α-Gal H-2 + β-ManNAc H-3] resonance, and a cross-peak between the pyruvate methyl and the α-Gal H-3. The slices through the NOESY spectrum produced the same data as the 1D n.O.e. difference spectra, but with much poorer resolution. Integration of the n.O.e. difference spectra showed intensities of −26% for the α-Gal H-1 to [α-Gal H-2 + β-ManNAc H-3] enhancement, and −8% for the Me to α-Gal H-3 enhancement, calibrated against the saturation of the irradiated signals. For a macromolecule, these enhancements are negative, with the difference

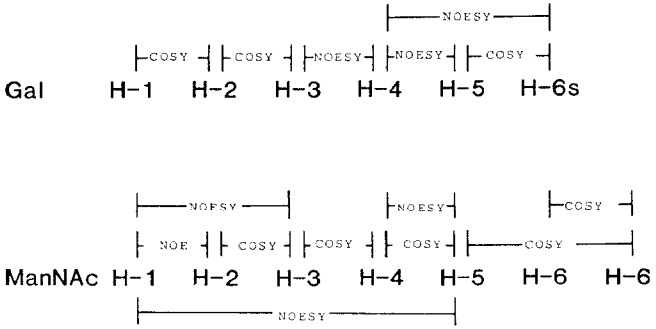

Fig. 1. Basis of the assignment of the resonances of the α-Gal and β-ManNAc residues. Using COSY-45, and phase-sensitive NOESY (mixing time of 50 ms), 1D n.O.e. difference spectra. Several inter-residue n.O.e.s (*e.g.*, α-GalNAc H-1 to α-Gal H-4) also confirmed the assignments. N.O.e.s used in the determination of the pyruvate acetal stereochemistry are not included.

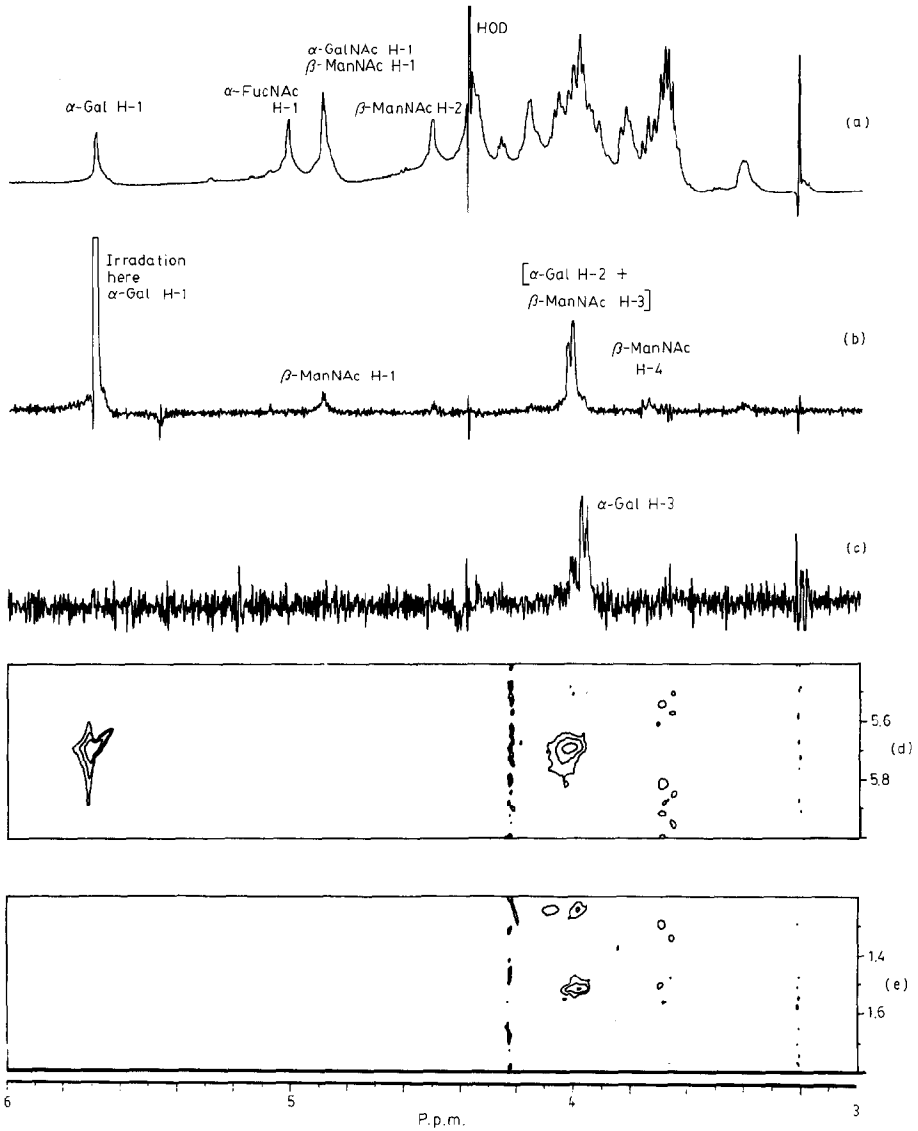

Fig. 2. (*a*) 500-MHz 1D ^{1}H-n.m.r. spectrum of pneumococcal S4 polysaccharide at 343 K, processed with Lorentzian-to-Gaussian resolution enhancement; (*b*) the 500-MHz n.O.e. difference spectrum obtained with pre-irradiation of the α-Gal H-1 resonance at 5.693 p.p.m., showing saturation of that resonance and the enhancements of [α-Gal H-2 + β-ManNAc H-3], β-ManNAc H-1, and β-ManNAc H-4 signals; (*c*) the 500-MHz n.O.e. difference spectrum obtained with pre-irradiation of the pyruvate methyl resonance at 1.52 p.p.m., showing the enhancement of the α-Gal H-3 resonance; (*d*) the partial 400-MHz phase-sensitive NOESY spectrum showing the α-Gal H-1 resonance on the diagonal and the α-Gal H-1/(α-Gal H-2 + β-ManNAc H-3) cross-peak, and (*e*) the partial 400-MHz phase-sensitive NOESY spectrum showing the pyruvate methyl/α-Gal H-3 cross-peak.

TABLE I

PARTIAL PROTON ASSIGNMENT OF THE POLYSACCHARIDE S4 AT 343 K

Residue	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*H-6'*
α-Gal	5.693	4.016	3.959	4.376	4.058	3.695	3.695
β-ManNAc	4.893	4.503	4.019	3.750	3.415	3.938	3.823

peaks showing the same phase as the saturated peak in the n.O.e. difference spectra and the cross-peaks the same phase as the diagonal in NOESYPH spectra. No attempt was made to integrate the cross-peak volumes in the NOESY spectra.

The *R* and *S* configurations[10] at pyruvate C-2 are shown in Fig. 3 for α-Gal in S4. The *S* configuration will lead to enhancement of the α-Gal H-3, and the observed n.O.e.s are illustrated by arrows. This residue was incorporated into a model of the conformation of the polysaccharide S4[11], and n.O.e. simulations were carried out using a program which takes into account the free rotation of the methyl group[12]. The n.O.e. at α-Gal H-3 produced by saturation of the pyruvate methyl group was estimated to be −10%, compared to the observed −8%. The expected ratio of n.O.e.s to α-Gal H-3 and α-Gal H-2 was estimated, from simulations, to be 9:1, in good agreement with Fig. 2c. Fig. 3b and n.O.e. simulations suggest that the *R* configuration would result in an enhancement at the α-Gal H-2 resonance. The local structure in the *Klebsiella* polysaccharides containing pyruvated β-GlcA is very similar and should produce the same pattern of n.O.e.s.

EXPERIMENTAL

The polysaccharide was obtained from Merck, Sharpe, and Dohme. N.m.r. experiments were performed[4] on a Bruker AM500 or AM400 spectrometer at an indicated* probe temperature of 343 K. The sample was deuterium-exchanged by lyophilisation of a solution in D_2O and partially depolymerised in the n.m.r. tube by sonication[13]. 1D N.O.e. difference experiments used a pre-irradiation time of

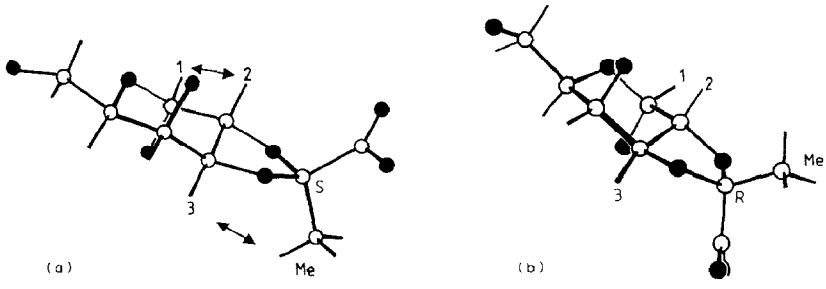

Fig. 3. Computer-generated models for the *S* (*a*) and *R* (*b*) 2,3-pyruvate acetals of α-Gal. The *S* configuration generates an n.O.e. to the α-Gal H-3 below the ring, whereas the *R* configuration should generate an n.O.e. to α-Gal H-2.

100 ms and a decoupler power of "35L" for pre-irradiation of the pyruvate methyl and "40L" for pre-irradiation of the α-Gal H-1. Inter-leaved accumulations of 32 scans up to a total of 320 (pyruvate methyl) or 1280 (α-Gal H-1) were collected. Quantitation used standard Bruker software and was normalised to the saturated peak. The phase-sensitive NOESY spectrum was obtained using the pulse sequence described by Bodenhausen *et al.*[9], with 512 time increments, and 32 scans of 2k data points per experiment over a spectral width of 2024 Hz, and zero-filled to a final digital resolution of 2.0 Hz/point in each domain. Mixing times of 100 or 50 ms were used, with a random variation of ±20 ms in order to suppress scalar coupling artefacts.

Molecular modelling was carried out on a ChemX system (Chemical Design Ltd., Oxford), run on a MicroVax II, and structures were optimised by the MM2 algorithm[14] (QCPE, Indiana University) optimised for carbohydrates[15]. The modelling was carried out on the free acids. N.O.e. simulations were carried out on a pyruvated decasaccharide fragment with NOEMOL[12] running on a SUN 3/160. The single correlation time of 7 ns was estimated from integration of intra-residue n.O.e.s and data fitting.

ACKNOWLEDGMENTS

Merck, Sharp, and Dohme are thanked for the polysaccharide, the M.R.C. Biomedical NMR Centre, Mill Hill for access to the high-field spectrometers, and Dr. Mark Forster (N.I.B.S.C.) for the n.O.e. simulation software.

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