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ISOLATION OF 9-*O*-(α-D-*N*-ACETYLNEURAMINYL)-β-D-*N*-ACETYLNEURAMINIC ACID BY PARTIAL ACID HYDROLYSIS, AND ITS CHARACTERISATION BY ^{13}C N.M.R.*†

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ABSTRACT

9-*O*-(α-D-*N*-Acetylneuraminyl)-β-D-*N*-acetylneuraminic acid (**1**) has been obtained in 16% yield by partial hydrolysis of the sialic acid homopolymer from the serogroup C, meningococcal polysaccharide. The disaccharide was purified by gel chromatography and paper chromatography and was obtained as its diammonium salt. Its structure was confirmed by ^{13}C-n.m.r. spectroscopy in comparison with related monomers (sialic acid and its methyl α- and β-glycosides). The free-acid forms of the monomers proved to be inadequate models for the assignment of the C-1, C-2, C-3, and C-4 resonances of the disaccharide (**1**) because of substantial upfield shifts in these resonances caused by the introduction of a monovalent ion (NH_4^+) at the carboxylic acid carbons (C-1 and C-1′). When the monovalent salts (Na^+, K^+, and NH_4^+) of the monomers were used as models, improved assignments could be made, permitting unambiguous assignment of the anomeric configurations of the disaccharide (**1**) based solely on the chemical shift of the carboxylate carbon atoms (C-1 and C-1′). Formation of the sodium salt of methyl α-D-*N*-acetylneuraminic acid also produced a significant chemical-shift difference (0.9 p.p.m.) at the remote C-8 position. This shift is attributed to an interaction (hydrogen bonding) between the axial carboxylate ion of the methyl α-anomer and the hydroxyl group at C-8.

INTRODUCTION

Partial acid-hydrolyses of sialic acid-containing polymers, leading to fragments containing intact glycosidic linkages between sialic acid residues, have not been previously reported. However, such oligosaccharides are formed by the acetolysis[1] and methanolysis[2] of gangliosides. This paper describes the partial acid-hydrolysis of an α-D-(2→9)-linked‡ sialic acid homopolymer[3] from *N. meningitidis* serogroup C,

*Dedicated to the memory of Professor J. K. N. Jones, F.R.S.

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‡The designation of the α-D configuration is based on the hydroxyl group at C-8 of the 5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid moiety. See ref. 4.

to give acceptable yields of its constituent disaccharide (**1**). The structure of **1** in the form of its diammonium salt is confirmed by ^{13}C n.m.r. spectroscopy, and it is established that it is necessary to utilize the carboxylate-ion form of the sialic acid monomers in order to obtain accurate assignments. This is due to large, downfield displacements of the chemical shifts of the carboxylic acid carbon atom (C-1) and those carbon atoms (C-2, C-3, and C-4) in its vicinity because of formation of the carboxylate ion. This effect has been documented for the alkylcarboxylic acids[5]. In addition we found a further downfield displacement of the chemical shift of C-8 that occurs in all sialic moieties having the α-D configuration, and this may be interpreted as being probably due to hydrogen-bonding between the hydroxyl group at C-8 and the axial carboxylate group at C-2. The possibility of such an interaction was not previously proposed as an explanation for the hydrogen bonding to the hydroxyl group at C-8, as determined from spin–lattice relaxation studies of the carboxylate forms of the methyl ketosides of sialic acid[6].

EXPERIMENTAL

Isolation and characterisation of 9-O-(α-D-N-*acetylneuraminyl*)-β-D-N-*acetylneuraminic acid* (**1**). — The *O*-deacetylated C polysaccharide (250 mg) was dissolved in 20mM hydrochloric acid (25 ml) and warmed on a water bath for one h at 72 ±2°. The solution was then immediately evaporated to dryness on a rotary evaporator at 40° and the residue was dissolved in 0.025M ammonium acetate buffer at pH 6.5 (2.0 ml). This solution was applied directly to a column of Sephadex G-15 and the column was eluted with the same ammonium acetate buffer. The elution profile is

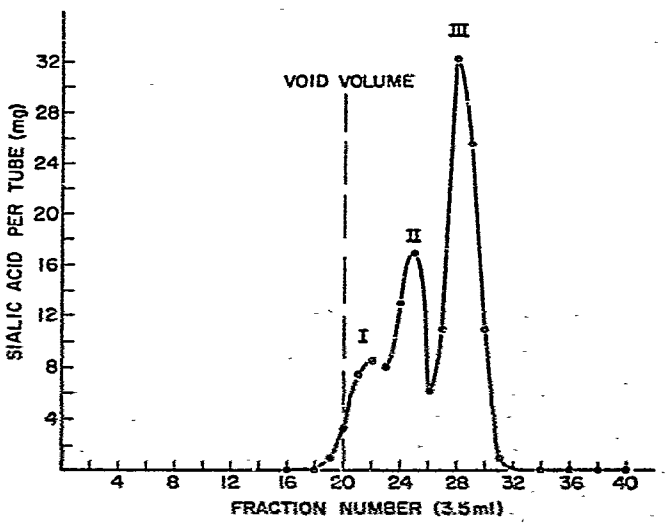

Fig. 1. Gel-filtration on Sephadex G-15 of the products of partial acid hydrolysis of the *O*-deacetylated C polysaccharide.

TABLE I

CARBON-13 CHEMICAL SHIFTS[a] OF POLYSACCHARIDES, THE DISACCHARIDE (1), AND RELEVANT SIALIC ACID MONOMERS

Compound	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-7*	*C-8*	*C-9*	*CH_3 ($NHCOCH_3$)*	*$C=O$ ($NHCOCH_3$)*	*OCH_3 (methyl glycoside)*
N-Acetylneuraminic acid (Na^+ salt)	176.0	97.6	40.6	68.5	53.5	71.7[e]	69.6	71.5[e]	64.5	23.3	176.0	—
Methyl β-D-*N*-acetylneuraminic acid[b]	174.1	100.6	40.6	67.8	53.1	71.2[e]	69.4	71.8[e]	64.7	23.3	176.1	52.1
Methyl β-D-*N*-acetylneuraminic acid (Na^+ salt)	175.9	101.7	41.1	68.4	53.3	71.4	69.7	71.4	64.8	23.4	176.0	51.8
Disaccharide 1 (Na^+ salt) obtained from serogroup C polysaccharide	174.9[d] 175.8	101.6[d] 97.5	41.0[d] 40.3	69.4[d] 68.3	53.0[d,e] 53.3[e]	73.6[d] 71.3	69.1[d,e] 69.4[e]	72.8[d] 70.2	63.8[d] 66.7	23.1[d] 23.1	176.1[d] 176.1	— —
Methyl α-D-*N*-acetylneuraminic acid (Na^+ salt)[c]	174.6	101.9	41.3	69.5	53.2	73.8	69.5	72.9	63.9	23.3	176.3	52.8
Methyl α-D-*N*-acetylneuraminic acid[b]	172.3	100.3	40.0	68.5	52.9	74.0	69.5	72.0	64.3	23.2	176.1	52.7
Deionized *O*-deacetylated serogroup C polysaccharide ($-CO_2H$)	172.5	100.1	40.6	68.7	53.1	73.9	69.3	70.9	66.6	23.4	176.1	—

[a]In p.p.m. from external tetramethylsilane. [b]Data obtained from Ref. 2. [c]The Li^+, K^+, and NH_4^+ salts gave almost identical shifts. [d]Carbon atoms of the nonreducing moiety of the disaccharide. Marked with primes in **1**. [e]Tentative assignments.

shown in Fig. 1 and indicates three major peaks (I, II, and III). The fractions corresponding to each peak were combined and lyophilized. Paper-chromatographic analysis of the lyophilized fractions in solvent A indicated that the material from peak II contained a major component at R_S 0.66, contaminated with a small amount of sialic acid. Peak I contained small quantities of higher oligosaccharides, whereas peak III contained pure sialic acid. In addition to chromatographic evidence, the material from peak III was further identified as it gave an identical ^{13}C n.m.r. spectrum to that of the sodium salt of sialic acid (Table I).

The material from peak II (105 mg) was further purified by paper-chromatographic analysis on Whatman 3 MM paper with solvent B. Guide strips were cut from the papers to locate the disaccharide (**1**) at R_S 0.66, and the areas of the papers corresponding to this component were eluted with water and lyophilized to yield the title disaccharide (**1**) in an overall yield of 16% (40 mg). The disaccharide could not be induced to crystallize and had $[\alpha]_D$ −11° (*c* 1.0, water).

Anal. Calc. (for the hydrated diammonium salt) $C_{22}H_{41}N_4O_{17}\cdot H_2O$: C, 41.2; H, 6.4; N, 8.7. Found: C, 41.5; H, 6.5; N, 8.6.

Determination of free sialic acid in the disaccharide gave a value of 30% for its total sialic acid content.

RESULTS AND DISCUSSION

^{13}C n.m.r.-spectral analysis of the disaccharide (**1**) *from the C polysaccharide.* — The ^{13}C chemical shifts of the individual carbon atoms of the disaccharide (**1**) are listed in Table I, and the spectrum is consistent only with the structure **1** shown in Fig. 2. Initially, the assignments were based on comparisons with the chemical shifts of the methyl α- and β-glycosides of sialic acid. For the most part, these monomer

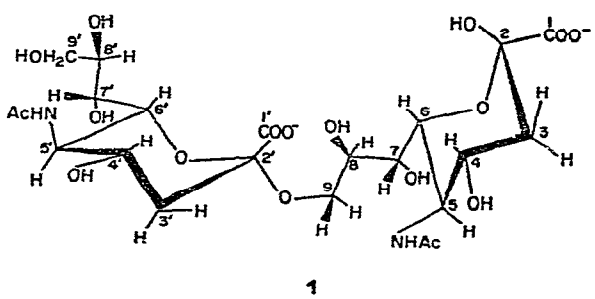

Fig. 2. Disaccharide (**1**) obtained by partial acid-hydrolysis of the *O*-deacetylated C polysaccharide.

residues served as adequate models, but some anomalies were noticeable in comparing the C-1, C-2, C-3, and C-4 resonances of **1** with those of the monomer residues (Table II). These anomalies had been previously reported in assigning the B and *O*-deacetylated C polysaccharides (obviously isolated as their sodium salts), and

were attributed to effects of the aglycon[3]. That these anomalies were actually due to the comparison of the sodium-salt form of the polysaccharides with the free-acid form of the monomers was ascertained by their virtual elimination on deionization of the *O*-deacetylated C polysaccharide (Table I). The disaccharide (**1**) was isolated as its diammonium salt but, because of its extreme acid lability, it was not converted into its free-acid form. Instead, the carboxylate-ion forms (sodium salts) of the monomers were used successfully as models in the assignment of **1** (Table II) after it had been established that change of the monovalent cation did not alter the chemical shifts of the monomers to any extent. Assignments were made for the non-reducing moiety of **1** by using the sodium salts of the methyl α- and β-ketosides as models, and the chemical shifts of the non-reducing moiety of **1** were almost identical to those of the α-anomer. The reducing moiety of **1** is a 9-*O*-substituted derivative of sialic acid and was therefore best assigned by using the sodium salt of sialic acid as a model. The fact that the linkage to the reducing moiety was at C-9 was ascertained by the downfield shift (2.2 p.p.m.) of C-9 and an upfield shift (1.3 p.p.m.) of C-8 in comparison with the monomer unit. These shifts are of similar magnitude to those originally found in assigning the identical linkage of the C polysaccharide[3]. The reducing moiety of **1** exists predominantly in the β configuration in aqueous solution, as has been found to be the case with free sialic acid[3] and other disaccharides having reducing end-groups of sialic acid[7]. This conclusion was ascertained from the similarity of the chemical shifts of the C-1, C-4, and C-6 resonances of **1** with the equivalent resonances of the sodium salt of sialic acid and its methyl β-ketoside.

TABLE II

^{13}C SHIELDING DIFFERENCES (p.p.m.)[a] BETWEEN THE DISACCHARIDE (**1**) AND METHYL α- AND β-D-*N*-ACETYLNEURAMINIC ACIDS AND THEIR SODIUM SALTS

Disaccharide (**1**) *and methyl glycosides of neuraminic acid* (Na^+ *salts*)	*C-1*	*C-2*	*C-3*	*C-4*
Non-reducing moiety of **1**, α anomer (CO_2Na)	+0.3	−0.3	−0.3	−0.1
Non-reducing moiety of **1**, β anomer (CO_2Na)	−1.0	−0.1	−0.1	+1.0
Reducing moiety of **1**, α anomer (CO_2Na)	+1.2	−4.4	−1.0	−1.2
Reducing moiety of **1**, β anomer (CO_2Na)	−0.1	−4.2	−0.8	−0.1
Reducing moiety of **1**, sialic acid (CO_2Na)	−0.2	−0.1	−0.3	−0.2

[a]Obtained from data in Table I; a positive difference indicates that the resonance in the oligosaccharide occurs at lower magnetic field (larger chemical-shift from Me_4Si).

Table II indicates that assignment of the anomeric configuration of both sialic acid moieties of **1** may be made by using the sodium salts of the monomer components. Thus, in addition to the C-4 and C-6 resonances (previously shown to be sensitive to change in anomeric configuration[3,7]), the C-1 resonance may also be used to deduce the anomeric configuration of a sialic acid aglycon, provided that comparisons are made only between the monovalent-salt forms of the monomers and **1**. This restriction also applies to the converse situation (Table I) with the free-acid

forms, and this observation has been previously made with the methyl α- and β-ketosides as models[8]. However, in comparison with our chemical-shift data, the chemical shifts of the carbonyl carbons cited[8] would seem to be more consistent with the carboxylate forms of the ketosides.

Some evidence for the existence of an aglycon effect was detected by comparing the chemical shifts of the sodium salts of sialic acid and its methyl β-glycoside with those of the reducing moiety of **1**. The large differences in chemical shift (Table II) between C-2 and C-3 of the sodium salt of the methyl β-ketoside, as compared with those of the reducing moiety of **1**, were largely eliminated by using the more-relevant sodium salt of sialic acid. As both of these monomers have the β-configuration[3], in the sodium-salt form, these differences are probably attributable to the different aglycons.

In addition to the differences in chemical shift of the C-1, C-2, C-3, and C-4 resonances caused by monovalent salt-formation of the methyl α- and β-glycosides of sialic acid, a further difference was also evident at the more remote C-8 position (Table I). This difference was fairly significant in the methyl α-anomer; sodium-salt formation caused a downfield shift of +0.9 p.p.m., with a similar shift occurring in the ammonium-salt form of the non-reducing moiety of **1** (+0.8 p.p.m.) compared to the free-acid form of the methyl α-ketoside. These differences in chemical shift are of sufficient magnitude to suggest that some interaction occurs between the carboxylate ion at C-2 and the hydroxyl group at C-8. Although reports of selective binding of calcium ions to sialic acid derivatives have appeared in the literature[9], we dismissed any interpretations involving direct interactions between the monovalent cation and the hydroxyl group at C-8 because this effect did not change with increasing size of cation (Li^+ to K^+). On this basis, we prefer to propose the possibility of hydrogen bonding between the carboxylate ion and the hydroxyl group at C-8. Other evidence to support this proposal is the enhancement of the effect by carboxylate-ion formation and its configurational dependence. These large, downfield shifts occur only on monovalent-salt formation of sialic acid derivatives in the α-configuration, and the difference in chemical shift between the sodium salts of the methyl α- and β-ketosides was a significant +1.5 p.p.m. The conformational dependence may be attributed to the greater accessibility to the C-8 hydroxyl group of the carboxylate ion of the α-anomer in comparison with that of the β-anomer. The crystal structure of β-D-*N*-acetylneuraminic acid dihydrate would certainly suggest that the equatorial carboxylate group of the β-anomer, in the $^1C_4$(D) conformation, would be the least accessible to the C-8 hydroxyl group[10]. Brown and co-workers[9] have suggested that the glycerol side-chain of sialic acid is in a favored conformation because of hydrogen bonding of the hydroxyl group at C-7 to the oxygen atom in the ring. However, on the basis of ^{13}C spin–lattice relaxation studies, Thornton and co-workers[6] have concluded that the motions of both C-7 and C-8 of the methyl α- and β-ketosides of sialic acid in their carboxylate forms are isotropic with the other ring carbon atoms. To account for this, they have proposed that, independent of configuration, hydrogen bonding occurs between the hydroxyl group at C-8 and the ring oxygen atom. On the

basis of our chemical-shift data, it would appear that, even though the motions of C-7 and C-8 are restricted in both anomers, the interpretation of hydrogen bonding to the hydroxyl group at C-8 must take into account the contribution of the carboxylate ion in the α anomer.

MATERIALS AND METHODS

The *Neisseria meningitidis* serogroup C polysaccharide was isolated and purified as previously described[11], and it was *O*-deacetylated by incubation for 4 h at 37° in 0.1M sodium hydroxide[3]. The free-acid form of the polysaccharide was produced by passage of a solution of the purified polysaccharide (isolated as its sodium salt) through Rexyn-101 (H^+) ion-exchange resin and subsequent lyophilization of the de-ionized solution. *N*-Acetylneuraminic acid (sialic acid) was obtained from Nutritional Biochemicals Ltd., Cleveland, Ohio. The methyl α- and β-ketosides were prepared according to the methods of Yu and Ledeen[12] and Kuhn *et al.*[13], respectively. The sodium salts of the methyl ketosides and free sialic acid were prepared by titration of a solution of the free acid to pH 7 with 0.01M sodium hydroxide, and subsequent lyophilization of the solution.

Paper-chromatographic analysis. — Paper chromatograms were run by the descending method on Whatman No. 1 and 3 MM papers with the following solvent systems (v/v): A, 54:8:18 2-propanol–acetic acid–water; and B, 5:3:2 ethyl acetate–1-propanol–water. Sugars were detected by alkaline silver nitrate[14] or periodate–thiobarbiturate[15] spray reagents. The mobilities are quoted relative to that of sialic acid (*N*-acetylneuraminic acid) ($R_S = 1.0$).

Gel filtration. — Gel filtration was performed on a Sephadex G-15 column (1.6 × 90 cm) with 0.025M ammonium acetate (pH 6.5) as buffer. The column effluent was monitored for sialic acid by the method of Svennerholm[16].

Analytical methods. — Determinations of free sialic acid were made by the thiobarbituric acid assay of Warren[17].

Carbon-13 n.m.r. spectroscopy. — Carbon-13 n.m.r. spectra were recorded in 10-mm tubes at 37° on a Varian CFT 20 spectrometer operating at 20 MHz in the pulsed, Fourier-transform mode with complete proton-decoupling. Chemical shifts are reported in p.p.m. downfield from external tetramethylsilane. Samples were 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. The compounds were used at 40–100 mg/ml concentration in deuterium oxide.

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