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N-Acetylglucosaminyltransferase Substrates Prepared from Glycoproteins by Hydrazinolysis of the Asparagine-*N*-acetylglucosamine Linkage. Purification and Structural Determination of Oligosaccharides with Mannose and *N*-Acetylglucosamine at the Non-reducing Termini

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Sixteen asparagine-linked oligosaccharides ranging in size from $(Man)_2(GlcNAc)_2(Fuc)_1$ to $(GlcNAc)_6(Man)_3(GlcNAc)_2$ were obtained from human α_1-acid glycoprotein and fibrinogen, hen ovomucoid and ovalbumin, and bovine fetuin, fibrin and thyroglobulin by hydrazinolysis, mild acid hydrolysis and glycosidase treatment. The oligosaccharides had *N*-acetylglucosamine at the reducing termini and mannose and *N*-acetylglucosamine residues at the non-reducing termini and were prepared for use as *N*-acetylglucosaminyltransferase substrates. Purification of the oligosaccharides involved gel filtration and high performance liquid chromatography on reverse phase and amine-bonded silica columns. Structures were determined by 360 MHz and 500 MHz proton nuclear magnetic resonance spectroscopy, fast atom bombardment-mass spectrometry and methylation analysis. Several of these oligosaccharides have not previously been well characterized.

Abbreviations: bis, bisecting GlcNAc; DMSO, dimethylsulfoxide; FAB, fast atom bombardment; Fuc, L-fucose; Gal, D-galactose; GLC, gas-liquid chromatography; GlcNAc or Gn, *N*-acetyl-D-glucosamine; HPLC, high performance liquid chromatography; Man or M, D-mannose; MES, 2-(*N*-morpholino)ethanesulfonate; MS, mass spectrometry; NMR, nuclear magnetic resonance; PIPES, piperazine-*N*,*N*'-bis(2-ethane sulfonic acid); the nomenclature of the oligosaccharides is shown in Table 1.

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The biological role of asparagine-linked complex carbohydrates (*N*-glycans) is uncertain although recent studies have suggested important functions for these substances in cell-cell interaction phenomena. The biosynthesis of highly branched *N*-glycans is potentially important to many biological problems such as differentiation and metastatic potential [1-5]. For example, baby hamster kidney cells transformed either by polyoma virus [1] or by Rous sarcoma virus [2] showed a two-fold increase in one of the *N*-acetylglucosaminyltransferases (GlcNAc-transferase V) involved in the synthesis of highly branched complex *N*-glycans. Lectin-resistant glycosylation mutants of a highly metastatic murine tumor cell line were shown to be deficient both in GlcNAc-transferase V activity and metastatic potential [3]. Induction of GlcNAc-transferase V action in clones of a non-metastatic murine mammary carcinoma cell line correlated strongly with acquisition of metastatic potential [3]. Induction of GlcNAc-transferase III, the enzyme which incorporates a bisecting GlcNAc in β(1-4)-linkage to the β-linked Man of *N*-glycans, has been reported in pre-neoplastic rat liver nodules [4].

In order to develop methods for studying the GlcNAc-transferases involved in the synthesis of highly branched complex *N*-glycans, we have purified and characterized potential substrates for these enzymes, i.e. a series of bisected and non-bisected oligosaccharides with reducing GlcNAc termini and Man and GlcNAc residues at the non-reducing termini. Oligosaccharides were prepared by hydrazinolysis of human α_1-acid glycoprotein and fibrinogen, hen ovalbumin and ovomucoid, and bovine fetuin, fibrin and thyroglobulin. Terminal sialic acid and galactose residues were removed by mild acid hydrolysis and glycosidase treatment. Purification involved a combination of gel filtration and high performance liquid chromatography (HPLC). Structures were determined by 360 and 500 MHz nuclear magnetic resonance (NMR) spectroscopy, fast atom bombardment (FAB)-mass spectrometry and methylation analysis. Several of these oligosaccharide structures are either novel or were previously not well characterized.

These oligosaccharides are useful as reference compounds for structural studies, and as standards and substrates for *N*-acetylglucosaminyltransferase assays. The methods previously available for the assay of the GlcNAc-transferases which initiate the synthesis of *N*-glycan branches were tedious and time consuming. The oligosaccharides described in this report have been used to develop rapid and accurate HPLC assays for six of these GlcNAc-transferases [6]. Application of this assay technique has resulted in the discovery of novel biosynthetic pathways [6], and a new enzyme activity, GlcNAc-transferase VI, which adds GlcNAc in β(1-4)-linkage to the Manα(1-6) arm of *N*-glycans [7].

Materials and Methods

Materials

NaB^3H_4 (319 Ci/mol) was purchased from New England Nuclear, Boston, MA, USA. Acetonitrile was obtained from Caledon Laboratories, Ont., Canada and Alltech Assoc. Inc, Deerfield, IL, USA. Anhydrous hydrazine and anhydrous dimethyl sulfoxide (DMSO) were from Pierce, Rockville, IL, USA; Bio-Gel P-4 (-400 mesh) and AG 50W-X8 (200-400 mesh) were from Bio-Rad, Richmond, CA, USA; Sephadex G-15 and Sephadex G-25 were from Pharmacia, Uppsala, Sweden; acetic anhydride, hen ovomucoid, hen

ovalbumin (grade V), bovine fetuin, bovine fibrin, and coffee bean α-galactosidase were from Sigma, St. Louis, MO, USA; 2-(*N*-morpholino)ethanesulfonate (MES), piperazine-*N*,*N*′-bis(2-ethanesulfonate) (PIPES), human fibrinogen and pronase were from Calbiochem-Behring, La Jolla, CA, USA; and human α_1-acid glycoprotein and bovine thyroglobulin glycopeptides were kind gifts from the Canadian Red Cross and Dr. Dale Cumming, Dept. of Medical Genetics, University of Toronto, respectively. Sep-Pak C_{18} cartridges were from Waters Assoc., Milford, MA, USA. Jack bean β-galactosidase (7 units/ml, 1 unit = 1 μmol/min) was prepared in this laboratory [8] by Jeanne Orr and Aviva Schachter, or was purchased from Sigma. Peptide *N*-glycanase F was kindly donated by Drs. A.L. Tarentino and T.H. Plummer, Albany, N.Y., and bovine testicular β-galactosidase was a generous gift from Dr. G.W. Jourdian, Ann Arbor, Michigan.

HPLC

HPLC separations were performed with an LKB system comprising a 2152 controller, 2150 pumps and a 2152 variable wavelength u.v. monitor. Columns were protected with a guard column filled with C_{18}-bound silica. The following columns were used under isocratic conditions as described below: (1) reverse phase Waters "Dextro-Pak" 10 μ C_{18} column (8 × 100 mm), used with a compression module from Waters, run in water at a flow rate of 1 ml/min; (2) normal phase 10 μ "carbohydrate" column from Waters (3.9 × 300 mm) containing primary amine-bonded silica, run with acetonitrile/water mixtures at ratios between 64:36 and 80:20 at a flow rate of 1 ml/min; (3) Spherisorb 5 μ amine column from Alltech Assoc. (4.6 × 250 mm) run with acetonitrile/water mixtures (70/30) at a flow rate of 0.7 ml/min; and (4) a semi-preparative 10 μ Magnum amine column (10 × 250 mm) from Alltech Assoc. run with acetonitrile/water mixtures from 50/50 to 55/45 at a flow rate of 1 ml/min. Pressures varied between 20 and 100 bar according to the type of column, the age of the column, the flow rate and the mobile phase concentration.

Carbohydrate Analyses

The presence of carbohydrate in eluates from HPLC or other chromatographic columns was monitored by absorbance at 195 or 207 nm; less than one nmol of GlcNAc-containing oligosaccharide can be detected by this method. Oligosaccharides in column eluates were also detected by the phenol/sulfuric acid assay [9] or on TLC plates by spraying samples with orcinol/sulfuric acid followed by heating. Oligosaccharides were monitored for purity by proton NMR spectroscopy. Sialic acid was measured by the resorcinol assay [10]. Quantitative amino sugar analysis was carried out on all purified oligosaccharides with the Waters Picotag HPLC method following hydrolysis of samples by the HCl vapour phase method and derivatization with phenylisothiocyanate. Norleucine was used as an internal standard. These analyses were performed in the laboratory of Dr. C. Hew, University of Toronto. The sugar composition of oligosaccharides {2,2} and {2,24} (see Table 1 for nomenclature) was determined by GLC following methanolysis of samples with dry methanolic HCl for 20 h at 80°C, and derivatization with trifluoroacetic acid for 30 min at 80°C [11]. Inositol served as internal standard. Separations were performed with a Varian Model 3700 gas chromatograph using a silicone OV 210 column (from Chromatographic Specialities) and helium as a carrier gas. The temperature was increased from 110°C to 210°C at 2°C/min.

Preparation of Oligosaccharides

The general procedure for preparation of oligosaccharides from glycoprotein was as follows. *O*-Linked oligosaccharides, if present, were removed by β-elimination. Sialic acid was removed by mild acid hydrolysis. Glycopeptides were prepared by pronase digestion of glycoprotein. Oligosaccharides were released from glycopeptides by hydrazinolysis/re-*N*-acetylation/acid hydrolysis [12, 13]. Oligosaccharides were trimmed down to terminal GlcNAc or mannose residues by sequential glycosidase digestions. Each of these procedures was followed by at least one intermediate purification step. The final purification involved several gel filtration and HPLC steps.

(1) Hen ovomucoid. Hen ovomucoid (5 g) was dissolved in 200 ml of 0.5 M HCl and heated at 80°C for 1 h. After neutralizing with NaOH, the glycoprotein was purified in four batches on a column of Sephadex G-25 (2.4 × 90 cm) equilibrated in water, and digested in 90 ml 0.1 M Tris-HCl, pH 8, containing 2 mM $CaCl_2$ and 250 mg pronase, for 48 h at 37°C. Another 250 mg of pronase were added after 24 h. The digest was passed in two batches through Sephadex G-25 (24 × 90 cm). About 75% of the glycopeptide preparation was lyophilized and dried over P_2O_5 for several days in four hydrolysis tubes. A total of 34 ml anhydrous hydrazine was added under dry Argon and the reaction allowed to proceed in sealed tubes for 25 h at 100°C. The tubes were cooled and frozen; hydrazine was removed by lyophilization. The residues were dissolved in a total of 32 ml of saturated $NaHCO_3$; about 2 g of solid $NaHCO_3$ and 1.6 ml acetic anhydride were added and the solution left 10 min at room temperature. After the addition of another 1.6 ml acetic anhydride, the solution was left for 30 min and then neutralized with solid $NaHCO_3$ to about pH 8. Oligosaccharides were purified in four batches on a column of Sephadex G-15 (3 × 80 cm) equilibrated in water. Hydrolysis of the β-hydrazide derivatives [12, 13] was carried out in 40 ml 0.1 MHCl for 1 h at 40°C. After neutralization with solid $NaCO_3$, the solution was passed through Sephadex G-15 (3 × 80 cm). The oligosaccharides were pooled, lyophilized and incubated in 10 ml 50 mM sodium citrate, pH 3.5, with four units of jack bean β-galactosidase at 37°C for 24 h; another three units of enzyme were added and the incubation was continued for 24 h. The mixtures were passed in four batches through a Bio-Gel P-4 column (1.6 × 84 cm) equilibrated in water. Partially included fractions were further separated by HPLC as described in the Results section followed by passage through a column of Sephadex G-25 (2.5 × 39 cm) equilibrated in water.

(2) Hen ovalbumin. Oligosaccharide {M5} (see Table 1 for oligosaccharide nomenclature and structures) was isolated by hydrazinolysis, re-*N*-acetylation and mild acid hydrolysis [12, 13] of $(Man)_5(GlcNAc)_2$-Asn glycopeptide prepared from hen ovalbumin as previously described [14]. {M5} was purified by HPLC followed by chromatography on Bio-Gel0P-4 (-400 mesh, 1.6 × 84 cm).

(3) Bovine fibrin. Oligosaccharides {0,2+F}, {M2+F}, and {M3+F} (Table 1) were prepared from a mono-sialylated bi-antennary oligosaccharide released from fibrin by hydrazinolysis/re-*N*-acetylation of the intact glycoprotein without prior pronase digestion (B. Bendiak and H. Schachter, unpublished results). The structure of this oligosaccharide was Galβ1-4GlcNAcβ1-2Manα1-6(sialyl-Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc, designated {GS}. Oligosaccharide {0,2+F} was prepared by sequential treatment of {GS} with a mixture of *Clostridium perfringens* β-galactosidase and β-*N*-acetylglucosaminidase [15], removal of sialic acid by mild acid hydrolysis (0.1 N

H_2SO_4 for 1 h at 80°C) and digestion with jack bean β-galactosidase (B. Bendiak and H. Schachter, unpublished results). Oligosaccharides {M2+F} and {M3+F} were prepared by removal of sialic acid from {GS} followed by digestion with *Clostridium perfringens* β-galactosidase, β-*N*-acetylglucosaminidase and α-mannosidase.

(4) Human fibrinogen. Glycopeptides (23 μmol) were prepared by pronase digestion of fibrinogen, followed by mild acid hydrolysis and β-galactosidase treatment, as described before [15]. Hydrazinolysis/re-*N*-acetylation/acid hydrolysis was carried out as described above for ovomucoid [12, 13]. Oligosaccharides were purified on Bio-Gel P-4 (-400 mesh, 1.6 × 84 cm), HPLC and Sephadex G-25 (2.5 × 39 cm).

(5) Bovine thyroglobulin. Glycopeptides prepared from bovine thyroglobulin were donated by Dr. D. Cumming, Department of Medical Genetics, University of Toronto. Sialic acid was removed by treatment at 80°C for 1 h in 5 ml 0.1 N H_2SO_4. After neutralization, glycopeptides were purified on Bio-Gel P-6 (2.5 × 90 cm) and α-linked galactose residues were removed by incubation with five units of coffee bean α-galactosidase in 1 ml 250 mM PIPES, pH 6.5, for 4 h at 37°C; a further five units were then added and incubation was continued for 4 h. The glycopeptides were purified on a column of Bio-Gel P-4 (200-400 mesh, 1 × 120 cm). Oligosaccharides were released by hydrazinolysis/re-*N*-acetylation/acid hydrolysis as described above for ovomucoid [12, 13], and incubated with 1 unit of jack bean β-galactosidase in 2 ml 50 mM sodium citrate, pH 3.5, for 24 h at 37°C, followed by addition of another unit of enzyme and incubation for a further 24 h. Oligosaccharides were purified by two passages through Bio-Gel P-4 (-400 mesh, 1.6 × 84 cm).

(6) Bovine fetuin. Bovine fetuin (5 g) was incubated in 200 ml 0.05 M KOH/1 M $NaBH_4$ for 14 h at room temperature and 12 h at 45°C, to remove *O*-linked glycans. The pH was adjusted to 5 with 4 M acetic acid and the mixture was passed through a column of AG 50W-X8 (200-400 mesh, H^+-form) to remove cations, followed by purification of the glycoprotein on a column of Sephadex G-25 (5.6 × 100 cm) equilibrated in water. Sialic acid was removed by mild acid hydrolysis and glycopeptides were prepared by pronase digestion, as described above for ovomucoid. Glycoprotein or glycopeptides were purified after each of these steps on Sephadex G-25 (5.6 × 100 cm). Oligosaccharides were released by hydrazinolysis/re-*N*-acetylation/acid hydrolysis [12, 13] and purified on Sephadex G-15 (1.2 × 90 cm) in three batches. Oligosaccharides were digested in 12 ml 50 mM sodium citrate, pH 3.5, with seven units of jack bean β-galactosidase for 23 h at 37°C; a further seven units of enzyme were added and incubation was continued for 43 h. Oligosaccharides were passed through Sephadex G-25 (5.6 × 100 cm) and digested with two units of bovine testicular β-galactosidase in 5 ml citrate-phosphate buffer, pH 4.3, for 24 h at 37°C to remove β(1-3)-linked galactose [16]; a further 2.8 units of enzyme were then added and incubation proceeded for another 24 h. Oligosaccharides were purified on Bio-Gel P-4 (-400 mesh) equilibrated in water, HPLC, and Sephadex G-25 (2.5 × 39 cm).

(7a) Human α_1-acid glycoprotein. Oligosaccharides released by peptide N-glycanase F. Human α_1-acid glycoprotein (50 mg) was treated with 20 ml 0.5 M HCl for 1 h at 37°C, neutralized and passed through a column of Sephadex G-25 (2.4 × 84 cm) equilibrated in water. The glycoprotein was digested with 1 mg chymotrypsin in 2 ml 50 mM Tris-HCl, pH 8.2, at 37°C for 3 h. After heating the mixture for 10 min at 100°C, it was passed

through a column of Sephadex G-25 (2.4 × 42 cm) equilibirated in water. Oligosaccharides were released by treatment with 50 units of peptide *N*-glycanase F [17] in 1 ml 0.25 M sodium phosphate, pH 8.5, at 37°C for 72 h. The pH was adjusted to 6, the enzyme was heat inactivated, and the oligosaccharides were lyophilized and incubated with 0.114 units of bovine epididymis α-fucosidase in 1.8 ml 0.2 M sodium acetate, pH 5, for 44 h at 37°C. The mixture was heat treated and passed through Sephadex G-25 (2.4 × 42 cm). Oligosaccharides were digested with 0.7 units of jack bean β-galactosidase in 1.6 ml 50 mM sodium citrate, pH 3.5, for 48 h at 37°C; 0.35 units of enzyme were added and the incubation was continued for another 48 h. The incubations were heat treated and oligosaccharides were purified on a column of Bio-Gel P-4 (-400 mesh, 1.6 × 84 cm) equilibrated in water.

(7b) Human α_1-acid glycoprotein. Oligosaccharides released by hydrazinolysis. Human α_1-acid glycoprotein (500 mg) was hydrolyzed with mild acid and digested with pronase, as described above for ovomucoid. Oligosaccharides were released by hydrazinolysis/re-*N*-acetylation/acid hydrolysis [12, 13]. Bovine epididymis α-fucosidase and jack bean β-galactosidase digestions were carried out as described above for the release of oligosaccharides by peptide *N*-glycanase F, except that the quantities were scaled up ten fold. Oligosaccharides were purified by Bio-Gel P-4 (-400 mesh, 1.6 × 84 cm), HPLC and Sephadex G-25 (2.5 × 39 cm).

Reduction of Oligosaccharides

Oligosaccharides were reduced to the *N*-acetylglucosaminitol form by incubating 300 nmol oligosaccharide for 4.5 h at room temperature in 0.5 ml 1 M $NaBH_4$. After the pH was adjusted to 5 with 4 M acetic acid, reduced oligosaccharides were purified on Sephadex G-25 (2.5 × 39 cm).

Proton NMR Spectroscopy

Samples were prepared by exchanging 2-3 times with 99.8% ^2H_2O (Aldrich, Milwaukee, WI, USA) and 2-3 times with 99.96% ^2H_2O (Merck, Sharp and Dohme, Montreal, Canada). Spectra were recorded at the Toronto Carbohydrate Research Centre from samples dissolved in 99.96% ^2H_2O with acetone as an internal standard, which was set at 2.225 ppm, relative to sodium 4,4-dimethyl-1-silapentansulfonate. Spectra were recorded at 300 K with a 360 MHz Nicolet spectrometer and at 300 and 343 K with a 500 MHz Bruker spectrometer equipped with an Aspect 3000 computer.

Fast Atom Bombardment Mass Spectrometry

FAB-MS was used to determine the molecular weight and purity of oligosaccharides. FAB-Mass spectra were recorded at the Toronto Carbohydrate Research Centre with a VG Analytical ZAB-SE double focusing mass spectrometer and VG 11/250 data system. Samples were dissolved in water and a thioglycerol matrix on a stainless steel target and bombarded by a Xenon atom beam (8 kV). An accelerating voltage of 8 kV and resolutions between 1000 to 2500 were used depending on the samples. Cesium iodide was used for calibration.

Methylation Analysis

Oligosaccharides (138 to 184 nmol) were reduced at room temperature for 4 h in 1.0 ml M $NaBH_4$/0.01 N NaOH, containing 6 mCi of NaB^3H_4 (New England Nuclear). The pH was adjusted to 5 with 4 M acetic acid and the mixtures were flash evaporated from acidified methanol. Reduced oligosaccharides were purified on a Sephadex G-25 column (2.5 × 39 cm), 200 nmol inositol was added as a carrier, and the mixtures were flash-evaporated and dried over P_2O_5. Permethylation was carried out by the method of Ciucanu and Kerek [18], as follows. Solid NaOH was pulverized with a mortar and pestle and dried over P_2O_5. All subsequent procedures were carried out under dry argon gas. The oligosaccharides were dissolved in 0.5 ml dry DMSO by stirring with a small magnet, dry powdered NaOH (40 mg) was added and stirring was continued for 10 min at room temperature. Ice-cold methyl iodide (0.2 ml) was added and the mixture was stirred fo 20 min at room temperature. The reaction was stopped by addition of 0.7 ml water. Permethylated oligosaccharides were purified by sequential elution from Sep-Pak C_{18} cartridges with acetonitrile and water mixtures according to the method of Waeghe *et al.* [19]. The fractions eluting from the Sep-Pak cartridges with 100% actonitrile were hydrolyzed in 0.15 ml 2 M trifluoroacetic acid at 100°C for 4 h. The trifluoroacetic acid was removed by nitrogen stream at 30°C, and the oligosaccharides were reduced in 0.2 ml 1% (w/v) NaB^2H_4 in 1 M ammonia/95% ethanol at room temperature for 2 h. The sample was acidified with acetic acid, and borate was removed by repetitive drying with a nitrogen stream from acidified methanol. The samples were peracetylated in 0.2 ml acetic anhydride at 100°C for 4 h, dried and analyzed by GLC-MS using a Hewlett-Packard 5985B quadrupole mass spectrometer coupled *via* a jet separator to a 5840A gas chromatograph equipped with a 25 m 5% phenyl methyl silicone 0.33 μ fused-silica capillary column. The temperature was raised from 80 to 150°C in 2 min and then to 310°C at a rate of 8°/min. The mass spectrometer was operated in the electron impact (EI) mode and was coupled to an HP-1000E computer, an HP 7906 disk drive, an HP 2648A graphics terminal and a 9876A Hewlett-Packard printer. Partially methylated alditol acetates were identified from their GLC retention times and diagnostic m/z values, as follows: terminal mannose at 10.33 min, m/z at 118, 129, 145, 161, 162, 205; 2-substituted mannose at 11.55 min, m/z at 129, 130, 145, 161, 190; 2,4-substituted mannose at 12.75 min, m/z at 130, 173, 190, 233; 2,6-substituted mannose at 13.22 min, m/z at 129, 130, 189, 190; 3,4,6-substituted mannose at 13.87 min, m/z at 118, 139, 160, 333; 2,4,6-substituted mannose at 14.15 min, m/z at 130, 159, 190, 201, 261; terminal GlcNAc at 14.50 min, m/z at 117, 129, 143, 145, 159, 161, 203, 205; 4-substituted GlcNAc at 15.37 min, m/z at 117, 129, 143, 159, 203, 233; and 4-substituted *N*-acetylglucosaminitol (GlcNAc-ol) at 12.75 min, the same position as 2,4-substituted Man but readily distinguished by the diagnostic m/z at 130, 142, 205 and 290.

Results

The nomenclature and structures of the sixteen oligosaccharides isolated and characterized in this study are shown in Table 1. Their purity, as judged from their NMR spectra, was greater than 80%. Major contaminations originated from bleeding of the HPLC silica based columns. Hydrazinolysis generally released about 70-90% of the oligosaccharides from glycopeptides.

Table 1. Structures, nomenclature and glycoprotein sources of oligosaccharides.

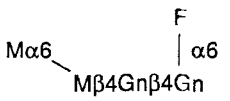
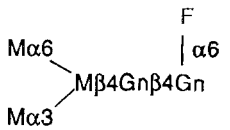
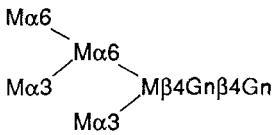
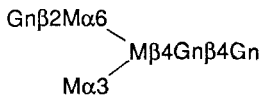
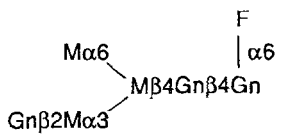
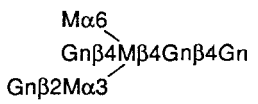
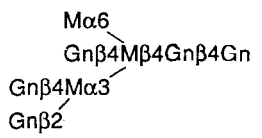
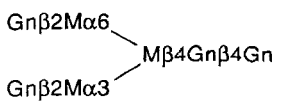
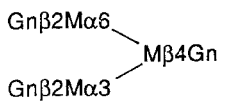
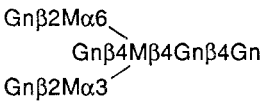
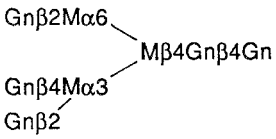
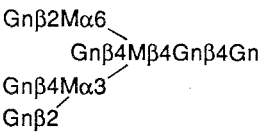
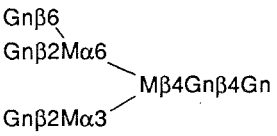
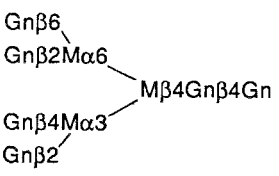
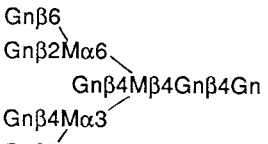
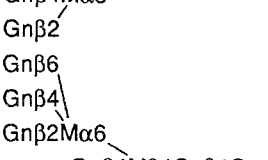
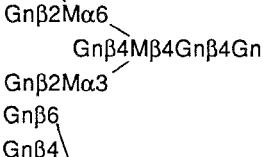
Short form	Structure[a]	Glycoprotein source	Fraction
{M2+F}		bovine fibrin	K1
{M3+F}		bovine fibrin	K2
{M5}		hen ovalbumin	
{2,0}		human fibrinogen	L1
{0,2+F}		bovine fibrin	
{bis-0,2}		hen ovomucoid	A2, C1
{bis-0,24}		hen ovomucoid	E1, H1
{2,2}		bovine thyroglobulin hen ovomucoid human fibrinogen,	T2 A1 L2
{2,2d}		hen ovomucoid bovine thyroglobulin	A1 T3

Table 1. (continued).

Short form	Structure[a]	Glycoprotein source	Fraction
{bis-2,2}		hen ovomucoid	D2, E2
{2,24}		bovine fetuin hen ovomucoid human α_1-acid-glycoprotein	N1 D1 O1
{bis-2,24}		hen ovomucoid	G1, H2, I1
{26,2}			
{26,24}		human α_1-acid-glycoprotein	O2
{bis-26,24}		hen ovomucoid	D3, G2
{bis-246,2}		hen ovomucoid	A3, B1
{bis-246,24}		hen ovomucoid	D4, F1

[a] M, mannose; Gn, GlcNAc; F, fucose; bis, bisected.

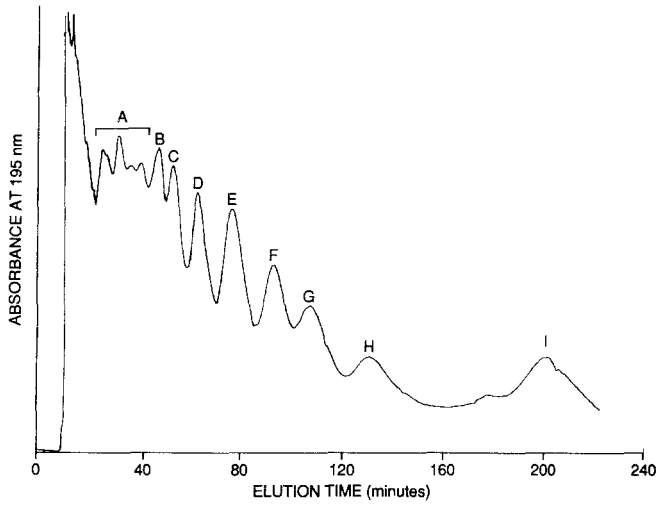


Figure 1. HPLC elution pattern of oligosaccharides released by hydrazinolysis from ovomucoid. A reverse phase (Dextro-Pak) column was used with water as the mobile phase at a flow rate of 1 ml/min. Oligosaccharides eluted in two widely separated peaks (Table 2). Each peak was subfractionated on a Magnum amine column (Figs. 2a-2i).

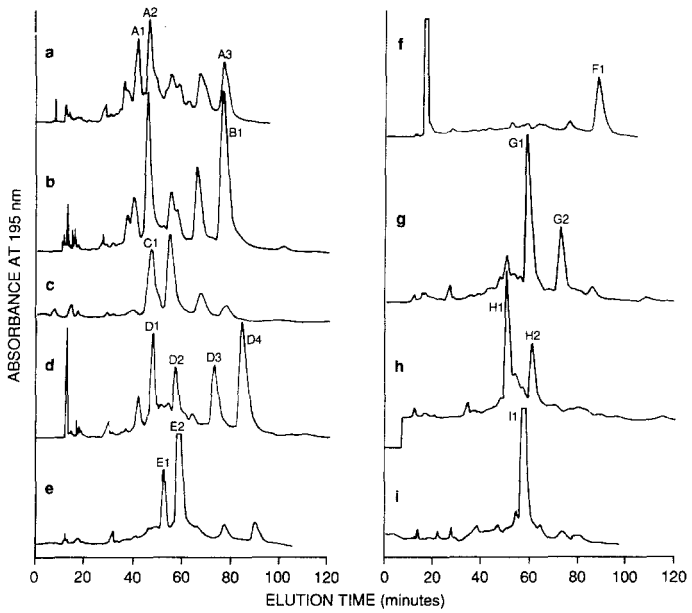


Figure 2. Subfractionation of ovomucoid oligosaccharides on a Magnum amine column with acetonitrile/water (50/50) as the mobile phase at a flow rate of 1 ml/min. (a) - (i) peaks A to I, respectively, from Fig. 1.

Table 2. Retention times on reverse phase Dextro-Pak column of ovomucoid oligosaccharide fractions[a].

Fraction	Retention time (min)	Oligosaccharide[b]
Peak A	16-39	{bis-0,2} {bis-246,2} {2,2} {2,2d}
Peak B	43	{bis-246,2}
Peak C	49	{bis-0,2}
PeakD	59	{bis-2,2} {bis-26,24} {bis-246,24} {2,24}[c]
Peak E	72	{bis-0,24} {bis-2,2}
Peak F	89	{bis-246,24}
Peak G	105	{bis-2,24} {bis-26,24}
Peak H	129	{bis-0,24} {bis-2,24}
Peak I	201	{bis-2,24}

[a] Ovomucoid oligosaccharides after hydrazinolysis were fractionated on a reverse phase Dextro-Pak column, eluted with water at a flow rate of 1 ml/min. The pressure was 21 bar.
[b] Oligosaccharides isolated from the indicated peaks A - I after subsequent chromatographic steps; for nomenclature refer to Table 1.
[c] The {2,24} oligosaccharide isolated from fetuin had the retention times 27 and 58 minutes.

Hen Ovomucoid Oligosaccharides

Oligosaccharides released from ovomucoid by hydrazinolysis were separated into four major fractions on Bio-Gel P-4 (not shown). The first peak was excluded from the column and represented about 20-30% of the total carbohydrate. It contained mainly unreacted glycopeptides as was evident from NMR signals at 2.011 ppm typical for the *N*-acetyl methyl group of β-GlcNAc attached to asparagine. The most included, fourth peak contained mainly salt and was discarded. The third peak contained relatively little carbohydrate and was not studied further. The second peak contained most of the carbohydrate. HPLC analysis of this peak using a reverse phase Dextro-Pak C_{18} column showed at least 13 absorbance peaks (Fig. 1). The retention times of these fractions and the oligosaccharides present in them are listed in Table 2. Peaks A to I (Fig. 1) were pooled, lyophilized, filtered and further sub-fractionated on a Magnum semi-preparative amine column (Figs. 2a-2i). The major sub-fractions are listed in Table 3. They were further purified on Sephadex G-25 (2.5 × 39 cm) and were subjected to extensive structural analysis.

Table 3. Relative retention times of oligosaccharides on HPLC, using amine columns.[a]

Oligosaccharide	Fractions	Retention times (min)	
		analytical[b]	Magnum[c]
GlcNAc		7	21
N-Acetyllactosamine		9	—
{M2+F}	K1	21	—
{M3+F}	K2	29	—
{M5}	ovalbumin	51	—
{2,0}	L1	36	—
{0,2+F}	fibrin	36	—
{bis-0,2}	A2,C1	52	46-47
{bis-0,24}	E1,H1	67	53
{2,2}	A1,L2+T2	49	42
{2,2d}	A1,T3	38	42
{bis-2,2}	D2,E2	73	56-59
{2,24}	D1,O1,N1	65	47
{bis-2,24}	G1,H2,I1	95	60-61
{26,24}	O2	105	56
{bis-26,24}	D3,G2	133	73
{bis-246,2}	A3,B1	163	75-77
{bis-246,24}	D4,F1	218	84-88

[a] Refer to Table 1 for nomenclature of oligosaccharides. Ovomucoid fractions A to I eluting from the Dextro-Pak column were concentrated and reinjected into HPLC. They were subfractionated on a Magnum amine column with acetonitrile/water mixtures at a flow rate of 1 ml/min. Major fractions were subjected to structural analysis and identified as the oligosaccharides indicated. The purity and size of oligosaccharides were assessed by their elution patterns on an analytical amine column.

[b] The analytical amine column was a Waters carbohydrate column and was run in acetonitrile/water, 70/30 by vol, at 1 ml/min.

[c] The Magnum column was run at a flow rate of 1 ml/min with acetonitrile/water, 50/50 by vol.

(a) Oligosaccharide {bis-0,2}. Fractions A2 and C1 eluting from the Magnum amine column at 47 and 46 min, respectively (Table 3, Figs. 2a and 2c) proved to be the same compound and represented about 10% of the total identified ovomucoid oligosaccharides. In the proton NMR spectrum, the reducing end is recognized by the signals at 5.183 and 2.038 ppm (Table 4) arising from the H-1 and *N*-acetyl methyl group of the α-GlcNAc residue, respectively. The anomeric H-1 resonance of the sub-terminal core β-GlcNAc is found at 4.606 and 4.598 ppm as 8 Hz doublets due to the effects of the α- and β-configurations of the GlcNAc at the reducing end. The bisecting GlcNAc, which is β(1-4)-linked to the β-linked Man of the trimannose core, is recognized by the 8 Hz doublet at 4.442 ppm due to H-1, and the presence of a characteristic multiplet at about 3.25 ppm (e.g., see Fig. 4b). Signals for the H-1/H-2 pairs of the Manα3- and Manα6- residues at 5.060/4.250 ppm and 4.968/3.998 ppm, respectively, indicate a bisected structure with a substituted Manα3- arm and an unsubstituted Manα6- arm. The H-2 signal of the β-Man is found at 4.189 ppm, consistent with the presence of a bisecting GlcNAc. The anomeric H-1 resonance of β-Man is hidden under the H^2HO signal at room temperature (Table 4) but is found at 4.741 ppm when the spectrum is recorded at higher temperature (Table 5). The Manα3- arm is substituted by a β(1-2)-linked GlcNAc with resonances for H-1 and the *N*-acetyl group at 4.535 ppm (8 Hz doublet) and at 2.067 ppm, respectively. These data

Table 4. Proton NMR chemical shifts (ppm) and coupling constants (Hz) recorded at 300°K.

		Oligosaccharide			
Hydrogens[a]		(1){M2+F}	(2){M3+F}	(3){M5}	(4){2,0}
H-1	M4,4	N.D.	N.D.	4.781	N.D
	M3,4,4	5.105	5.100	5.096	5.098
	M6,4,4	—	4.914	4.879	4.917
	M3,6,4,4	—	—	5.093	—
	M6,6,4,4	—	—	4.907	—
	Gn α	5.178	5.182	5.186	5.188
	β	N.D.	N.D.	N.D.	N.D.
	Gn4	N.D.	N.D.	4.595	4.61
	Gn4,4,4	—	—	—	—
	Gn2,3,4,4	—	—	—	—
	Gn4,3,4,4	—	—	—	—
	Gn2,6,4,4	—	—	—	4.551
	Gn4,6,4,4	—	—	—	—
	Gn6,6,4,4	—	—	—	—
	F6	4.899	4.899	—	—
H-2	M4,4	4.231	4.257	4.252	4.255
	M3,4,4	4.068	4.067	4.068	4.065
	M6,4,4	—	3.972	4.145	4.108
	M3,6,4,4	—	—	4-060	—
	M6,6,4,4	—	—	3-983	—
CH_3	Gn α	2.039	2.038	2.038	2.038
	Gn β	2.039	2.038	2.038	2.038
	Gn4	2.075	2.092	2.064	2.079[d]
	Gn4,4,4	—	—	—	—
	Gn2,3,4,4	—	—	—	—
	Gn4,3,4,4	—	—	—	—
	Gn2,6,4,4	—	—	—	2.050[d]
	Gn4,6,4,4	—	—	—	—
	Gn6,6,4,4	—	—	—	—
	F6	1.226	1.22	—	—

		Oligosaccharide			
Hydrogens[a]		(5){0,2+F}	(6){bis-0,2}	(7){bis-0,24}	(9){2,2d}[b]
H-1	M4,4	N.D.	N.D.	N.D.	N.D
	M3,4,4	5.119	5.060	5.056	5.115
	M6,4,4	4.919	4.968	4.965	4.920
	M3,6,4,4	—	—	—	—
	M6,6,4,4	—	—	—	—
	Gn α	5.180	5.183	5.185	5.212[c]
	β	N.D.	N.D.	N.D.	4.725[c]
	Gn4	4.61	4.606(8.30)	4.605(8.00)	—
			4.598(8.10)	4.596(7.95)	—
	Gn4,4,4	—	4.442(8.15)	4.439(8.35)	—
	Gn2,3,4,4	4.553(8.35)	4.535(8.05)	4.519(8.45)	4.554(8.28)
	Gn4,3,4,4	—	—	4.517(8.45)	—
	Gn2,6,4,4	—	—	—	4.554(8.28)
	Gn4,6,4,4	—	—	—	—
	Gn6,6,4,4	—	—	—	—

	F6	4.9	—	—	—
H-2	M4,4	4.255	4.189	4.158	4.251
	M3,4,4	4.189	4.250	4.287	4.188
	M6,4,4	3.971	3.998	3.991	4.108
	M3,6,4,4	—	—	—	—
	M6,6,4,4	—	—	—	—
CH_3	Gnα	2.039	2.038	2.037	—
	Gnβ	2.039	2.038	2.037	—
	Gn4	2.094	2.078[e]	2.076[f]	2.052[c](α,β)
	Gn4,4,4	—	2.065[e]	2.062[f]	—
	Gn2,3,4,4	2.053	2.067[e]	2.063[f]	2.052
	Gn4,3,4,4	—	—	2.080[f]	—
	Gn2,6,4,4	—	—	—	2.052
	Gn4,6,4,4	—	—	—	—
	Gn6,6,4,4	—	—	—	—
	F6	1.216	—	—	—

		Oligosaccharide			
Hydrogens[a]		(8){2,2}	(10){bis-2,2}	(11){2,24}	(12){bis-2,24}
H-1	M4,4	N.D.	N.D.	4.76	4.684
	M3,4,4	5.117	5.058	5.109	5.055
	M6,4,4	4.920	5.001	4.914	4.998
	Gn α	5.189	5.185	5.185	5.191
	β	N.D.	N.D.	N.D.	4.716
	Gn4	4.61	4.609	4.61	4.609
			4.599		4.598
	Gn4,4,4	—	4.465(8.28)	—	4.463(8.25)
	Gn2,3,4,4	4.555(7.92)	4.557(8.28)	4.536(8.28)	4.539(8.50)
	Gn4,3,4,4	—	—	4.519(8.28)	4.515(8.45)
	Gn2,6,4,4	4.555(8.28)	4.549(8.28)	4.551(8.28)	4.544(8.40)
	Gn4,6,4,4	—	—	—	—
	Gn6,6,4,4	—	—	—	—
H-2	M4,4	4.251	4.175	4.214	4.142
	M3,4,4	4.192	4.245	4.214	4.281
	M6,4,4	4.112	4.144	4.109	4.142
CH_3	Gnα	2.038	2.036	2.038	2.038
	Gnβ	2.039	2.036	2.038	2.038
	Gn4	2.079	2.083	2.078	2.080,2.078
	Gn4,4,4	—	2.065	—	2.062
	Gn2,3,4,4	2.053	2.057	2.050	2.057
	Gn4,3,4,4	—	—	2.078	2.081
	Gn2,6,4,4	2.050	2.048	2.050	2.048
	Gn4,6,4,4	—	—	—	—
	Gn6,6,4,4	—	—	—	—

		Oligosaccharide			
Hydrogens[a]		(13){26,24}	(14){bis-26,24}	(15){bis-246,2}	(16){bis-246,24}
H-1	M4,4	N.D.	4.719	N.D.	4.702
	M3,4,4	5.118	5.060	5.068	5.070
	M6,4,4	4.861	4.908	4.889	4.887
	Gnα	5.187	5.186	5.189	5.188
	β	N.D.	4.696	N.D.	4.678
	Gn4	4.607	4.60	α4.597	α4.595
				β4.588	β4.586

	Gn4,4,4	—	4.489(8.10)	4.444(8.20)	4.442(8.10)
	Gn2,3,4,4	4.542(6.84)[g]	4.569(8.65)[h]	4.539(8.20)[i]	4.540(8.35)σ
	Gn4,3,4,4	4.521(7.92)[g]	4.522(8.55)[h]	—	4.516(8.55)[j]
	Gn2,6,4,4	4.563(8.28)[g]	4.575(8.35)[h]	4.555(8.05)[i]	4.556(8.30)[j]
	Gn4,6,4,4	—	—	4.597(8.30)[i]	4.585(8.45)[j]
	Gn6,6,4,4	4.530(7.92)[g]	4.516(8.50)[h]	4.515(8.40)[i]	4.516(8.55)[j]
H-2	M4,4	4.215	4.143[k]	4.164[l]	4.148[m]
	M3,4,4	4.215	4.272[k]	4.238[l]	4.280[m]
	M6,4,4	4.092	4.122[k]	4.165[l]	4.148[m]
CH_3	Gnα	2.039	2.038	2.037	2.038
	Gnβ	2.039	2.041	2.037	2.040
	Gn4	2.079	2.082[n]	2.087[o]	2.082[p]
	Gn4,4,4	—	2.064[n]	2.065[o]	2.064[p]
	Gn2,3,4,4	2.054	2.064[n]	2.067[o]	2.064[p]
	Gn4,3,4,4	2.079	2.082[n]	—	2.088[p]
	Gn2,6,4,4	2.048	2.082[n]	2.093[o]	2.093[p]
	Gn4,6,4,4	—	—	2.122[o]	2.122[p]
	Gn6,6,4,4	2.039	2.038[n]	2.042[o]	2.041[p]

[a] The numbers appearing after the sugar residue indicate the linear sequence of glycosidic linkages from the nonreducing to the reducing end. M, Mannose; Gn, GlcNAc; F, Fucose.
[b] {2,2d} has only one reducing GlcNAc in the core.
[c] Signal of reducing GlcNAc attached to β-Man.
[d-p] Resonances with these superscripts cannot be unambiguously assigned by classical chemical shift comparison methods. Thus these assignments may have to be interchanged.
N.D. = not determined.

are consistent with those reported for Manα6(GlcNAcβ2Manα3)(GlcNAcβ4)Manβ4GlcNAcβ4(Fucα6)GlcNAc-Asn (compound 66) by Carver and Grey [20]. However, the H-2 of Manα6- was assigned by Carver and Grey [20] to a peak at 3.962 ppm; more recent re-evaluation of the spectrum of compound 66 indicates that this hydrogen has a chemical shift of 3.998 ppm (Table 4). The chemical shift for this hydrogen moves 0.03 ppm downfield when a bisecting GlcNAc is introduced. The molecular weight of pooled fractions A2 and C1 was determined by FAB-MS as 1316 (Table 6). The structure of the oligosaccharide in fractions A2 and C1 is therefore {bis-0,2} (Table 1).

(b) Oligosaccharides {2,2} and {2,2d}. Fraction A1, eluting from the Magnum amine column at 42 min (Table 3), shows NMR parameters that are, within a range of 0.005 ppm, identical to those reported by Vliegenthart *et al.* [21] for the reducing bi-antennary oligosaccharide {2,2d} with only one GlcNAc in the core (structure in Table 1), and to those reported in this study for {2,2d} isolated from thyroglobulin (see below and Tables 4 and 5). The diagnostic resonance is seen at 5.212 ppm (Table 4) due to the α-anomer of the single reducing GlcNAc residue in the core. Oligosaccharide {2,2d} from hen ovomucoid is either a breakdown product from the hydrazinolysis procedure, or a product of endogenous endo-hexosaminidase action with preference for {2,2}. No other oligosaccharides with only one GlcNAc in the core were found in the major ovomucoid fractions. In addition to {2,2d}, fraction A1 appears to contain undegraded {2,2} (Table 1), as deduced from NMR and FAB-MS studies (data for {2,2} isolated from other glycoproteins is shown below).

Table 5. Proton NMR chemical shifts (ppm) and coupling constants (Hz) recorded at 343°K.

		Oligosaccharide			
Hydrogens[a]		(1){M2+F}	(2){M3+F}	(3){M5}	(4){2,0}
H-1	M4,4	4.774	4.772	4.774	4.762
	M3,4,4	5.129	5.127	5.120	5.122
	M6,4,4	—	4.921	4.882	4.910
	M3,6,4,4	—	—	5.120	—
	M6,6,4,4	—	—	4.917	—
	Gnα	5.188	5.190	5.199	5.197
	β	N.D.	4.706	4.715	4.711
	Gn4	N.D.	4.679	4.61	4.63(8.35)
	Gn4,4,4	—	—	—	—
	Gn2,3,4,4	—	—	—	—
	Gn4,3,4,4	—	—	—	—
	Gn2,6,4,4	—	—	—	4.573(8.35)
	Gn4,6,4,4	—	—	—	—
	Gn6,6,4,4	—	—	—	—
	F6	4.898	4.894	—	—
H-2	M4,4	4.215	4.236	4.230	4.229
	M3,4,4	4.073	4.076	4.078	4.072
	M6,4,4	—	3.979	4.131	4.092
	M3,6,4,4	—	—	4.078	—
	M6,6,4,4	—	—	3.988	—
CH_3	Gnα	2.045	2.046	2.044	2.044
	Gnβ	2.048	2.046	2.044	2.044
	Gn4	2.076	2.090	2.069	2.078[d]
	Gn4,4,4	—	—	—	—
	Gn2,3,4,4	—	—	—	—
	Gn4,3,4,4	—	—	—	—
	Gn2,6,4,4	—	—	—	2.052[d]
	Gn4,6,4,4	—	—	—	—
	Gn6,6,4,4	—	—	—	—
	F6	1.2	1.2	—	—

		Oligosaccharide			
Hydrogens[a]		(5){0,2+F}	(6){bis-0,2}	(7){bis-0,24}	(9){2,2d}[b]
H-1	M4,4	4.770	4.741	4.729	4.765
	M3,4,4	5.139	5.056	5.055	5.137
	M6,4,4	4.921	4.959	4.956	4.909
	M3,6,4,4	—	—	—	—
	M6,6,4,4	—	—	—	—
	Gnα	5.189	5.196	5.196	5.224[c]
	β	4.704	4.709	4.709	N.D.
	Gn4	4.678	4.625(7.55)	4.63(7.90)	N.D.
	Gn4,4,4	—	4.456(8.25)	4.451(8.30)	—
	Gn2,3,4,4	4.580	4.543(8.25)	4.550(8.50)	4.58
	Gn4,3,4,4	—	—	4.524(8.35)	—
	Gn2,6,4,4	—	—	—	4.58
	Gn4,6,4,4	—	—	—	—
	Gn6,6,4,4	—	—	—	—
	F6	4.896	—	—	—

H-2	M4,4	4.232	4.184	4.148(3.30)	N.D.
	M3,4,4	4.182	4.236	4.271	N.D.
	M6,4,4	3.975	3.995	3.99	N.D.
	M3,6,4,4	—	—	—	—
	M6,6,4,4	—	—	—	—
CH_3	Gnα	2.044	2.043	2.043	—
	Gnβ	2.044	2.043	2.043	—
	Gn4	2.088	2.075[e]	2.075[f]	2.053[c](α,β)
	Gn4,4,4	—	2.068[e]	2.065[f]	—
	Gn2,3,4,4	2.052	2.066[e]	2.065[f]	2.053
	Gn4,3,4,4	—	—	2.078[f]	—
	Gn2,6,4,4	—	—	—	2.053
	Gn4,6,4,4	—	—	—	—
	Gn6,6,4,4	—	—	—	—
	F6	1.224	—	—	—

		Oligosaccharide			
Hydrogens[a]		(8){2,2}	(10){bis-2,2}	(11){2,24}	(12){bis-2.24}
H-1	M4,4	4.761	4.711	4.749	4.701
	M3,4,4	5.134	5.059	5.129	5.058
	M6,4,4	4.911	4.983	4.908	4.982
	Gnα	5.200	5.197	5.196	5.197
	β	4.714(8.00)	4.711	4.710	4.711
	Gn4	4.635(8.45)	4.637(8.05)	4.629(6.5)	4.635(7.90)
	Gn4,4,4	—	4.481(8.25)	—	4.479(8.10)
	Gn2,3,4,4	4.577(8.30)	4.563(8.30)	4.563(8.28)[g]	4.552(8.50)[h]
	Gn4,3,4,4	—	—	4.563(8.28)[g]	4.535(8.60)[h]
	Gn2,6,4,4	4.584(8.30)	4.554(8.25)	4.574(8.64)[g]	4.561(8.35)[h]
	Gn4,6,4,4	—	—	—	—
	Gn6,6,4,4	—	—	—	—
H-2	M4,4	4.226	4.179	4.195	4.145[i]
	M3,4,4	4.183(1.7,3.3)	4.237	4.195	4.30
	M6,4,4	4.095	4.125	4.095	4.125[i]
CH_3	Gnα	2.044	2.043	2.045	2.043
	Gnβ	2.044	2.043	2.045	2.043
	Gn4	2.078	2.079	2.076	2.079[k]
	Gn4,4,4	—	2.066[j]	—	2.065[k]
	Gn2,3,4,4	2.052	2.064[j]	2.052	2.065[k]
	Gn4,3,4,4	—	—	2.076	2.079[k]
	Gn2,6,4,4	2.052	2.055[j]	2.052	2.054[k]
	Gn4,6,4,4	—	—	—	—
	Gn6,6,4,4	—	—	—	—

		Oligosaccharide			
Hydrogens[a]		(13){26,24}	(14){bis-26,24}	(15){bis-246,2}	(16){bis-246,24}
H-1	M4,4	4.749	4.699	4.707	4.697
	M3,4,4	5.134	5.061	5.069	5.068
	M6,4,4	4.860	4.913	4.901	4.897
	Gnα	5.196	5.197	5.197	5.198
	β	4.712	4.711	4.712	4.713
	Gn4	4.63	4.63(7.50)	4.631(8.15)	4.629
				4.620	4.620
	Gn4,4,4	—	4.485(8.35)	4.457(7.90)	4.453(8.50)

	Gn2,3,4,4	4.564(8.28)[l]	4.551(8.50)[m]	4.531(8.45)[n]	4.552(8.55)[o]
	Gn4,3,4,4	4.564(8.28)[l]	4.551(8.50)[m]	—	4.530(8.60)[o]
	Gn2,6,4,4	4.588(9.72)[l]	4.569(8.75)[m]	4.568(8.50)[n]	4.562(8.10)[o]
	Gn4,6,4,4	—	—	4.578(8.40)[n]	4.569(8.15)[o]
	Gn6,6,4,4	4.564(8.28)[l]	4.551(8.50)[m]	4.531(8.45)[n]	4.530(8.60)[o]
H-2	M4,4	N.D.	4.141[p]	4.164	4.161
	M3,4,4	N.D.	N.D.	4.227	4.258
	M6,4,4	4.082	N.D.[p]	N.D.	N.D.
CH_3	Gnα	2.046	2.044	2.043	2.044
	Gnβ	2.046	2.044	2.043	2.044
	Gn4	2.076	2.080[r]	2.088[s]	2.080[t]
	Gn4,4,4	—	2.061[r]	2.061[s]	2.060[t]
	Gn2,3,4,4	2.050[q]	2.066[r]	2.067[s]	2.067[t]
	Gn4,3,4,4	2.076[q]	2.086[r]	—	2.089[t]
	Gn2,6,4,4	2.050[q]	2.086[r]	2.093[s]	2.094[t]
	Gn4,6,4,4	—	—	2.112[s]	2.113[t]
	Gn6,6,4,4	2.046[q]	2.044[r]	2.048[s]	2.044[t]

[a] The numbers appearing after the residue indicate the sequence of sugar linkages from the nonreducing to the reducing end. M, Man; Gn, GlcNAc; F, Fucose.
[b] {2,2d} has only one reducing GlcNAc in the core.
[c] Signal of reducing GlcNAc attached to β-Man.
[d-t] Resonances with these superscripts cannot be unambiguously assigned by classical chemical shift comparison methods. Thus these assignments may have to be interchanged as a result of future studies.
N.D. = not determined.

(c) Oligosaccharide {bis-2,2}. Fractions D2 and E2 (Table 3) contained about 9% of the total identified ovomucoid oligosaccharides. The oligosaccharide in both fractions was identified as {bis-2,2} (Table 1). The NMR resonances of the trimannose core, the GlcNAcβ2-residues and the bisecting GlcNAc (Tables 4 and 5) of {bis-2,2} are very similar to those previously reported for the corresponding glycopeptide [22] and the corresponding oligosaccharide with only one core GlcNAc [21]. Compared to {2,2} (Tables 4 and 5), the presence of the bisecting GlcNAc in {bis-2,2} causes major shifts in the resonances of H-1 and H-2 of all three mannoses, but only minor shifts for the H-1 of the two antennary GlcNAcβ2-residues. FAB-MS data (Table 6) confirmed the oligosaccharide structure of {bis-2,2}.

(d) Oligosaccharide {2,24}. The NMR data for fraction D1 are identical to those of the reducing oligosaccharide {2,24} (Table 1) isolated from fetuin and α_1-acid glycoprotein (Tables 4 and 5). The chemical shifts for the H-1 and H-2 resonances of the tri-mannose structure and H-1 and *N*-acetyl methyl for the three antennary GlcNAc residues are virtually identical to the signals previously reported for the corresponding glycopeptide [23, 25]. The H-3 signal for Manα3- is found as a doublet of doublets at 4.041 ppm ($J_{2,3}$ = 3.24 Hz; $J_{3,4}$ = 9.00 Hz). This indicates a 4-substitution of the Manα3-residue [21]. The triantennary galactosylated glycopeptide (compound 9, Vliegenthart *et al.* [21] shows very similar chemical shifts for H-1 and H-2 of all three mannoses, and the H-3 of Manα3-; the *N*-acetyl methyl signals for the antennary GlcNAc residues of the galactosylated glycopeptide are also very similar to {2,24}, but all three H-1 signals are shifted 0.026-0.034 ppm downfield as expected due to galactosylation [24].

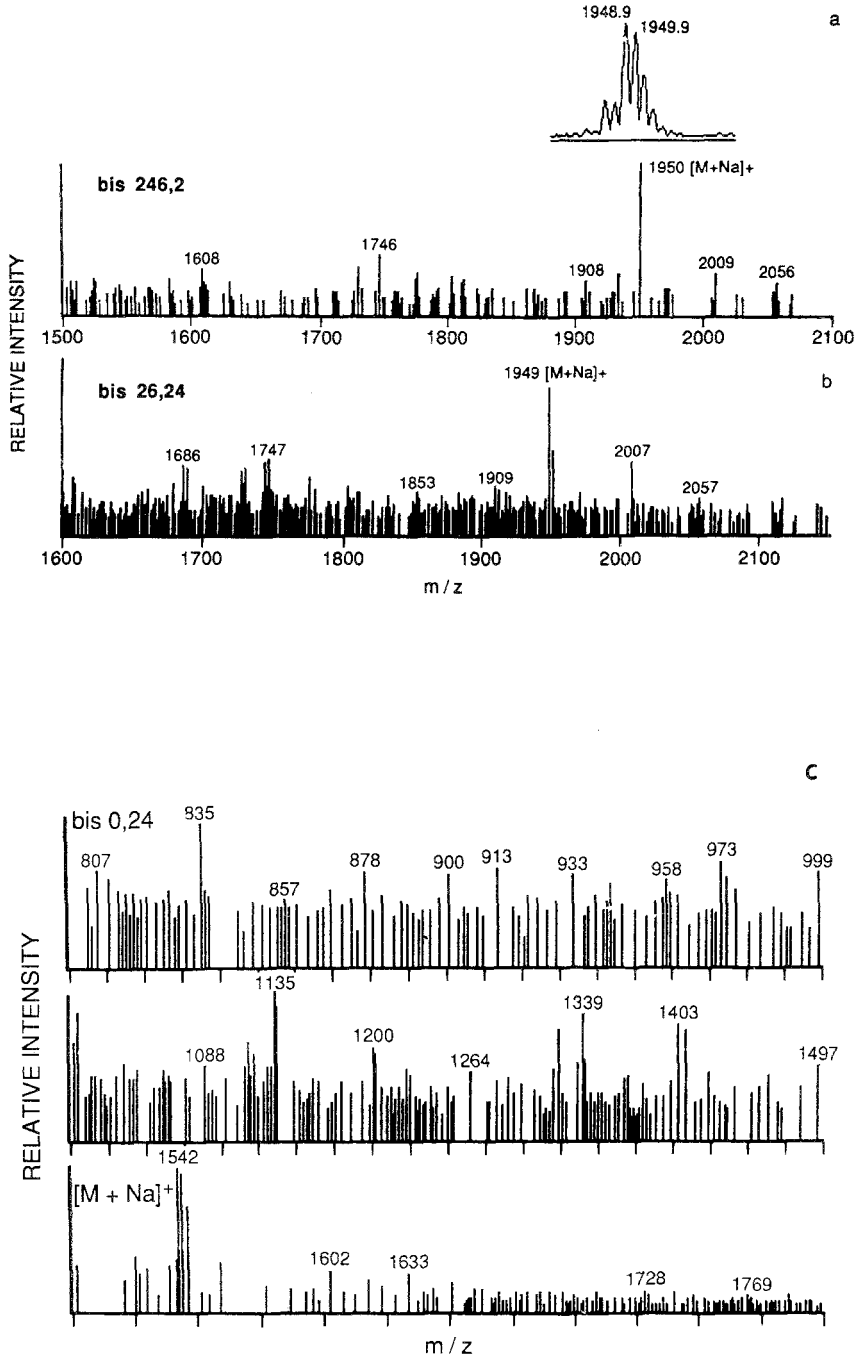
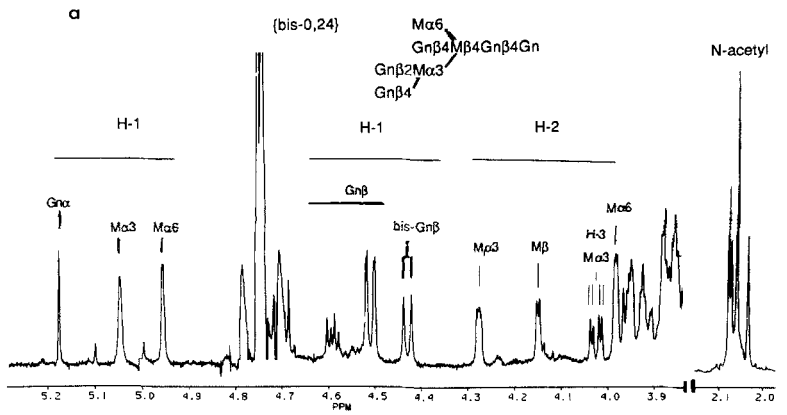
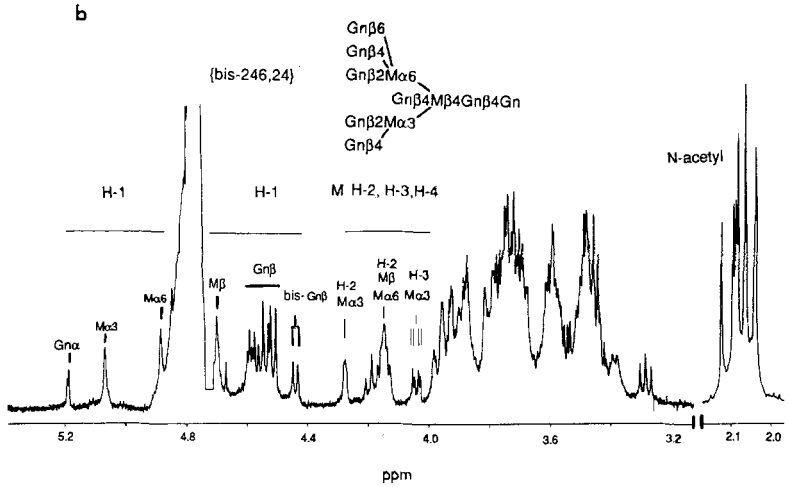
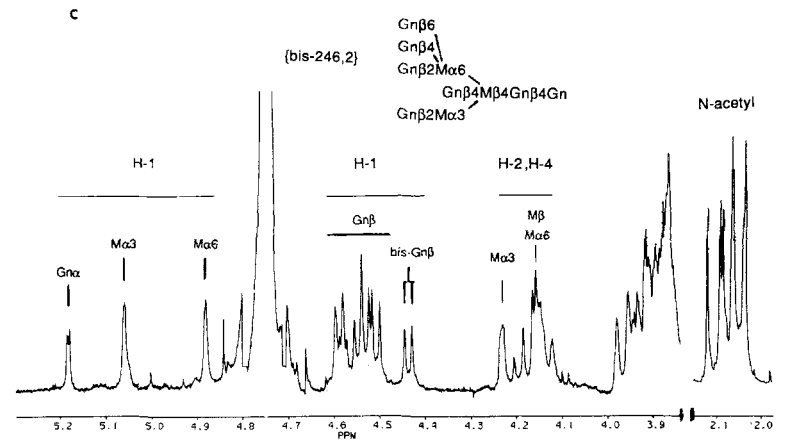


Figure 3. FAB-MS spectra of three oligosaccharides from ovomucoid. (a) {bis-246,2}; (b) {bis-26,24}; (c) {bis-0,24}. The insert in Fig. 3a shows an expansion of the spectrum at m/z 1950 for {bis-246,2}. See Table 1 for oligosaccharide nomenclature.


a
{bis-0,24}
Mα6
Gnβ4Mβ4Gnβ4Gn
Gnβ2Mα3
Gnβ4
N-acetyl
H-1
H-1
H-2
Gnβ
Gnα
Mα3
Mα6
bis-Gnβ
Mβ3
Mβ
H-3
Mα6
Mα3
5.2
5.1
5.0
4.9
4.8
4.7
4.6
4.5
4.4
4.3
4.2
4.1
4.0
3.9
2.1
2.0
PPM


b
Gnβ6
Gnβ4
{bis-246,24}
Gnβ2Mα6
Gnβ4Mβ4Gnβ4Gn
Gnβ2Mα3
Gnβ4
N-acetyl
H-1
H-1
M H-2, H-3,H-4
Gnβ
Mβ
Mα6
bis-Gnβ
H-2
Mα3
H-2
Mβ
Mα6
H-3
Mα3
Gnα
Mα3
5.2
4.8
4.4
4.0
3.6
3.2
2.1
2.0
ppm


c
Gnβ6
Gnβ4
{bis-246,2}
Gnβ2Mα6
Gnβ4Mβ4Gnβ4Gn
Gnβ2Mα3
N-acetyl
H-1
H-1
H-2,H-4
Gnβ
Mβ
Mα6
Gnα
Mα3
Mα6
bis-Gnβ
Mα3
5.2
5.1
5.0
4.9
4.8
4.7
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3.9
2.1
2.0
PPM

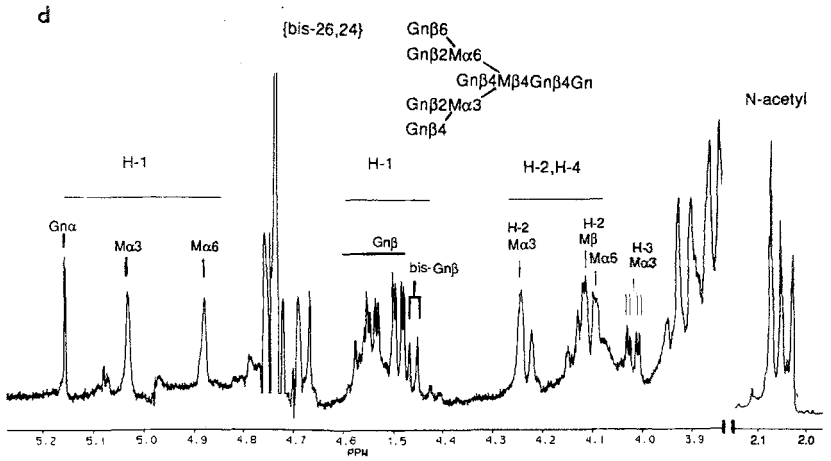

Figure 4. 500 MHz proton NMR spectra of reducing oligosaccharides isolated from ovomucoid, recorded at room temperature with acetone (set at 2.225 ppm) as an internal standard. (a) {bis-0,24}; (b) {bis-246,24}; (c) {bis-246,2}; (d) {bis-26,24}. See Table 1 for oligosaccharide nomenclature.

(e) Oligosaccharide {bis-2,24}. The oligosaccharide in the three fractions G1, H2 and I1 (Table 3) was identified as {bis-2,24} (Table 1) and represents about 38% (the major component) of identified ovomucoid oligosaccharides. The NMR spectra (Tables 4 and 5) were very similar to those reported previously for bisected tri-antennary glycopeptide from chicken ovotransferrin [25] and for the corresponding oligosaccharide [21]. Insertion of a bisecting GlcNAc into {2,24} causes the resonances of H-1 and H-2 of all three mannoses to shift significantly (compare {2,24} and {bis-2,24} in Tables 4 and 5). As in {2.24}, 4-substitution of the Manα3- arm is indicated by the appearance of a doublet of doublets at 4.044 ppm due to the H-3 of Manα3- ($J_{2,3}$ = 3.22 Hz; $J_{3,4}$ = 9.13 Hz). FAB-MS data of pooled fractions G1, H2 and I1 (Table 6) confirm the structure of {bis-2,24}.

(f) Oligosaccharide {bis-0,24}. The oligosaccharide in fractions E1 and H1 represents about 10% of identified ovomucoid oligosaccharides. NMR spectra of E1 and H1 were identical and the fractions were pooled. The 500 MHz proton NMR spectrum of pooled E1 and H1, recorded at room temperature, is shown in Fig. 4a. The NMR parameters (Tables 4 and 5) are similar to those of the glycopeptide AC-CC reported by Ceccarini *et al.* [26]. Two antennary GlcNAc residues are present (8 Hz doublets at 4.519 and 4.517 ppm due to H-1) in addition to the bisecting GlcNAc (H-1 8 Hz doublet at 4.439 ppm). The signals from the H-1, H-2 and H-3 of the α3-linked mannose (5.056, 4.287 and 4.034 ppm, respectively) are almost identical to those of AC-CC [26], and of oligosaccharide {bis-2,24} (Tables 4 and 5), suggesting that the Manα3-residue is substituted by two GlcNAc residues in β(1-2)- and β(1-4)-linkages. The chemical shift of the H-2 of Manα6- at 3.991 ppm strongly suggests that this residue is unsubstituted [20]; a similar conclusion can be drawn from comparing the chemical shifts for the H-1 and H-2 of Manα6- for oligosaccharides {bis-0,2} and {bis-0,24} (Tables 4 and 5). FAB-MS data (Table 6, Fig. 3c) of

Table 6. Fast atom bombardment mass spectrometry of oligosaccharides. About 1 to 2 nmol of oligosaccharide sample, dissolved in water, was run in a thioglycerol matrix with VG Analytical ZAB-SE double focusing FAB-mass spectrometer.

Compound[a]		Fraction[b]	Major m/z observed	Ion composition	Molecular weight
1.	{M5}	ovalbumin	1235	$[M_5Gn_2+H]+$	1234
2.	{M2+F}	K1	917	$[M_2Gn_2F+Na]+$	894
3.	{M3+F}	K2	1079	$[M_3Gn_2F+Na]+$	1056
4.	{2,0}	L1	1136	$[M_3Gn_3+Na]+$	1113
5.	{0,2+F}	fibrin	1281	$[M_3Gn_3F+Na]+$	1258
			1297	$[M_3Gn_3F+K]+$	1258
6.	{bis-0,2}	A2+C1	1339	$[M_3Gn_4+Na]+$	1316
7.	{bis-0,24}	E1+H1	1543	$[M_3Gn_5+Na]+$	1520
8.	{2,2}	L2,T2	1339	$[M_3Gn_4+Na]+$	1316
9.	{2,2d}	T3	1136	$[M_3Gn_3+Na]+$	1113
10.	{bis-2,2}	D2+E2	1543	$[M_3Gn_5+Na]+$	1520
11.	{2,24}	O1,N1	1542	$[M_3Gn_5+Na]+$	1519
12.	{bis-2,24}	G1+H2+I1	1746	$[M_3Gn_6+Na]+$	1723
13.	{26,24}	O2	1746	$[M_3Gn_6+Na]+$	1723
14.	{bis-26,24}	D3+G2	1949	$[M_3Gn_7+Na]+$	1926
15.	{bis-246,2}	A3,B1	1949	$[M_3Gn_7+Na]+$	1926
16.	{bis-246,24}	D4+F1	2152	$[M_3Gn_8+Na]+$	2129

[a] M, Man; Gn, GlcNAc; F, Fucose.
[b] The plus sign indicates that samples were pooled prior to analysis.

pooled E1 and H1 fractions support the bisected bi-antennary structure. Methylation analysis of the reduced oligosaccharide indicates the presence of terminal mannose, 2,4-substituted mannose, 3,4,6-substituted mannose, terminal GlcNAc and 4-substituted GlcNAc. The combined data prove the major oligosaccharide in E1 and H1 to be {bis-0,24} (Table 1).

(g) Oligosaccharide {bis-246,24}. Identical NMR spectra (Fig. 4b) were obtained for fractions D4 and F1 (Table 3) which represented about 8% of identified ovomucoid oligosaccharides. The NMR parameters ({bis-246,24} in Tables 4 and 5) were very similar to those previously reported [27] for bisected penta-antennary reduced oligosaccharide corresponding to {bis-246,24} (Table 1). The chemical shifts for the H-1 resonances of the terminal non-reducing GlcNAc residues observed in this work are identical to those reported by Paz-Parente *et al.* [27] to within 0.011 ppm, but resonances cannot be assigned to particular linkages with certainty, because the required NMR experiments, such as nuclear Overhauser enhancements, have not been carried out as yet. The resonance at 2.122 ppm (Table 4) is present in all spectra of oligosaccharides in which the Manα6-arm is tri-substituted (β2, β4 and β6) but not in those in which the Manα6- arm is mono-substituted (β2) or bi-substituted (β2 and β6); this signal therefore may be attributed to the *N*-acetyl methyl of GlcNAcβ4-linked to Manα6. Methylation analysis of the reduced oligosaccharide in D4 and F1 detected partially methylated alditol acetate derivatives from 2,4-substituted, 2,4,6-substituted and 3,4,6-substituted mannose, terminal GlcNAc and 4-substituted GlcNAc. The FAB-MS spectrum of pooled fraction D4 and F1 (Table 6) confirmed the structure of the oligosaccharide as {bis-246,24}.

(h) Oligosaccharide {bis-246,2}. The oligosaccharides in fractions A3 and B1 had identical NMR spectra and were therefore pooled. These fractions comprise about 6% of the identified ovomucoid oligosaccharides. Fig. 4c shows the 500 MHz proton NMR spectrum of the pooled fractions and the NMR parameters are listed in Tables 4 and 5. The presence of the bisecting GlcNAc is indicated by the characteristic multiplet at 3.3 ppm, the GlcNAc H-1 resonance at 4.444 ppm (8 Hz doublet), and the upfield chemical shift of the β-Man H-1 at 4.707 ppm (recorded at high temperature, Table 5). Signals from the reducing *N*-acetylchitobiose core and four antennary β-GlcNAc residues are present. The *N*-acetyl methyl signal at 2.122 ppm suggests the presence of GlcNAcβ4 linked to the Manα6- arm. Comparing {bis-246,24} and {bis-246,2} (Table 4), one of the antennary GlcNAc H-1 signals is missing in {bis-246,2}. Since the doublets at 4.02 to 4.04 ppm due to the H-3 of a 4-substituted Manα3 are not seen, it is concluded that the Manα3- arm in {bis-246,2} is mono-substituted by GlcNAcβ2 and the Manα6 arm is tri-substituted by GlcNAcβ2, β4 and β6 residues. The FAB-MS spectrum of fraction A3 is shown in Fig. 3a. Molecular weight determination by FAB-MS (Table 6) confirmed that the oligosaccharide in fractions A3 and B1 has the size of a bisected tetra-antennary oligosaccharide. Methylation analysis of pooled fractions A3 and B1 detected partially methylated alditol acetate derivatives of 2-substituted, 2,4,6-substituted and 3,4,6-substituted mannose, as well as 4-substituted and terminal GlcNAc, and 4-substituted GlcNAc-ol. The combined data are consistent with the structure of the major oligosaccharide in both fractions A3 and B1 being {bis-246,2} (Table 1).

(i) Oligosaccharide {bis-26,24}. Fractions D3 and G2 (Table 3) comprised about 8% of the total identified ovomucoid oligosaccharides. The NMR spectra of the two fractions were identical ({bis-26,24} in Tables 4 and 5). The 500 MHz proton NMR spectrum of pooled fractions D3 and G2 is shown in Fig. 4d. The presence of a bisecting GlcNAc is indicated by the characteristic multiplet at 3.3 ppm, and the chemical shift for H-1 of β-linked Man at 4.699 ppm (recorded at high temperature, Table 5). The chemical shift for the H-1 of the bisecting GlcNAc at 4.489 ppm (8 Hz doublet) is further downfield than in any of the other bisected structures, and lies in the region of galactose H-1 resonances. Signals are detected for the *N*-acetylchitobiose core and for four antennary β-GlcNAc residues. The absence of a resonance at 2.122 ppm and the significant shift of the H-1 signal of the Manα6-residue compared to {bis-246,24} suggest the absence of GlcNAcβ4-linked to Manα6. Methylation analysis of reduced pooled D3 and G2 fractions detected partially methylated alditol acetate derivatives of 2,4-substituted, 2,6-substituted and 3,4,6-substituted mannose, terminal and 4-substituted GlcNAc, and 4-substituted GlcNAc-ol. The total ion current pattern for the GC separation of partially methylated alditol acetates is shown in Fig. 6. The FAB-MS spectrum of pooled D3 and G2 is shown in Fig. 3b. The molecular weight is the same as that of {bis-246,2} (Table 6). The data indicate that the major oligosaccharide in fractions D3 and G2 is {bis-26,24} (Table 1).

Hen Ovalbumin Oligosaccharide {M5}.

Pure ovalbumin oligosaccharide {M5} (Table 1) was obtained by fractionation on HPLC using a Magnum amine column followed by chromatography on Bio-Gel P-4. NMR data (Tables 4 and 5) and FAB-MS data (Table 6) are consistent with the structure of {M5} with a reducing *N*-acetylchitobiose core. The chemical shift values for H-1 and H-2 of all five

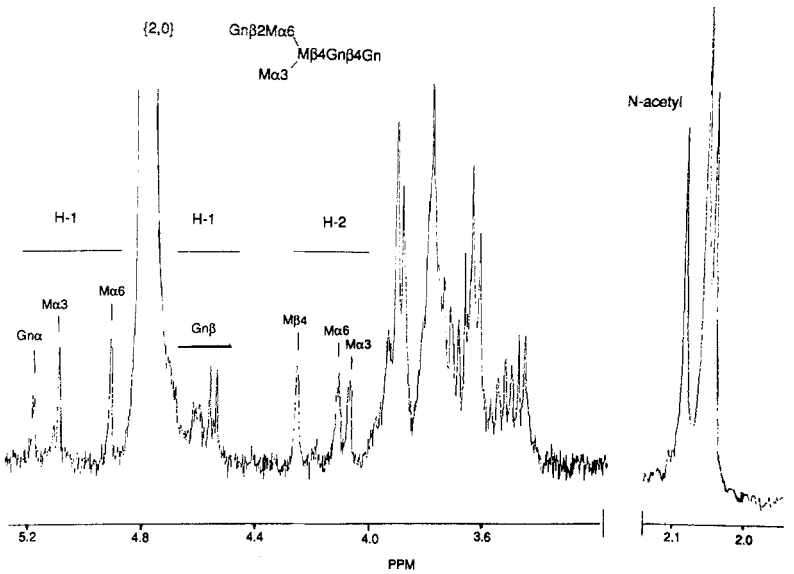

Figure 5. 360 MHz proton NMR spectrum of {2,0} (for nomenclature, see Table 1) recorded at room temperature with acetone (set at 2.225 ppm) as an internal standard.

mannose residues are almost identical to those previously reported for the corresponding glycopeptide [21, 28, 34] and were assigned accordingly.

Bovine Fibrin Oligosaccharides {M2+F}, {M3+F} and {0,2+F}

Fibrin oligosaccharides were fractionated on a Magnum amine column into at least six peaks (data not shown). The two major peaks, K1 at 30 min and K2 at 35 min, were analyzed by NMR. The chemical shift values for H-1 and H-2 of the mannoses in K2 ({M3+F} in Tables 4 and 5) are identical to those of the corresponding glycopeptide reported previously [22]. K2 has a reducing *N*-acetylchitobiose core and fucose α(1-6)-linked to the reducing GlcNAc. The chemical shift values for fucose in a reducing oligosaccharide (H-1 at 4.899 ppm, H-5 at 4.108 ppm, and the methyl group at 1.226 ppm, Tables 4 and 5) differ significantly from values found for the corresponding glycopeptide [21, 22]. The FAB-MS spectrum (Table 6) confirmed the structure of K2 as {M3+F} (Table 1). The molecular weight of K1 (Table 6) is one hexose less than K2. The NMR spectrum of K1 ({M2+F} in Tables 4 and 5) is similar to that of K2 except that the signals for α6-linked mannose are missing and there are upfield shifts of the H-2 signal of the β-mannose (0.0026 ppm) and of the *N*-acetyl methyl group of the sub-terminal GlcNAc of the core (0.0017 ppm). The data are compatible with the structure {M2+F} for K1.

The NMR data for oligosaccharide {0.2+F} from fibrin reveal an *N*-acetylchitobiose core with fucose (Tables 4 and 5). The NMR parameters for H-1 and H-2 of the three mannose residues and for H-1 of the single antennary GlcNAc are similar to those previously reported for the corresponding glycopeptide [22]. The FAB-MS data (Table 6) confirm the structure of oligosaccharide {0.2+F}.

Human Fibrinogen Oligosaccharides {2,2} and {2,0}

Fibrinogen oligosaccharides were fractionated on Bio-Gel P-4 followed by HPLC on an analytical amine and a Magnum amine column. Two major fractions L1 and L2, were characterized (Table 3). The NMR parameters of L2 ({2,2} in Tables 4 and 5) are identical within 0.004 ppm to those previously reported for glycopeptide GnGn [22, 29]. L2 has a reducing *N*-acetylchitobiose core without fucose. Fraction L2 contained Man and GlcNAc in a ratio of about 3:4 as determined by GLC. FAB-MS analysis (Table 6) confirmed that fraction L2 contains {2,2} (Table 1). FAB-MS indicated that L1 is one *N*-acetylhexosamine residue smaller than L2 (Table 6). The NMR spectra of L1 ({2,0} in Tables 4 and 5 and Fig. 5) are different from those of {2,2}, {2,2d}, and {0,2+F}; the parameters resemble those of compound GM reported by Grey *et al.* [22]. Fraction L1 contains a reducing *N*-acetylchitobiose core with a single β(1-2)-linked antennary GlcNAc (H-1 signal at 4.551 ppm and *N*-acetyl methyl signal at 2.050 ppm). The chemical shift values for H-1 and H-2 of the α3-linked mannose at 5.098 and 4.065 ppm, respectively, indicate that this residue is unsubstituted [22]. The H-2 signal at 3.972 ppm in {M3+F}, typical for an unsubstituted α6-Man, has shifted in L1 to 4.108 ppm (Table 4), indicating that the α6-Man is substituted by GlcNAc. The structure of fraction L1 is therefore {2,0} (Table 1).

Bovine Thyroglobulin Oligosaccharides {2,2} and {2,2d}

Fractionation of oligosaccharides isolated from thyroglobulin [30] on Bio-Gel P-4 and by HPLC on a Magnum amine column resulted in three fractions T1, T2 and T3. T1 was a minor fraction and contained a mixture of reducing oligosaccharides, including {2,24}, {2,2} and possibly {26,2} (Table 1). Attempts to isolate {26,2} were not successful. The major fraction T2 contained {2,2} (Table 1) as indicated by NMR spectra (Tables 4 and 5) and FAB-MS (Table 6). The minor fraction T3 contained {2,2d} (Table 1), i.e. {2,2} with only one core GlcNAc, as indicated by NMR spectra (Tables 4 and 5) and FAB-MS (Table 6).

Bovine Fetuin Oligosaccharide {2,24}

Fetuin oligosaccharides [31, 32] separated on Bio-Gel P-4 into a major peak N1 and two minor peaks N2 and N3. These peaks were sub-fractionated by HPLC. Sub-fractions from N3 did not contain sufficient material for NMR identification. Fraction N2 seemed to contain a mixture of {2,2} and {2,24} with one or two GlcNAc residues in the core. The major fraction N1 was {2,24} (Table 1), as indicated by HPLC analysis (Table 3), NMR spectra (Tables 4 and 5) and FAB-MS (Table 6).

Human α_1-Acid Glycoprotein Oligosaccharides {2,24} and {26,24}

Oligosaccharides released from α_1-acid glycoprotein [33], either by hydrazinolysis or by *N*-glycanase, eluted as poorly resolved peaks from Bio-Gel P-4. HPLC using an analytical amine column and acetonitrile/water, 65/35 by vol, as the mobile phase separated oligosaccharides released by either method into at least four to six fractions with similar retention times but in different proportions.

Fractions with similar retention times were pooled. The NMR spectra indicated that the two major hydrazinolysis fractions, O1 at 42 min and O2 at 53 min, were relatively pure {2,24} and {26,24} (Table 1), respectively. The other fractions were mixtures and contained

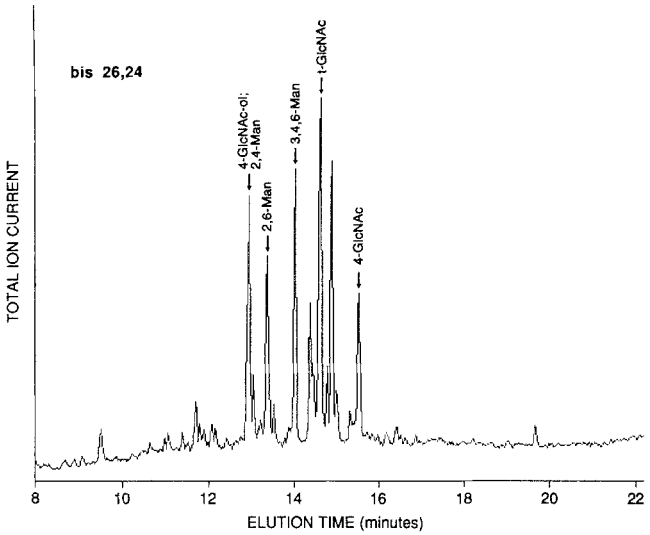

Figure 6. Methylation analysis of {bis-26,24} (see Table 1 for nomenclature). Total ion current chromatographic pattern of partially methylated alditol acetates eluted by gas chromatography. The main derivatives detected were from 2,4-substituted mannose and 4-substituted GlcNAc-ol (eluting at the same position at 12.95 min); 2,6-substituted mannose (13.40 min); 3,4,6-substituted mannose (14.02 min); terminal GlcNAc (14.67 min); 4-substituted GlcNAc (15.55 min). The small peak at 11.7 min showed a mass spectrum characteristic of a derivative from 2-substituted mannose (m/z 129, 130, 161, 190). This peak was found in varying amounts in all samples and was probably due to an artifact of the methylation procedure.

some galactose. The ratio of O1 to O2 was approximately 3:1. The NMR parameters (Tables 4 and 5) of O1 were identical to oligosaccharide {2,24} isolated from fetuin or ovomucoid (see above). With exception of signals for the *N*-acetylchitobiose core, NMR parameters of O2 ({26,24} in Tables 4 and 5) are similar to those of the corresponding galactosylated tetra-antennary glycopeptide {26,24} (compound 13 in Vliegenthart *et al.* [21]), after adjusting the shift of the antennary GlcNAc H-1 resonance by 0.03 ppm to compensate for the absence of galactose substitution. The following chemical shift values diagnostic for a tetra-antennary structure were detected for O2: (a) the appearance of doublets for the H-3 of the α3-Man at 4.047 ppm, indicating 4-substitution on the Manα3 arm, (b) an upfield shift of the H-1 of the α6-Man to 4.861 ppm from 4.914 ppm in {2,24} due to 6-substitution of the Manα6 arm. It is interesting to note that the observed chemical shift values for {bis-26,24} are very different from those for {26,24} (Tables 4 and 5), indicating major conformational changes upon the introduction of the bisecting GlcNAc residue. The HPLC elution times (Table 3) and FAB-MS data (Table 6) confirm that fractions O1 and O2 have the structures {2,24} and {26,24}, respectively.

Reduced {2,2}, {bis-2,2} and {2,24}

The NMR spectra, recorded at 300 K, of oligosaccharides {2,2}, {bis-2,2} and {2,24} after the reduction step were similar to those of the unreduced compounds. Reduction of the terminal core GlcNAc was apparent by loss of the signal at 5.18 ppm due to the H-1 of the α-GlcNAc, and by the change of the H-1 resonance of the β4-linked core GlcNAc to a single doublet at 4.636 ppm ($J_{1,2}$ = 8.45 Hz).

Oligosaccharides {2,2} and {2,24} and all bisected ovomucoid oligosaccharides showed two widely separated peaks on HPLC using the reverse phase Dextro-Pak column (Table 2). The ratio of elution times was always approximately 2 to 3, and the relative amounts of material in the early and late peaks varied from 2:5 to 3:2. When either the early or late peak was concentrated and re-injected into HPLC, the material always eluted as two well resolved peaks on the reverse phase column. The amine column does not show this phenomenon and gives only single peaks for pure reducing oligosaccharides (Table 3). When oligosaccharides {2,2}, {bis-2,2} and {2,24} were reduced and analyzed on HPLC using the reverse phase column, only a single peak was observed for each of these oligosaccharides. These findings suggest that the α- and β-anomers of a pure oligosaccharide are resolved on the reverse phase column. The elution times of the anomers are significantly different indicating that mutarotation is greatly inhibited even though elution is carried out in water. The pattern of elution on the reverse phase column is not related to the carbohydrate size nor to the primary structure in any recognizable way. For example, {bis-2,24} elutes at 105/201 min (Table 2), whereas both a larger ({bis246,24}) as well as a smaller oligosaccharide ({bis-2,2}) elute earlier (59/89 min and 59/72 min, respectively). The two isomers {bis-246,2} and {bis-26,24} elute at very different times, i.e. at 16/43 min and 59/105 min, respectively.

The interaction of oligosaccharides with the amine column is probably ionic or based on hydrogen bonding. On an amine column, the elution time is proportional to the size of the carbohydrate (Table 3). Isomers elute in patterns that are not predictable from the primary oligosaccharide structure. Isomers with long retention times such as the pair {bis-246,2} and {bis-26,24} usually separate better than smaller oligosaccharides (Table 3).

Discussion

We have purified and identified 16 mannose- and GlcNAc-terminating reducing oligosaccharides as listed in Table 1, and presented their FAB-MS and proton NMR spectra. These oligosaccharides include most of the major intermediates in the biosynthetic pathways to highly branched *N*-glycans [5, 6]. The bisected and non-bisected versions of oligosaccharides {2,2}, {2,24} and {26,24}, either as shown in Table 1 or with sialyl, galactosyl and fucosyl substituents on their antennae, are the most common structures found in glycoproteins.

We have prepared these oligosaccharides for use as GlcNAc-transferase substrates [6]. Reducing oligosaccharides were prepared because of the ease with which these can be coupled to solid supports for affinity chromatography and because they can be readily labelled by reduction with NaB^3H_4. Oligosaccharides are preferable to glycopeptides because they can be more readily purified by HPLC. We have used these oligosaccharides to develop HPLC assay procedures for all six GlcNAc-transferases involved in the initiation of *N*-glycan branches [6]. We have used the HPLC assay to detect a novel branching enzyme activity, GlcNAc-transferase VI, which adds a GlcNAc in β(1-4)-linkage to the α6-mannose in {bis-26,24} [6, 7]. Oligosaccharide {bis-26,24} is a specific substrate for GlcNAc-transferase VI [6].

Many of the oligosaccharides isolated in this report have been previously characterized as the corresponding glycopeptides [20-23, 25, 28, 34], as oligosaccharides with a single reducing GlcNAc residue in the core [21], as reduced oligosaccharides [27, 35-39], or as fluorescent derivatives [40]. Compounds {bis-26,24}, {bis-246,2} and {bis-0,24} have been reported to occur in ovomucoid by Yamashita *et al.* [36]. However, the FAB-MS and NMR spectra of {bis-246,2} and {bis-26,24} have not yet been reported. Further, none of the oligosaccharides listed in Table 1 has previously been purified and characterized by NMR spectroscopy and FAB-MS as reducing oligosaccharides with a complete *N*-acetylchitobiose core.

Oligosaccharide {2,0} has been suggested to occur in Fowl Plague virus hemagglutinin [39] but the fraction containing {2,0} and {0,2} was not purified to homogeneity nor were the oligosaccharides characterized by NMR; the presently accepted pathway for the synthesis of *N*-glycans [5, 6] requires that a GlcNAc be added in β(1-2)-linkage to the Manα3 arm before it is added to the Manα6 arm, thereby making {2,0} an impossible biosynthetic intermediate. Our view is that compounds like {2,0} are formed by glycosidase digestion *in vivo* or during purification.

GlcNAc-terminating (truncated) oligosaccharides may also serve as standards in the isolation and identification of cell surface oligosaccharides. Truncated oligosaccharides occur in mutant Chinese hamster ovary cells [41, 42] and in wheat germ agglutinin resistant mutant cells derived from a highly metastatic murine tumour cell line [43, 44]. Using {26,24} as a standard, we have shown that this structure occurs in another wheat germ agglutinin resistant cell line with lowered metastatic potential, D36W25-1 (I Brockhausen, JW Dennis and JP Carver, unpublished data).

Since the purpose of this study was to prepared oligosaccharides in amounts sufficient to serve as enzyme substrates, only the major structures from ovomucoid, fetuin and α_1-acid glycoprotein were isolated. Many other oligosaccharide fractions were detected but in amounts insufficient for a complete structural analysis. There is good correlation between the major ovomucoid oligosaccharide structures (this study and [27] and [35-37], and the branching GlcNAc-transferase activities found in hen oviduct membranes [6, 23, 45]). However, there are exceptions. For example, it is possible to synthesize {26,2} glycopeptide or oligosaccharide *in vitro* [1, 6, 46, 47] but it was not possible to isolate {26,2} from any of the glycoproteins in this study. This suggests that, *in vivo*, either alternative pathways are more significant or {26,2} is rapidly converted to other oligosaccharides. Another possibility is that degradation of oligosaccharides may take place *in vivo* or during the isolation procedure. For example, as mentioned above, degradation may explain the isolation of {2,0} from fibrinogen since {2,0} cannot be made by GlcNAc-transferase *in vitro* [5, 48-50]. The addition and subsequent removal of antennary GlcNAc has been suggested to occur *in vivo* during the biosynthesis of the plant glycoproteins phaseolin and phytohemagglutinin [51].

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