Structural Elucidation of the 3-Deoxy-D-*manno*-octulosonic Acid Containing Meningococcal 29-e Capsular Polysaccharide Antigen Using Carbon-13 Nuclear Magnetic Resonance†

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ABSTRACT: The capsular polysaccharide antigen from *Neisseria meningitidis* serogroup 29-e contains equimolar quantities of 2-acetamido-2-deoxy-D-galactose and 3-deoxy-D-*manno*-octulosonic acid (KDO), the latter of which is rarely found in biopolymers other than lipopolysaccharides. Carbon-13 nuclear mangetic resonance in conjunction with other chemical data indicated that the polysaccharide is composed of an alternating sequence of these two residues, the linkages being at C-3 of galactosamine and C-7 of KDO in the α-D and β-D configuration, respectively. The native 29-e polysaccharide is O-acetylated, the *O*-acetyl groups being located at C-4 and C-5 of the KDO residues. Assignments of the signals in the ^{13}C nuclear magnetic resonance spectrum of the 29-e polysaccharide were made by consideration of those in the spectra of the monomer models, which necessitated the first recorded syntheses of methyl-α- and -β-D-3-deoxy-*manno*-octulopyranosonic acid. Like the methyl α- and β-D-ketosides of sialic acid (Na^+ salts), the equivalent methyl α- and β-D-ketosides of KDO exhibit large chemical shift differences in the exocyclic C-8 position dependent on anomeric configuration. This can again be attributed to hydrogen bonding between the axial carboxylate group of the methyl β-D anomer of KDO (C1 conformation) and the primary hydroxyl group at C-8. This phenomenon is also exhibited by the β-D-linked KDO units of the 29-e polysaccharide.

The isolation of the 29-e serogroup of the meningococci was first reported by Evans et al. (1968). In continuation of our studies on the meningococcal polysaccharide antigens (Bundle et al., 1974a; Bhattacharjee et al., 1975, 1976), we have completed a structural determination of the 29-e polysaccharide which confirms the above serological classification. The structure of the 29-e polysaccharide is unique, being a partially O-acetylated 2→3-β-linked disaccharide sequence of 7-*O*-α-D-2-acetamido-2-deoxygalactopyranosyl-3-deoxy-D-*manno*-octulosonic acid. The locations of the *O*-acetate substituents are restricted to C-4 and C-5 of the 3-deoxy-D-*manno*-octulosonic acid (KDO) residues. The characterization of KDO as a major component of the capsular polysaccharide emphasizes further its uniqueness. This characterization together with some preliminary structural data were reported in a preliminary communication (Bhattacharjee et al., 1974) and at that time it was thought to be the first identification of KDO in a biopolymer other than a lipopolysaccharide. However, almost simultaneously Taylor (1974) reported the identification of KDO as the major component of an acidic polysaccharide from a rough strain of *E. coli* (LP 1092). The presence of KDO in the 29-e polysaccharide, and the relatively limited knowledge of the basic chemistry of this complex molecule, made the immediate application of conventional methylation techniques difficult. However, as with the sialic acid containing meningococcal polysaccharides (Bhattacharjee et al., 1975, 1976), it was possible to determine the structure of the 29-e polysaccharide by relying heavily on the nondestructive technique of ^{13}C nuclear magnetic resonance. The use of this technique necessitated the first recorded synthesis of the methyl α- and β-glycosides of KDO as model compounds. The successful assignments of the individual carbons of these KDO methyl glycosides were aided by comparing some of their structural similarities with the previously assigned methyl glycosides of sialic acid (Bhattacharjee et al., 1976). A number of steric similarities between the KDO and sialic acid methyl glycosides have also been established, which extend beyond them both being 2-keto-3-deoxy acids, and which could possibly have some biological significance.

Experimental Procedure

Materials

The cells of *N. meningitidis* serogroup 29-e (strain 550) were obtained from the culture collection of the Laboratory Center for Disease Control, Ottawa, and were grown on a chemically defined medium (Bundle et al., 1974b). The capsular polysaccharide was isolated and purified using procedures identical with those previously described for other meningococcal polysaccharides (Bundle et al., 1974b). The 29-e polysaccharide was de-O-acetylated by treatment with 0.1 M NaOH solution at 37 °C for 4 h, deionized by passage of an aqueous solution through Rexyn 101 (H^+) ion-exchange resin, with subsequent lyophilization of the eluate, and reduced using sodium borohydride treatment of the carbodiimide complex of the carboxyl groups (Taylor and Conrad, 1973). D-Galactosamine HCl was obtained from Pfanstiehl Laboratories Inc., and *meso*-erythritol from Nutritional Biochemicals Corp., Cleveland, Ohio. 3-Deoxy-D-*manno*-octulosonic acid (KDO) was synthesised conveniently and in acceptable yields (5–8%) by the method of Hershberger and Binkley (1968) and was obtained as its crystalline ammonium salt. Diazomethane was prepared from *N,N'*-dimethyl-*N,N'*-dinitrosoterephthalamide obtained from the Aldrich Chemical Co., Milwaukee, Wis.

Analytical Methods

Amino sugars were detected using a Technicon auto analyzer and their quantitation was carried out by the Elson-

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Morgan method (Rondle and Morgan, 1955). Quantitation of KDO was performed using the method of Weisbach and Hurwitz (1959). Paper chromatographic analysis was performed using the descending method on Whatman No. 1 and 3MM papers using the following solvent systems (v/v): (A) butan-1-ol:pyridine:0.1 M HCl (5:3:2); (B) propan-2-ol:acetic acid:water (54:8:18). Compounds were detected with alkaline silver nitrate (Trevelyan et al., 1950) and periodate-thiobarbiturate (Warren, 1960) spray reagents. Thin-layer chromatographic analysis (TLC) was carried out on precoated silica gel plates (Merck, Darmstadt) and compounds were detected by their charring in an oven at 110 °C following the application to the plates of a 5% H_2SO_4/ethanol spray reagent. Silica gel 60 (Merck, Darmstadt) was used for the column chromatography.

Optical rotations are recorded at 23 ± 1° on a Perkin-Elmer 141 polarimeter and melting points were recorded on a Fisher-Johns apparatus and are uncorrected. Solutions were concentrated below 50 °C under diminished pressure.

Periodate oxidations were performed on 10 mg of the 29-e polysaccharide using 0.02 M sodium metaperiodate solution (5 mL). The periodate uptake was measured spectrophotometerically (Aspinall and Ferrier, 1967) and the formaldehyde estimated by the method of Nash (1953).

Gas-liquid chromatography (GLC) was performed on an F & M Model 402 gas chromatograph fitted with a flame ionization detector. The column used was 2% OV17 on Chromosorb G (100–200 mesh) at 210 °C. The retention volume was quoted relative to that of methyl 2,3,4-tri-*O*-methyl galactopyranoside (T_G). The 70-eV mass spectra were recorded on a Finnigan 3100D gas chromatograph mass spectrometer (GLC–MS) connected on-line to a computer. The gas chromatograph was used with a column of 3% SE30 on Gas Chrom Q at 230 °C, and the ion-source temperature was 90 °C.

^{13}C NMR. ^{13}C NMR spectra were recorded in 12-mm or 10-mm tubes at 37 °C on a Varian XL-100-15 spectrometer operating at 25.16 MHz or a Varian CFT20 spectrometer operating at 20 MHz, respectively, both in the pulsed Fourier transform mode with complete proton decoupling. Chemical shifts are reported in parts per million (ppm) downfield from external tetramethylsilane, contained in a coaxial inner tube of outside dimension 5 mm, and the ^2H resonance of deuterium oxide was used as a field-frequency lock signal. All the monomers and polysaccharides were run as deuterium oxide solutions at concentrations of 50–100 mg per mL. The solution of the ammonium salt of KDO was allowed to equilibrate (24 h) before the ^{13}C NMR analysis was started.

Proton NMR. Proton magnetic resonance (^1H NMR) spectra were measured at 37 °C on a Varian HR 100A spectrometer using 3-(trimethylsilyl)propanesulfonic acid (Na^+ salt) as an internal standard. Samples were lyophilized (twice) from 99.7% D_2O and run in the same solvent. The apparent first-order coupling constants (Hz) were measured directly.

Synthetic Procedures: Methyl 2,4,5,7,8-Penta-O-acetyl-D-3-deoxy-manno-octulosonate. Crystalline KDO (NH_4^+ salt) (250 mg) was dissolved in pyridine (4 mL) and stirred with the dropwise addition of acetic anhydride (3 mL). Stirring was continued for 18 h. Ice-water was added to the reaction mixture and it was evaporated to a syrup, the residual pyridine being eliminated by co-evaporation (three times) from water. The dry residue was dissolved in water and passed through a small column of Dowex 50 (H^+) ion-exchange resin at 4 °C and the effluent and washings were immediately lyophilized. The dry lyophilized powder was dissolved in dry methanol and esterified using a freshly prepared ether solution of diazomethane. Following concentration to a syrup (400 mg), the methyl ester derivative was purified by application to a silica gel column (2 × 40 cm) using benzene–methanol (20:1) as the eluent. Fractions (10 mL) were collected and those containing the major component (16–21) (determined by TLC analysis) were concentrated to a syrup, which crystallized.[1] Recrystallization from ethanol–light petroleum (bp 30–60 °C) gave large crystals (200 mg) of mp 152–153 °C and $[\alpha]_D$ + 97° (*c*, 0.9 in MeOH). Analysis for the methyl ester penta-*O*-acetate derivative. Anal. Calcd for $C_{19}H_{26}O_{13}$: C, 49.35; H, 5.67. Found: C, 49.70; H, 5.59.

Methyl 4,5,7,8-Tetra-O-acetyl-2-chloro-2,3-dideoxy-α-D-manno-octulosonate. The penta-*O*-acetyl methyl ester derivative (280 mg) was dissolved in acetyl chloride (10 mL) in a glass-tube and cooled in an acetone–solid carbon dioxide bath. Dry HCl was passed through the solution for a few minutes and the tube was sealed and kept at room temperature for 4 h. The tube was recooled, the seal was broken, and it was allowed to equilibrate slowly with room temperature. The reaction mixture was then transferred to a round-bottomed flask and concentrated to a syrup. Further coevaporations (five times) of the syrup from benzene gave a syrup which was finally dried in a vacuum desiccator. It could not be induced to crystallize even after purification on a silica gel column (2 × 40 cm) using benzene–acetone (10:1) as the eluent which yielded on concentration a syrup (282 mg). It had $[\alpha]_D$ +138° (*c* 2.8 in $CHCl_3$), gave one spot on TLC analysis in the above solvent mixture and gave a ^{13}C NMR spectrum in $CDCl_3$ indicative of the presence of only one anomer. Anal. Calcd for: $C_{17}H_{23}O_{11}Cl$: C, 46.52; H, 5.24; Cl, 8.8. Found: C, 45.97; H, 5.39; Cl, 8.4.

It was tentatively assigned the α-D configuration because the equilibrium conditions of this type of reaction have been shown in the formation of other pyranosyl chlorides to yield the thermodynamically stable α-D anomer with the anomeric chloro group in an axial orientation (Horton and Turner, 1965). Although by comparison KDO is complicated by having an addition polar anomeric substituent (carboxylate group), it would appear that, whatever the effect of the carboxylate group, under equilibrium conditions the above general rules would probably still apply, resulting in an axial orientation of the anomeric chloro group. Some evidence for this can be deduced from equilibrium experiments described in this paper using the methoxy aglycone. When the methyl β-D-glycoside of KDO (Figure 3) was treated with methanolic HCl, it was found that the formation of the methyl α-D-glycoside (axial methoxy aglycone) was exclusively favored.

Methyl-β-D-3-deoxy-manno-octulosonic Acid. The acetochloro derivative (300 mg) was dissolved in dry methanol (10 mL) in an aluminum foil covered flask and with the addition of drierite (0.25 g) and silver carbonate (1.0 g) was stirred magnetically for 18 h. The reaction mixture was filtered through "Celite", and the residue was washed with methanol. Concentration of the methanol solution gave a syrup (250 mg) of $[\alpha]_D$ + 59° (*c* 5.0 in $CHCl_3$), the ^{13}C NMR spectrum (in $CDCl_3$) of which indicated the presence of only the β-D anomer.

The above acetate derivative was dissolved in a small volume of acetone and aqueous 0.1 M NaOH was added to the point of turbidity. The mixture was left to stand for 24 h at room temperature during which time further additions (twice) were made of the 0.1 M NaOH solution. The solution was deionized by passage through Rexyn 101 (H^+) ion-exchange resin at 4

[1] Having obtained seed crystals, it was possible to omit the purification step using the silica gel column in subsequent preparations.

°C and the product was converted to the sodium salt by titration of the acidic solution to pH 7.0 using 0.02 M NaOH. Lyophilization of the solution yielded a white powder (150 mg) which could not be induced to crystallize from ethanol–ether solution. It has $[\alpha]_D$ + 47° (*c* 2.0 in H_2O) and the nine-resonance ^{13}C NMR spectrum is only consistent with it being the methyl β-D anomer (chemical shifts listed in Table I). The ^1H NMR spectrum at 100 MHz (shown in Figure 1) gave a complex group of signals centered on δ 3.80 (6 protons) a singlet at δ 3.26 (3 protons, $-OCH_3$) a quartet at δ 2.38 (1 proton, H3e), and a triplet at δ 1.74 (1 proton, H3a). The coupling constants for the latter two protons (H3e and H3a) are *J* (3 ax, 3 eq) = 12.2 Hz, *J* (3 ax, 4) = 12.2 Hz, and *J* (3 eq, 4) = 4.3 Hz.

Methyl-α-D-3-deoxy-manno-octulosonic Acid. The tetra-*O*-acetyl methyl ester derivative of methyl β-D-3-deoxyoctulosonic acid from the previous preparation (100 mg) was treated with 0.1 M sodium methoxide in methanol (50 mL) and left overnight. The solution was passed through a column of Dowex 50 (H^+) ion-exchange resin at −20 °C and the column eluate and washings were concentrated to a syrup (60 mg), which was shown by ^{13}C NMR spectroscopy to be a 3:1 mixture of the respective β-D- and α-D-methyl glycoside methyl ester derivatives. This could be easily ascertained by the well-resolved carbon signals of the methyl glycoside group of the β-D anomer (52.7 ppm) and the α-D anomer (52.2 ppm). The anomeric mixture was equlibrated in 0.1 M MeOH/HCl (20 mL) for 12 h at 55 °C. Concentration of the solution gave a syrup $[\alpha]_D$ + 76° (*c* 1.8 in MeOH), the ^{13}C NMR spectrum of which showed no trace of the signals of the methyl β-D anomer. The syrup was treated with 0.1 M NaOH for 3 h and deionized by passage through Rexyn 101 (H^+) ion-exchange resin, and the solution was titrated to pH 7.0 using 0.02 M NaOH before being lyophilized. The white power (50 mg) could not be induced to crystallize from ethanol–ether solution. It had $[\alpha]_D$ + 79° (*c* 0.5 in H_2O) and the nine-resonance ^{13}C NMR spectrum is only consistent with it being the methyl α-D anomer (chemical shifts listed in Table I).

Characterization of the Component Sugars of the 29-e Polysaccharide 3-Deoxy-D-manno-octulosonic Acid (KDO). Paper chromatographic analysis of the hydrolysate from a small scale hydrolysis of the 29-e polysaccharide using 0.5 M HCl at 80 °C for 3 h in solvents A and B showed the presence of a compound which cochromatographed with 2-acetamido-2-deoxygalactose and another major component of slower mobility. The latter gave a positive red coloration with thiobarbiturate spray reagent and had a mobility identical with that of authentic KDO in solvents A and B. Quantitative estimation of KDO in the polysaccharide indicated that it contained 53% by weight of KDO. The hydrolysate from a large scale (100 mg) hydrolysis using identical conditions was applied to a column (1.4 × 8 cm) of Rexyn 201 (carbonate form) and the column washed with water to remove neutral sugars. The acidic component was then eluted with 0.5 M ammonium bicarbonate solution and the eluate was lyophilized a number of times (three times) using further additions of water. Crystallization of the residue from water–ethanol gave crystals of ammonium 3-deoxy-D-*manno*-octulosonate (14 mg) of mp 120–122 °C undepressed on a mixture with an authentic sample.

2-Amino-2-deoxy-D-galactose. The 29-e polysaccharide (100 mg) was hydrolyzed in 1 M HCl (15 mL) at 100 °C for 4 h. The aqueous acid was removed under reduced pressure and the residue was left under vacuum over KOH pellets. The residue was dissolved in water (2 mL) and applied to a column (1.4 × 8 cm) of Rexyn 201 (acetate form) ion-exchange resin. The acidic components were removed by the column and the eluate and water washings were lyophilized to give the salt of 2-amino-2-deoxy-D-galactose (46 mg), which failed to crystallize. It had $[\alpha]_D$ + 74° (*c* 0.78 in water), gave lyxose (identified by paper chromatographic analysis) on ninhydrin degradation (Stoffyn and Jeanloz, 1954), and was characterized as its crystalline hydroxynaphthaldehyde derivative (Jolles and Morgan, 1940) (mp 178–180 °C).

Smith Degradation of the 29-e Polysaccharide. Periodate oxidation of the de-O-acetylated 29-e polysaccharide resulted in the rapid consumption of 1 mol of periodate per mol of KDO. The uptake of periodate at the following times was: 1 h, 0.45 mol; 2 h, 0.83 mol; 4 h, 1.0 mol; 24 h, 1.0 mol. No formaldehyde was released.

The 29-e polysaccharide (100 mg) was dissolved in 0.1 M sodium metaperiodate (10 mL) and left for 4 h. The excess periodate was destroyed by the addition of ethylene glycol and the solution was dialyzed. Sodium borohydride (100 mg) was added to the dialyzed solution and the solution was left overnight. It was deionized by passage through Rexyn 101 (H^+) ion-exchange resin and the eluate was concentrated to dryness. The boric acid was removed by repeated (six times) concentrations of the residue solubilized in methanol. The dry residue was hydrolyzed with 1 M HCl at 100 °C for 2 h and the hydrolysate was concentrated to dryness. Paper chromatographic analysis of the hydrolysate in solvent B indicated the presence of unoxidized 2-acetamido-2-deoxygalactose and erythritol. The components were isolated by paper chromatography using solvent B and guide strips were cut from the chromatograms to locate the components and the sections were eluted with water. Concentration of the two eluates gave (i) a syrup which cochromatographed with authentic 2-acetamido-2-deoxygalactose and (ii) a syrup which when recrystallized from aqueous ethanol gave crystals of $[\alpha]_D$ 0° (in water) and mp 118–120 °C, undepressed on a mixture with an authentic sample of *meso*-erythritol. In an earlier communication this compound had been erroneously reported as exhibiting a measurable specific rotation (Bhattacharjee et al., 1974).

Methylation of the 29-e Polysaccharide. The 29-e polysaccharide was partially methylated using the procedure of Haworth and Leitch (1918) in aqueous solution. A fully methylated product was obtained using the same procedure except that the partially methylated polysaccharide was initially dissolved in tetrahydrofuran–water (5:1) before the addition of the reagents. The fully methylated product was methanolyzed using 1 M methanolic HCl in sealed tubes at 100 °C for 16 h. The solvent was evaporated off under reduced pressure and the methanolysate was re-N-acetylated using silver carbonate and acetic anhydride (Bhattacharjee et al., 1976). The re-N-acetylated product was then treated with aqueous 0.02 M NaOH to remove any *O*-acetyl groups and neutralized with 0.02 M sulfuric acid and concentrated to dryness. GLC analysis of the methanolysate gave only one nonacidic component peak at T_G 3.5. This component was identified as 2-acetamido-2-deoxy-4,6-di-*O*-methyl-D-galactopyranoside as it had a mass spectrum identical with that previously shown to be characteristic of this 4,6-di-*O*-methyl derivative (Heyns et al., 1967).

Results and Discussion

Assignments of the Resonances in the Monomer Units. Both the methyl α- and β-D-3-deoxy-*manno*-octulosonic acids were synthesized by relatively straightforward methods: the methyl β-D anomer by complete inversion of the configuration from the α-D-chloro derivative using silver carbonate and the methyl α-D anomer exclusively by the equilibration of the methyl β-

TABLE I: Carbon-13 Chemical Shifts of the De-O-acetylated Group 29-e Polysaccharide and Relevant Monomers.[a]

	KDO moiety								
	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	OCH_3 (methyl glycoside)
De-O-acetylated serogroup 29-e polysaccharide (Na^+ salt)	174.5	103.9	36.1	68.4[b]	66.8[b]	73.3	78.9	63.8	
Methyl-β-D-3-deoxy-*manno*-octulosonic acid (Na^+ salt)	174.8	102.4	35.5	68.6[b]	66.5[b]	74.6	70.3	65.2	52.9
Methyl-α-D-3-deoxy-*manno*-octulosonic acid (Na^+ salt)	176.5	102.5	35.2	67.4[b]	67.1[b]	72.5	70.5	64.2	51.9
α-D-3-Deoxy-*manno*-octulosonic acid (Na^+ salt)	177.9	97.6	34.8	67.8[b]	67.4[b]	72.4	70.5	64.2	

[a] In parts per million (ppm) from external tetramethylsilane. [b] Tentative assignments.

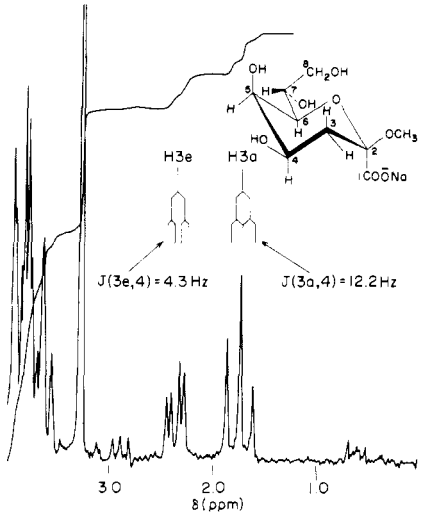


FIGURE 1: The 100-MHz NMR spectrum and assignment of the geminal H-3 protons of methyl-β-D-3-deoxy-*manno*-octulosonic acid (Na^+ salt).

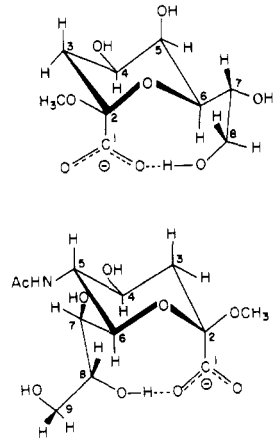


FIGURE 2: Comparative steric arrangement of the carboxylate form of the molecules of methyl-β-D-3-deoxy-*manno*-octulosonic acid (upper) and methyl-α-D-*N*-acetylneuraminic acid (lower).

D-glycoside in methanolic HCl. Each product of these syntheses gave simple nine resonance ^{13}C NMR spectra indicating that they were by this criterion, anomerically pure. The chemical shift assignments for both anomers are listed in Table I. The characteristic C-1, C-2, and C-3 resonances were readily assigned by comparison with previous assignments made on the equivalent carbons of the methyl glycosides of sialic acid (Bhattacharjee et al., 1975). In addition the resonance at 65.2 ppm was assigned to the exocyclic hydroxymethyl carbon (C-8) of the methyl β-D anomer by the appearance of a triplet of relative intensity 1:2:1 in its ^1H-^{13}C coupled spectrum (Bhattacharjee et al., 1975). Although KDO has many structural similarities with sialic acid, in order to maintain the equatorial orientation of its bulky exocyclic chain at C-6 it has to adopt the opposite conformation (C1). This can be deduced from the ^1H NMR spectrum of the methyl β-D-glycoside shown in Figure 1. Integration of the spectrum indicates the presence of the expected 11 protons and, although it is complex and not completely amenable to analysis, the signals of the geminal H-3 protons are clearly resolved. The vicinal couplings to the geminal protons must originate from the H-4 proton and the values of these constants are only consistent with an axial-axial and axial-equatorial arrangement of these protons, similar to those found in the sialic acid molecule (Brown et al., 1975), but in this case dictating the opposite 1C conformation. Confirmation of the preferred C1 conformation of KDO and its methyl glycosides can also be found in their ^{13}C NMR spectra. In the C1 conformation, C-3 of the KDO derivatives is shielded by the 1:3 axially disposed hydroxyl group on C-5 (Figure 2); this shielding does not occur on C-3 in the sialic acid derivatives in their 1C conformation (Bhattacharjee et al., 1975). In comparison with the chemical shifts of C-3 of the sialic acid methyl glycosides (Bhattacharjee et al., 1975), this shielding results in substantial upfield displacements (4.0–4.5 ppm) of the C-3 resonance of the methyl glycosides of KDO (Table I). Because of the conformational difference between sialic acid and KDO the methyl β-D-glycoside or KDO is sterically related to the methyl α-D-glycoside of sialic acid (see Figure 2). In both these molecules the carboxylate group is axially oriented and the chemical shift of C-1 of the methyl β-D-glycoside of KDO (174.8 ppm) is almost identical with that of the methyl α-D-glycoside of sialic acid at 174.6 ppm (Jennings and Bhattacharjee, 1977). Conversely the opposite anomers of the two molecules above also have similar C-1 chemical shifts (Jennings and Bhattacharjee, 1977). Therefore as in the case of the sialic acid methyl glycosides (Jennings and Bhattacharjee, 1977) the C-1 resonance of the KDO methyl glycosides is displaced significantly (1.7 ppm) by change in anomeric configuration, and can be used to assign the anomeric configuration. A further examination of the methyl β-D-glycoside of KDO and the methyl α-D-glycoside of sialic acid (Figure 2) indicates more extensive spatial similarities between these molecules. Except for the orientation of C-4 and ignoring the exocyclic chain carbons all the other carbons of the pyranose ring are spatially equivalent. On this basis of similarity it was also possible to assign the C-6 and C-4 resonances of KDO. It has been previously established in the sialic acid methyl glycosides that these carbons are the most sensitive to change in anomeric configuration (Bhattacharjee et al., 1975; Jennings and Bhattacharjee, 1977). Thus in the methyl β-D

anomer of KDO the resonance at 74.6 ppm was assigned to C-6, it being displaced upfield by 2.1 ppm with change in anomeric configuration. A similar displacement (2.4 ppm) with change in anomeric configuration occurs on C-6 of the sialic acid methyl glycosides (Bhattacharjee et al., 1975; Jennings and Bhattacharjee, 1977). Using a similar reasoning the resonance at 68.6 ppm was assigned to C-4 as this was displaced upfield by 1.2 ppm, a value in close agreement with the equivalent methyl glycosides of sialic acid both quantitatively and in direction. However, this last assignment must be regarded as tentative because the opposite assignment of the resonance at 66.5 ppm to C-4 also results in a displacement of approximately the same value but in the opposite direction. In like fashion it was impossible to assign the C-4 and C-5 resonances of the methyl α-D-glycoside of KDO with any certainty because of the closeness of their chemical shifts. However, an extremely tentative assignment was made of the resonance at 67.1 ppm to C-5; this results in the lowest possible displacement of the C-5 resonance with change in anomeric configuration, and this is consistent with the established lack of sensitivity of C-5 of the sialic acid methyl glycosides with change in anomeric configuration (Bhattacharjee et al., 1975; Jennings and Bhattacharjee, 1977). A similar lack of sensitivity is also found in the equivalent C-4 position of the hexapyranoses (Perlin et al., 1970; Dorman and Roberts, 1970). Although the C-4 and C-5 resonances are only tentatively assigned, they are not critical for any arguments used for structural determinations on the 29-e polysaccharide. The remaining unassigned resonance of the methyl β-D anomer at 70.3 ppm was assigned to C-7 as it has its counterpart at 70.5 ppm in the ^{13}C NMR spectrum of the methyl α-D anomer. This is consistent with the resonances of the exocyclic chain not being expected to be sensitive to change in anomeric configuration except in exceptional circumstances (see following sections and Jennings and Bhattacharjee (1977)). Confirmation of this assignment can also be obtained from chemical shift assignments made on the 29-e polysaccharide (see following sections). The similarities on the spatial arrangement of the molecules of the methyl β-D-glycoside of KDO and the methyl α-D-glycoside of sialic acid also extend to the carbons of their exocyclic chains in addition to their pyranose ring carbons because of proposed hydrogen bonding between their axial carboxylate groups and the hydroxyl groups on C-8 of both molecules. All these similarities are illustrated in the comparative arrangement of the two molecules in Figure 2. It has been previously proposed by Jennings and Bhattacharjee (1977) on the basis of the anomeric configuration dependency of the C-8 chemical shifts of the sodium salts of the methyl α- and β-sialic acids that such a hydrogen bonding does exist. Similarly this dependency also occurs in the equivalent C-8 position of the sodium salts of the KDO methyl glycosides producing a similar downfield displacement of 1.0 ppm in going from the methyl α-D to the methyl α-D anomer (Table I). The significance of all these (spatial) steric similarities in these two important biological molecules is not known but could possibly be related to their biological functionality.

The assignment of the α-D configuration to the predominant form of KDO in solution was made by virtue of the fact that except for the C-1, C-2, and C-3 resonances (differing aglycones) all the other major signals in the ^{13}C NMR spectrum were almost identical with those of methyl α-D-glycoside of KDO (Table I). None of the signals associated with the other anomer could be detected at this resolution but the spectrum was still complex. This is probably due to the presence of five- and six-membered lactone rings formed from the open chain form of the molecule. Evidence for this is the presence of discernible signals at 86.8, 86.3, 45.8, and 44.7 ppm. The former two are probably due to C-4 and C-5 of the two different lactone rings and the latter two to C-3 of the different lactone rings.

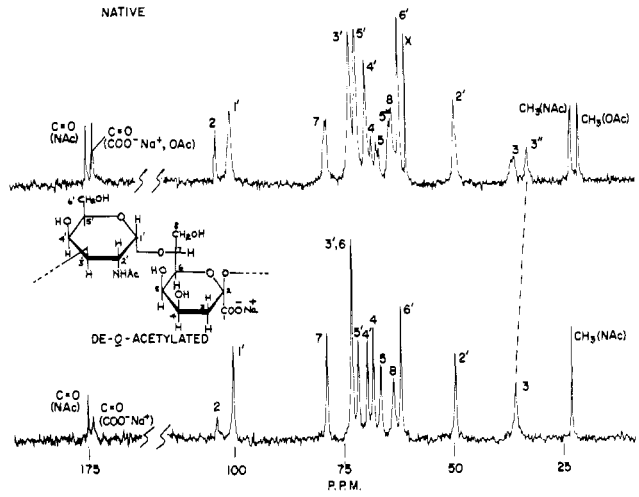
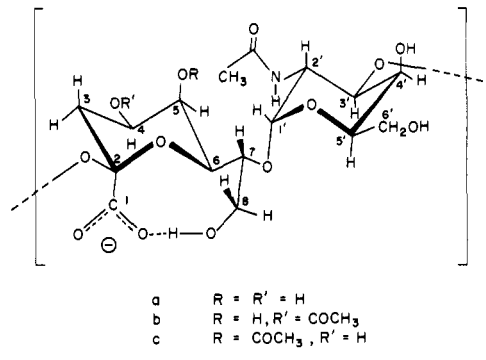


FIGURE 3: Fourier transformed ^{13}C NMR spectra of the native (upper) and de-O-acetylated (lower) 29-e polysaccharide in D_2O, pH 7, 31 °C, taken with acquisition time 0.45 s, pulse angle 90°, and spectral width of 5 kHz; number of accumulated force induction decays: 221 000 (upper) and 114 900 (lower).



a R = R′ = H
b R = H, R′ = $COCH_3$
c R = $COCH_3$, R′ = H

FIGURE 4: Disaccharide repeating unit of the 29-e polysaccharide.

Structure and Configuration of the 29-e Polysaccharide. Chemical analysis of the de-O-acetylated 29-e polysaccharide $[\alpha]_D + 90°$ (*c* 1.0 in H_2O) indicated that it contained 2-acetamido-2-deoxy-D-galactose and 3-deoxy-D-*manno*-octulosonic acid in a 1:1 molar ratio. In addition the ^{13}C NMR spectrum shown in Figure 3 shows two distinct anomeric signals at 103.9 and 100.2 ppm. The absence of multiplicity in these signals (Bhattacharjee et al., 1976) is consistent with the polysaccharide having an alternating disaccharide sequence as depicted in Figure 4. The signal at 103.9 ppm was assigned to C-2 of the KDO moiety by virtue of its low intensity. This is due to C-2 of KDO not having any α protons and consequently obtaining less nuclear Overhauser enhancement (Kuhlman et al., 1970). A Smith degradation (Goldstein et al., 1959) was also very informative in the release of unoxidized 2-acetamido-2-deoxygalactose and erythritol from the oxidized KDO residues indicating that the two linkages were at C-3′ or C-4′ of the galactosamine moiety and C-7 or C-8 of the KDO moiety and C-7 or C-8 of the KDO moiety. The remaining structural problems can be easily solved using ^{13}C NMR. This is accomplished by making a comparison of the chemical shifts of the carbons of the polysaccharide with those of the model compounds, the methyl α- and β-D-glycosides of KDO and the α- and β-D anomers of 2-acetamido-2-deoxygalactopyranose.

TABLE II: Carbon-13 Chemical Shifts of the De-O-acetylated Group 29-e Polysaccharide and Relevant Monomers.[a]

	Hexosamine moiety							
	C-1′	C-2′	C-3′	C-4′	C-5′	C-6′	CH_3 ($NHCOCH_3$)	C=O ($NHCOCH_3$)
2-Acetamido-2-deoxy-β-D-galactopyranose	96.5	54.9	72.3	69.0	76.3	62.2	23.4	175.8
2-Acetamido-2-deoxy-α-D-galactopyranose	92.2	51.4	68.6	69.7	71.6	62.4	23.2	176.1
De-O-acetylated serogroup 29-e polysaccharide (Na^+ salt)	100.2	49.9	73.3	69.7	71.8	62.2	23.4	175.6

[a] In parts per million (ppm) from external tetramethylsilane.

TABLE III: Chemical Shifts of the KDO Moiety of 29-e Most Influenced by Modification of the Carboxyl Group (C-1).[a]

Polysaccharides	C-1	C-2	C-3	C-8
Reduced 29-e polysaccharide		103.0	33.5	62.8
Native 29-e polysaccharide (Na^+ salt)	174.6	103.9	36.3	63.9
Deionized 29-e polysaccharide	172.3	102.1	35.8	63.0

[a] In parts per million (ppm) from external tetramethylsilane.

The chemical shifts of the galactosamine moiety of the 29-e polysaccharide together with those of 2-acetamido-2-deoxy-α- and β-D-galactopyranose are shown in Table II. It can be readily seen that the chemical shifts of the C-4′ and C-5′ signals are almost identical with those of the α-D form of the monomer unit thus establishing the α-D configuration of the linkage between the galactosamine and KDO residues. In addition the upfield displacement of the highly characteristic C-2′ signal by 1.5 ppm in comparison with that of 2-acetamido-2-deoxy-α-D-galactopyranose is indicative that it is β to a linkage point (Bundle et al., 1973, 1974a) and the fact that C-3 was the linkage point was unambiguously confirmed by methylation analysis. The signal at 73.7 ppm in the ^{13}C NMR spectrum of the polysaccharide was assigned to C-3′, indicating that the C-3 resonance undergoes a downfield displacement of 4.7 ppm on being linked in the polysaccharide. The actual signal at 73.3 ppm in the spectrum integrates for two carbons and the other of these signals was assigned to C-6 of the KDO moiety.

The chemical shifts of the signals associated with the KDO moiety of the 29-e polysaccharide are listed in Table I and a comparison of these chemical shifts with those of the methyl α- and β-D-glycosides of KDO indicates that the KDO moiety is in the β-D configuration. This can be ascertained readily by the fact that the chemical shift of the carbon of the carboxylate group (C-1) of the KDO moiety of the polysaccharide is almost identical with C-1 of the methyl β-D-glycoside of KDO and therefore must be axially oriented. The chemical shift of C-4 is also consistent with this analysis, but the C-6 position usually very characteristic of the anomeric configuration is ambiguous in this case due to the fact that it is vicinal to the linkage position (C-7). This β effect is well documented (Bundle et al., 1974a) and means that the C-6 undergoes an upfield chemical shift displacement of 1.4 ppm. Confirmation of the point of linkage (C-7) can also be obtained by the fact that C-8 also suffers a similar upfield displacement (1.4 ppm) which is consistent with it also being vicinal to the carbon involved in the linkage (C-7). On this evidence the resonance at 78.9 ppm was assigned to C-7 representing a downfield displacement of 8.6 ppm in this resonance due to linkage at this position. Because C-8 was vicinal to the linkage position, it was not possible to ascertain by chemical shift data, as in the case of the monomers, whether the axial carboxylate groups of the β-D-KDO units of the polysaccharide are also hydrogen bonded to the hydroxyl group at C-8 (Figure 4). Some evidence for this type of hydrogen bonding, however, could be deduced from the ^{13}C NMR spectra of both the deionized and reduced carboxylate forms of the 29-e polysaccharide. Both these modifications to C-1 in addition to changing the chemical shifts of the carbons in the vicinity of C-1 (C-2 and C-3) also substantially changed the chemical shift (1.0 ppm) of the exocyclic C-8 position without changing the chemical shifts of the other carbons to any comparable extent (Table III).

Location of the O-Acetyl Groups in the 29-e Polysaccharide. The ^{13}C NMR spectrum of the native 29-e polysaccharide $[\alpha]_D + 75°$ (*c* 1.2 in H_2O) is shown in Figure 3, and due to the effect of substituent *O*-acetyl groups on the chemical shifts of the KDO residues is more complex than that of the de-O-acetylated 29-e polysaccharide. However, these effects can be used to locate the *O*-acetyl groups (Bundle et al., 1973; Bhattacharjee et al., 1975). The degree of O-acetylation could not be readily obtained using resonance height intensity data (Bundle et al., 1973) because of multiplicity in the *N*-acetyl CH_3- signal and the closeness of those individual signals; however, a comparison of the total relative area of this signal as determined by planimeter with these of the *O*-acetyl CH_3- signal indicated that they were in the approximate ratio of 1:0:0.7, respectively. On this basis the polysaccharide contains 0.7 mol of *O*-acetyl per 2-acetamido-2-deoxygalactose residue and thus per KDO residue. The critical signal in locating the *O*-acetyl groups is the multiplet signal of C-3 of KDO in the spectrum of the native polysaccharide. The three signals indicate the presence of three different disaccharide repeating units, differing only in the position of O-acetylation of the KDO moiety. The three different repeating units (a, b, and c) are shown in Figure 4. One of these C-3 signals has a chemical shift identical with that of C-3 in the de-O-acetylated polysaccharide and represents unacetylated KDO residues (a). These residues must be present as only 70% of the KDO residues could possibly be O-acetylated. Another signal (C-3″) has been displaced upfield by 2.8 ppm, and previous work has indicated that an upfield shift of this magnitude is due to C-3″ being vicinal (β) to the *O*-acetyl substituent (Bundle et al., 1973; Bhattacharjee et al., 1975). This signal represents about 40% of the area of the three signals and must be due to approximately 40% of the KDO residues having an *O*-acetyl substituent on C-4 (b). The third C-3 signal is displaced downfield by approximately 0.5 ppm and this displacement could be due C-3 of some of the KDO moieties being γ to an *O*-acetate substituent on C-5 (repeating unit c). These γ shifts have been previously described (Bhattacharjee et al., 1975) and some confirmatory evidence for this tentative assignment can be found in an equivalent displacement in the other signal (C-7) γ to C-5. Although it can be seen that a large proportion

of the C-4 and C-5 signals has been displaced in the spectrum of the native 29-e polysaccharide due to the presence of *O*-acetyl groups, these displacements cannot be measured because definitive assignments on the relocated signals cannot be made due to the complexity of the signals in this area of the spectrum.

It is interesting to note the complexity of the CH_3- (acetamido) signal in the native polysaccharide in comparison with that of the de-O-acetylated polysaccharide (Figure 3). Due to the remoteness of this group from the C-4 and C-5 positions of the KDO moiety, it would be difficult to attribute the small displacement on this signal to any through-bond proximity effect of the *O*-acetate groups (Figure 4). However, if the disaccharide repeating unit were to adopt a conformation similar to that depicted in Figure 4 the through-space interaction between the CH_3- (acetamido) group and the *O*-acetyl group on C-5, and to some extent to that on C-4, would be maximized. Due to the presence of the three types of repeating unit (a, b, and ç), this interaction could possibly lead to three different CH_3- (acetamido) signals. A close examination of the ^{13}C NMR spectrum of the native polysaccharide (Figure 3) indicates the presence of three such signals. The signal marked X in the spectrum of the native 29-e polysaccharide is due to an impurity and has been identified in a number of other meningococcal preparations (Bundle et al., 1974a; Bhattacharjee et al., 1976).

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