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Porcine submaxillary mucin contains α2→3- and α2→6-linked *N*-acetyl- and *N*-glycolyl-neuraminic acid

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Four acidic trisaccharides have been obtained by alkaline borohydride reductive cleavage (β-elimination) of a fraction of porcine submaxillary mucin precipitating at 60–75% ethanol. Their structures have been investigated using the techniques of methylation analysis involving gas-liquid chromatography/mass spectrometry along with high-resolution ^{1}H-NMR analysis. Two of the four oligosaccharides, B1–B4, contain *N*-acetylneuraminic acid (NeuAc) while two contain N-glycolylneuraminic acid (NeuGc). The following structures are proposed for the acidic trisaccharide fraction: (B1) NeuAcα(2→3)Galβ(1→3)GalNAcol, (B2) NeuGcα(2→3)Galβ(1→3)GalNAcol, (B3) Galβ(1→3)[NeuAcα(2→6)]GalNAcol and (B4) Galβ(1→3)[NeuGcα(2→6)]GalNAcol, (GalNAcol = reduced *N*-acetylgalactosamine.

These oligosaccharides were present in a molar ratio of 69:22:4:5. Although oligosaccharide B4 has previously been found in porcine submaxillary mucin B1, B2 and B3 have not. Furthermore, oligosaccharide B2 is a novel structure.

The oligosaccharide structures of porcine submaxillary mucin and their biosynthesis have been the subject of many investigations [1–9]. Some questions have, however, remained unanswered. For instance, Rearick et al. [7] have reported the characterization of porcine submaxillary β-galactoside α2→3-sialyltransferase, to form the sequence NeuAcα(2→3)-Galβ(1→3)GalNAc, although this structural element had not been reported in porcine submaxillary mucin (PSM). In addition, a study by Sherblom and Dahlin [10] showing that both NeuAc and NeuGc were present in tumour cell glycoproteins, along with the report of Buscher et al. [11] on the biosynthesis of NeuGc in porcine submaxillary glands and the considerable controversy which has surrounded the oligosaccharide structures of PSM [3–5] prompted a further investigation.

The mucin was precipitated from porcine submaxillary gland extracts using a higher alcohol concentration than that employed by earlier investigators. Oligosaccharides were released by β-elimination, and their structures were determined by high-resolution ^{1}H-NMR spectroscopy and methylation analysis. Four sialic-acid-containing trisaccharides, three of which had not previously been described in PSM and one of which is novel, were found.

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Abbreviations. GalNAcol, reduced *N*-acetylgalactosamine; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; HPLC, high-pressure liquid chromatography; GLC-MS, gas-liquid chromatography/mass spectrometry; PSM, porcine submaxillary mucin.

Enzymes. β-Galactoside α2→3-sialyltransferase (EC 2.4.99.4); NeuAc:α-*N*-acetylgalactosaminide α2→6-sialyltransferase (EC 2.4.99.3); *N*-acetylneuraminate monooxygenase (EC 1.14.99.18).

MATERIALS AND METHODS

Isolation of oligosaccharides

Mucin was isolated from porcine submaxillary glands according to de Salegui and Plonska [12] except that additional precipitates were obtained: following the removal of the 48–54% and 54–60% ethanol precipitates by centrifugation, the mucin solution was brought to 75% (v/v) ethanol, allowed to stand at 4°C for 16 h and centrifuged at 27000 × *g* for 45 min. The resultant precipitate was dissolved in H_2O, dialyzed against deionized water and lyophilized.

Reduced oligosaccharides were obtained from the 60–75% ethanol precipitate by β-elimination according to published procedures [13, 14] and fractionated by gel filtration on a column (1.6 × 200 cm) of Bio-Gel P-4 (200–400 mesh), equilibrated and eluted with 0.05 M ammonium acetate, pH 5.2. The flow rate was 15 ml/h and fractions of 4 ml were collected and assayed for sialic acid by the resorcinol method [15].

Oligosaccharides were separated from the pooled fractions by preparative HPLC on a Spectra Physics SP 8700 liquid chromatograph, equipped with a Rheodyne 7105 injection valve and a Hewlett-Packard HP 1040A diode-array detector operating at 195 nm. Chromatograms were recorded with a Hewlett-Packard HP 3390A integrator. Chromatography was performed on a column (4 × 250 mm) of Lichrosorb-NH_2 (particle size 5 μm, Merck) at an ambient temperature of 20°C

and a pressure of 11 MPa, essentially as described previously [16]. The mobile phase consisted of a mixture of acetonitrile (Lichrosorb grade, Merck) and deionized distilled water containing 15 mM potassium phosphate, pH 5.2. Starting at a ratio of 4:1, the acetonitrile concentration was decreased in a linear gradient at a rate of 0.5%/min. The flow rate was 2 ml/min. Individual peaks were lyophilized and desalted on columns (0.7 × 50 cm) of Bio-Gel P-4 (200–400 mesh) equilibrated and eluted with 0.05 M ammonium acetate, pH 5.2. The flow rate was 15 ml/h and fractions of 1 ml were collected and assayed for sialic acid [17]. Those containing sialic acid were pooled and lyophilized.

Identification of sialic acids

Constituent sialic acids were identified as follows.

a) Samples (50 nmol) were hydrolyzed (0.1 M CF_3CO_2H, 100 µl, 1 h, 80 °C), lyophilized and injected on HPLC using the equipment and conditions described above.

b) Samples (25 nmol) were subjected to methanolysis [18] with 1 ml 0.5 M MeOH/HCl for 1 h at 80 °C under N_2 in a sealed tube, evaporated under a stream of N_2, and dried in a vacuum dessicator. They were then derivatized to the Me_3Si ethers [19] in 40 µl pyridine/bis(trimethylsilyl)-trifluoroacetamide (1:1, v/v) for 1 h at room temperature.

The products were identified using a Hewlett-Packard 5993 gas chromatograph/quadrupole mass spectrometer/computer combination equipped with an ultra-performance capillary column (12.5 m × 0.2 mm i.d.) containing crossed-linked methylsilicone. Helium was used as a carrier gas at a pressure of 50 kPa. The initial column temperature was 140 °C rising 5 °/min to 240 °C which was held for 10 min (programme A). Mass spectra were recorded at 70 eV at an ion source temperature of 200 °C and a pressure of 1.33 mPa.

Carbohydrate composition

The carbohydrate compositions were estimated essentially as has been described [20]. Samples (25 nmol) were subjected to methanolysis with 1 ml 1 M MeOH/HCl for 16 h at 80 °C under N_2 in a sealed tube, evaporated under a stream of N_2 and dried in a vacuum dessicator. They were reacetylated by dissolving in 500 µl MeOH containing 2% pyridine (by vol.) and adding 20 µl acetic anhydride. After 20 min at room temperature, the samples were evaporated and then de-*O*-acetylated with 500 µl 0.2 M MeOH/NH_3 for 20 min at 50 °C. Following evaporation and drying, the Me_3Si derivatives were formed [19] and these were then analyzed by GLC-MS as described above but using the following column temperature programme: initial temperature 140 °C rising 2 °/min to 200 °C and then 5 °/min to 240 °C which was held for 4 min (programme B).

Methylation analysis

The lyophilized oligosaccharides (50 nmol) were suspended in 200 µl dry dimethyl sulphoxide in Teflon-lined screw-capped tubes under N_2 [21]. An equal volume of 2 M potassium methylsulfinylcarbanion/*tert*-butoxide [22] was added and the samples kept for 30 min at room temperature. Methyl iodide (300 µl) was then added with cooling. After 30 min the excess of reagent was removed under a stream of N_2 and the samples lyophilized. Salts were removed [23] by dissolving the samples in 1 ml chloroform/methanol (2/1, v/v), containing 5% (v/v) water, and by passing them through small columns containing 1 g Sephadex G-25 superfine, previously equilibrated in the same solvent mixture for 20 min. The columns were eluted with solvent (3 ml), the eluate was reduced in volume by evaporation under a stream of N_2 and finally dried by lyophilization. The products were then dissolved in 1 ml 0.5 M MeOH/HCl in Teflon-lined screw-capped tubes and heated for 20 h at 80 °C under N_2 [24]. After drying and acetylation with 50 µl pyridine/acetic anhydride (1/1, v/v) for 3 h at room temperature, in the dark, the samples were analyzed by GLC-MS [25] as described above, but using the following column temperature programme: initial temperature 55 °C rising 25 °/min to 130 °C, then 4 °/min to 214 °C and finally 8 °/min to 230 °C (programme C).

For mild hydrolysis of oligosaccharides B3 and B4 the conditions used for HPLC identification of sialic acids were employed.

^{1}H-NMR spectroscopy

Prior to ^{1}H-NMR spectroscopic analysis samples B1–B4 were repeatedly treated with ^2H_2O (99.96 atom% ^{2}H, Aldrich, Milwaukee, WI) at p^{2}H 7 and room temperature, and lyophilized.

Samples B1 and B4 were investigated by ^{1}H-NMR spectroscopy at 360 MHz, using a Brucker HX-360 spectrometer (SON hf-NMR facility, University of Groningen, The Netherlands). The ^{1}H-NMR spectrum of B3 was recorded at 400 MHz on a Bruker MSL-400 machine (Department of Physics, Vrije Universiteit, Amsterdam, The Netherlands), while sample B2 was investigated at 500 MHz on a Bruker WM-500 spectrometer (SON hf-NMR facility, University of Nijmegen, The Netherlands). Further experimental details have been described [26]. Chemical shifts (δ) are expressed downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ = 2.225 ppm in ^2H_2O at 27 °C).

RESULTS

Isolation of oligosaccharides

Approximately 10% of the total mucin-bound sialic acid was precipitated in the 60–75% ethanol fraction. Oligosaccharides released by β-elimination gave, after fractionation on Bio-Gel P-4, three main peaks (Fig. 1). Only the broad peak B was investigated further in this study. Using HPLC two major and two minor components were identified (Fig. 2A) in the ratios 69.2:22.2:3.8:4.8 (ultraviolet absorbance at 195 nm). Four acidic oligosaccharides (B1, B2, B3 and B4) were isolated by preparative HPLC. Following desalting, quantification using the Warren procedure [17] gave the ratios 67.8:21.2:4.8:6.2. It is interesting to note that quantification of oligosaccharides having equal numbers of chromophores (carbonyl functions) using HPLC with an ultraviolet detector operating at 195 nm gives essentially the same result as the Warren assay.

Composition of oligosaccharides

Analysis of the oligosaccharides after mild hydrolysis, using HPLC, showed oligosaccharides B1 and B3 to be composed of NeuAc and a disaccharide eluting with the same volume as standard Galβ(1→3)GalNAcol (Fig. 2B). Oligosaccharides B2 and B4, however, were shown to be composed of NeuGc and the same disaccharide (Fig. 2C). The sialic acid

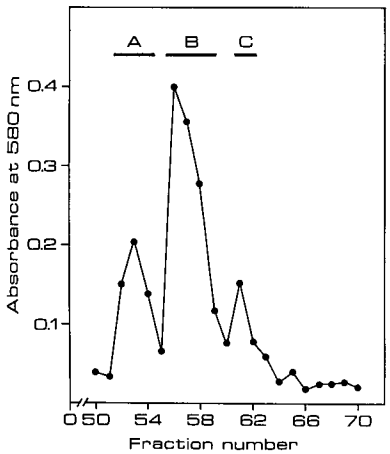

Fig. 1. *Fractionation of the products of β-elimination by gel filtration.* Oligosaccharides were applied to a column (1.6 × 200 cm) of Bio-Gel P-4 (200–400 mesh), equilibrated and eluted at 37°C with 0.05 M ammonium acetate, pH 5.2, at a flow rate of 15 ml/h. Fractions of 4 ml were collected and assayed for sialic acid [15]. The bars show the fractions which were collected; only peak B was further investigated in this study

species was also identified in each case using GLC-MS giving the same result (Table 1). Each of the four oligosaccharides was shown to be composed of equimolar amounts of sialic acid, galactose and *N*-acetylgalactosaminitol using GLC-MS (Table 1).

Methylation analysis

The molar ratios of the various partially methylated monosaccharides obtained on methanolysis and acetylation of the permethylated oligosaccharide-alditols are given in Table 2. These data confirm that each is an acidic trisaccharide.

From the substitution patterns of the Gal and GalNAcol residues of B1 and B2 it can be deduced that in both oligosaccharides Gal is glycosidically linked to position 3 of GalNAcol, and sialic acid to position 3 of Gal.

From the substitution patterns of the Gal and GalNAcol residues of B3 and B4 it can be deduced that Gal is a terminal residue and that GalNAcol is substituted at positions 3 and 6 in each case. In order to determine the linkage position of Gal to GalNAcol, B3 and B4 were permethylated following mild acid hydrolysis to remove sialic acid. Since methylation analysis showed in each case the presence of 1,4,5,6-Me_4GalNAcNMe-ol and the absence of 1,3,4,5-Me_4GalNAcNMe-ol (data not shown) it can be deduced that Gal is linked to GalNAcol at position 3. It follows, then, that sialic acid is linked to GalNAcol at position 6 in B3 and B4.

Based on these results the following structures can be proposed: (B1) NeuAc2→3Gal1→3GalNAcol, (B2) NeuGc2→3Gal1→3GalNAcol, (B3) Gal1→3[NeuAc2→6]GalNAcol and (B4) Gal1→3[NeuGc2→6]GalNAcol.

^{1}H-NMR spectroscopy

In order to determine the anomeric configurations of the linkages and to determine independently the primary structures of the four oligosaccharides, they were investigated using high-resolution ^{1}H-NMR spectroscopy. The chemical shifts of pertinent structural-reporter groups of B1–B4 have

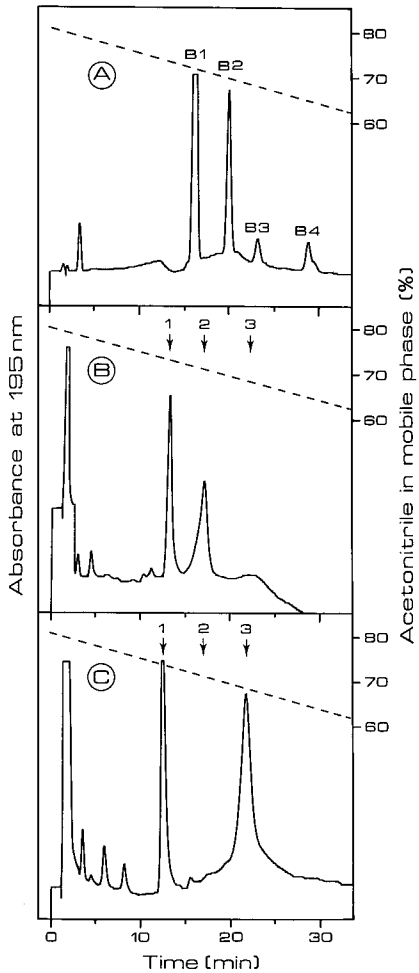

Fig. 2. *HPLC separation of oligosaccharides.* (A) Isolation of the four sialylated trisaccharides constituting peak B of the Bio-Gel P-4 fractionation (Fig. 1). (B, C) Separation of the products of mild acid hydrolysis of oligosaccharide B1 (B) and B2 (C) and identification of the sialic acid residue. The arrows indicate the elution positions of (1) Galβ1→3GalNAcol, (2) NeuAc and (3) NeuGc. For details of the HPLC system see Materials and Methods

Table 1. *Carbohydrate composition of oligosaccharide-alditols obtained from porcine submaxillary mucin*
The differentiation of NeuAc and NeuGc was made by HPLC and GLC-MS analyses. The molar sugar composition was calculated relative to GalNAcol

Oligosaccharide-alditol	Monosaccharide residues			
	GalNAcol	Gal	NeuAc	NeuGc
	mol/mol oligosaccharide			
B1	1.00	0.96	1.12	0
B2	1.00	1.12	0	1.14
B3	1.00	1.19	1.13	0
B4	1.00	1.13	0	1.13

Table 2. *Molar ratios of monosaccharide methyl ethers present in the methanolysates of the permethylated reduced oligosaccharides from porcine submaxillary mucin*
Me-2,3,4,6-Me_4Gal = methyl 2,3,4,6-*O*-methyl galactoside, etc.; GalNAcNMe-ol = *N*-acetyl-*N*-methyl galactosaminitol, etc.

Oligosaccharide-alditol	Per-*O*-acetylated derivative of					
	Me-2,3,4,6-Me_4Gal	Me-2,4,6-Me_3Gal	1,4,5,6-Me_4Gal-NAcNMe-ol	1,4,5-Me_3Gal NAcNMe-ol	Me-4,7,8,9-Me_4Neu-AcNMe	Me-4,7,8,9-Me_4Neu-GcNMe
	mol/mol oligosaccharide					
B1	0	1.00[a]	1.04	0	1.02	0
B2	0	1.00[a]	0.95	0	0	0.98[b]
B3	1.00[a]	0	0	1.13	1.00	0
B4	1.00[a]	0	0	1.01	0	0.99[b]

[a] Relative to Me-2,4,6-Me_3Gal and Me-2,3,4,6-Me_4Gal respectively.
[b] Combined values of the NeuGc and NeuAC derivatives, a small amount of the latter was formed by deacylation during methanolysis [38].

Table 3. *^{1}H-NMR chemical shift data for NeuAc- and NeuGc-containing trisaccharide-alditols obtained from porcine submaxillary mucin*
Chemical shifts are reported relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (using internal acetone at δ = 2.225 ppm) in ^2H_2O at 27 °C. Data for B1 and B4 were acquired at 360 MHz, for B2 at 500 MHz, and for B3 at 400 MHz. n.d. value could not be determined by inspection of the spectrum

Reporter group	Residue	Chemical shift in compound			
		B1	B2	B3	B4
		ppm			
H2	GalNAcol	4.390	4.389	4.379	4.380
H3		4.074	4.073	4.060	4.061
H4		3.498	3.495	3.538	3.541
H5		4.187	4.188	4.244	4.249
H6′		n.d.	n.d.	3.492	3.497
NAc		2.046	2.045	2.047	2.049
H1	Galβ(1→3)	4.547	4.547	4.474	4.477
H3		4.122	4.132	n.d.	n.d.
H3*ax*	NeuXα(2→3)	1.800	1.817	—	—
H3*eq*		2.774	2.787	—	—
X = NAc		2.034	—	—	—
X = NGc		—	4.122	—	—
H3*ax*	NeuXα(2→6)	—	—	1.693	1.711
H3*eq*		—	—	2.729	2.746
X = NAc		—	—	2.033	—
X = NGc		—	—	—	4.123

been listed in Table 3. As a typical example the ^{1}H-NMR spectrum of B2 is presented in Fig. 3.

The chemical shifts found for H2 and H5 of GalNAcol in both B1 and B2 occurring at δ = 4.39 ppm and 4.19 ppm, respectively, are indicative of mono-substitution of the GalNAcol residue by Gal in β1→3 linkage. In contrast, the chemical shifts found for H2, H5 and H6′ of GalNAcol in both B3 and B4 occurring at δ = 4.38 ppm, 4.25 ppm and 3.49 ppm, respectively, are indicative of di-substitution of the GalNAcol residue by Gal in β1→3 linkage and sialic acid in α2→6 linkage [26, 27]. Furthermore, the chemical shifts of H3*ax* and H3*eq* of the sialic acid residues (see Table 3) indicate that sialic acid is linked α2→3 to Gal in B1 and B2, while sialic acid is linked α2→6 to GalNAcol in B3 and B4 [26]. Thus ^{1}H-NMR analysis indicates a linear structure for trisaccharides B1 and B2 compared to the branched structures for their isomers B3 and B4, in agreement with the data obtained from methylation analysis (Table 2).

The structural difference within each pair of compounds (B1/B2 and B3/B4) lies in the nature of the substituent group at C5 of the sialic acid residue (see Table 1). The 5Ac and 5Gc groups are differentiated using ^{1}H-NMR spectroscopy in two ways: firstly, the appearance of the *N*-acetyl methyl singlet at $\delta \approx$ 2.03 ppm, equal in intensity to that of the GalNAcol methyl group at $\delta \approx$ 2.05 ppm in the spectra of B1 and B3 indicates the presence of NeuAc in these compounds. In contrast, the spectra of B2 and B4 reveal only one *N*-acetyl methyl resonance at $\delta \approx$ 2.05 ppm belonging to GalNAcol. In addition, however, they contain an *N*-glycolyl methylene singlet (intensity 2H) at $\delta \approx$ 4.12 ppm attributable to a NeuGc residue in the trisaccharides (Fig. 3). Secondly, a characteristic difference in chemical shift is noted for both H3*ax* and H3*eq* between the NeuAc-containing compounds B1 and B3 and their NeuGc-containing isomers B2 and B4. The NeuGc H3 signals are observed at a 0.017-ppm increment downfield from those of NeuAc, when comparing B2 to B1, or B4 to B3 (see Table 3). A similar observation has been made [26] comparing NeuGcα(2→3)Galβ(1→4)Glc to NeuAcα(2→3)Galβ(1→4)Glc.

Combining the results of chemical (Tables 1 and 2) and ^{1}H-NMR spectroscopic analyses (Table 3), the following structures for B1 – B4 can be proposed: (B1) NeuAcα(2→3)-Galβ(1→3)GalNAcol, (B2) NeuGcα(2→3)Galβ(1→3)Gal-NAcol, (B3) Galβ(1→3)[NeuAcα(2→6)]GalNAcol and (B4) Galβ(1→3)[NeuGcα(2→6)]GalNAcol.

The order of elution of the oligosaccharides on HPLC in comparison to Bio-Gel P-4 is also of interest. Using HPLC the two linear oligosaccharides, B1 and B2, eluted before the two branched oligosaccharides, B3 and B4, with the NeuAc-containing oligosaccharide eluting first for each pair (see Fig. 2A). Using Bio-Gel P-4, however, it was observed (results not shown) that the two NeuAc-containing oligosaccharides eluted together and slightly before the NeuGc-containing pair in the broad peak B.

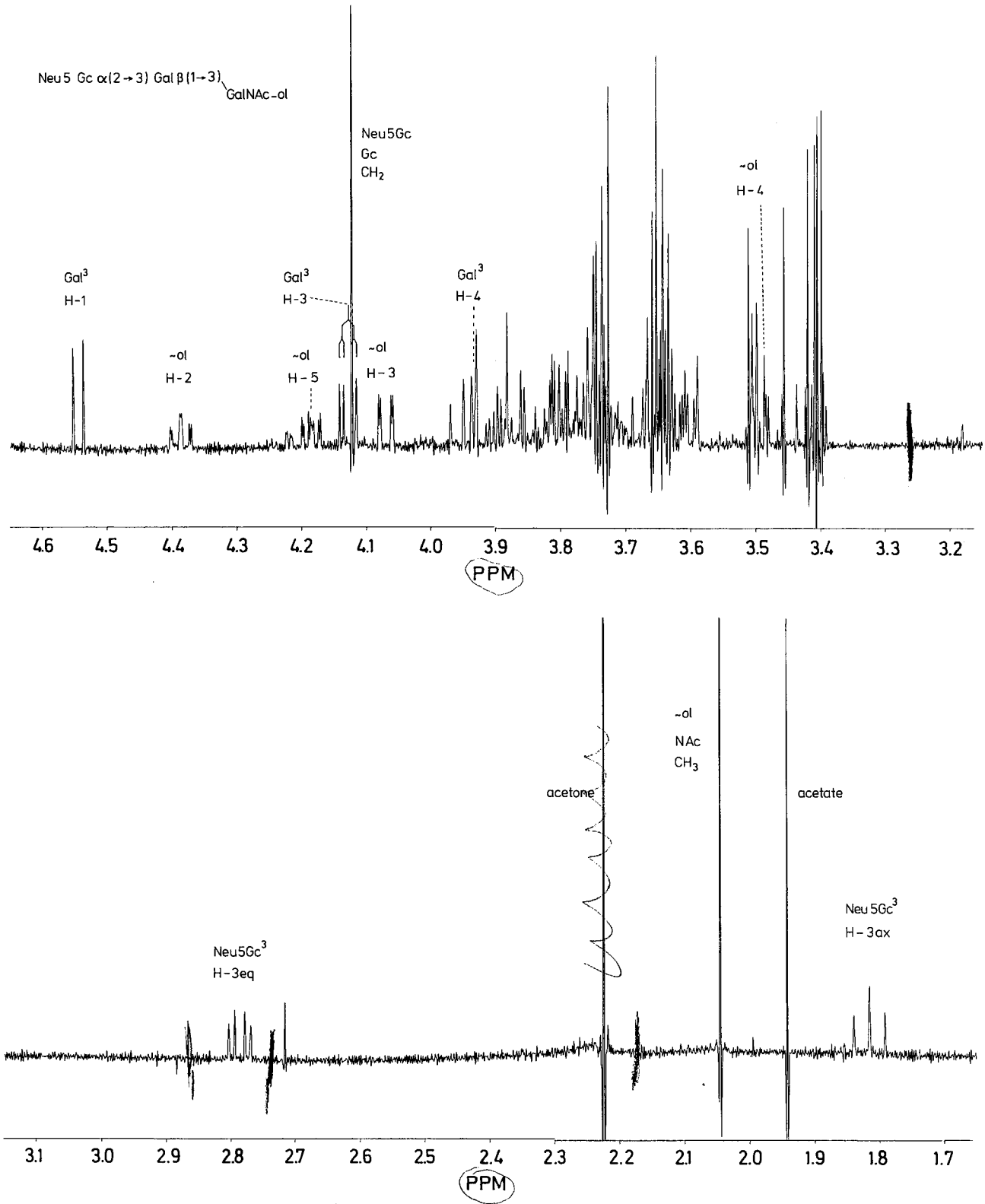

Fig. 3. *500-MHz ^{1}H-NMR spectrum, recorded in ^2H_2O at p^{2}H 7 and 27°C, of NeuGcα(2→3)Galβ(1→3)GalNAcol, oligosaccharide B2, obtained from porcine submaxillary mucin*

DISCUSSION

We report here on the structures of four acidic trisaccharides isolated from PSM. Previous structural work [5] had indicated the occurrence of only one acidic trisaccharide in PSM: B4 in this study. Although it has been known for some time that about 90% of the sialic acid on PSM is NeuGc [28] there have been no reports of NeuAc-containing oligosaccharides in PSM. We report here two such structures, B1 and B3. B1 has previously been reported in glycoproteins such as fetuin [13, 29], glycophorin [30], epiglycanin [31] and

κ-casein [27, 32], but not in salivary gland mucins, while B3 has not previously been found in mucins but is probably present in ovine submaxillary mucin [33]. In addition, a fourth sialic-acid-containing trisaccharide, B2, was found. This is a novel oligosaccharide of which only preliminary ^{1}NMR data have been reported[1].

The isolation of B1 and B2 which contain sialic acid linked α2→3 to Galβ(1→3)GalNAcol as the major oligosaccharides in the trisaccharide fraction is consistent with the occurrence of the β-galactoside α2→3-sialyltransferase in porcine submaxillary glands reported by Rearick et al. [7]. The supposed absence of products of this enzyme has received considerable comment [7, 34]. Our results now clearly show that this enzyme functions in the *in vivo* synthesis of NeuAc(Gc)-α2→3Gal-containing oligosaccharide chains on PSM.

That both NeuAc- and NeuGc-containing oligosaccharides can occur in the same glycoprotein is of particular interest [35]. In 1985 Sherblom and Dahlin [10] showed that both species were present in tumour cell glycoproteins and so the proportions of each present in PSM are worth consideration. The four acidic oligosaccharides isolated from the 60–75% ethanol fraction are present in the approximate ratio 69:22:4:5. This fraction represents approximately 10% of the total sialic acid on PSM[2]. The 55–60% ethanol fraction contains approximately 5% of the total sialic acid on PSM[2]. The only sialic acid found previously [5] in the 48–54% ethanol fraction was NeuGc linked α2→6 to GalNAcol. This moiety, therefore, represents approximately 90.5% of total sialic acid when B4 in this study is included. Therefore, in the total mucin more than 75% of sialic acid which is α2→3-linked to Gal is NeuAc while more than 95% of sialic acid linked α2→6 to GalNAc is NeuGc. This preferential occurrence of NeuAc in α2→3 linkage to Gal and of NeuGc in α2→6 linkage to GalNAc can be the result, potentially, of at least three different effects.

In our study we did not obtain information on the composition of the protein backbone of the mucin fraction precipitated at 60–75% ethanol. It is highly unlikely, however, that the amino acid sequence directs the preference for the different sialic acids since it has been shown that PSM having only NeuGc in α2→6 linkage to GalNAc, after desialylation, was an excellent acceptor for β-galactoside α2→3-sialyltransferase [7, 36] as well as α-*N*-acetylgalactosaminide α2→6-sialyltransferase [9] using CMP-NeuAc as the sugar donor.

The second possibility of preference of the α2→3- or α2→6-sialyltransferase for CMP-NeuAc or CMP-NeuGc (shown to be present in porcine submaxillary glands in a ratio of 6:4 by Buscher et al. [11]) can be discounted since in a recent study by Higa and Paulson [37] it has been demonstrated that both the α2→3- and α2→6-sialyltransferase transfer NeuAc and NeuGc from the respective cytidylglycosides to the Galβ1→3GalNAcα1→Ser(Thr) chains of antifreeze glycoprotein at essentially the same rate.

The third possibility would be a preferential action of the monooxygenase, the enzyme which converts NeuAc to NeuGc, on sialic acid residues in α2→6 linkage to GalNAc. The results of our report are in agreement with the suggestion that the predominant factor in determining the linkage distribution of sialic acids in porcine submaxillary mucin is the preferential action of the monooxygenase on NeuAc linked α2→6 to GalNAc rather than on NeuAc linked α2→3 to Gal. Whether this is the result of a specific recognition of the NeuAc linked α2→6 to GalNAc by the enzyme remains to be established.

[1] Preliminary ^{1}H-NMR data for the novel oligosaccharide B2 has been included in a review article [26]. The authors were unaware of the correct origin of this compound.

[2] The structures of the oligosaccharides in this fraction are currently under investigation.

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