Carbohydrate Research, 191 (1989) 279–293
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

THE COMPLETE STRUCTURE OF THE CAPSULAR POLYSACCHARIDE FROM *Streptococcus sanguis* 34*

CHITRANANDA ABEYGUNAWARDANA†, C. ALLEN BUSH†,
Department of Chemistry, Illinois Institute of Technology, Chicago, Illinois 60616 (U.S.A.)

SUSAN S. TJOA, PAUL V. FENNESSEY,
NIH Clinical Mass Spectrometry Resource, University of Colorado Health Sciences Center, Denver, Colorado 80262 (U.S.A.)

AND MICHAEL R. MCNEIL
Department of Microbiology, Colorado State University, Fort Collins, Colorado 80523 (U.S.A.)
(Received March 29th, 1988; accepted for publication, July 8th, 1988)

ABSTRACT

A complete structure for the capsular polysaccharide of *Streptococcus sanguis* 34, which is responsible for coaggregation of this bacterium with *Actinomyces viscosus* T14V, an important step in the formation of dental plaque, is proposed, based partly on the ^{1}H-n.m.r. spectrum, which was assigned by 2-dimensional COSY, homonuclear Hartmann–Hahn spectroscopy, and nuclear Overhauser effects. A phosphoric diester linkage was identified from the ^{31}P-n.m.r. spectrum, and the linkage was determined from long range ^{1}H–^{31}P correlation spectroscopy. The proposed structure is supported both by methylation analysis before and after dephosphorylation and by g.l.c.–m.s. of the phosphorylated monosaccharides as their trimethylsilyl derivatives, isolated by partial hydrolysis of the polysaccharide. The structure is composed of repeating linear hexasaccharide units joined by a phosphoric diester linkage, *i.e.*, [→PO_4^-→6)-α-D-Gal*p*NAc-(1→3)-β-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→6)-β-D-Gal*f*-(1→6)-β-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-(1→]$_n$.

INTRODUCTION

A polysaccharide from the capsule of *Streptococcus sanguis* 34 has been identified as the material that interacts with a lectin on *Actinomyces viscosus* T14V to cause coaggregation[1]. The polysaccharide–lectin interaction is an essential step in the coaggregation between these two bacterial species in the early steps leading to formation of dental plaque. The polysaccharide consists of hexasaccharide repeating-units linked through phosphoric diester bonds[2]. The structure of the re-

*This research was supported in part by NSF grant DMB-8514721 to C.A.B. and the NIH Clinical Mass Spectrometry Research Resource in Denver was supported in part by NIH grant RR-001152.
†Present address: Department of Chemistry, University of Maryland Baltimore County, Baltimore, MD 21228, U.S.A.

0008-6215/89/$ 03.50

duced hexasaccharide was established[3] as **1** by a combination of mass spectrometry, ^{1}H-n.m.r. spectrometry, and chemical and enzymic degradation.

During the initial isolation and preliminary identification experiments, it was determined that the polysaccharide was associated with a phosphoric ester, one of the linkages of which was susceptible to mild alkaline hydrolysis, whereas the other required strong alkali or hydrofluoric acid for cleavage[2]. Presumably, the former linkage is connected to a glycosidic carbon atom and the latter to a nonglycosidic carbon atom of the hexasaccharide. This report describes experiments to test this hypothesis, as well as the identification of the position of the phosphoric diester linkage, thus establishing the complete structure of the *S. sanguis* 34 capsular polysaccharide. In addition to various chemical degradation procedures, we have applied several high-field n.m.r. techniques for an unambiguous assignment of most of the nonexchangeable proton and phosphorus resonances in the polysaccharide.

α-D-Gal*p*NAc-(1→3)-β-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→6)-β-D-Gal*f*-(1→6)-β-D-Gal*p*NAc-(1→3)-D-Galol
1

EXPERIMENTAL

Materials. — The sugar phosphates were purchased from Sigma Chemical Co. (St. Louis, MO 63178). All chemicals were either analytical reagents or h.p.l.c. grade. The following is a list and source for each of the major chemicals used: $NaBH_4$, 48% HF, and acetic anhydride (T. J. Baker Chem. Co. Phillipsburg, NJ 08865), $NaBD_4$ (99%; Stohler/Kor Stable Isotopes, Cambridge, MA 02142), NaH (60%; Aldrich Chem. Co. Inc., Milwaukee, WI 53233), and methyl iodide (Sigma). The capsular polysaccharide was isolated from *S. sanguis* 34 cells and was purified as described previously[2].

Chemical degradation and derivatization. — Experiments were devised to test whether the polysaccharide contained any free reducing terminal sugars. Linkage analysis of the dephosphorylated oligosaccharide was performed by methylation analysis, following dephosphorylation of the polysaccharide. The intact polysaccharide (2 mg) was treated with 2M $NaBD_4$. After 16 h at room temperature, the excess $NaBD_4$ was removed by the method of Albersheim *et al.*[4]. The crude product was then treated with 48% HF at 4° to cleave the phosphoric diester linkage, *N*-acetylated with 3% acetic anhydride in acetone, reduced with $NaBH_4$ to form alditols at any newly exposed reducing ends, and the excess borate was removed as described earlier[4]. The oligoglycosylalditol was methylated and hydrolyzed, and the resulting partially methylated monosaccharides were reduced with $NaBH_4$, *N*- and *O*-acetylated with acetic anhydride, and analyzed by g.l.c. and g.l.c.–m.s.

In a second series of experiments, the polymer was methylated before HF

cleavage of the phosphoric diester. The intact polysaccharide (50 μg) was treated with methyl iodide in the presence of 4M sodium methylsulfinylmethide[5] (NaH in dimethyl sulfoxide). After removal of the excess anion, the phosphate linkages were hydrolyzed with 48% HF at 4° for 24 h. The crude product was *N*-acetylated with 3% acetic anhydride in acetone (50°, 2 h) and hydrolyzed[6] with 0.25M H_2SO_4 in 80% acetic acid for 20 h at 80° (ref. 7). Enough NaOH was added to the mixture to neutralize H_2SO_4, and the excess acetic acid and water were removed by evaporation. The mixture of partially methylated monosaccharides was reduced to the corresponding alditols with 0.25M $NaBH_4$ and acetylated for g.l.c. and g.l.c.–m.s. analysis.

A third series of experiments was designed to isolate phosphorylated monosaccharides by partial hydrolysis of the polymer, and to detect them as their trimethylsilyl derivatives by mass spectrometry. The polysaccharide (50 μg) was treated with 2M trifluoroacetic acid for 1 h at 120°. The resulting monosaccharides and monosaccharide phosphates were treated with pyridine, bis(trimethylsilyl)-trifluoroacetamide and chlorotrimethylsilane to form the trimethylsilyl ethers and esters. This mixture was subjected to g.l.c. and g.l.c.–m.s. analysis to establish the presence and identity of all phosphorylated monosaccharides. As a control, this method was applied to D-galactose 6-phosphate, D-glucose 6-phosphate and 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate, and 2-amino-2-deoxy-D-glucose 6-phosphate.

Gas–liquid chromatography. — All samples were analyzed with a capillary gas chromatograph (Carlo Erba or Pye), fitted with a DBI column (30 m × 0.25 mm o.d.) from J & W Scientific. The injector port of both instruments was modified to fit a van den Berg dry injector[7], and the heated zone was held at 250°. Samples (1–2 μL) were placed on the injector tip, and the solvent was evaporated before the tip was lowered into the thermal zone. The detector, or transfer line for the mass spectrometer, was also held at 250°. The g.l.c. oven was programmed for 4 min at an initial isothermal temperature of 160°, and then heated to 250° at 2°/min. The relative retention index (methylene units) was determined for each component by the addition of hydrocarbons to each sample and interpolation of each unknown peak between those of two hydrocarbons. Mass spectra were obtained on a Vacuum Generator (VG) MM-16, low-resolution mass spectrometer. Samples were introduced with a van den Berg injector and a capillary g.l.c. column as described above. The instrument was operated in the electron-impact mode with an ionization potential of 70 eV. The source was held at 200° and a *m/z* range of 700 to 20 was scanned in a repetitive manner. Data were acquired on a VG 2000 data system.

Nuclear magnetic resonance methods. — For n.m.r. studies, the polymer (0.5 mg) was treated three times with D_2O (99.8 atom% D), followed by lyophilization. The final solution was prepared by dissolving the sample in high purity (99.96 atom% D) D_2O (350 μL;MSD Isotopes, St. Louis, MO 63116). The ^{1}H-n.m.r. spectra were recorded with a 300-MHz Nicolet NT-300 n.m.r. spectrometer equipped with a 293C pulse programmer. The observed chemical shifts are re-

ported relative to the signal of internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) by use of acetone as an internal standard (2.225 p.p.m. downfield from the signal of DSS). The scalar-coupled peaks were assigned by COSY and 1D-HOHAHA (Homonuclear Hartman–Hahn Coherence Transfer) experiments[8,9]. The COSY spectrum was recorded in the magnitude mode using the $90° - t_1 - 90° - Acq$ pulse sequence with appropriate phase cycling to obtain N-type peaks. A constant delay of 10 ms was used before and after the mixing pulse to reduce the antiphase character of the cross-peak multiplets[10]. The 2-D data set was transferred *via* high speed, parallel-data link to a DEC VAX computer for processing with the FTNMR program of D. Hare (Infinity Systems, Seattle, Washington). Prior to the Fourier transformation, the data were apodized by a sinebell function in each dimension. The final data matrix consisted of 512 × 512 real data points in the two dimensions. The 1D-HOHAHA spectra were recorded by the pulse sequence (*1*),

$$180°_{sel} - 90° - (SL_y - 60°_{-y} - 300°_y - SL_{-y} - 60°_y - 300°_{-y})_n - Acq \quad (1)$$

where SL_y and SL_{-y} denote spin locking along the y and $-y$ axes, respectively, and $180°_{sel}$ is a selective inversion pulse[9]. A 3-ms pulse was used for the spin-lock field and the number of cycles n was chosen to be 20 with a total propagation time of 120 ms for the spin magnetization. The experiment was carried out in the low power rf mode with an ENI 411LA power amplifier to amplify the transmitter power. For spectral widths of ±800.0 Hz, rf power levels of 1–2 W proved adequate to effectively spin-lock the resonances. The 90°-pulse widths were approximately 55 μs depending on the power levels used. 1D-Nuclear Overhauser effects (n.O.e) were measured by the difference method where the on-resonance irradiated spectrum was subtracted from the one in which the irradiation frequency was off-resonance shifted by use of a 3-s saturation pulse. 80.96-MHz ^{31}P-n.m.r. spectra were recorded with a Varian XL-200 spectrometer at Purdue University Biomedical Magnetic Resonance Laboratory. The ^{31}P–^{1}H shift-correlation spectrum was obtained by use of a COLOC (correlation spectroscopy *via* long-range coupling) experiment with a 16-step phase cycling[11]. A spectral width of 300 Hz was used along the ^{31}P axis with a digital resolution of 2.43 Hz. Forty-eight points (t_1's) were acquired along the ^{1}H axis over a spectral width of 481 Hz (5.6–3.2 p.p.m.). In the ^{1}H-n.m.r. spectrum, the upfield methyl resonances were allowed to fold over to optimize the digital resolution along t_1. The ^{31}P-n.m.r. chemical shifts are reported relative to the external reference signal of 85% H_3PO_4 contained in a sealed capillary tube.

RESULTS

The ^{1}H-n.m.r. spectrum of the polysaccharide (Fig. 1a) is closely related to that of the hexasaccharide **1** which has been previously reported[3]. In addition to the most downfield resonance at δ 5.493 (Fig. 1a), there are five anomeric protons which were readily identified by comparison with their corresponding anomeric

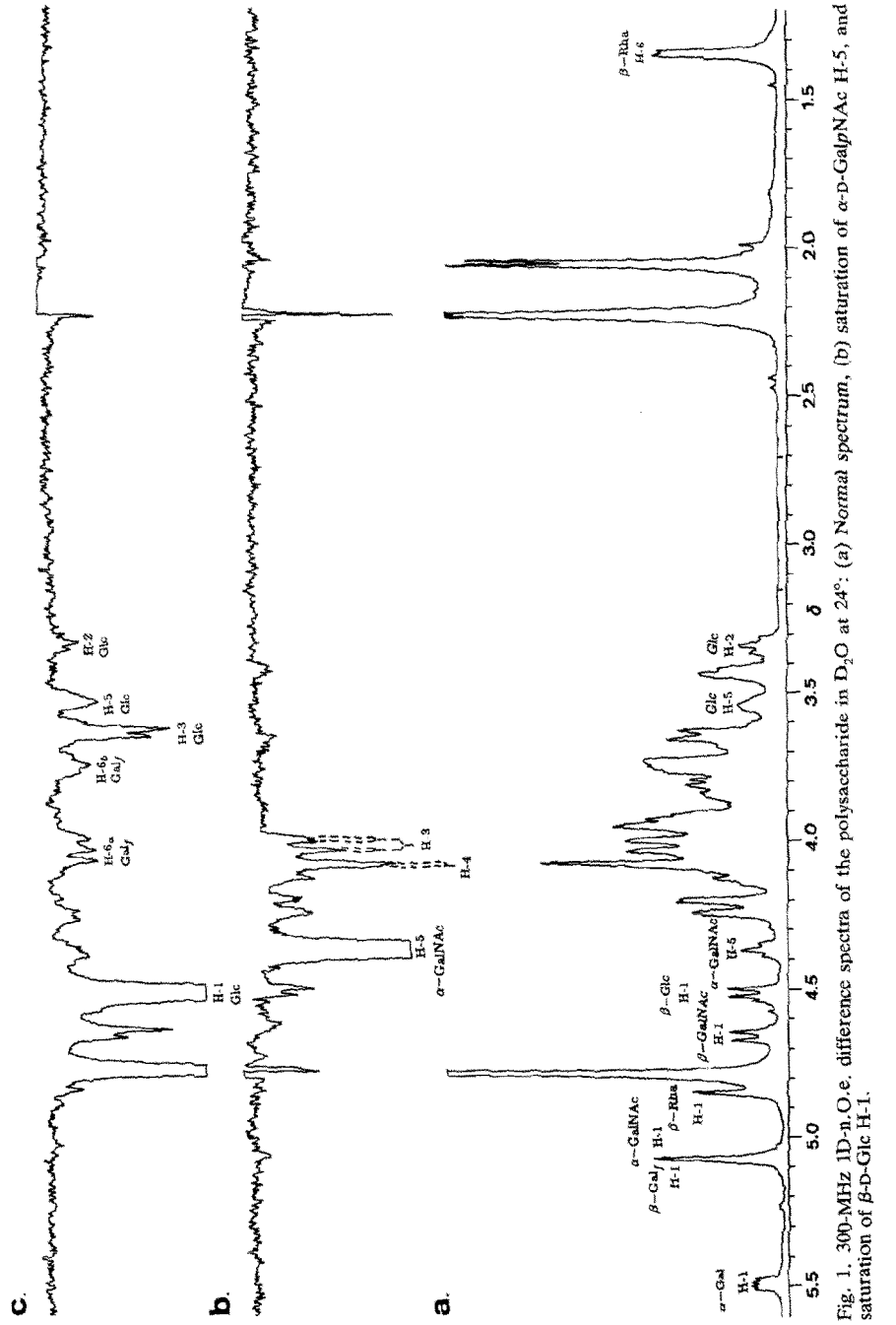

Fig. 1. 300-MHz 1D-n.O.e. difference spectra of the polysaccharide in D_2O at 24°: (a) *Normal spectrum*, (b) saturation of α-D-Gal*p*NAc H-5, and (c) saturation of β-D-Glc H-1.

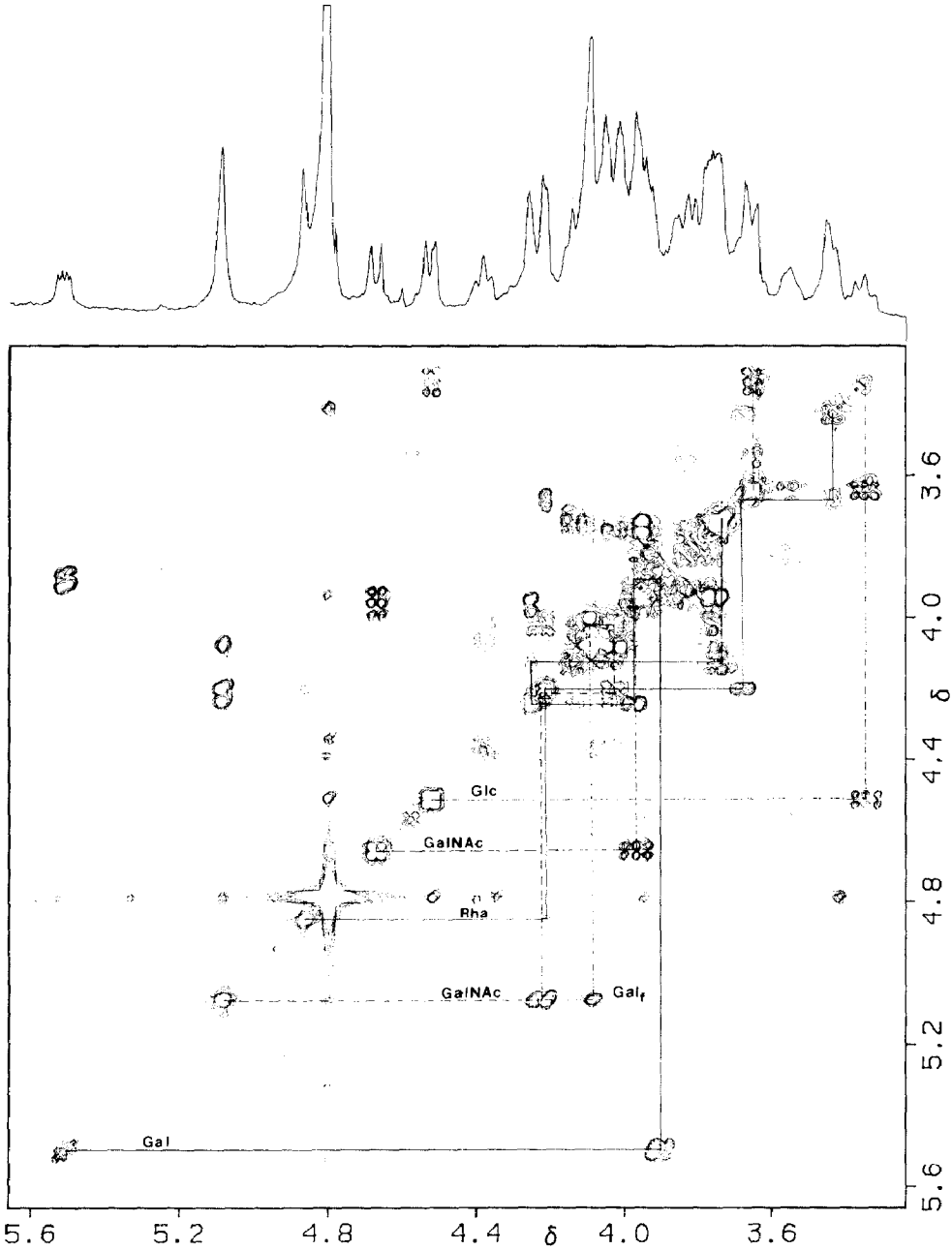

Fig. 2. 300-MHz partial ^{1}H-COSY spectrum of the polysaccharide in D_2O at 24°. The full spectrum of 512 × 512 real data points over a spectral width of ±700.28 Hz (digital resolution 2.74 Hz).

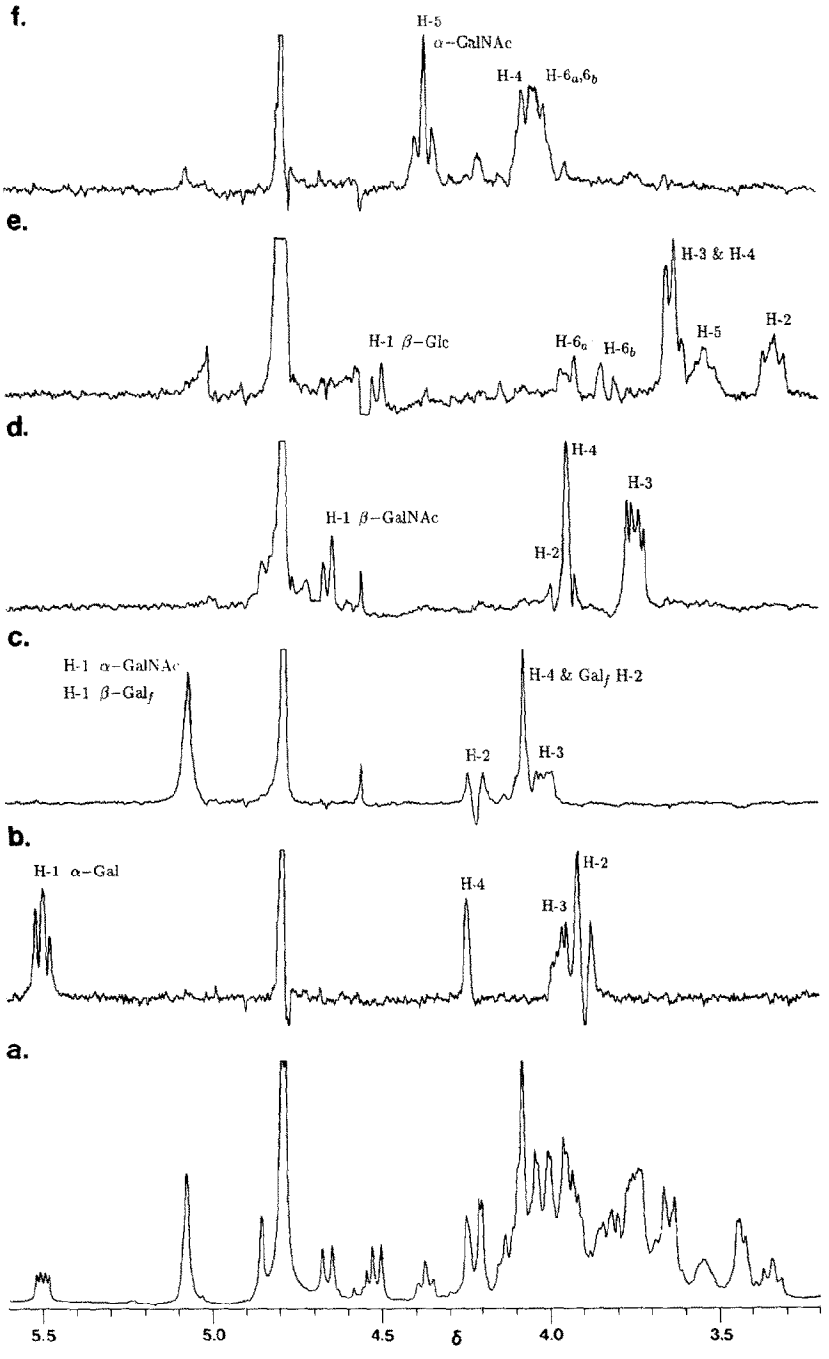

Fig. 3. 300-MHz partial 1D-HOHAHA spectra of the polysaccharide in D_2O at 24°. Selective excitation of: (a) Normal spectrum, (b) α-D-Gal H-1, (c) anomeric protons of both α-D-Gal*p*NAc and β-D-Gal*f*, (d) β-D-Gal*p*NAc H-1, (e) β-D-Glc H-1, and (f) α-D-Gal*p*NAc H-5. A total propagation time of 120 ms was used in all these difference spectra.

signals in hexasaccharide **1**. The quartet at δ 5.493 (J 3.3 and 6.9 Hz) was assigned as the anomeric ^{1}H-resonance of an α-D-galactopyranosyl phosphate residue by comparison with the anomeric proton signal of α-D-galactopyranosyl 1-phosphate (quartet at δ 5.486 with J 3.6 and 7.5 Hz). This assignment was confirmed by ^{1}H–^{31}P n.m.r. spectroscopy to be discussed below. Although the resonances assigned to α-D-Gal*p*NAc and to the β-D-galactofuranoside units overlap at 24°, the temperature-dependence of the chemical shifts allowed their observation separately at 70°, where they showed the same multiplet structure as observed in the hexasaccharide[3]. The chemical shifts of the anomeric resonances of the D-glucose and L-rhamnose units are similar in the polymer and hexamer, and the downfield chemical shift of β-D-Gal*p*NAc H-1 (0.117 p.p.m.) in the polysaccharide relative to the hexasaccharide **1** resulted consequently from the presence in **1** of D-galactitol and in the polysaccharide of α-D-galactopyranosyl phosphate. A significant dependence of the chemical shifts of H-1 and -2 of β-D-Gal*p*NAc unit on the anomeric configuration of the adjacent sugar unit has been observed in related systems[12].

The COSY spectrum of the polysaccharide in D_2O at 24° is shown in Fig. 2. All the corresponding H-2 resonances and the scalar-coupled network for the β-L-Rha skeleton protons were readily assigned in the COSY spectrum. Starting from the H-1 resonance, the positions of H-2, -3, -4, and -5 for each sugar residue were identified sequentially by tracing out the cross-peak connectivities from the 2D-COSY contour map. However, several strongly coupled resonances precluded continuing the assignment by a COSY experiment (α-D-Gal H-2 and -3, β-D-Glc*p* H-3

TABLE I

300-MHz ^{1}H-N.M.R. CHEMICAL SHIFTS[a] OF THE CELL WALL POLYSACCHARIDE FROM *Streptococcus sanguis* 34 IN D_2O AT 24°

Residue	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6a,6b*	*N-Ac*[b]
α-Gal*p*NAc	5.068	4.216	4.015	4.075	4.367	4.06, 4.01	2.058
	(3.5)	(11.0)	(4.7)	(<1.0)	(6.6)		
β-Rha*p*	4.846	4.199	3.666	3.422	3.431	1.342	
	(<1.0)	(5.5)	(10.2)	(6.3)	(6.5)		
β-Glc*p*	4.508	3.335	3.641	3.63	3.541	3.943, 3.824	
	(7.8)	(7.4)			(6.5)	(−12.5)	
β-Gal*f*	5.068	4.075				4.05, 3.75[c]	
	(<1.0)						
β-Gal*p*NAc	4.653	3.959	3.746	3.947			2.042
	(8.6)	(11.0)	(4.3)	(<1.0)			
α-Gal*p*	5.493	3.893	3.967	4.241	4.13	3.73	
	(3.3, 6.9[d])	(11.0)	(4.3)	(<1.0)			

[a]Chemical shifts are with reference to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) with acetone as an internal standard (2.225 p.p.m. downfield from the signal of DSS). Although the spin connectivities were established by COSY, wherever possible accurate chemical shifts (±0.003 p.p.m.) were obtained either by 1D-difference decoupling or 1D-HOHAHA experiments. For strongly coupled resonances, however, accuracy is ±0.01 p.p.m. Coupling constants (Hz) are given in parentheses. [b]Assigned by chemical shift analogy[3] with hexasaccharide **1**. [c]Assignments based on n.O.e. and unpublished ^{1}H–^{13}C correlation experiments. [d]J_{P-H} coupling constant.

and -4, and α-D-Gal*p*NAc H-3 and -4), and these resonances were assigned by the 1D-HOHAHA technique (Fig. 3). In the β-D-Glc*p* residue, the connectivity of H-1 to H-3 could be easily identified by corresponding cross-peaks in the COSY spectrum, but the cross-peak for H-3 and -4 of this residue was not observed owing to strong coupling. Figure 3e shows the spectrum of the β-D-Glc*p* residue obtained in 1D-HOHAHA experiment by inversion of β-D-Glc*p* H-1. Because of the large coupling between the ring protons of the D-glucose residue, all the proton signals including those of H-6 were observed in the spectrum. Some of these spin-coupled resonances exhibited a distorted-multiplet shape due to partial dispersive character[9]. The large complex resonance centered at δ 3.64 was assigned to the strongly coupled H-3 and H-4, whereas the signal at δ 3.54 was assigned to D-Glc*p* H-5. Even though the diagonal peak for H-5 was absent, the corresponding cross-peaks for H-4 and the two H-6 protons were clearly visible in the COSY spectrum. These assignments are summarized in Table I.

Fig. 3b shows the partial HOHAHA spectrum of the α-D-Gal*p* residue, and connectivity up to the H-4 resonance could easily be assigned. But the connectivity of H-1 to H-5, and further to H-6, was not observed owing to the small scalar coupling between H-4 and H-5 ($J_{4,5}$ <1.0 Hz). This is true for all the D-Gal*p* and D-Gal*p*NAc residues in the polymer. But in the case of the α-D-Gal*p* residue, the corresponding cross-peak for H-4 and -5 is clearly visible in the COSY spectrum. The two strongly coupled H-6 protons and H-5 contribute to the large cross-peak in the COSY spectrum. Thus, the complete assignment from H-1 to H-6 in the α-D-Gal*p* residue could be obtained. In the β-D-Gal*p*NAc residue, connectivity up to H-4 could easily be obtained by use of COSY and 1D-HOHAHA experiments (Fig. 3d). For the β-D-galactofuranoside residue, the resonance of H-2 was assigned in the COSY spectrum, but the other protons of that residue could not be readily identified by either COSY or HOHAHA experiments. The HOHAHA-difference spectrum obtained by irradiation of the overlapping anomeric resonances of α-D-Gal*p*NAc H-1 and β-D-Gal*f* H-1 is shown in Fig. 3c. The peak at δ 4.075 resulted from the overlapping of two sharp resonances (β-D-Gal*f* H-2 and α-D-Gal*p*NAc H-4). Even though it is fairly close to the diagonal, the corresponding cross-peak between H-3 and H-4 of α-D-Gal*p*NAc was readily identified by its characteristic lineshape.

The one-proton triplet at δ 4.367, for which no obvious correspondence is seen in the spectrum of the hexasaccharide, showed corresponding cross-peaks to δ 4.06 and 4.01. The assignment of this downfield triplet to α-D-Gal*p*NAc H-5 is supported by the observation of large intraresidue n.O.e. (Fig. 1b) to H-4 and to H-3. The weak cross-peak between H-4 and H-5 was not observed in the COSY spectrum owing to the small coupling constant. Although the H-5 resonance of a nonreducing terminal α-D-Gal*p*NAc group is expected to be in the region[13] between δ 4.16 and 4.27, the ^{1}H-n.m.r. spectrum of the polymer showed three ^{1}H signals in this region, all of which were unambiguously assigned to α-D-Gal*p* H-4 (δ 4.241), α-D-Gal*p*NAc H-2 (δ 4.219), and β-L-Rha*p* H-2 (δ 4.207). The unusual, downfield chemical shifts of α-D-Gal*p*NAc H-5 and two H-6 protons will be discussed below.

Although a connectivity beyond H-2 for the β-D-Gal*f* residue was not observed in the COSY and 1D-HOHAHA spectra owing to strong coupling between the furanose-ring protons, some tentative assignment evidence was obtained in the n.O.e. experiments. Irradiation of β-D-Glc*p* H-1 showed negative n.O.e. (Fig. 1c) at β-D-Glc*p* H-2, -3, and -5, as well as at additional resonances which we tentatiely assigned to H-6 of β-D-Gal*f*. The assignment given in Table I is also supported by 1D-difference decoupling data. In general, irradiation of the anomeric signal showed a strong n.O.e. to the proton attached to the carbon atom involved in the intrasaccharide linkage[14,15]. Since a strong interresidue n.O.e. to protons other than the one attached to C-1 involved in the glycosidic linkage of oligosaccharides[13,16] has also been observed, this assignment is marked as tentative in Table I.

There are two minor resonances in the anomeric region of the polymer, and the corresponding cross-peaks to the H-2 protons are seen in the COSY spectrum. The chemical shifts of these two resonances (δ 5.23 and 4.56) are in good agreement with those of the reducing terminal D-Gal residue in the disaccharide β-D-Gal*p*NAc-(1→4)-D-Gal reported by Donald and Feeney[12], and they may arise from the reducing terminal D-Gal residue rather than from minor constituents of the polymer.

The ^{1}H-coupled, 80-MHz ^{31}P-n.m.r. spectrum of the polymer showed a broad

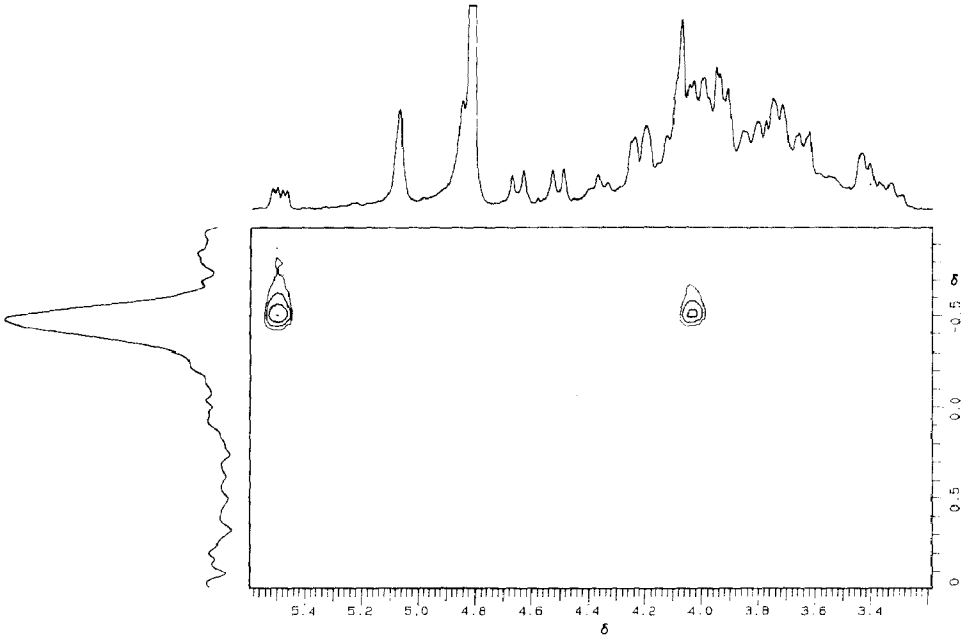

Fig. 4. 80.96-MHz 2D ^{31}P–^{1}H correlation spectrum of the polymer in D_2O obtained by the COLOC[11] pulse sequence. Data were obtained from a 48 × 256 matrix. ^{1}H- and ^{31}P-chemical shifts are relative to internal DSS with acetone as an internal standard, and external reference signal of 85% H_3PO_4, respectively. The regular 200-MHz ^{1}H-spectrum and the projection of the matrix on the ^{31}P axis are shown along the sides.

line (FWHM = 7.5 Hz) centered at δ −0.543. Since this linewidth is greater than that in the ^{1}H-n.m.r. spectra, the coupling constants between ^{31}P and protons are not observed. This ^{31}P-chemical shift is characteristic of a phosphoric diester linkage, which is consistent with the proposal that the polysaccharide is composed of repeating hexasaccharide subunits joined by phosphoric diester linkages[17]. Therefore, it should be possible to determine explicitly the position of the phosphoric diester linkage by ^{1}H–^{31}P long-range coupling correlation[18]. Fig. 4 shows the two-dimensional heteronuclear correlation *via* long range coupling (COLOC) spectrum of the polymer. The cross section at the chemical shift of the ^{31}P resonance showed a coupling from phosphorus to proton resonances centered at δ 4.03, in addition to the resonance at δ 5.49 previously assigned to the anomeric proton of α-D-galactopyranosyl phosphate.

The anomeric aldehyde group of any free sugar residue not bound glycosidically to a phosphate group or another sugar residue is reduced to the corresponding deuterated alditol residue by treatment of the intact polymer with $NaBD_4$, as described in the experimental section. Of the six partially methylated, partially acetylated alditols detected by g.l.c.–m.s. in this procedure, none was found to contain deuterium. After treatment of the polymer with 48% HF, the glycosidic phosphate linkages were cleaved and the reducing terminal D-galactose residues were then reduced by $NaBH_4$. No measurable deuterium incorporation was found by mass spectrometry, which is consistent with the n.m.r. result that the proportion of free reducing terminal D-galactose residue was very small.

Comparison of the results of linkage analysis done by methylation of the intact polymer and complete hydrolysis of the polymer with the results of methylation preceded by hydrolysis of the phosphoric diester linkage with 48% HF would reveal

TABLE II

METHYLATION ANALYSIS OF THE *S. sanguis* 34 POLYSACCHARIDE

Compound	*Methylation, followed by HF hydrolysis of phosphate linkage*	*HF hydrolysis of phosphate linkage, followed by methylation*
1,3,5-Tri-*O*-Ac-2,4,6,-tri-*O*-Me-galactitol (3-substituted Gal*p*)	+	−
3-*O*-Ac-1,2,4,5,6-penta-*O*-galactitol (3-substituted, reducing terminal Gal*o*l)	−	+
1,3,5-Tri-*O*-Ac-2,4-di-*O*-Me-rhamnitol (3-substituted Rha*p*)	+	+
1,4,6-Tri-*O*-Ac-2,3,5-tri-*O*-Me-galactitol (6-substituted Gal*f*)	+	+
1,4,5-Tri-*O*-Ac-2,3,6-tri-*O*-Me-glucitol (4-substituted Glc*p*)	+	+
1,5,6-Tri-*O*-Ac-3,4-di-*O*-Me-2-*N*-(Me)Ac-galactitol (6-substituted Gal*p*NAc)	+	+
1,5-Di-*O*-Ac-2-3,4,6-tri-*O*-Me-*N*-(Me)Ac-galactitol (non red. terminal Gal*p*NAc)	−	+

the position of the phosphate linkage. Table II lists the partially methylated alditol acetate compounds resulting from these two analytical procedures, the details of which are given in the Experimental section. The relevant features of the results, which are consistent with those reported[3] previously for the isolated hexasaccharide **1**, may be summarized as follows. First, when HF treatment preceded reduction and methylation, 1,2,4,5,6-penta-*O*-methylgalactitol was detected for the reducing terminal D-galactose residue as was seen for the hexasaccharide **1**. This compound was replaced by 2,4,6-tri-*O*-methylgalactose when methylation preceded hydrolysis of the phosphoric diester linkage, which indicated that the α-D-galactose residue is in the pyranoside ring form and is linked at C-1 as a phosphoric ester. These conclusions are consistent with the n.m.r. results, which further indicated that the D-galactopyranosyl phosphate residue is in the α-D-anomeric configuration. Second, methylation followed by hydrolysis gave one fewer product, as 3,4,6-tri-*O*-methyl-GalNAcol was absent. This was interpreted as indicating that the phosphoric ester is linked to C-6 of α-D-Gal*p*NAc residue, which becomes the nonreducing terminal group of the hexasaccharide. Another source of 3,4-di-*O*-methyl-GalNAcol was the internal β-D-Gal*p*NAc residue which is substituted at C-6 by the β-D-galacto-furanoside residue. Thus, the methylation results were consistent with the interpretation of the n.m.r. results, which imply a linear polymer having a phosphoric diester linkage between C-1 of the α-D-galactopyranosyl and C-6 of the α-D-Gal*p*NAc residues.

In order to further confirm the position of the phosphoric diester linkage, the polysaccharide was partially hydrolyzed under conditions that cleave glycosides and glycosyl phosphates, but not all the phosphate esters linked to hydroxyl groups. As controls, the hydrolysis procedure was tested on D-galactose 6-phosphate, D-glucose 6-phosphate, and 2-acetamido-2-deoxy-D-glucose-6-phosphate, as well as α-D-galactopyranosyl and 2-acetamido-2-deoxy-D-glucopyranosyl phosphates. Using the hydrolytic procedure described in the Experimental section, we found 50, 55, and 56% hydrolysis of the sugar 6-phosphate esters, respectively, and complete hydrolysis of the glycosyl phosphate esters. The 2-acetamido-2-deoxy-α-D-

TABLE III

PRODUCTS OF PARTIAL HYDROLYSIS OF THE POLYSACCHARIDE

	G.l.c. retention times (methylene units)	
Product[a]	*Polymer hydrolysis product*	*Authentic standard*
Rhamnose	16.84, 17.37	16.84, 17.39
2-Amino-2-deoxygalactose	18.71, [b]	[c]
Galactose	18.99, 19.47	18.99, 19.48
Glucose	19.29, 20.37	19.31, 20.40
2-Amino-2-deoxygalactose 6-phosphate	24.28	[c]

[a]Identified by g.l.c. retention time and by mass spectrum of the trimethylsilyl derivatives. [b]Not detected. [c]Not measured.

glucopyranosyl phosphate was completely *N*-deacetylated by this procedure. The products of partial hydrolysis of the intact polymer, identified by g.l.c.–m.s. of the trimethylsilyl derivatives, are listed in Table III. The presence of 2-amino-2-deoxygalactose 6-phosphate among the hydrolysis products of the polysaccharide confirmed the presence of the 6-phosphate linkage to this residue in the polymer. The only source for this product would be the α-D-Gal*p*NAc residue, since the β-D-Gal*p*NAc residue is substituted at C-6 by the β-D-Gal*f* residue.

DISCUSSION

The ^{31}P-n.m.r. spectra and the long-range coupling correlation to the protons indicated that the hexasaccharide repeating units are joined by a phosphoric diester bond linked to the anomeric carbon of the α-D-galactopyranosyl residue and to a carbon atom that is linked to a proton resonating at δ 4.03. The linkage of the α-D-galactopyranosyl phosphate group cannot be to β-D-Glc*p*, β-L-Rha*p*, or α-D-Gal*p* residues since all the protons of these residues have been assigned and none is in the δ 4.03 region of the spectrum. Furthermore, the linkage cannot be to β-D-Gal*p*NAc, since only H-5 and two H-6 protons were not assigned for that residue, and the structure of the hexasaccharide repeating-unit indicated that neither position could be involved in the linkage.

The candidate assignments for the phosphoric diester linkage consistent with the n.m.r. data include C-3 and C-5 of the β-D-Gal*f*, and C-3 or C-6 of the α-D-Gal*p*NAc residue. The chemical shift of H-3 of the α-D-Gal*p*NAc residue (δ 4.015) is typical for a residue not substituted at that position, whereas the chemical shifts of both H-5 and two H-6 protons of the α-D-Gal*p*NAc residue are well downfield of their normal position in nonreducing terminal D-Gal*p*NAc groups[13]. Although the signals for two H-6 protons of nonreducing terminal D-Gal*p*NAc groups are found at δ 3.75, they were assigned at δ 4.01 and 4.06 in this polymer, and H-5, which is normally found at δ 4.15–4.25, was assigned at δ 4.370 in this polymer. Thus, we interpret the P–H cross-peak at δ 4.03 in Fig. 4 as coupling of the phosphate group to the two H-6 resonances of α-D-Gal*p*NAc which were not resolved at 200 MHz and at the low digital resolution of the COLOC experiment. The phosphoric diester linkage most consistent with the n.m.r. data is to C-6 of an α-D-Gal*p*NAc residue, a conclusion that is supported by ^{13}C-n.m.r. spectroscopy and the assignments of the galactofuranoside resonances based on ^{1}H–^{13}C correlation data[19].

Several chemical evidences point to a phosphoric diester linkage at the α-D-Gal*p* residue. The ease of the hydrolysis of approximately 50% of the phosphate groups with 0.07M NaOH at 50° for 100 h is in agreement with such an ester. The observation that reduction of the intact polysaccharide with $NaBD_4$ did not result in deuterium incorporation suggested that the α-D-Gal*p* residue is protected. Furthermore, the methylation experiment resulted in the expected products from a compound having one phosphoric ester linkage on C-1 of the α-D-Gal*p* residue.

The linkage analysis in which the permethylation preceded hydrolysis of the phosphoric diester lacked a derivative characteristic of a terminal Gal*p*NAc group. Our interpretation of these results is that this Gal*p*NAc residue is phosphorylated at C-6, yielding a derivative identical to that from the β-D-Gal*p*NAc residue which is substituted at C-6 position by the β-D-Gal*f* residue. Since only one Gal*p*NAc product from the methylation of the intact polysaccharide was found, we concluded that both Gal*p*NAc residues are substituted at C-6. Further support for our assignment of the phosphate linkage to C-6 of α-D-Gal*p*NAc residue was provided by the isolation of 2-amino-2-deoxygalactose 6-phosphate from the partial hydrolysis products of the polymer (Table III). Both the n.m.r. data and the methylation analysis support structure **2** for the repeating unit of the capsular polysaccharide of *Streptococcus sanguis* 34.

$$[\rightarrow PO_4^- \rightarrow 6)\text{-}\alpha\text{-D-Gal}p\text{NAc-}(1\rightarrow 3)\text{-}\beta\text{-L-Rha}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 6)\text{-}\beta\text{-D-Gal}f\text{-}(1\rightarrow 6)\text{-}\beta\text{-D-Gal}p\text{NAc-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\text{-}]_n$$

2

Although ribitol phosphates are known to occur in many bacterial polysaccharides, phosphoric diester linkages between pyranosides are less common in the backbone of bacterial polysaccharides[20]. Among the capsular polysaccharides of streptococcal species, glycosyl phosphoric diester linkages in the backbone are found in *Streptococcus pneumoniae* types 19 and 20, structures which are not known[21] to be immunologically related to *S. sanguis* 34. It is not clear whether the phosphoric diester linkage in *S. sanguis* 34 is involved in either immunological reactivity or in activity as a lectin receptor for actinomyces. Koga *et al.*[22] have reported a preliminary analysis of a *S. sanguis* strain (ATCC 10557) which cross-reacts with the actinomyces lectin. Although these workers reported a low phosphate content for the ATCC 10557 strain, we have evidence that this polymer is linked by phosphoric diester bonds similar to those of the *S. sanguis* 34 polysaccharide[19]. Details of the correlation of the lectin-receptor activity, immunological reactivity, and the structures of the capsular polysaccharide of *S. sanguis* strains will require further structural studies.

ACKNOWLEDGMENTS

The samples of *S. sanguis* 34 polysaccharide was generously provided by Dr. F. C. McIntire. The authors thank the Purdue University Biological Magnetic Resonance Laboratory supported by NIH grant RR-01077 for the ^{31}P-n.m.r. spectroscopy.

REFERENCES

1 J. MIZUNO, J. O. CISAR, A. E. VATTER, P. V. FENNESSEY, AND F. C. MCINTIRE, *Infect. Immun.*, 40 (1983) 1204–1213.

2 F. C. McIntire, L. K. Crosby, A. E. Vatter, J. O. Cisar, M. R. McNeil, C. A. Bush, S. S. Tjoa, and P. V. Fennessey, *J. Bacteriol.*, 170 (1988) 2229–2235.
3 F. C. McIntire, C. A. Bush, S. S. Wu, S. C. Li, Y. T. Li, M. McNeil, S. S. Tjoa, and P. V. Fennessey, *Carbohydr. Res.*, 166 (1987) 133–143.
4 P. Albersheim, D. J. Nevins, P. D. English, and A. Karr, *Carbohydr. Res.*, 5 (1967) 340–345.
5 T. J. Waeghe, A. D. Darvill, M. McNeil, and P. Albersheim, *Carbohydr. Res.*, 123 (1983) 281–304.
6 W. Stoffel and P. Hanfland, *Hoppe-Seyler's Z. Physiol. Chem.*, 354 (1973) 21–31.
7 P. M. J. van den Berg and T. P. H. Cox, *Chromatographia*, 5 (1972) 301–305.
8 A. Kumar, G. Wagner, R. R. Ernst, and K. Wüthrich, *Biochem. Biophys. Res. Commun.*, 96 (1980) 1156–1163.
9 D. G. Davis and A. Bax, *J. Am. Chem. Soc.*, 107 (1985) 7197–7198.
10 A. Kumar, R. V. Hosuy, and K. Chandrasekhar, *J. Magn. Reson.*, 60 (1984) 1–6.
11 H. Kessler, C. Griesinger, J. Zarbock, and H. R. Loosli, *J. Magn. Reson.*, 57 (1984) 331–336.
12 A. S. R. Donald and J. Feeney, *Biochem. J.*, 236 (1986) 821–828.
13 V. K. Dua, B. N. N. Rao, S. S. Wu, V. E. Dube, and C. A. Bush, *J. Biol. Chem.*, 261 (1986) 1599–1608.
14 J. R. Brisson and J. P. Carver, *J. Biol. Chem.*, 258 (1983) 1431–1434.
15 T. A. W. Koerner, J. H. Prestegard, P. C. Demon, and R. K. Yu, *Biochemistry*, 22 (1983) 2687–2690.
16 R. U. Lemieux, K. Bock, L. T. Delbaere, S. Koto, and V. S. Rao, *Can. J. Chem.*, 58 (1980) 631–652.
17 D. G. Gorenstein, *Annu. Rev. Biophys. Bioeng.*, 10 (1981) 355–386.
18 D. G. Gorenstein, in D. G. Gorenstein (Ed.), *Phosphorus-31 NMR. Principles and Applications*, Academic Press, 1984, New York.
19 C. Abeygunawardana, C. A. Bush, and J. O. Cisar, unpublished results.
20 H. J. Jennings, *Adv. Carohydr. Chem. Biochem.*, 41 (1983) 155–208.
21 J. C. Richards, M. B. Perry, and D. J. Carlo, *Can. J. Biochem. Cell Biol.*, 61 (1983) 178–190.
22 T. Koga, N. Okahashi, T. Yamamoto, J. Mizuno, M. Inoue, and S. Hamada, *Infect. Immun.*, 42 (1983) 696–701.