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Note

Characterization of the group A streptococcal polysaccharide by two-dimensional ^{1}H-nuclear-magnetic-resonance spectroscopy*

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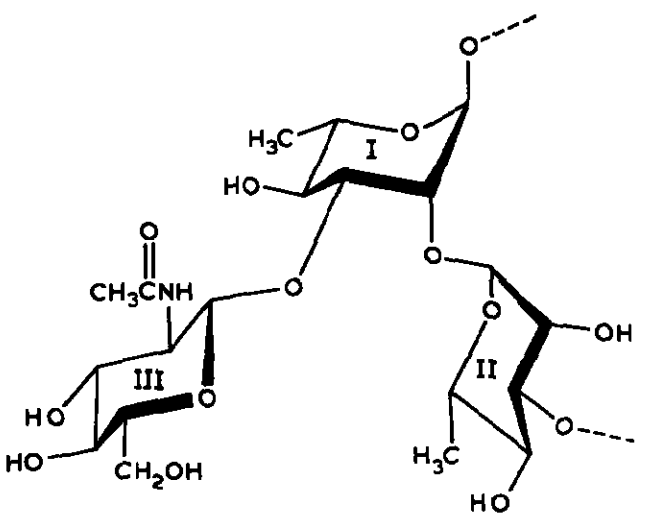
The group-specific polysaccharide of group A streptococcus has played a central role in a very large number of studies to probe the mechanism of immunity and the genetics of antibody response[1,2]. This is because this polysaccharide, like other streptococcal group antigens, was found to elicit antibody responses of extremely restricted heterogeneity in certain strains of rabbits and mice.

Methylation analysis and periodate oxidation studies indicated that the group



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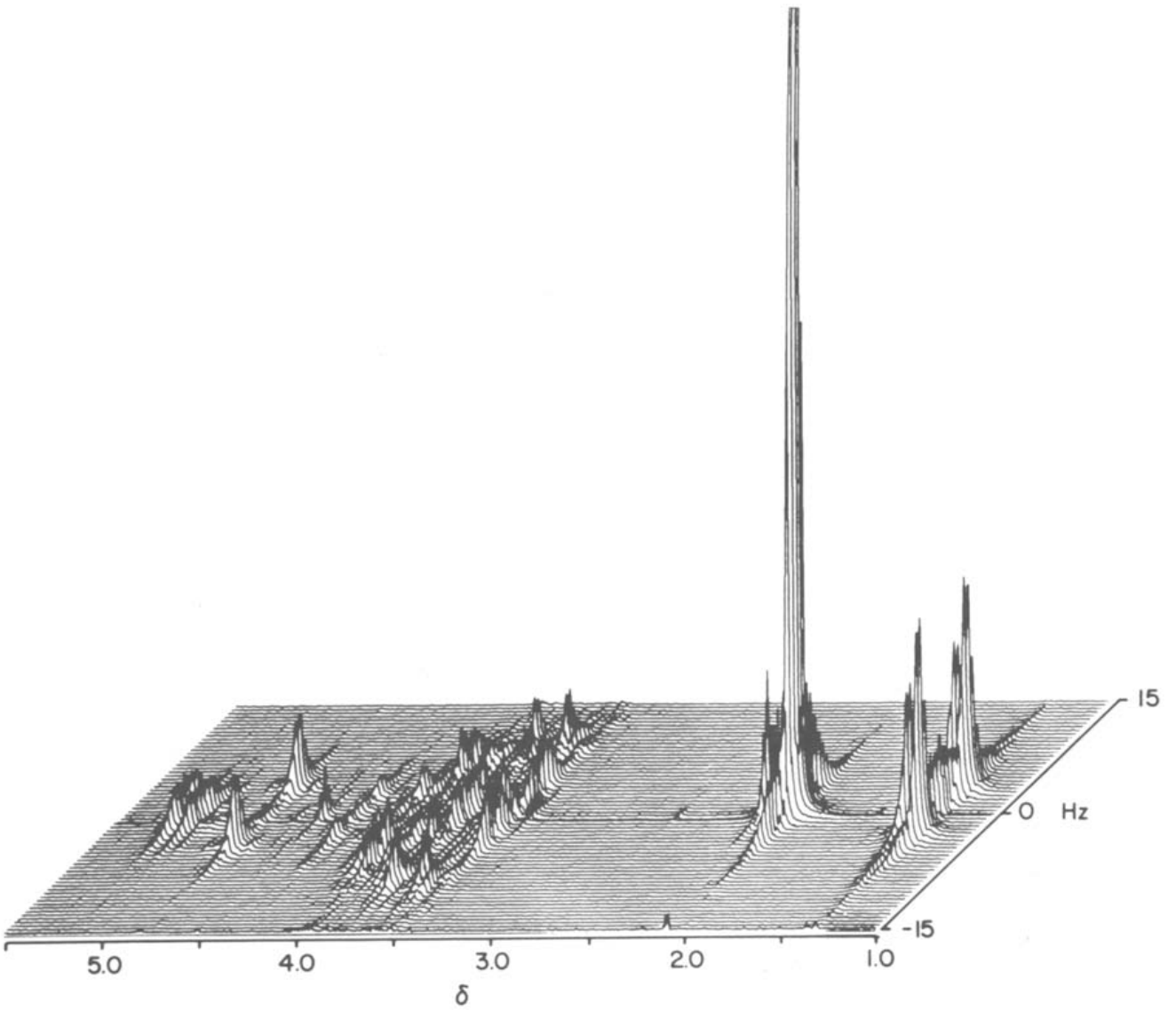

Fig. 1. Stacked plot of 2D–*J*-resolved ^{1}H-n.m.r. spectrum of the group A streptococcal polysaccharide (**1**).

A streptococcal polysaccharide (**1**) consists of a backbone of alternating α-(1→2)- and α-(1→3)-linked L-rhamnopyranose units and side units of 2-acetamido-2-deoxy-β-D-glucopyranosyl groups linked to O-3 of the L-rhamnose of the backbone[3]. The group A-variant streptococcal polysaccharide, which lacks the 2-acetamido-2-deoxy-β-D-glucopyranosyl groups, was studied by high-resolution n.m.r. spectroscopy[4]. Sequential, selective-decoupling experiments made it possible to obtain two sets of proton-resonance assignments corresponding to the two L-rhamnopyranosyl residues. The sets were assigned to a residue of L-rhamnopyranose linked either at O-2 or O-3, based upon the results of ^{13}C-heteronuclear-decoupling experiments. A similar approach was not feasible for the more complex group A streptococcal polysaccharide, because of the more complicated ^{1}H-n.m.r. spectrum of this polysaccharide.

This report describes the application of several two-dimensional n.m.r. methods[5–10] for an unambiguous assignment of all the nonexchangeable proton resonances of the group A streptococcal polysaccharide. A two-dimensional *J*-resolved experiment was carried out in order to sort out the complicated, over-

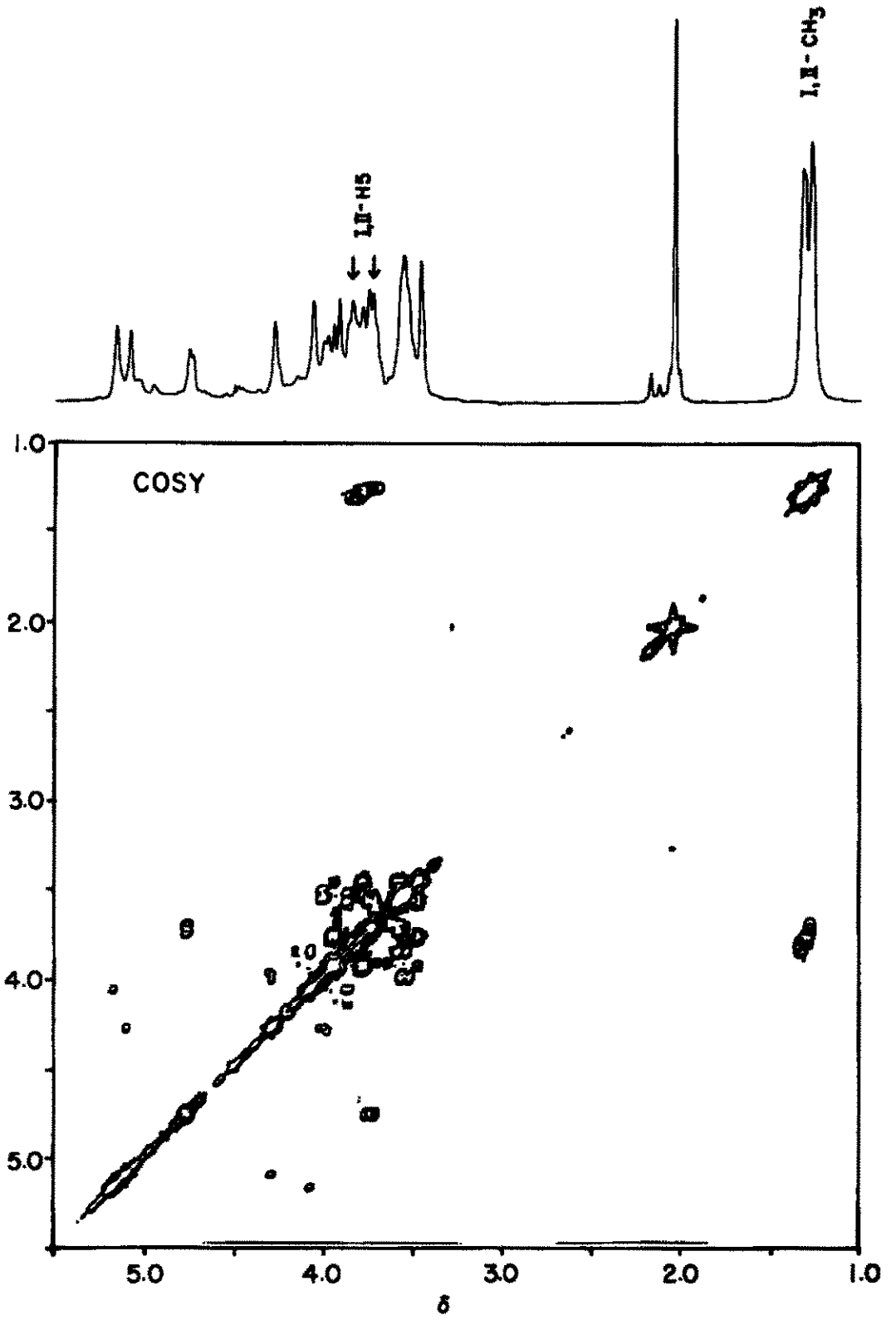

Fig. 2. 400-MHz, ^{1}H-2D–COSY contour map of the group A streptococcal polysaccharide (**1**) with the one-dimensional, high-resolution spectrum at the top.

lapping coupling-patterns of the 400-MHz ^{1}H-n.m.r. spectrum, and the stacked plot of the 2D *J*-resolved spectrum is shown in Fig. 1.

The resonance assignments based on the connectivities involving the methyl groups observed in a 2D–COSY experiment are illustrated in Fig. 2. The *N*-acetyl methyl resonance occurred at δ 2.04 and was easily identified by its characteristic chemical-shift position and by the absence of any COSY cross peaks at δ 2.04 (the amide proton rapidly exchanges with the solvent deuterium and, hence, does not contribute to the ^{1}H-n.m.r. spectrum). The remaining two methyl resonances from

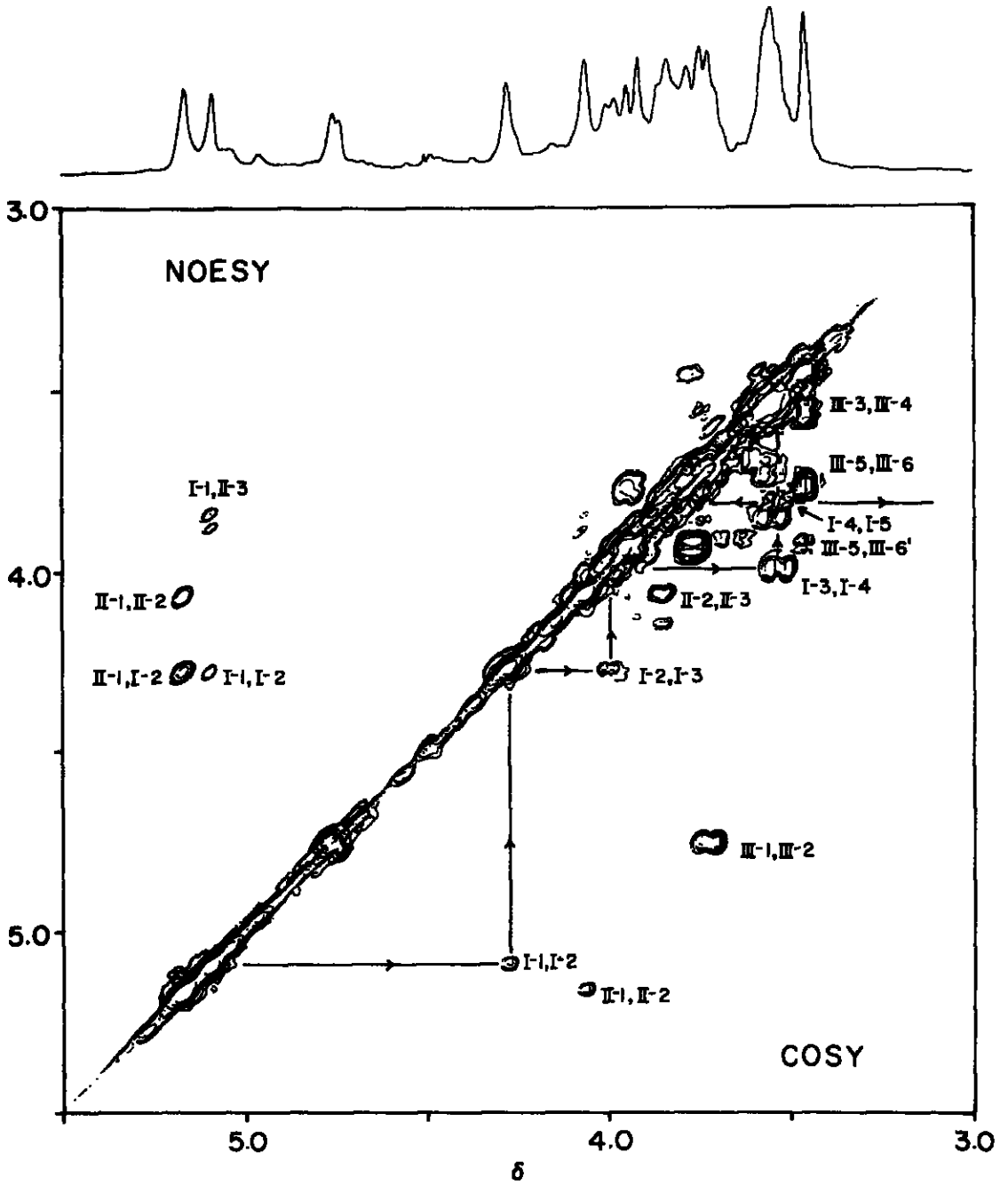

Fig. 3. Combined ^{1}H-2D–COSY contour map (lower right) and 2D–NOESY contour map (upper left) of the group A streptococcal polysaccharide (**1**). The expansion of the region from δ 5.5–3.0 is shown together with the corresponding 1D spectrum at the top.

the L-rhamnose units I and II appeared at δ 1.27 and 1.31, respectively, and the positions of the H-5 protons to which these methyl protons are coupled was readily identified from the COSY cross-peaks.

Fig. 3 shows a combination contour map for the region δ 5.5–3.0 with the 2D–NOESY on the upper left and 2D–COSY at the lower right. The resonances from the H-1 protons appear at δ 5.17, 5.09, and 4.75. Starting from the H-1 resonance of each sugar, the positions of the H-2,3,4,5 protons for each sugar residue were identified sequentially by tracing out the cross-peak connectivity networks for each sugar unit from the 2D–COSY contour map. The 2D–COSY cross-peak for the H-4,5 protons of sugar unit III were not observed owing to the overlapping of the resonance signals. The resonances at δ 3.77 and 3.94 are coupled to each other with a coupling constant of -12.1 Hz, which is typical for methylene

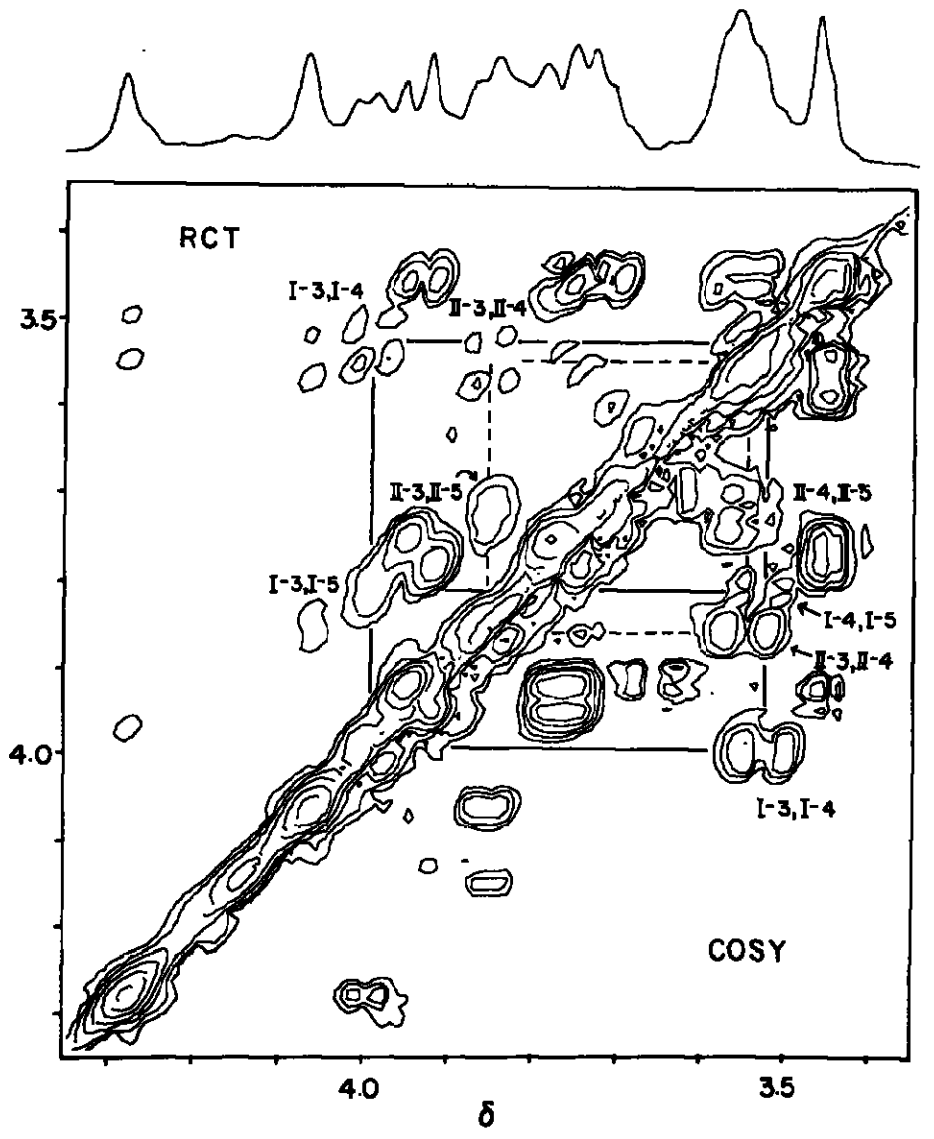

Fig. 4. Expanded region of the combined ^{1}H-2D–COSY map and the 2D-relayed coherence transfer experiment map of the group A streptococcal polysaccharide (**1**).

protons. Thus, these two resonances were assigned to H-6,6′ of the 2-acetamido-2-deoxy-D-glucopyranosyl group. The resonance assignment for H-5 of this group was then identified by its connectivity to the signals for H-6,6 in the 2D–COSY spectrum, and further confirmed by spin-decoupling experiments.

An additional confirmation of the sequential assignments just described was provided by the 2D–RCT experiment. The results obtained by use of this procedure are shown in the upper-left side of Fig. 4, with the corresponding 2D–COSY spectrum in the lower-right side. One difficulty encountered in tracing out the sequential-connectivity network among protons in the conventional 2D–COSY spectrum was the proper identification of some of the cross peaks arising from the H-5 protons in the crowded spectral-region δ 4.1–3.4. This problem was overcome by use of 2D–RCT spectroscopy which showed the connectivities between the signals for H-3 and H-5; these are identified in Fig. 4.

The unambiguous assignment of the remaining two sets of resonances to the L-rhamnose residues was also based on the 2D–NOESY spectrum shown in Fig. 3. The primary structure of the Group A polysaccharide suggested that the L-rhamnose units I and II could be expected to show the following, inter-residue

TABLE I

^{1}H-CHEMICAL SHIFTS (δ) AND COUPLING CONSTANTS (Hz) OF THE GROUP A STREPTOCOCCAL POLYSACCHARIDE (1)

Unit	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*H-6'*
I	5.09	4.27	4.00	3.52	3.81	1.31	
	$J_{1,2}$ 2.4	$J_{2,3}$ 3.4	$J_{3,4}$ 9.5	$J_{4,5}$ 9.9	$J_{5,6}$ 6.4		
II	5.17	4.07	3.85	3.56	3.73	1.27	
	$J_{1,2}$ 2.2	$J_{2,3}$ 3.7	$J_{3,4}$ 9.8		$J_{5,6}$ 6.4		
III	4.75	3.72	3.57	3.46	3.46	3.77	3.94
	$J_{1,2}$ 8.3	$J_{2,3}$ 10.5	$J_{3,4}$ 8.5		$J_{5,6}$ 5.8	$J_{6,6'}$ −12.1	
					$J_{5,6'}$ 2.4		

n.O.e. contacts: between II-H-1 and I-H-2, and between I-H-1 and II-H-3. Such predictions are largely based on the theoretical energy calculations of Lemieux and Koto[11]. These characteristic, intersugar through-space connectivities would therefore greatly facilitate the unambiguous assignment of resonances for the two L-rhamnose units. Such through-space contacts were, in fact, observed in the 2D–NOESY spectrum of group A polysaccharide and are shown in the upper-left section of Fig. 3. A strong NOESY cross-peak was seen between an H-1 signal (at δ 5.17) of one of the L-rhamnose units and the H-2 signal (at δ 4.27) of the other L-rhamnose unit. The H-1 signal (at δ 5.09) of this latter sugar unit showed a strong NOESY cross-peak with the H-3 signal (at δ 3.85) of the former sugar. Based on this observation, the signals of the former sugar were assigned to the L-rhamnose unit II and the signals of the latter sugar to the L-rhamnose unit I. In addition to these inter-residue cross-peaks, the 2D–NOESY spectrum also showed several intra-residue cross-peaks.

Table I lists the resonance assignments and coupling constants based on the above described results. The chemical shifts of the 2-acetamido-2-deoxy-D-glucopyranosyl group confirms the β-D configuration and the large coupling-constants for $J_{1,2}$, $J_{2,3}$, and $J_{3,4}$ indicate the $^4C_1$(D) conformation. Similarly, the small $J_{1,2}$ values for the α-linked L-rhamnopyranosyl residues indicate that they are both present in the $^1C_4$(L) conformation.

EXPERIMENTAL

Group A polysaccharide. — Group-specific polysaccharide from strain J17A4 group A streptococci was a gift from Dr. John E. Coligan of the Laboratory of Immunogenetics, National Institute of Health, Bethesda, Maryland. It was isolated essentially as described by Krause[12]. Briefly, group A polysaccharide was extracted from acetone-dried streptococci by treatment with formamide at 180°. After extraction, acidified ethanol (19:1 ethanol–M HCl; 2 vol.) was added and the mixture centrifuged. The polysaccharide was recovered from the supernatant by

precipitation with acetone (5 vol.). Further purification was achieved by passing an aqueous solution of the material through columns of Dowex 2 (OH^-) and Dowex 50 (H^+) ion-exchange resins. The n.m.r. studies were carried out on a sample (20 mg) dissolved in D_2O (0.5 mL).

N.m.r. methods. — N.m.r. spectra were recorded with a 9.4-Tesla Bruker WH-400 spectrometer operating in the F.t. mode. The spectrometer was equipped with an Aspect-2000 computer, interfaced with a Diabolo 31 disk drive. All spectra were recorded at 55° for solutions in D_2O in the presence of internal sodium 4,4-dimethyl-4-sila-[2,2,3,3-$^2H_4$]pentanoate as standard. The residual solvent peak was presaturated with a 2-s, weak radio-frequency (RF) pulse in both the 1- and 2-dimensional n.m.r. experiments. The 2D *J*-resolved experiment used a 32 × 2048-data matrix in the time domain. Two-dimensional, chemical-shift correlated spectroscopy (2D–COSY), the relayed coherence-transfer (RCT) experiment[13–15], and the NOESY experiments were all performed with data matrices of 256 × 1024-data points, then zero filled to yield a 512 × 512 data-point matrix. The free-induction decays in both dimensions were multiplied by the sine-bell window function prior to Fourier transformation in order to enhance resolution of the 2D-n.m.r. spectra.

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