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Structural studies on the *O*-linked carbohydrate chains of human platelet glycocalicin

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Glycocalicin (140 kDa), constituting the main part of glycoprotein Ib (160 kDa), was released from the human platelet membrane by the action of a Ca^{2+}-dependent protease, present in the platelet cytoplasm and liberated during sonication of the platelet suspension. After activation of the protease by Ca^{2+}, the sonicated plateled suspension was subjected to differential centrifugation. The supernatant was applied to a column of wheat germ agglutinin linked to Sepharose 4B; glycocalicin was eluted from the column with 2.5% (w/v) *N*-acetylglucosamine.

Glycocalicin was found to contain 40% carbohydrate by weight, representing *N*- as well as *O*-glycosidically linked carbohydrate chains. The *O*-glycosidic chains were split off by alkaline cleavage in the presence of ^{3}H-labelled $NaBH_4$. The liberated ^{3}H-labelled oligosaccharide-alditols were fractionated on a DEAE-Sephadex A-25 column. The structures of the oligosaccharide-alditols were investigated by 500-MHz ^{1}H-NMR spectroscopy. The major compound was identified as NeuAcα(2→3)Galβ(1→3)[NeuAcα(2→3)Galβ(1→4)GlcNAcβ(1→6)]GalNAc-ol. Two minor compounds were found to be NeuAcα(2→3)Galβ(1→3)[NeuAcα(2→6)]GalNAc-ol and NeuAcα(2→3)Galβ(1→3)GalNAc-ol.

Platelet membrane glycoproteins play functional roles in vital processes such as platelet aggregation and adhesion [1]. Glycoprotein Ib (160 kDa) is one of the main membrane glycoproteins. By the action of a Ca^{2+}-dependent protease, present in the platelet cytoplasm, glycocalicin (140 kDa), constituting the main part of glycoprotein Ib [2, 3], can be cleaved from the platelet surface [4]. Glycoprotein Ib/glycocalicin is probably the receptor for Von Willebrand's factor in the presence of ristocetin [5] and for platelet adhesion to the vessel wall [6]. The carbohydrate chains of glycoprotein Ib may be involved in the aforementioned interaction phenomena.

Glycocalicin was reported to have a carbohydrate content of 60% (w/w) comprising *N*- as well as *O*-glycosidically linked carbohydrate chains [7–9]. The major *O*-linked carbohydrate chain, isolated by Judson et al. [8], was shown to be a hexasaccharide-alditol containing *N*-acetylneuraminic acid, galactose, *N*-acetylglucosamine and *N*-acetylgalactosaminitol in the molar ratio 2:2:1:1. Here we describe the elucidation, by 500-MHz ^{1}H-NMR spectroscopy, of the primary structures of the prevalent *O*-glycosidic carbohydrate chains of glycocalicin. A preliminary account of this study has been presented [10]. During the preparation of the manuscript of this paper a report by Tsuji et al. [9] appeared describing the structure of the main *O*-glycosidic chain, derived by methylation analysis in combination with enzymic degradation studies.

MATERIALS AND METHODS

Isolation of glycocalicin from human platelets

Platelets (40 units), obtained from the Central Laboratory of the Blood Transfusion Service of the Swiss Red Cross [2], were resuspended in 80 ml 10 mM Tris/HCl buffer, pH 8.0, containing 20 mM $CaCl_2$. The suspension was cooled to 4 °C and sonicated for 2 min with a B-30 sonifier (Branson Sonic Power Company, Danbury, USA) (output control 7, 50% duty cycle; pulsed mode). After incubating the suspension for 1 h at room temperature, it was subjected to differential centrifugation (9000 × *g* for 20 min followed by 100000 × *g* for 1 h, 4 °C). The supernatant was mixed with EDTA (4 mM final concentration) and sodium deoxycholate (0.5% final concentration) and subsequently applied to a 50 ml WGA-Sepharose 4B column [11] equilibrated with 10 mM Tris/HCl buffer, pH 8.0, containing 0.5% (w/v) sodium deoxycholate. The column was washed with the same buffer until the flow-through peak had eluted and the absorbance of the effluent at 280 nm had returned to the base line; then the column was washed with 50 ml buffer without deoxycholate. Glycocalicin was eluted with 2.5% (w/v) *N*-acetylglucosamine in 10 mM Tris/HCl buffer, pH 8.0. dialyzed against water and lyophilized. The purity was checked by SDS/polyacrylamide gel electrophoresis [12].

Alkaline borohydride treatment

Glycocalicin (10 mg) was treated with 4 ml 0.1 M NaOH containing 148.5 mg $NaBH_4$ and 2.7 mg ^{3}H-labelled $NaBH_4$ (spec. act. 12.9 GBq/mmol; New England Nuclear, USA), final activity 925 MBq. After 20 h at 40 °C the solution was acidified to pH 5.0 with 4 M acetic acid and applied to a column (20 × 2 cm) of Dowex 50WX8, H^+ form (100–200 mesh). The column was washed with 180 ml water and the eluate lyophilized. Boric acid was removed by co-evaporation with methanol under reduced pressure. The mixture of oligosaccharide-alditols obtained was subjected to preparative descending chromatography on Whatman 3MM paper for

Abbreviations. SDS, sodium dodecyl sulfate; WGA, wheat germ agglutinin; NOE, nuclear Overhauser enhancement.

24 h at room temperature using *n*-butanol/ethanol/water (4:1:1, v/v) as solvent system. The ^{3}H-labelled material present at the origin was recovered from the paper with water.

Fractionation of the oligosaccharide-alditols

The lyophilized mixture of ^{3}H-labelled oligosaccharide-alditols (14.8 kBq) was dissolved in 0.05 M pyridine/acetate buffer, pH 5.0, and applied to a column (60 × 1.5 cm) of DEAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden), equilibrated with the same buffer. The column was eluted with 400 ml of a linear concentration gradient of 0.05 – 1.0 M pyridine/acetate buffer, pH 5.0 [13]. Fractions of 4 ml were collected at a flow rate of 24 ml/h and assayed for radioactivity. ^{3}H-labelled peaks were pooled, lyophilized and desalted on a column (90 × 2.5 cm) of Bio-Gel P-6 (Bio-Rad, Richmond, USA) using water as eluent at a flow rate of 20 ml/h.

Analytical methods

SDS/polyacrylamide gel electrophoresis was performed in 8% polyacrylamide gels according to Laemmli [12]. The gels were stained with the periodic acid/Schiff reagent [14] or by using the silver nitrate staining method of Morrisey [15].

Sugar analysis was carried out by gas-liquid chromatography on a CPsil5 WCOT fused silica capillary column (25 m × 0.32 mm i.d.) using a Varian Aerograph 3700 gas chromatograph. The trimethylsilylated methyl glycosides were prepared by methanolysis, *N*-(re)acetylation and trimethylsilylation [16, 17].

Prior to ^{1}H-NMR spectroscopic analysis the desalted samples were repeatedly treated with ^2H_2O (99.996 atom% ^{2}H, Aldrich, Milwaukee, USA) at p^{2}H 7 and room temperature. 500-MHz ^{1}H-NMR spectra were recorded using a Bruker WM-500 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands) operating in the pulsed Fourier-transform mode at a probe temperature of 27 °C [18, 19]. Resolution enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation [20]. Chemical shifts (δ) are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone ($\delta = 2.225$ ppm in ^2H_2O at 27 °C).

RESULTS AND DISCUSSION

Preparation of glycocalicin

Washed human platelets were resuspended in 10 mM Tris/HCl buffer, pH 8.0, containing 20 mM $CaCl_2$, and sonicated. During sonication the platelets were disrupted and the Ca^{2+}-dependent protease, present in the cytoplasm, was released and activated by Ca^{2+}. After an hour the suspension was subjected to differential centrifugation. To the final supernatant EDTA and sodium deoxycholate were added and the solution was then applied to a WGA-Sepharose 4B column. After desorption by 2.5% *N*-acetylglucosamine the fractions containing glycocalicin were pooled, dialyzed against water and lyophilized. The purity of this glycocalicin fraction was checked by SDS/polyacrylamide gel electrophoresis. Upon staining with the periodic acid/Schiff and silver nitrate reagents, the gels showed only one band (see Fig. 1).

Sugar analysis of the glycocalicin preparation indicated the presence of Gal, GlcNAc, GalNAc, NeuAc, Man and Fuc.

Fig. 1. *SDS/polyacrylamide gels of glycocalicin after affinity chromatography on WGA-Sepharose 4B.* Lane A, silver-nitrate-stained gel [15]; lane B, periodic acid/Schiff-stained gel [14]

Table 1. *Carbohydrate content and molar composition of glycocalicin and its carbohydrate chains released by alkaline borohydride reduction* The molar ratios for the intact glycoprotein were calculated relative to 1 mol GalNAc; those for the mixture obtained after alkaline borohydride reduction, relative to 1 mol GalNAc-ol

Monosaccharide	Molar ratio in glycocalicin		
	present study	Okumura et al. [7]	after alkaline borohydride reduction
Fuc	0.4	0.3	0.2
Man	0.7	0.2	0.9
Gal	2.4	2.6	3.4
GalNAc	1	1	–
GalNAc-ol	–	–	1
GlcNAc	6.3(1.4)[a]	1.2	2.2
GlcNAc-ol	–	–	2.3
NeuAc	2.0	2.1	3.0
	% (w/w)		
Carbohydrate content	40	60	

[a] The GlcNAc content of this preparation is high because of incomplete removal of GlcNAc from the WGA-Sepharose column procedure. After prolonged dialysis the GlcNAc content is 1.4

Molar ratios are presented in Table 1 together with the data published by Okumura et al. [7]. As is evident from the table the sugar composition of both preparations are very similar but the mannose content of the preparation described here is significantly higher and the total carbohydrate content is somewhat lower (40% w/w). The difference in carbohydrate content may point to a difference in length of the isolated polypeptide chain.

Isolation and characterization of oligosaccharide-alditols

Alkaline borohydride reductive treatment of glycocalicin resulted in a mixture of ^{3}H-labelled oligosaccharide-alditols.

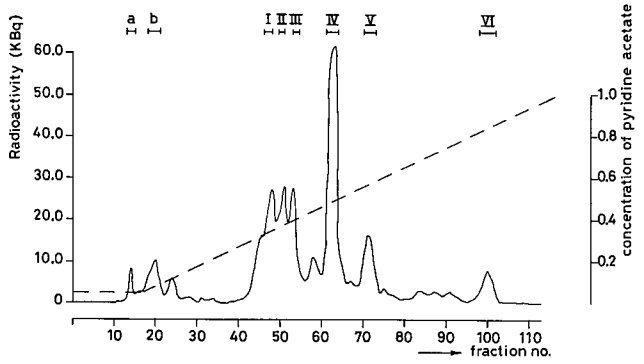

Fig. 2. *Fractionation pattern of the ^{3}H-labelled oligosaccharide-alditols derived from glycocalicin on DEAE-Sephadex A-25 (60 × 1.5 cm).* The column was eluted with a linear concentration gradient (----) of 0.05 – 1.0 M pyridine/acetate buffer, pH 5.0. Fractions of 4 ml were collected at a flow rate of 24 ml/h and assayed for ^{3}H radioactivity. Based on activity counting, 100% recovery was recorded. Fractions were pooled according to the bars

Table 2. *Carbohydrate content and molar composition of the desalted oligosaccharide-alditol fractions I – VI of the DEAE-Sephadex A-25 column*
The molar ratios are calculated relative to 1 mol GalNAc-ol

Mono-saccharide	Molar ratio in fraction					
	I	II	III	IV	V	VI
Man	3.8	6.7	1.5	0.2	0.5	0.5
Gal	10.4	14.0	3.6	2.3	1.8	3.4
Glc[a]	2.8	4.5	1.3	+	0.7	1.2
GlcNAc	3.5	12.0	1.4	1.2	0.4	1.4
GalNAc-ol	1	1	1	1	1	1
NeuAc	2.6	10.0	1.6	1.9	2.1	2.2
	nmol					
Total amount GalNAc-ol	8	3	21	128	29	11

[a] Glucose material present in the fractions is probably derived from the DEAE-Sephadex A-25 column material

The carbohydrate composition of this mixture (see Table 1) shows that, besides GalNAc-ol, GlcNAc-ol appeared in the sugar analysis, which stems for the greater part from the incomplete removal of *N*-acetylglucosamine used in the affinity column procedure. The occurrence of Man besides GalNAc-ol suggests that *N*-glycosidic carbohydrate chains have also been released.

In Fig. 2 the fractionation pattern of the oligosaccharide-alditols on DEAE-Sephadex A-25 has been depicted. The carbohydrate compositions of fractions I – VI, obtained after desalting on Bio-Gel P-6, are given as molar ratios in Table 2. The total amount of *O*-glycosidic alditols in fractions I – VI is expressed as the molar amount of GalNAc-ol. It should be mentioned that radioactivity may lead to an overestimation of these amounts, due to aspecific ^{3}H-labelling of material different from oligosaccharide-alditols (compare Fig. 2). The two rapidly eluting peaks, a and b, in the neutral region, contained mainly GlcNAc-ol but no GalNAc-ol as was evident from sugar analysis. This implies that no asialo *O*-glycosidic carbohydrate chains occur in the cleavage product of glycocalicin.

Fractions I – VI, as well as the mixture of oligosaccharide-alditols, were subjected to 500-MHz ^{1}H-NMR spectroscopy. The spectra of the mixture and the major fraction IV are depicted in Fig. 3 and 4, respectively. The chemical shifts of the structural-reporter groups of the constituting monosaccharides of fractions III, IV and V, together with those of some reference oligosaccharide-alditols [21,22], are listed in Table 3.

The spectrum of fraction IV (Fig. 4) shows that this sample contains a highly pure oligosaccharide-alditol with a mucin-type structure ending in a 3,6-disubstituted GalNAc-ol residue. The chemical shift of H-2 of GalNAc-ol ($\delta = 4.387$ ppm) indicates the presence of Gal in $\beta(1\rightarrow3)$-linkage to GalNAc-ol [22]. The set of H-5 and H-6 chemical shifts of GalNAc-ol (δH-5 = 4.265 ppm and δH-6 = 3.928 ppm) points to the presence of GlcNAc $\beta(1\rightarrow6)$-linked to GalNAc-ol [21,22]. Based on the carbohydrate composition of fraction IV (Table 2), the occurrence of three H-1 doublets at $\delta \approx 4.54$ ppm having $J_{1,2} \approx 8.0$ Hz indicates that a second Gal residue is present in β-linkage. This Gal residue is $\beta(1\rightarrow4)$-linked to GlcNAc because of the presence of the GlcNAc H-6 signal at $\delta = 4.004$ ppm. Such a value points to a Gal$\beta(1\rightarrow4)$GlcNAc $\beta(1\rightarrow\cdot)$ moiety (cf. [21,22]).

Both Gal residues are substituted by NeuAc in $\alpha(2\rightarrow3)$-linkage. This is evident from the presence of two coinciding NeuAc H-3*ax* triplets, at $\delta = 1.800$ ppm, and of two H-3*eq* signals of equal intensity, at $\delta = 2.755$ ppm and $\delta = 2.775$ ppm [18]. Further evidence for these structural elements stems from the resonance positions of the Gal H-1 signals, since they are both shifted downfield as compared to D (see Table 3). This fits the empirical chemical shift rules for $\alpha(2\rightarrow3)$-sialylation of *N*-acetyllactosamine branches in *N*-glycosidic glycopeptides [18, 19]. Moreover, the Gal H-3 signals coincide at $\delta = 4.113$ ppm. Therefore, the structure of the main oligosaccharide-alditol IV has been identified as:

$$\begin{matrix} & & \text{NeuAc}\alpha(2\rightarrow3)\text{Gal}\beta(1\rightarrow3)\diagdown \\ \text{NeuAc}\alpha(2\rightarrow3)\text{Gal}\beta(1\rightarrow4)\text{GlcNAc}\beta(1\rightarrow6)\diagup & & \text{GalNAc-ol} \end{matrix}$$

The assignment of the three doublets at $\delta \approx 4.54$ ppm could not be obtained on the basis of their chemical shifts and coupling constants, only. However, the 1,3-diaxial relation between H-1 and H-3 of β-linked Gal allows to identify the Gal anomeric protons by means of an NOE effect upon presaturation of the H-3 signals, at $\delta = 4.113$ ppm. The doublets at $\delta = 4.529$ ppm ($J_{1,2} = 8.1$ Hz) and $\delta = 4.545$ ppm ($J_{1,2} = 8.4$ Hz) showed an NOE effect. In consequence, the H-1 doublet at $\delta = 4.550$ ppm ($J_{1,2} = 8.3$ Hz) belongs to GlcNAc. The Gal^3 and Gal^4 H-1 signals could be distinguished from each other by comparison of their shifts with those of the monosialo analogue E (see Table 3).

The assignment of the H-3*eq* signal at $\delta = 2.775$ ppm to NeuAc^{3,3} is based on the data for the reference compounds B, C and E (see Table 3). Thus the H-3*eq* signal at $\delta = 2.755$ ppm belongs to NeuAc^{3,4}, a value frequently observed for NeuAc$\alpha(2\rightarrow3)$Gal$\beta(1\rightarrow4)$GlcNAc$\beta(1\rightarrow\cdot)$ elements in *N*-glycosidic chains [18,19]. A similar reasoning applies to the *N*-acetyl signals at $\delta = 2.033$ ppm (NeuAc^{3,3}) and at $\delta = 2.031$ ppm (NeuAc^{3,4}). Attachment of NeuAc in $\alpha(2\rightarrow3)$-linkage to *N*-acetyllactosamine is expected to give rise to a well-known effect on the position of the *N*-acetyl signal of GlcNAc ($\Delta\delta = -0.003$ ppm) (compare [19]). For this reason the singlet at $\delta = 2.062$ ppm is assigned to GlcNAc.

The main peaks in the spectrum of fraction V are superimposable with those of reference compound C (see

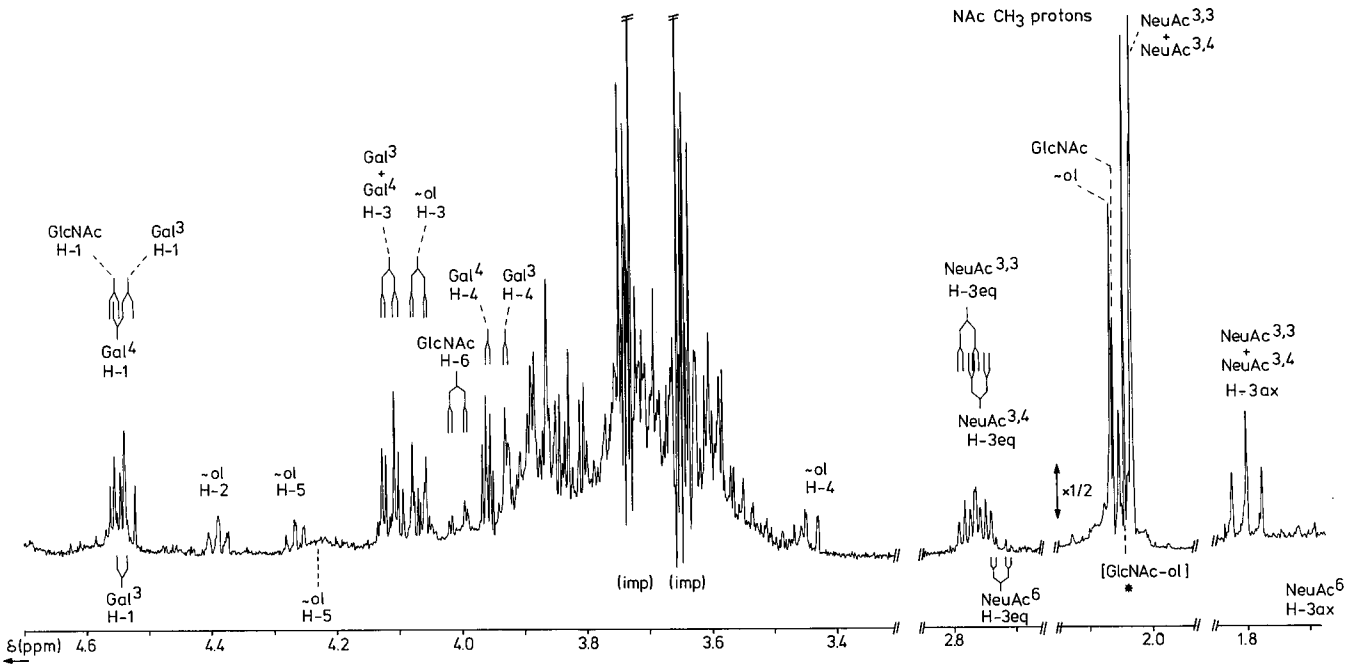

Fig. 3. *500-MHz ^{1}H-NMR spectrum, recorded in ^2H_2O at p^{2}H 7 and 27 °C, of the mixture of oligosaccharide-alditols obtained after alkaline borohydride treatment of glycocalicin*

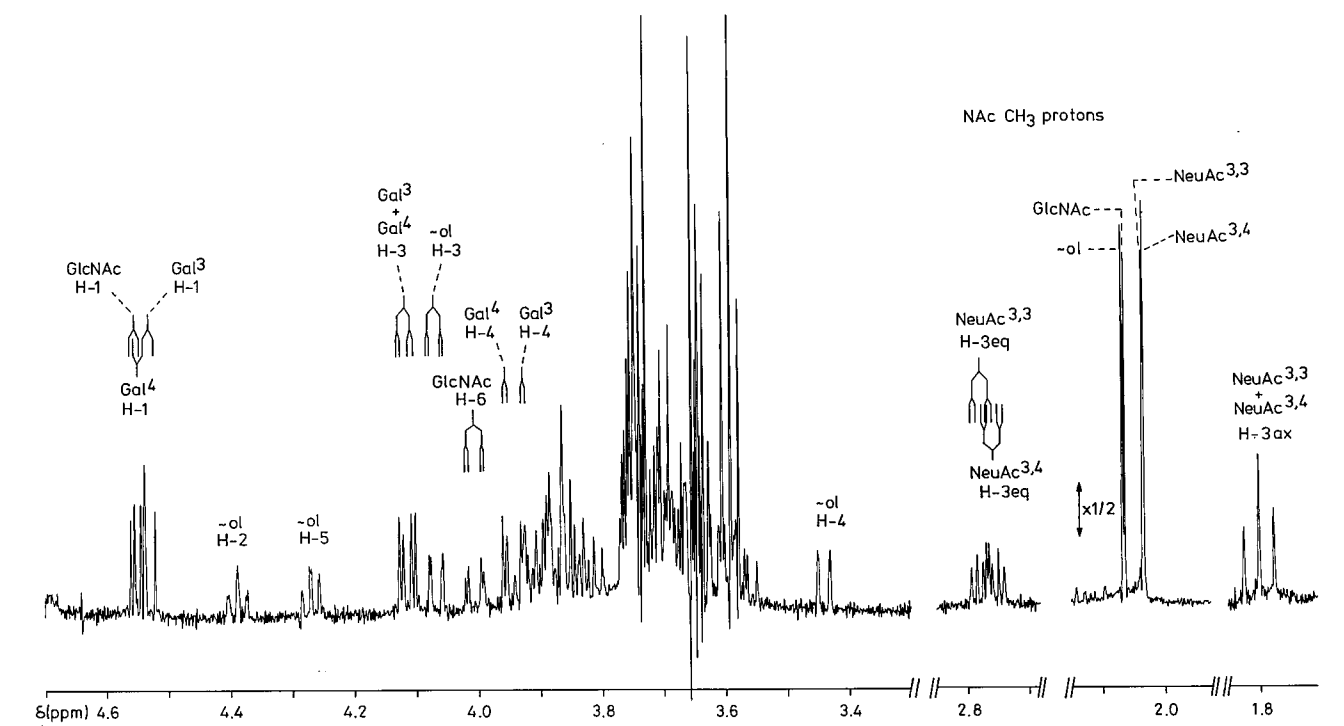

Fig. 4. *500-MHz ^{1}H-NMR spectrum, recorded in ^2H_2O at p^{2}H 7 and 27 °C, of the purified hexasaccharide-alditol, fraction IV, from glycocalicin*

Table 3). The following characteristic features may be mentioned. Two sets of NeuAc H-3*ax* and H-3*eq* signals with equally high intensity are observed. One of them (δH-3*ax* = 1.799 ppm, δH-3*eq* = 2.773 ppm) is specific for NeuAcα(2→3)-linked to Gal^3. The other (δH-3*ax* = 1.692 ppm, δH-3*eq* = 2.725 ppm) is indicative of NeuAcα(2→6)-linked to GalNAc-ol [18,21]. The spectrum shows in the *N*-acetyl proton region two signals in the intensity ratio of 1:2. The singlet at δ = 2.041 ppm is assigned to GalNAc-ol and that at δ = 2.031 ppm belongs to the NeuAc residues. This led to the

Table 3. *^1H Chemical shifts of structural reporter group protons of constituent monosaccharides of the fractions III, IV and V obtained from glycocalicin together with those of reference oligosaccharide-alditols A – E [21, 22]*

The first superscript at the name of a sugar residue indicates to which position of the adjacent monosaccharide it is glycosidically linked. A second superscript is used to discriminate between identically linked residues, by indicating the type of the next linkage in the sequence. Chemical shifts are in ppm downfield from internal 4,4-dimethyl-4-silapentane-1-sulfonate in ^4H_2O at 27 °C acquired at 500 MHz. In the table heading structures are represented by short-hand symbolic notation (cf. [19, 22]): ◇ = GalNAc-ol; ■— = Gal; ●— = GlcNAc; △— = NeuAcα(2→3) and ○— = NeuAcα(2→6). n.d. = not determined

Residue	Reporter group	Chemical shift in compound							
		A	B	III	C	V	D	E	IV
		ppm							
GalNAc-ol	H-2	4.395	4.390	4.387	4.378	4.378	4.394	4.390	4.387
	H-3	4.065	4.074	4.072	4.067	4.067	4.060	4.072	4.066
	H-4	3.507	3.498	3.500	3.524	n.d.	3.465	3.456	3.441
	H-5	4.196	4.187	4.198	4.240	4.232	4.282	4.272	4.265
	H-6	3.69	3.68	n.d.	3.84	n.d.	3.931	3.927	3.928
	H-6′	3.628	3.65	n.d.	3.475	3.471	3.7	3.7	n.d.
	NAc	2.050	2.046	2.045	2.042	2.041	2.067	2.066	2.065
Gal^3	H-1	4.478	4.547	4.545	4.541	4.541	4.465	4.534	4.529
	H-3	3.671	4.122	4.123	4.117	4.105	3.66	4.116	4.113
	H-4	3.901	3.931	3.927	3.927	3.925	3.900	3.922	3.928
GlcNAc	H-1	–	–	–	–	–	4.560	4.559	4.550
	H-6	–	–	–	–	–	3.998	3.993	4.004
	NAc	–	–	–	–	–	2.064	2.066	2.062
Gal^4	H-1	–	–	–	–	–	4.470	4.470	4.545
	H-3	–	–	–	–	–	3.68	3.7	4.113
	H-4	–	–	–	–	–	3.925	3.931	3.956
NeuAc^{3,3}	H-3*ax*	–	1.800	1.800	1.800	1.799	–	1.801	1.800
	H-3*eq*	–	2.774	2.771	2.774	2.773	–	2.774	2.775
	NAc	–	2.034	2.033	2.032	2.031	–	2.033	2.033
NeuAc^6	H-3*ax*	–	–	–	1.692	1.692	–	–	–
	H-3*eq*	–	–	–	2.723	2.725	–	–	–
	NAc	–	–	–	2.032	2.031	–	–	–
NeuAc^{3,4}	H-3*ax*	–	–	–	–	–	–	–	1.800
	H-3*eq*	–	–	–	–	–	–	–	2.755
	NAc	–	–	–	–	–	–	–	2.031

conclusion that fraction V contains the tetrasaccharide-alditol:

NeuAcα(2→3)Galβ(1→3)
NeuAcα(2→6)
GalNAc-ol

Although sugar analysis (Table 2) shows the presence of additional carbohydrate material in fraction V, this could not be recognized in the NMR spectrum.

The spectrum of fraction III matches that of the linear trisaccharide-alditol B (see Table 3). Therefore it can be concluded that fraction III contains a constituent with the structure NeuAcα(2→3)Galβ(1→3)GalNAc-ol. Sugar analysis (Table 2) suggests the additional presence of *N*-type carbohydrates which could not be deduced from the spectrum.

The spectra of fractions I, II and VI show no signals arising from other structural elements than those present in fractions III, IV and V. Signals stemming from *N*-glycosidic chains could not be detected in the spectra of fractions I, II and VI, although the latter must contain a certain amount of *N*-glycosidic carbohydrate chains (see Table 2).

The spectrum of the mixture of oligosaccharide-alditols (Fig. 3) contains all the signals, even the smaller ones, that were found in one or more of the spectra of fractions I – VI. This implies that recording a spectrum before fractionation may give relevant information about the composition of the mixture. This information can be useful for the fractionation strategy.

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