Eur. J. Biochem. *168*, 679–685 (1987)

Characterization of *N*-linked gluco-oligomannose type of carbohydrate chains of glycoproteins from the ovary of the starfish *Asterias rubens* (L.)

Pieter DE WAARD, Johannis P. KAMERLING, Herman VAN HALBEEK, Johannes F. G. VLIEGENTHART and Jan J. S. BROERTJES
Departments of Bio-Organic Chemistry and Chemical Animal Physiology, Utrecht University

(Received April 8/June 22, 1987) – EJB 87 0418

Glycoproteins were isolated from the ovary of the starfish *Asterias rubens* (L.). After delipidation, sugar analysis revealed the presence of mannose, glucose and *N*-acetylglucosamine in a molar ratio of 9.0:1.3:2.3. Subsequently, hydrazinolysis, re-*N*-acetylation, reduction and high-voltage paper electrophoresis were carried out, resulting in a mixture of neutral oligosaccharide alditols which was fractionated on Bio-Gel P-4. The alditols, investigated by 500-MHz ^{1}H-NMR spectroscopy, turned out to be of the oligomannose type or of the gluco-oligomannose type containing 9 mannose and 1–3 glucose residues. The most abundant compounds were established to be:

```
Manα1→2Manα1→6
                \
                 Manα1→6
                /        \
Manα1→2Manα1→3            \
                           Manβ1→4GlcNAcβ1→4GlcNAc-ol
                          /
Manα1→2Manα1→2Manα1→3
```

and

```
Manα1→2Manα1→6
                \
                 Manα1→6
                /        \
Manα1→2Manα1→3            \
                           Manβ1→4GlcNAcβ1→4GlcNAc-ol
                          /
Glcα1→3Manα1→2Manα1→2Manα1→3
```


Vitellogenesis in oviparous animals takes place by deposition of yolk precursors in the oocytes. Generally vitellogenins are synthesized in the liver or in organs with a similar function [1, 2]. Recently the occurrence of vitellogenic substances in the eggs and ovaries of the starfish *Asterias rubens* (L.) has been reported [3, 4]. Spermatozoa and testes did not contain these products. The complex compounds, isolated from ovary homogenates, turned out to be glycolipoproteins having a lipid content of about 60% [5]. Sugar analysis of the delipidated fractions revealed the presence of Man, GlcNAc and Glc only. Since the occurrence of *N*-linked gluco-oligomannose type of carbohydrate chains in the starfish *A. rubens* (L.) was hypothesized [5], the characterization of the neutral carbohydrate chains of one of the delipidated glycoproteins is reported in more detail. A preliminary communication has appeared [6].

Correspondence to J. P. Kamerling, Afdeling Bio-Organische Chemie, Rijksuniversiteit Utrecht, Transitorium III, Postbus 80.075, NL-3508 TB Utrecht, The Netherlands

Abbreviations. Man, D-mannose; GlcNAc, *N*-acetyl-D-glucosamine; GlcNAc-ol, *N*-acetyl-D-glucosaminitol; Glc, D-glucose.

MATERIALS AND METHODS

Isolation of vitellogenic substances

Specimens of *A. rubens* (L.) were collected from the Wadden Sea (The Netherlands). The ovaries, removed just prior to spawning, were stored at −20°C until use. Subsequently homogenates were fractionated on a Sepharose 2B column connected in series to a Sepharose 4B column and purified on a Sephacryl S-400 column, essentially as described earlier [4]. The glycolipoproteins obtained in this way were delipidated according to [7].

Hydrazinolysis procedure and fractionation of carbohydrate chains

Hydrazinolysis was carried out according to [8]. Briefly, thoroughly dried glycoprotein (60 mg) was suspended in

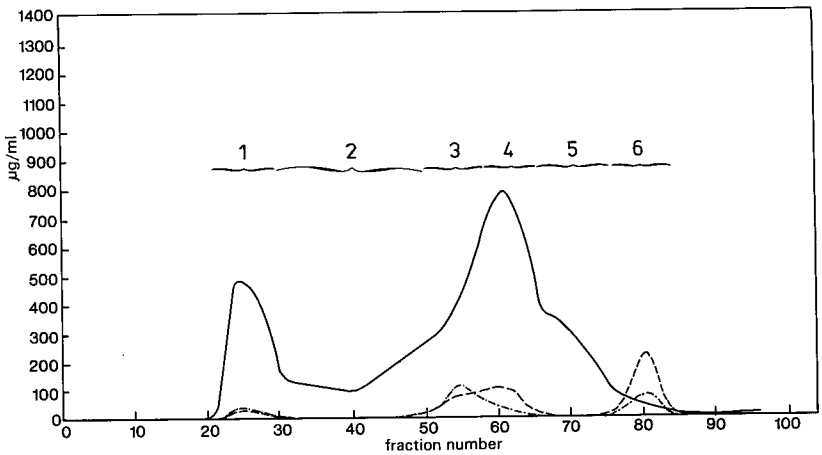


Fig. 1. *Typical elution pattern of the supernatant from a homogenate of ovaries, obtained before spawning from* A. rubens *(L.) on Sepharose 2B (85 × 2.6 cm) and 4B (85 × 2.6 cm) columns connected in series.* As eluent a 10 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 6.8, containing 1 mM EDTA and 0.2% NaN_3 was used. Elution was carried out at 4°C and at a flow rate of 15 ml/h. (——) Protein (595 nm); (–·–·–) lipid (436 nm); (– – – –) carbohydrate (585 nm). For details, see [3]. The quantitative estimation of lipid is disturbed by azide [13]

0.5 ml anhydrous hydrazine and heated for 8 h at 100°C. After evaporation of hydrazine, the material was re-*N*-acetylated and reduced. For the reduction with ^{3}H-labelled $NaBH_4$ 20% of the sample was dissolved in 200 µl 0.08 M NaOH and treated with $NaBH_4$ containing 1.7 mCi NaB^3H_4 in 200 µl *N,N*-dimethylformamide. The remaining part was reduced with NaB^2H_4. To facilitate the detection of oligosaccharide alditols, ^{3}H-labelled analogs were added. High-voltage paper electrophoresis (Whatman 3MM paper, 70 V/cm, 90 min) was carried out at pH 5.4 in a buffer consisting of pyridine/acetic acid/water (3:1:387, by vol.). The neutral oligosaccharide fraction was applied to two connected columns (100 × 2 cm each) of Bio-Gel P-4 (under 400 mesh; Bio-Rad) using water as eluent at a flow rate of 30 ml/h at 55°C [9]. Radioactivity was measured with a Minaxi Tri-Carb 4000 Series (Packard) liquid scintillation counter.

Sugar analysis

Glycoproteins or oligosaccharide alditols were subjected to methanolysis (1.0 M methanolic HCl, 24 h, 85°C) followed by gas-liquid chromatography of the trimethylsilylated (re-*N*-acetylated) methyl glycosides on a capillary CPsil5CB WCOT fused silica column (0.34 mm × 25 m; Chrompack) [10].

500-MHz ^{1}H-NMR spectroscopy

Oligosaccharide alditols were repeatedly exchanged in ^2H_2O (99.96 atoms ^{2}H/100 atoms, Aldrich) with intermediate lyophilization. ^{1}H-NMR spectra were recorded on a Bruker WM-500 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands) operating at 500 MHz in the Fourier-transform mode at a probe temperature of 27°C. Resolution enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation [11]. Chemical shifts (δ) are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured indirectly relative to acetone in ^2H_2O ($\delta = 2.225$ ppm) [12].

RESULTS AND DISCUSSION

A typical elution pattern of the supernatant from a homogenate of ovaries of the starfish *A. rubens* (L.), obtained before spawning, on Sepharose 2B and 4B columns connected in series, is presented in Fig. 1. By comparison of elution patterns of homogenates of eggs, female gonads before and after spawning, spermatozoa and male gonads before and after spawning, the major yolk proteins turned out to be fractions 2, 3 and 4 [3]. Fraction 3 and part of fraction 4 are dissociation products of fraction 2. Fraction 3 was further purified on Sephacryl S-400 and then delipidated. Sugar analysis of purified fraction 3 demonstrated the occurrence of Man, Glc and GlcNAc residues in a molar ratio of 9.0:1.3:2.3. The carbohydrate content was established to be 4%.

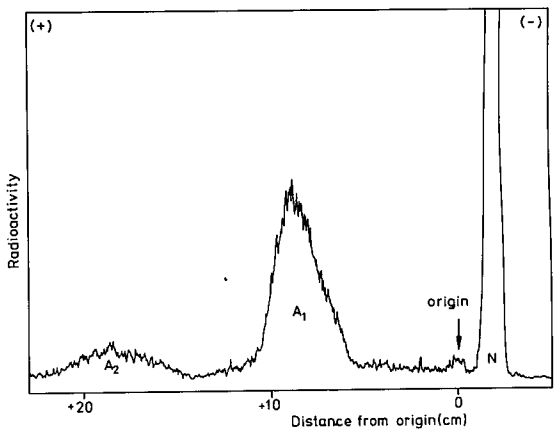


Fig. 2. *High-voltage paper electrophoresis of the mixture of ^{3}H-labelled oligosaccharide alditols, obtained from the yolk glycoprotein of* A. rubens *(L.) by the hydrazinolysis procedure.* Paper electrophoresis was carried out on Whatman 3MM paper at pH 5.4, using a buffer consisting of pyridine/acetic acid/water (3:1:387, by vol.), and at a potential of 70 V/cm for 90 min. N, neutral fraction; A_1 and A_2, acidic fractions

To characterize the structures of the *N*-linked carbohydrate chains, the glycoprotein material was subjected to the hydrazinolysis procedure [8]. After re-*N*-acetylation and reduction the released oligosaccharide alditols were separated by high-voltage paper electrophoresis at pH 5.4. As is evident from the analytical electropherogram, presented in Fig. 2, the mixture of oligosaccharide alditols consisted of a neutral fraction (66%) and of two acidic fractions (25% and 9% respectively). Because of the absence of sialic acid, the acidity

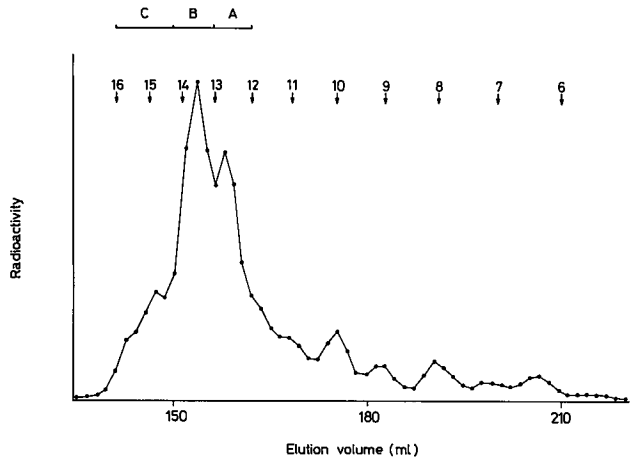


Fig. 3. *Bio-Gel P-4 column chromatography of the mixture of neutral ^{3}H-labelled oligosaccharide alditols derived from* A. rubens *(L.) yolk glycoprotein.* The column was eluted with bidistilled water at 55°C. Fractions of 1.3 ml were collected at a flow rate of 30 ml/h and assayed for ^{3}H radioactivity. The arrows at the top indicate the eluting positions of glucose oligomers derived from a dextran hydrolysate. The numbers indicate the glucose units. In preparative runs, fractions A, B and C were pooled as indicated

Table 1. *Survey of the sizes of the neutral oligosaccharide alditols derived from the yolk glycoprotein of* A. rubens *(L.) as estimated on Bio-Gel P-4 (under 400 mesh)*
For the calibration of the column a series of glucose oligomers derived from a dextran hydrolyzate were used. The column was eluted with bidistilled water at 55°C. Fractions of 1.3 ml were collected at a flow rate of 30 ml/h and assayed for ^{3}H-radioactivity. For comparison, literature values have been included [9]. In addition the ratios of the different compounds are indicated in percentages

Compounds	Size in Glc units	Size from literature values	Amount
			%
$Man_2GlcNAcGlcNAc$-ol	6.4	–	2.8
$Man_3GlcNAcGlcNAc$-ol	7.1	7.3	2.5
$Man_4GlcNAcGlcNAc$-ol	8.0	–	4.3
$Man_5GlcNAcGlcNAc$-ol	9.1	9.0	3.8
$Man_6GlcNAcGlcNAc$-ol	10.0	9.9	7.2
$Man_7GlcNAcGlcNAc$-ol	11.0	10.9	5.2
$Man_8GlcNAcGlcNAc$-ol	11.9	11.9	5.7
$Man_9GlcNAcGlcNAc$-ol	12.6	12.3	24.9
$Glc_1Man_9GlcNAcGlcNAc$-ol	13.5	–	29.4
$Glc_2Man_9GlcNAcGlcNAc$-ol	14.7	–	7.8
$Glc_3Man_9GlcNAcGlcNAc$-ol	15.4	–	6.5

has to be ascribed to acidic (in)organic substituents. These fractions are still under investigation.

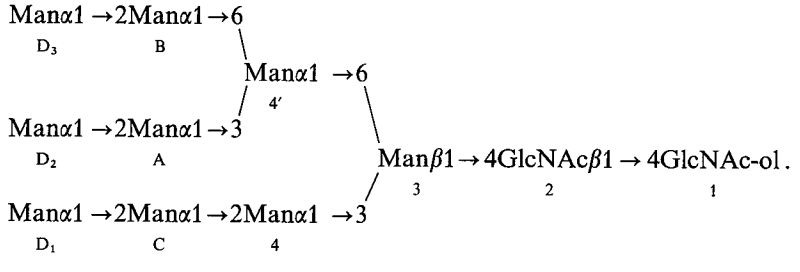
The mixture of neutral oligosaccharide alditols (Man:Glc:GlcNAc:GlcNAc-ol = 9.0:1.5:0.7:0.7) was fractionated on Bio-Gel P-4. The analytical elution profile of this mixture is shown in Fig. 3. On the basis of sugar analysis data and literature values of the eluting positions of model $Man_xGlcNAcGlcNAc$-ol substances relative to those of dextran hydrolysate oligomers [9], the chromatogram suggests the presence of a series of oligomannose-type carbohydrate chains varying from $Man_2GlcNAcGlcNAc$-ol up to and including $Man_9GlcNAcGlcNAc$-ol (Table 1). Besides these compounds even larger structures occur. In preparative runs fractions containing the high-molecular-mass oligosaccharide alditols were pooled as indicated in Fig. 3. If necessary, rechromatography was carried out.

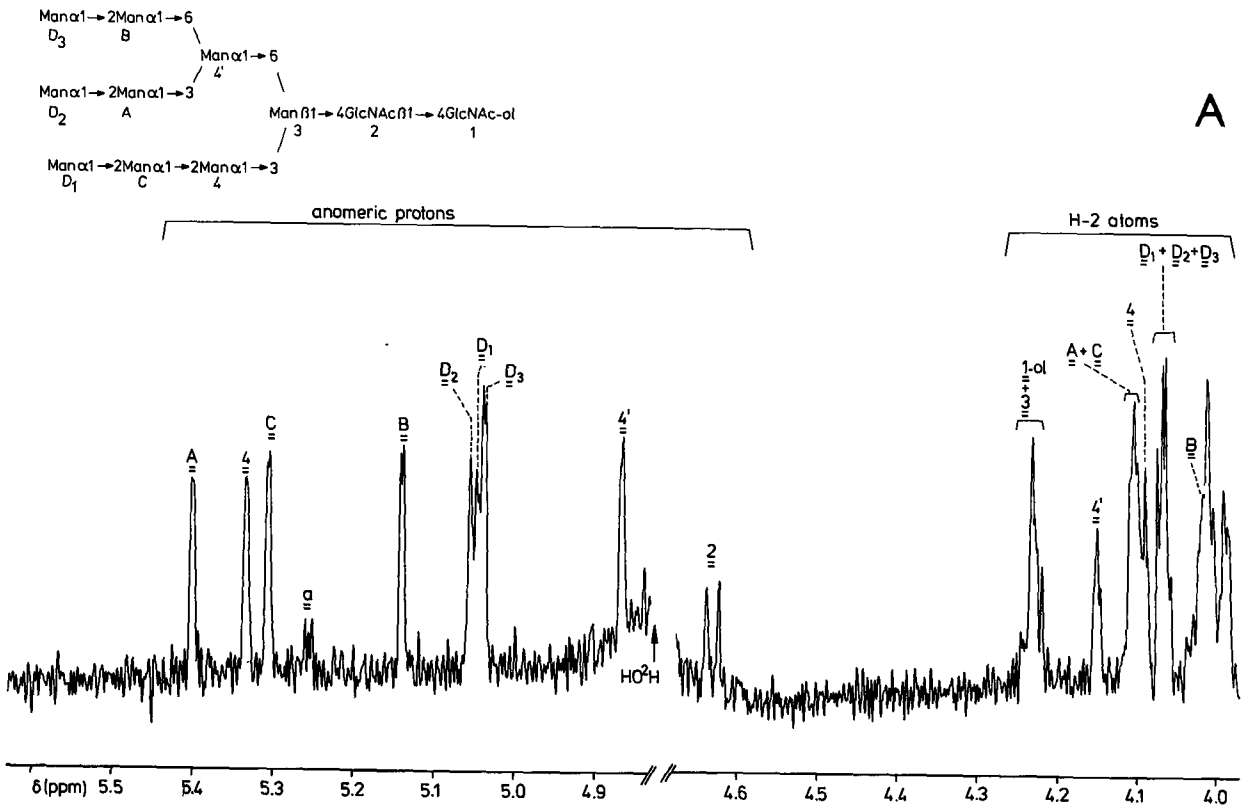
The fractions A, B and C were subjected to 500-MHz ^{1}H-NMR spectroscopy. The chemical shifts of the structural-reporter-group protons of the compounds from fractions A, B and C are summarized in Table 2, together with the data of some reference compounds [14–16].

In Fig. 4A the ^{1}H-NMR spectrum of fraction A is depicted. Comparison of the ^{1}H-NMR data of fraction A with those of $Man_9GlcNAcGlcNAc$-ol, obtained from prostaglandin endoperoxide synthase (R_1) [14] (Table 2), established the following structure as one of the major carbohydrate chains in the starfish glycoprotein material:

In the spectrum a low-intensity signal is present at $\delta = 5.256$ ppm, indicating the presence of a small amount of contamination with fraction B (see below).

The ^{1}H-NMR spectrum of fraction B is shown in Fig. 4B. The spectral features of the compound present in fraction B have much in common with those reported for Man_9-GlcNAcGlcNAc-ol (see Table 2). Besides the signals of the structural-reporter groups of the nine mannose units (H-1 and H-2) and the GlcNAc-2 (H-1, NAc) and GlcNAc-ol-1 (H-2, NAc) residues, in the anomeric region an additional doublet ($J_{1,2} = 4.0$ Hz) is observed at $\delta = 5.256$ ppm, having an equal intensity to the other anomeric signals. In view of the sugar analysis data this doublet is assigned to an α-linked Glc residue (Glc-a). Comparison of the ^{1}H-NMR data of $Man_9GlcNAcGlcNAc$-ol (fraction A) and fraction B shows that only the H-1 and H-2 signals of Man-D_1 differ in chemical shift, indicating that Glc-a is linked to Man-D_1. The attachment leads to a specific small upfield shift ($\Delta\delta = -0.007$ ppm) for Man-D_1 H-1; as a result the signal now coincides with that of Man-D_3 H-1 (compare Fig. 4A and B). The Man-D_1 H-2 signal undergoes a downfield shift ($\Delta\delta = +0.165$ ppm) to $\delta = 4.235$ ppm, being the resonance position of the H-2 signals of Man-3 and GlcNAc-ol-1. The chemical shift of Man-D_1 H-2 was established by a selective ^{1}H-decoupling experiment on Man-D_1 H-1. The downfield shift observed for the Man-D_1 H-2 signal is similar to that observed for Man-3 H-2 in the case of extension with α1→3-linked Man-4 [12].





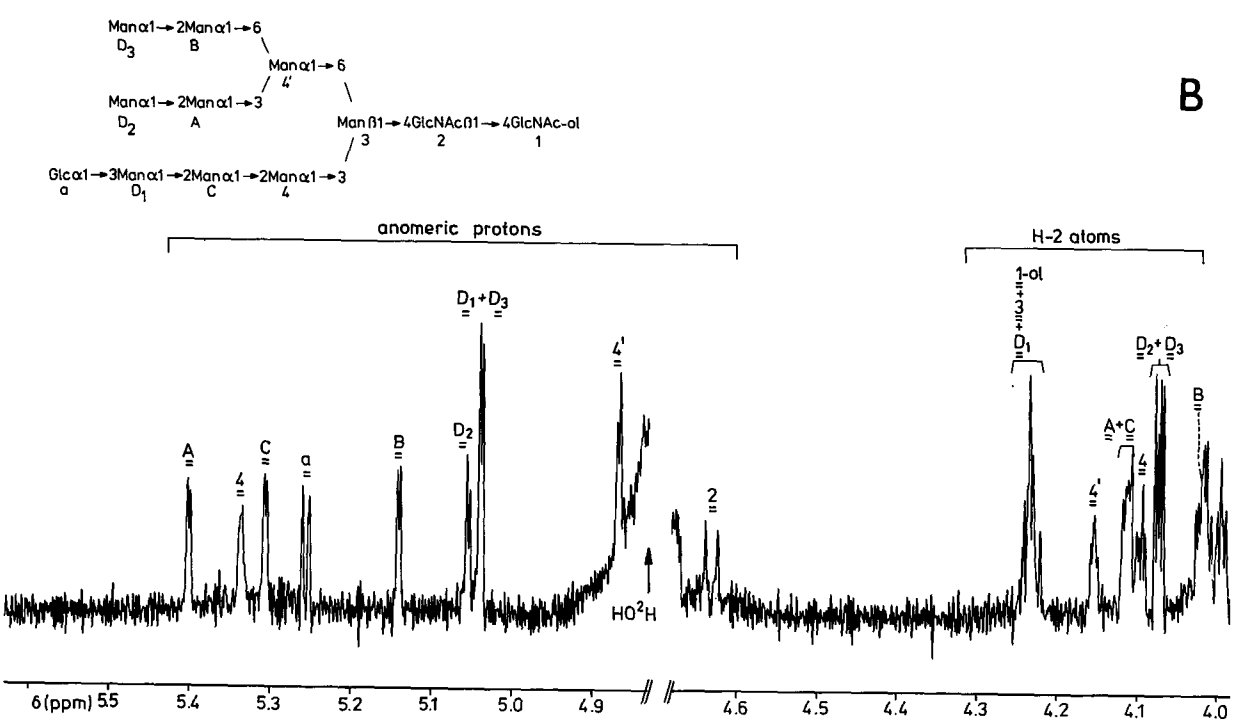


Fig. 4. *Structural-reporter-group regions of the resolution-enhanced 500-MHz* ^{1}*H-NMR spectra of oligosaccharide alditol fractions derived from* A. rubens *(L.) yolk glycoprotein. (A) Fraction A; (B) Fraction B; (C) Fraction C.* The figures and letters in the spectra refer to the corresponding residues in the structures. The quartet at $\delta = 4.10$ ppm, marked by ∅, stems from a frequently occurring, unknown non-carbohydrate, non-protein contaminant

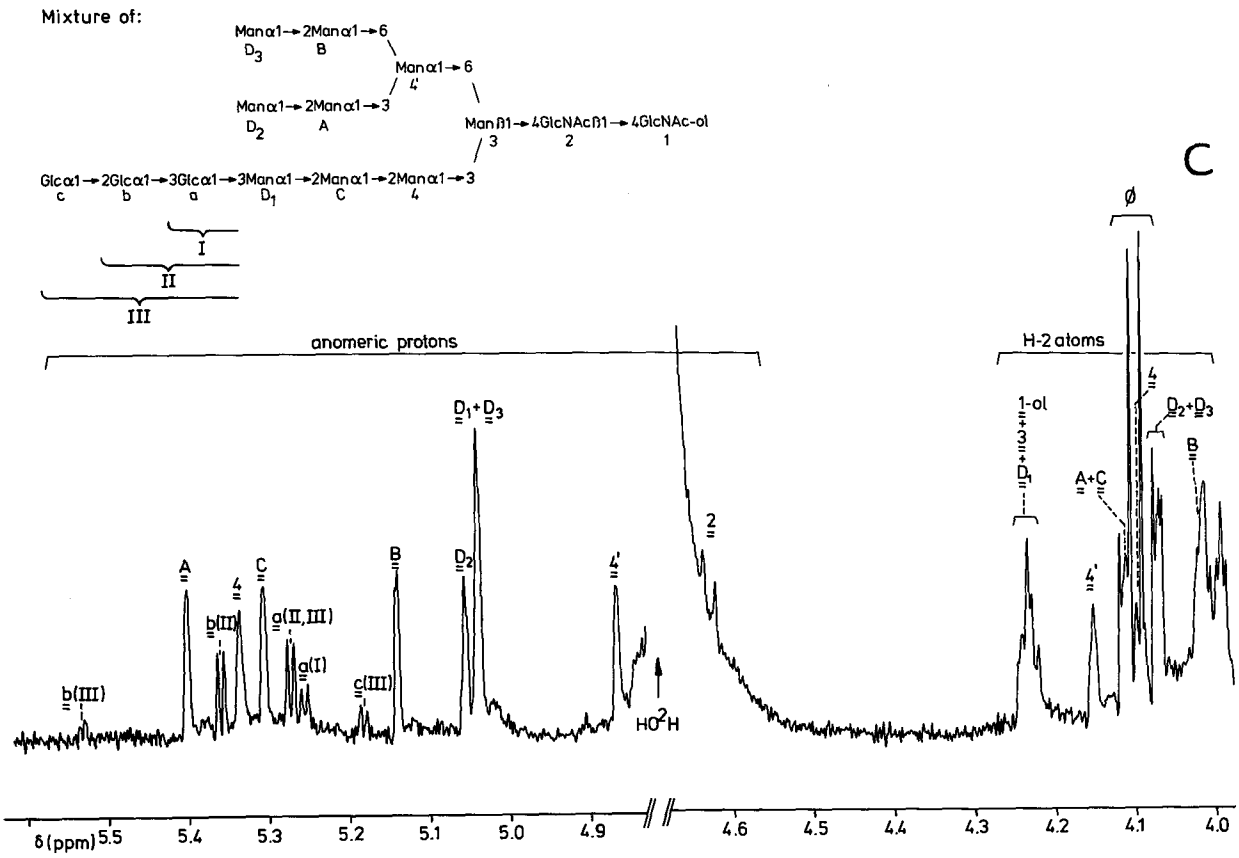
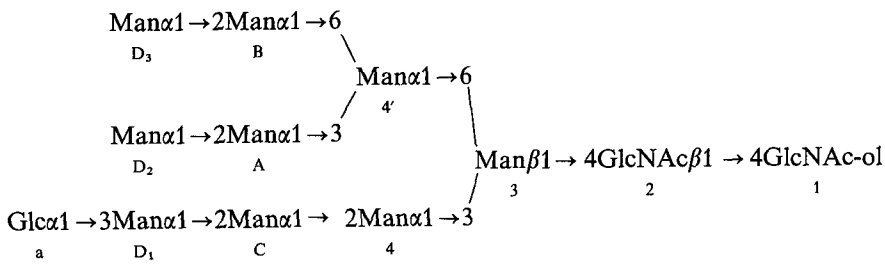


Fig. 4C

Therefore, the compound present in fraction B has the structure:



The ^{1}H-NMR spectrum of fraction C (Fig. 4C) shows the presence of a mixture of compounds having a $Man_9GlcNAcGlcNAc$-ol unit in common. This is evident from the chemical shift values of the structural-reporter-group signals of the nine Man residues, the GlcNAc-2 and the GlcNAc-ol-1 unit (Table 2). Furthermore, five doublets occur in the α-anomeric region with coupling constants $J_{1,2}$ = 4.0 Hz, but differing in intensity. Taking into account the sugar analysis data, these signals are assigned to α-Glc residues. The signal at $\delta = 5.183$ ppm ($J_{1,2} = 4.0$ Hz) corresponds to H-1 of an α1←2-linked terminal Glc residue in the reference compounds Glcα1→2Glcα1→3Glcα1→3Manα1→2Manα1→2Man (R$_4$) [15], $Glc_3Man_{10}GlcNAc$ derived from the cell-wall mannoprotein of a *Saccharomyces cerevisiae* mutant (R$_2$) [16] and $Glc_3Man_9GlcNAc_2$ from porcine thyroid gland [17]. On the same basis, signals at $\delta = 5.531$ ppm ($J_{1,2} = 4.0$ Hz) and $\delta = 5.273$ ppm ($J_{1,2} = 4.0$ Hz) can be assigned to the anomeric protons of α1→3-linked Glc residues. From the low intensity of the signals of Glc-b ($\delta = 5.531$ ppm) and Glc-c ($\delta = 5.183$ ppm) it can be concluded that fraction C contains as a minor component:

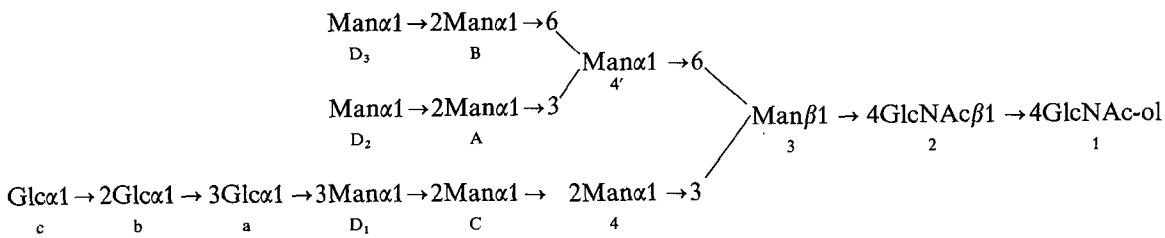


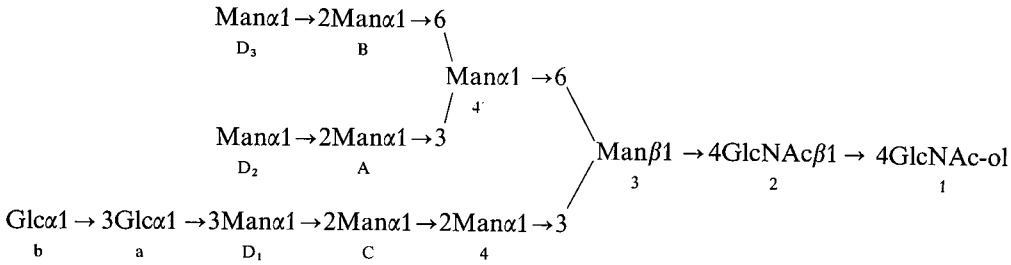
Table 2. ^{1}*H-chemical shifts of structural-reporter groups of the constituent monosaccharides for the oligosaccharide alditol fractions A, B and C derived from the yolk glycoprotein of* A. rubens *(L.), together with those for the reference compounds* $R_1 - R_4$

R_1 corresponds to fraction F1 from sheep prostaglandin endoperoxide synthase [14], R_2 and R_3 are derived from a *S. cerevisiae* mannoprotein [16], whereas R_4 is a synthesized hexasaccharide from [15]. Chemical shifts are given at 27°C, in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate in ^2H_2O. Compounds are represented by a letter/figure symbolic notation

Reporter group	Residue	Chemical shift in							
		R_1	A	B	C		R_2[a]	R_3[a]	R_4[b]
		D_3-B 4′ D_2-A 3-2-1ol D_1-C-4	D_3-B 4′ D_2-A 3-2-1ol D_1-C-4	D_3-B 4′ D_2-A 3-2-1ol a-D_1-C-4	D_3-B 4′ D_2-A 3-2-1ol b-a-D_1-C-4	D_3-B 4′ D_2-A 3-2-1ol c-b-a-D_1-C-4	c-b-a-D_1-	b-a-D_1-	c-b-a-D_1-
		ppm							
NAc	1-ol	2.055	2.055	2.055	2.055	2.055			
	2	2.067	2.068	2.068	2.068	2.068			
H-1	2	4.630	4.630	4.630	4.630	4.630			
	3	4.77	n.d.	n.d.	n.d.	n.d.			
	4	5.333	5.333	5.337	5.336	5.336			
	4′	4.867	4.866	4.867	4.867	4.867			
	A	5.400	5.402	5.401	5.401	5.401			
	B	5.140	5.142	5.140	5.140	5.140			
	C	5.307	5.305	5.306	5.306	5.306			
	D_1	5.045	5.045	5.038	5.040	5.040	5.04	n.d	5.049
	D_2	5.054	5.052	5.054	5.056	5.056			
	D_3	5.038	5.038	5.038	5.040	5.040			
	a	–	–	5.256	5.273	5.273	5.27	5.26	5.283
	b	–	–	–	5.359	5.531	5.52	5.34	5.543
	c	–	–	–	–	5.183	5.17	–	5.197
H-2	1-ol	4.232	4.23	4.23	4.23	4.23			
	3	4.232	4.23	4.23	4.23	4.23			
	4	4.094	4.091	4.094	4.095	4.095			
	4′	4.153	4.152	4.153	4.153	4.153			
	A	4.104	4.108	4.112	4.113	4.113			
	B	4.020	4.015	4.019	4.018	4.018			
	C	4.104	4.108	4.112	4.113	4.113			
	D_1	4.071	4.070	4.235	4.23	4.23			
	D_2	4.068	4.070	4.071	4.072	4.072			
	D_3	4.064	4.070	4.071	4.072	4.072			

[a] Recorded at 40°C (acetone, $\delta = 2.217$ ppm).
[b] Sodium 2,2,3,3-tetra-deutero-4,4-dimethyl-4-silapentanoate as internal standard (20°C).

The intensity of the signal of Glc-a H-1 ($\delta = 5.273$ ppm) is too high to be explained by the presence of this structure only. Therefore, another structure with an α-Glc residue with a similar chemical shift must be present in fraction C. Comparison with the ^{1}H-NMR data of the reference compound $Glc_2Man_{10}GlcNAc$ (R_3) [16] reveals that the anomeric proton of the α1→3-linked Glc-a residue has an equivalent chemical shift. The signal of the H-1 of the terminal Glc-b residue of this reference compound is in accordance with the signal at $\delta = 5.359$ ppm, having about the same intensity as the signal for Glc-a H-1. Therefore the main component of fraction C is:



Finally the chemical shift of the fifth α-Glc H-1 signal at $\delta = 5.256$ ppm is identical to that of terminal α1→3-linked Glc-a H-1 as discussed for fraction B. So $Glc_1Man_9GlcNAc$-GlcNAc-ol is present as a second minor component in fraction C.

^{1}H-NMR analysis of the remaining fractions containing oligomannose-type carbohydrate chains with fewer than nine mannose residues (data not shown) reveals that the only structure present with eight mannose residues is Man_8-GlcNAcGlcNAc-ol lacking the Man-D_3 residue specifically. This observation suggests that in *A. rubens* (L.) the processing of $Man_9GlcNAc_2$ starts with the enzymatic removal of Man-D_3.

The occurrence of Glc-containing oligomannose-type carbohydrate chains as a natural part of glycoproteins in the ovary of a starfish is surprising. Usually the Glc units are removed in the early processing steps of the asparagine-linked carbohydrate chain biosynthesis [18]. Since the removal of Glc-b and Glc-c is a faster process than the removal of the Glc-a unit [19, 20], it can be anticipated that $Glc_1Man_9GlcNAcGlcNAc$-ol occurs as main component. The latter is considered to be a key intermediate in processing the gluco-oligomannose type of carbohydrate chains [21]. After completion of the manuscript, a study appeared concerning the carbohydrate chains of a major glycoprotein present in the egg jelly coat of the starfish *A. amurensis*, showing similar findings with respect to the high-molecular-mass neutral oligosaccharide alditols [22].

This investigation was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO) and the Netherlands Foundation for Cancer Research (KWF, grant UUKC 83-13).

REFERENCES

1. Wallace, R. A. (1978) in *The vertebrate ovary* (Jones, R. E., ed.), pp. 469–502, Plenum Press, New York.
2. Engelmann, F. (1979) *Adv. Insect Physiol. 14*, 49–108.
3. Broertjes, J. J. S., De Waard, P. & Voogt, P. A. (1984) *J. Mar. Biol. Ass. UK 64*, 261–269.
4. Broertjes, J. J. S., De Waard, P. & Voogt, P. A. (1984) *Marine Biol. Lett. 5*, 99–104.
5. Broertjes, J. J. S., De Waard, P., Kamerling, J. P. & Voogt, P. A. (1985) *J. Mar. Biol. Ass. UK 65*, 79–83.
6. De Waard, P., Van Halbeek, H., Broertjes, J. J. S., Kamerling, J. P. & Vliegenthart, J. F. G. (1985) in *Glycoconjugates, Proc. VIIIth Int. Symp.* (Davidson, E. A., Williams, J. C. & Di Ferrante, N. M., eds), vol. 1, pp. 23–24, Praeger, New York.
7. Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.
8. Takasaki, S., Mizuochi, T. & Kobata, A. (1982) *Methods Enzymol. 83*, 263–268.
9. Yamashita, K., Mizuochi, T. & Kobata, A. (1982) *Methods Enzymol. 83*, 105–127.
10. Kamerling, J. P. & Vliegenthart, J. F. G. (1982) *Cell Biol. Monogr. 10*, 95–125.
11. Ernst, R. R. (1966) *Adv. Magnet. Res. 2*, 1–135.
12. Vliegenthart, J. F. G., Dorland, L. & Van Halbeek, H. (1983) *Adv. Carbohydr. Chem. Biochem. 41*, 209–374.
13. Broertjes, J. J. S., De Waard, P. & Voogt, P. A. (1983) *Experientia (Basel) 39*, 594–595.
14. Mutsaers, J. H. G. M., Van Halbeek, H., Kamerling, J. P. & Vliegenthart, J. F. G. (1985) *Eur. J. Biochem. 147*, 569–574.
15. Ogawa, T., Nukada, T. & Kitajima, T. (1983) *Carbohydr. Res. 123*, C12–C15.
16. Tsai, P. K., Ballou, L., Esmon, B., Schekman, R. & Ballou, C. E. (1984) *Proc. Natl Acad. Sci. USA 81*, 6340–6343.
17. Ronin, C., Van Halbeek, H., Mutsaers, J. H. G. M. & Vliegenthart, J. F. G. (1987) *Glycoconj. J.*, in the press.
18. Berger, E. G., Buddecke, E., Kamerling, J. P., Kobata, A., Paulson, J. C. & Vliegenthart, J. F. G. (1982) *Experientia (Basel) 38*, 1129–1162.
19. Kornfeld, S., Li, E. & Tabas, I. (1978) *J. Biol. Chem. 253*, 7771–7778.
20. Hubland, S. C. & Robbins, P. W. (1979) *J. Biol. Chem. 254*, 4568–4576.
21. Ronin, C., Stannard, B. S., Rosenbloom, I. L., Magner, J. A. & Weintraub, B. D. (1984) *Biochemistry 23*, 4503–4510.
22. Endo, T., Hoshi, M., Endo, S., Arata, Y. & Kobata, A. (1987) *Arch. Biochem. Biophys. 252*, 105–112.