

Primary structure of 21 novel monoantennary and diantennary N-linked carbohydrate chains from α_D -hemocyanin of *Helix pomatia*

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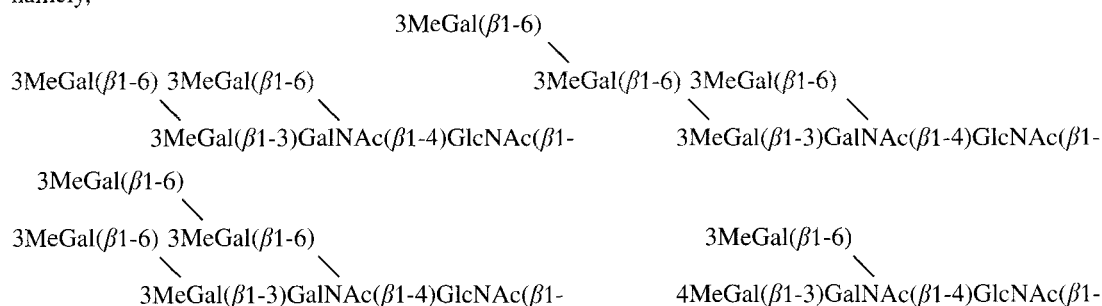
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The primary structures of 21 novel monoantennary and diantennary N-glycans of the glycoprotein α_D -hemocyanin (α_D -Hc) of *Helix pomatia* have been determined. Outer oligosaccharide fragments (antennae) were released from the glycoprotein by Smith degradation of an α_D -Hc pronase digest. The major antenna, obtained following HPLC fractionation on Lichrosorb-NH₂, was characterized using ¹H-NMR spectroscopy, fast-atom-bombardment mass spectrometry, and linkage analysis, and corresponds to a pentasaccharide fragment. The intact carbohydrate chains of α_D -Hc were released with peptide-N⁴-(N-acetyl- β -glucosaminyl)asparagine amidase-F digestion, separated from the protein on Bio-Gel P-100, and subfractionated on Bio-Gel P-4. A portion of subfractions was reduced with sodium borodeuteride, and the non-reduced and reduced samples were further fractionated on CarboPac PA-1, Lichrosorb-NH₂/Lichrosphere-NH₂, and/or Lichrosphere-C₁₈. Purified oligosaccharides and oligosaccharide-alditols were analyzed using 500/600-MHz ¹H-NMR spectroscopy. In total, four novel types of antenna were identified, namely,



which are all attached to O-2 of α Man residues of the trimannosyl-N,N'-diacetylchitobiose core element, which is generally β -1,2-xylosylated and α -1,6-fucosylated, Man(α 1-6)[Man(α 1-3)] $\pm$ Xyl(β 1-2)]Man(β 1-4)GlcNAc(β 1-4)[$\pm$ Fuc(α 1-6)]GlcNAc.

Keywords: glycoprotein; *Helix pomatia*; hemocyanin; N-linked carbohydrate chains.

Hemocyanin (Hc) is the high-molecular-mass (4.5×10^7 to 9×10^3 kDa) copper-containing oxygen-transporting protein that is freely dissolved in the hemolymph of several arthropod and mollusc species [1–3]. Most Hcs are glycoproteins, but to date,

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Abbreviations. Hc, hemocyanin; α_D -Hc, α_D -hemocyanin; 3MeGal, 3-O-methyl-D-galactose; 4MeGal, 4-O-methyl-D-galactose; PNGase-F, peptide-N⁴-(N-acetyl- β -glucosaminyl)asparagine amidase-F; IU, international unit; HOHAHA, homonuclear Hartmann-Hahn; ROESY, rotating-frame nuclear Overhauser enhancement spectroscopy; MLEV, composite pulse devised by Malcolm Levitt; WEFT, water-eliminated Fourier transform; 1D, one-dimensional; 2D, two-dimensional; FAB, fast-atom-bombardment; HPAEC, high-pH anion-exchange chromatography; PAD, pulsed amperometric detection; Me₃Si, trimethylsilyl; ROE, rotating-frame nuclear Overhauser enhancement.

Enzymes. Peptide-N⁴-(N-acetyl- β -glucosaminyl)asparagine amidase F (EC 3.5.1.52); pronase (EC 3.4.24.31).

solid information with respect to the significance of glycosylation is not available. It is notable that there are large differences in carbohydrate content and in monosaccharide composition of the Hcs from arthropods and molluscs [4].

The carbohydrate content of arthropod Hcs is usually low, and only D-Man and D-GlcNAc are detected [4]. Structural analysis of the glycan chains of the Hcs of *Androctonus australis* [5], *Panulirus interruptus* [6, 7] and *Astacus leptodactylus* [8] revealed N-linked glycans of the oligomannose type, which may also be sulfated [7].

The carbohydrate content of mollusc Hc is higher than that of the Hcs of most arthropods, and furthermore, especially the Hcs of *Helix pomatia* and *Lymnaea stagnalis* contain monosaccharides not usually found in animal glycoproteins [*H. pomatia*: D-Xyl and 3-O-methyl-D-galactose (3MeGal); *L. stagnalis*: D-Xyl, 3-O-methyl-D-mannose (3MeMan), and 3MeGal] [4, 9, 10]. Analyses of the Hc glycans from both molluscs demonstrated for the first time the presence of xylose-containing N-glycans in animal species [10–12]. Their low-molecular-mass glycans

consist of $\text{Xyl}(\beta 1-2)[\text{Man}(\alpha 1-6)][\text{Man}(\alpha 1-3)]\text{GlcNAc}(\beta 1-4)\text{GlcNAc}$, with a partially α -1,6-fucosylated Asn-linked GlcNAc residue (in *H. pomatia*) or with 3-*O*-methylated α Man residues (in *L. stagnalis*). The structure determination of the high-molecular-mass carbohydrate chains of *L. stagnalis* Hc revealed unusual antennae linked to O-2 of the α Man residues, namely, $\text{Fuc}(\alpha 1-2)\text{Gal}(\beta 1-3)\text{GalNAc}(\beta 1-4)\text{GlcNAc}(\beta 1-4)$ and $3\text{MeGal}(\beta 1-3)\text{GalNAc}(\beta 1-4)\text{GlcNAc}(\beta 1-4)$ [13].

Hemocyanin of *H. pomatia* (molecular mass about 9×10^3 kDa) consists of two types, designated α ($\alpha_D + \alpha_N$) and β (β_C) [14–16]. In contrast to α_N -Hc and β_C -Hc, under certain conditions α_D -Hc can be dissociated into 1/2-molecules. The amino acid compositions of both forms are very similar [17]. α_D -Hc and α_N -Hc give rise to similar monosaccharide ratios, but β_C -Hc gave higher values for 3MeGal, GlcNAc and GalNAc [18]. In order to study the physiological relevance of glycosylation of the secreted protein, a knowledge of the primary structures of the glycans is a prerequisite. In our previous study of the glycosylation of α_D -Hc of *H. pomatia* [11], which showed a carbohydrate content of about 9% (by mass) [19], it was shown that only N-linked carbohydrate chains occur, consisting of low-molecular-mass core structures (see above) and of very complex high-molecular-mass antennary glycans, containing, in addition to the Xyl, Man, GlcNAc and Fuc residues, 3MeGal and GalNAc as well. The primary structural analysis of the monoantennary and diantennary N-glycans of α_D -Hc are the subject of this report.

EXPERIMENTAL PROCEDURES

Materials. α_D -Hemocyanin (α_D -Hc) was isolated as described [20], lyophilized in the presence of a 2.75-fold excess of sucrose over glycoprotein (by mass), and stored at -20°C . Its purity was established using SDS/PAGE [21], and a single band at a molecular mass of about 450 kDa was detected. Peptide- N^4 -(*N*-acetyl- β -glucosaminyl)asparagine amidase-F (PNGase-F) from *Flavobacterium meningosepticum* and pronase from *Streptomyces griseus* were obtained from Boehringer Mannheim.

Preparation of copper-free α_D -hemocyanin. To prepare suitable starting material, lyophilized portions of α_D -Hc/sucrose (570, 3260 and 3000 mg, containing 152, 870 and 800 mg α_D -Hc, respectively) were dialyzed at 4°C as follows. α_D -Hc/sucrose was dissolved in 5 ml 50 mM Tris/HCl, pH 7.0, and the blue solution was dialyzed for a total of 48 h against 2 l of the same buffer, with three changes of buffer. Copper was removed from α_D -Hc by dialysis for a total of 24 h against 2 l 50 mM Tris/HCl, pH 7, containing 10 mM CaCl_2 and 10 mM KCN, with three changes of buffer, resulting in a colorless opalescent solution.

Pronase digestion. Copper-free α_D -Hc (152 mg) was denatured by dialysis for 3 h at 4°C against 2 l 100 mM NaHCO_3 , and subsequently for 2.5 h against 2 l 6 M urea. Finally, the solution was dialyzed for a total of 48 h against 2 l 100 mM NH_4OAc , pH 8.0, with three changes of buffer. A solution of pronase (80 mg) in 10 ml 100 mM NH_4OAc , pH 8.0, containing 15 mM CaCl_2 , was kept for 1 h at 40°C , and subsequently stored at 4°C . The heat treatment was carried out to inactivate any contaminating glycosidases. Aliquots (2.5 ml) of the Pronase solution were added to denatured α_D -Hc in 20 ml of the same buffer at 0, 4, 24, and 48 h. The incubation was carried out for 70 h at 40°C , maintaining the pH between 7.4 and 8.0. To inactivate the proteolytic activity, the solution was held for 2 min at 100°C , and, after concentration, passed through a column (100 cm $\times$ 2 cm) of Bio-Gel P-6 (200–400 mesh, Bio-Rad)

using water as eluent at a flow rate of 18 ml/h. The orcinol/ H_2SO_4 -positive fractions were pooled and lyophilized. The residue was dissolved in 20 ml 100 mM NH_4OAc , pH 8.0, containing 15 mM CaCl_2 , and subjected to a second pronase digestion followed by fractionation. The final glycopeptide mixture was lyophilized.

Smith degradation. A solution of the α_D -Hc glycopeptide mixture (10 mg) in 0.1 M NaOAc buffer (25 ml; pH 5.5), containing 8 mM NaIO_4 , was held for 48 h at 4°C in the dark [22]. After destroying the excess periodate with ethylene glycol (125 μ l; 4°C , 24 h), the pH was adjusted to 7.5 with 0.1 M NaOH. The oxidized material was reduced with NaBH_4 (105 mg) for 24 h at 4°C , and the pH was adjusted to 5.5 with 4 M HOAc. The mixture was concentrated, and boric acid was removed by co-evaporation with methanol under reduced pressure (5×20 ml). An aqueous solution of the residue was desalted on a column (47 cm $\times$ 2.5 cm) of Bio-Gel P-2 (200–400 mesh, Bio-Rad) and lyophilized. The residue was dissolved in 300 μ l 90% (by vol.) formic acid, and held for 1 h at 40°C . After removal of formic acid by evaporation and co-evaporation with water (20 ml) under reduced pressure at 30°C , the residue was taken up in water (10 ml) and the pH was adjusted to 7.5 with 0.1 M NaOH. A second reduction was performed by addition of 41 mg NaBH_4 (24 h, 5°C), and the mixture was prepared as described above. The residue was fractionated on a Mono Q HR 5/5 anion-exchange column (Pharmacia FPLC system) by elution with water (6 ml), followed by a linear concentration gradient of NaCl (0 to 0.5 M in 10 ml). The neutral oligosaccharide fraction was lyophilized, and subfractionated on a Lichrosorb- NH_2 10- μ m column (25 cm $\times$ 0.46 cm, Chrompack) using a Kratos Spectroflow 400 HPLC system (ABI Analytical). Elutions were carried out isocratically with a mixture of acetonitrile and water (3:1, by vol.) at a flow rate of 1.5 ml/min, and monitoring the effluent at 206 nm with a Spectroflow 783 programmable absorbance detector (ABI Analytical).

Methylation analysis. Oligosaccharide S2 (about 80 μ g), generated by Smith degradation, was pertrideuteromethylated according to [23], using $\text{C}^2\text{H}_5\text{I}$ and solid NaOH in dimethylsulfoxide. An aliquot (30%) was analyzed by fast-atom-bombardment (FAB)-MS. The remaining material (70%) was hydrolyzed with 4 M trifluoroacetic acid (250 μ l) for 4 h at 100°C , and, after concentration, the obtained mixture of partially trideuteromethylated monosaccharides was reduced in 0.25 M NaB^2H_4 (100 μ l) for 2 h at room temperature. After concentration, co-concentration with methanol, and acetylation, the partially trideuteromethylated alditol acetates were analyzed using GLC-MS [24, 25].

Monosaccharide analysis. Sucrose-free samples of α_D -Hc containing 40 ng carbohydrate were subjected to methanolysis (1.0 M methanolic HCl, 24 h, 85°C) followed by re-*N*-acetylation. After trimethylsilylation, the samples were analyzed using GLC on a CP-Sil5 CB WCOT fused-silica capillary column (25 m $\times$ 0.34 mm, Chrompack) [24].

PNGase-F digestion. Copper-free α_D -Hc (870 mg and in a separate batch 800 mg) was dialyzed against 2 l 50 mM Tris/HCl, pH 7.0, containing 50 mM EDTA and 0.02% (mass/vol.) NaN_3 , for a total of 48 h at 4°C , with three changes of buffer. To 1-ml portions of the α_D -Hc solution (30 ml) were added 200 μ l 12.5% (mass/vol.) SDS and 6 μ l 2-mercaptoethanol, and the mixtures were held for 10 min at 100°C . After cooling, 8–15 μ l PNGase-F (1.5–3 U) was added to the samples, and the solutions were incubated for 24 h at room temperature using an end-over-end mixer. In each case, the percentage of released oligosaccharides was measured using a concanavalin-A binding assay [26]. The 30 incubation mixtures were pooled and subsequently fractionated.

Fractionation of oligosaccharides. The PNGase-F digests derived from both copper-free α_D -Hc portions (870 mg and 800 mg) were fractionated on a column (95 cm $\times$ 2 cm) of Bio-Gel P-100 (200–400 mesh, Bio-Rad) eluted with 25 mM NH_4HCO_3 at a flow rate of 10 ml/h. The carbohydrate-positive fractions (orcinol/ H_2SO_4 assay) were pooled, lyophilized, and an aqueous solution of the residue was passed through a combined anion-/cation-exchange column (AG 1-X2/OAc[−] form and AG 5OW-X2/H⁺ form, 200–400 mesh, Bio-Rad), and lyophilized again. The oligosaccharide mixture was fractionated on a column (100 cm $\times$ 1.0 cm) of Bio-Gel P-4 (200–400 mesh, Bio-Rad) eluted with water at a flow rate of 8.8 ml/h, yielding four carbohydrate-positive fractions, denoted AI–AIV.

Subfractionation of Bio-Gel P-4 fraction AIV, containing low-molecular-mass oligosaccharides, was carried out on a Lichrosorb-NH₂ 10- μ m column (25 cm $\times$ 0.46 cm) using a Kratos Spectroflow 400 HPLC system. Elutions were performed isocratically with a mixture of acetonitrile and water (76:24, by vol.) at a flow rate of 1.5 ml/min, and monitoring at 206 nm.

Subfractionation of the Bio-Gel P-4 fractions AII and AIII, derived from the 870-mg α_D -Hc portion, was carried out on a CarboPac PA-1 column (250 mm $\times$ 4 mm) using a Dionex Bio-LC gradient pump and a PAD II detector (HPAEC-PAD). In the case of fraction AII, the elution was carried out isocratically with 15 mM NaOH (1 ml/min). The elution of fraction AIII was performed isocratically with 50 mM NaOH for 20 min, followed by a linear concentration gradient from 50 mM to 100 mM NaOH in 20 min (1 ml/min). Detection was performed using the following pulse potentials and durations for the PAD detector: E_1 0.05 V, 300 ms; E_2 0.65 V, 60 ms; E_3 −0.95 V, 180 ms. Fractions were collected and neutralized immediately with 0.2 M HCl, lyophilized, and desalted on a column (47 cm $\times$ 2.5 cm) of Bio-Gel P-2 (200–400 mesh). In some cases, HPAEC fractions were further separated on Lichrosorb-NH₂. For specific data, see Results.

Fractionation of oligosaccharide alditols. The Bio-Gel P-4 fractions AII and AIII, derived from the 800-mg α_D -Hc portion, were reduced in 25 ml aqueous NaB^2H_4 (1 mg/ml; 4 h, room temperature). After adjustment of the pH to 5.0 with cold (4°C) 2 M HOAc, the solutions were concentrated under reduced pressure at 30°C. Subsequently, boric acid was removed by co-evaporation with methanol under reduced pressure (5 $\times$ 20 ml), and desalting was carried out on a column (47 cm $\times$ 2.5 cm) of Bio-Gel P-2 (200–400 mesh). The reduced Bio-Gel P-4 fractions AII and AIII, designated BII and BIII, respectively, were subfractionated on a reverse-phase Lichrosphere-C₁₈ column (25 cm $\times$ 0.46 cm, Chrompack) using a Kratos Spectroflow 400 HPLC system. Elution programs applied were as follows: BII, 5.0% (by vol.) aqueous methanol for 5 min, followed by a linear concentration gradient to 9.0% (by vol.) aqueous methanol in 60 min; BIII, 2.5% (by vol.) aqueous methanol for 5 min, followed by a linear concentration gradient to 14.4% (by vol.) aqueous methanol in 60 min. Elutions were performed at a flow rate of 1.5 ml/min, and monitored at 206 nm.

The BII subfractions were further separated on a Lichrosphere-NH₂ column (25 cm $\times$ 0.46 cm, Chrompack) by elution with a mixture of water and acetonitrile, using a linear concentration gradient from 34.0% to 38.0% (by vol.) water in 60 min, at a flow rate of 1.5 ml/min. In a similar way the BIII subfractions were further separated using a linear concentration gradient from 30.0% to 34.0% (by vol.) water in 60 min.

Mass spectrometry. GLC-MS analysis was carried out on a JEOL JMS-AX505W mass spectrometer equipped with a Hewlett Packard 5980 gas chromatograph (Bijvoet Center, Department of Mass Spectrometry, Utrecht University) using helium as the carrier gas. Partially trideuteromethylated alditol

acetates were analyzed on a CP Sil5 CB capillary column (25 m $\times$ 0.32 mm, Chrompack), using on-column injection and the following temperature program: 2 min at 140°C, then at 6°C/min to 230°C, 10 min at 230°C. Trimethylsilylated methyl glycosides were analyzed on an SE-54 capillary column (30 m $\times$ 0.25 mm, Chrompack) using the temperature program: 2 min at 90°C, then at 30°C/min to 140°C, 2 min at 140°C, followed by 4°C/min to 240°C, 10 min at 240°C. Electron impact mass spectra were recorded using linear scanning from m/z 50–500 in 1 s at an accelerating voltage of 3 kV.

Positive-ion FAB mass spectra were obtained using MS1 of a JEOL JMS-SX/SX102A tandem mass spectrometer (Bijvoet Center, Department of Mass Spectrometry, Utrecht University) operated at 10 kV accelerating voltage. The FAB gun was operated at 6 kV accelerating voltage with an emission current of 10 mA and using xenon as the bombarding gas. Spectra were scanned at a speed of 30 s for the full mass range specified by the accelerating voltage used, and were recorded and averaged using JEOL COMPLEMENT software on a Hewlett Packard HP9000 data system. Thioglycerol was used as the matrix.

¹H-NMR spectroscopy. Carbohydrate samples were exchanged twice in ²H₂O (99.9 atom/100 atom ²H, IC Chemikalien GmbH) with intermediate lyophilization, and finally dissolved in 450 μ l ²H₂O (99.96 atom/100 atom ²H, IC Chemikalien GmbH). ¹H-NMR measurements were carried out on a Bruker AM-500 or AMX-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University) at 500 MHz or on a Bruker AMX-600 spectrometer (NSR Center, SON NMR facility, University of Nijmegen, The Netherlands; Bijvoet Center, Department of NMR Spectroscopy, Utrecht University) at 600 MHz. The probe temperature was usually 300 K, but in some cases a lower temperature was chosen, as indicated in the text. Suppression of the ¹HO²H signal was performed by a modified water-eliminated Fourier transform (WEFT) sequence [27] in one-dimensional (1D) experiments or by presaturation for 1 s in two-dimensional (2D) experiments. 2D homonuclear Hartmann-Hahn (HOHAHA) spectra were recorded with MLEV-17 mixing sequence cycles at a field strength of 9.3–8.6 kHz (54–58 ms, 180° pulse width) and an effective spinlock time of 100–120 ms. 2D rotating-frame NOE spectroscopy (ROESY) spectra were recorded at a field strength of 2.3 kHz (110 ms, 90° pulse width) with the offset at 5.5 ppm and a spinlock time of 150–200 ms. 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 (δ 2.225) [28]. Resolution enhancement was achieved for 1D spectra by Lorentzian-to-Gaussian transformation and for 2D spectra by multiplication with phase-shifted sine-bells in both directions, prior to Fourier transformation.

RESULTS

General. Monosaccharide analysis, following conversion into trimethylsilylated (Me₃Si) methyl glycosides, of the *H. pomatia* α_D -Hc preparation revealed the presence of Fuc, Xyl, 3MeGal, 4MeGal, Man, Gal, GlcNAc, and GalNAc in the molar ratio 0.6:1.0:2.7:0.1:3.0:0.5:2.1:1.0. The minor component, 4MeGal, has not been described in our previous work [9, 11], and its identification is based on GLC-MS of the trimethylsilylated methanolysate. The resolved low-intensity GLC peak with R_{mannitol} 0.68 gives rise to the same electron impact mass spectrum as the major GLC peak (R_{mannitol} 0.67) of reference methyl 4MeGal. The electron impact mass spectra contain relevant ions at m/z 377 (M–Me–MeOH), m/z 303 (M–OMe–Me₃SiOH), m/z 204 (Me₃SiO–C²H–C²H–OSiMe₃), m/z 159

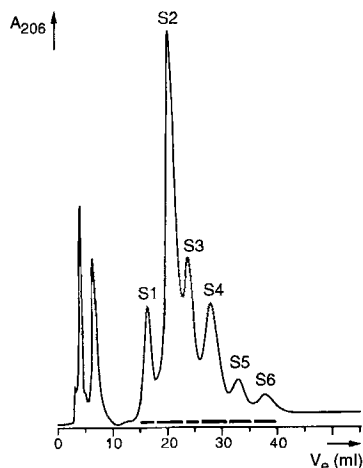


Fig. 1. Fractionation pattern (detection at 206 nm) of oligosaccharide fragments generated by Smith degradation of a pronase digest of α_n -Hc of *H. pomatia* on a Lichrosorb-NH₂ column. The column was eluted isocratically with a mixture of acetonitrile and water (3:1, by vol.) at a flow rate of 1.5 ml/min.

(MeO-C⁴H = C²H-C²H-OSiMe₃), and m/z 146 (MeO-C⁴H-C²H-OSiMe₃). The ion at m/z 204 (intensity $I_m > 1.0$) is much more intense than the ions at m/z 159 ($I_m = 0.2$) and m/z 146 ($I_m = 0.1$). The relative intensity pattern of these three ions is typical for a 4-*O*-methylated methyl aldohexopyranoside [29].

The occurrence of a series of very complex high-molecular-mass 3MeGal-containing N-glycans in *H. pomatia* α_n -Hc was already suggested in our previous study [11]. Taking this information into account, a glycopeptide mixture obtained on pronase digestion was used as a starting material from which oligosaccharide fragments corresponding to the antennae of the N-glycans were prepared by means of Smith degradation. Having established the structure of the major fragments, a set of ¹H-NMR structural reporters could be deduced, vital for the NMR analysis of the PNGase-F released intact carbohydrate chains and their corresponding alditols.

Structural determination of oligosaccharide fragments generated by Smith degradation of a pronase digest. The material obtained following Smith degradation was fractionated on Mono Q, and the neutral fraction was subfractionated on Lichrosorb-NH₂ (Fig. 1), yielding six carbohydrate-containing fractions, denoted S1–S6. In the context of this study only the MS and ¹H-NMR analysis of the structurally related fractions S2 and S3 will be described.

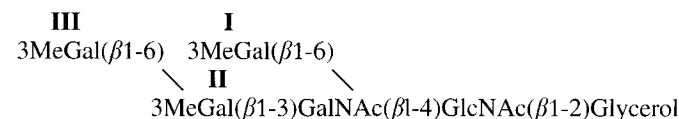
The positive-ion mode FAB mass spectrum from underivatized fraction S3 contained intense [M+Na]⁺ and [M+K]⁺ pseudomolecular ions at m/z 1049 and 1065, respectively, corresponding to a composition of (MeHex)₃HexNAc₂Glycerol. In addition to this major component, analogous pseudomolecular ions at m/z 1121 and 1137 indicate the presence of a minor amount of compound S2 (see below).

The 1D ¹H-NMR spectrum of fraction S3 (Fig. 2A) reveals the presence of five β -anomeric signals at δ 4.675 (³ $J_{1,2}$ 8.2 Hz), 4.580 (³ $J_{1,2}$ 8.6 Hz), 4.528 (³ $J_{1,2}$ 7.8 Hz), 4.474 (³ $J_{1,2}$ 7.8 Hz), and 4.446 (³ $J_{1,2}$ 7.9 Hz), three *O*-methyl singlets at δ 3.450, 3.442, and 3.420, and two *N*-acetyl singlets at δ 2.054 and 2.044, pointing to the occurrence of three MeGal and two HexNAc residues. 2D HOHAHA and 2D ROESY experiments were carried out to elucidate the complete peak pattern, and the ¹H-NMR data are compiled in Table 1. Based on the assignments, the presence of one GlcNAc, one GalNAc [30] and three 3MeGal residues was established. On the HOHAHA H-1 tracks (Fig. 2B) at δ 4.580,

4.528, 4.474, and 4.446 the typical H-2,3,4 cross-peak patterns belonging to aldohexoses with the *galacto*-configuration are observed. The presence of intraresidual H-3,OMe and H-4,OMe rotating-frame Overhauser enhancement (ROE) connectivities for three of the four H-3,4 signals in the ROESY spectrum (Fig. 2C) made a distinction between 3MeGal (I, δ 4.528; II, δ 4.474; and III, δ 4.446) and GalNAc (δ 4.580) possible. The observation of the intraresidual ROE cross-peaks H-3,OMe and H-4,OMe of the three 3MeGal residues shows that the most favorable staggered conformation around the O-3–C-3 bond ($\phi_{\text{Me-O3-C3-H3}}$) is -60° . It should be noted that both H-3 and H-4 of the 3MeGal residues resonate outside the bulk region, thereby acting as structural-reporter-group signals. In addition to H-1,2,3, the HOHAHA H-4 tracks of the 3MeGal/GalNAc residues reveal the H-5 signals. The assignments of the H-5 signals are confirmed by H-1,H-5 and H-4,H-5 cross-peaks in the ROESY spectrum. The H-6a,6b signals could be only partially assigned (Table 1). Finally, on the HOHAHA H-1 track of the remaining GlcNAc residue (δ 4.675) the H-2,3,4,5,6 cross-peak pattern is observed and, in combination with the results from the ROESY analysis, assignments of the various protons were made.

The interresidual ROE contacts between the various monosaccharide residues give evidence for the sequence of the monomers and the glycosidic linkages involved (for supporting linkage analysis data, see fraction S2). The interresidual ROE connectivities 3MeGal-III H-1,3MeGal-II H-6a, 3MeGal-III H-1,3MeGal-II H-6b, and 3MeGal-III H-1,3MeGal-II H-5 (weak) indicate a 3MeGal-III(β 1-6)3MeGal-II element. The interresidual ROE connectivities 3MeGal-I H-1,GalNAc H-6a, 3MeGal-I H-1,GalNAc H-6b, and 3MeGal-I H-1,GalNAc H-5 show a 3MeGal-I(β 1-6)GalNAc sequence. Furthermore, a strong interresidual cross-peak between 3MeGal-II H-1 and GalNAc H-3 leads to a 1,3-linkage between these residues, whereas the GalNAc(β 1-4)GlcNAc element is evidenced by a GalNAc H-1, GlcNAc H-4 connectivity.

Based on these data, and taking into account that Smith degradation may be expected to yield a 2-substituted glycerol unit (derived from 2-substituted Man; see fraction S2), the following structure for fraction S3 is proposed:



For the major fraction S2 the positive-ion mode FAB mass spectrum, obtained from pertrideuteromethylated fraction S2 (Fig. 3), contains an intense [M+H]⁺ pseudomolecular ion at m/z 1354, corresponding to a composition of (MeHex)₃HexNAc₂X (X = unknown with a mass of 197 after pertrideuteromethylation), accompanied by two less intense pseudomolecular ions at m/z 1371 ([M+NH₄]⁺) and m/z 1376 ([M+Na]⁺). Intense A⁺-type sequence ions [31] are observed at m/z 1156 [(MeHex)₃HexNAc₂]⁺ and m/z 902 [(MeHex)₃HexNAc]⁺, indicating the partial sequence (MeHex)₃-HexNAc-HexNAc-X. The fragment ion at m/z 1156 is accompanied by an ion at m/z 1121, generated by β -elimination of trideuteromethanol, showing that C-3 of the charged HexNAc residue is substituted by OC²H₃ [32]. The fragment ion at m/z 902 is accompanied by an ion at m/z 447, arising by β -elimination of (MeHex)₂, indicating that C-3 of the charged HexNAc residue is substituted by MeHex-MeHex. Furthermore, β -cleavage of terminal MeHex and MeHex-MeHex from the A⁺-type ion at m/z 902 gives rise to the ions at m/z 675 and m/z 465, respectively. These β -cleavage ions are accompanied (at m/z 703 and 493) by ions which result from cross-ring cleavages and which typically arise together with β -cleavage ions [33].

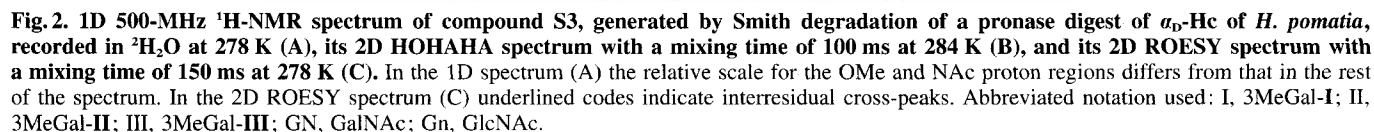
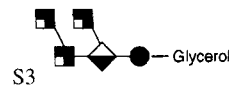
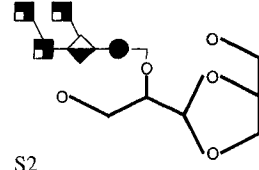
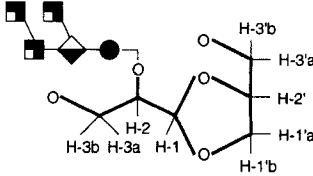
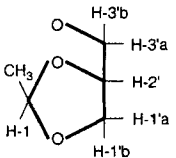


Table 1. ^1H chemical shifts of the monosaccharide constituents of compound S3 (at 284 K) and S2 (at 290 K), generated by Smith degradation of a pronase digest of *H. pomatia* α -Hc. Chemical shifts are given relative to internal acetone (δ 2.225) in $^2\text{H}_2\text{O}$ at p ^2H 7 [28]. Compounds are represented by short-hand symbolic notation: (●) GlcNAc; (◆) GalNAc; (■) 3MeGal. For numbering of the monosaccharide residues, see text. n.d., not determined.

Compound/constituent	Chemical shift of							
	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	OMe/NAc
 S3	ppm							
GlcNAc-Gn	4.675	3.742	3.771	3.650	3.546	3.831	n. d.	2.044 ^a
GalNAc-GN	4.580	4.070	3.925	4.225	3.987	4.103	3.923	2.054 ^a
3MeGal-I	4.528	3.515	3.361	4.195	3.667	3.773	n. d.	3.450
3MeGal-II	4.474	3.526	3.334	4.260	3.818	4.049	3.890	3.420
3MeGal-III	4.446	3.531	3.334	4.219	3.678	3.786	n. d.	3.442
 S2								
GlcNAc-Gn	4.774	3.732	3.786	3.662	3.541	3.824	n. d.	2.052
GalNAc-GN	4.591	4.069	3.918	4.214	3.994	4.105	3.920	2.052
3MeGal-I	4.496	3.518	3.349	4.186	3.665	3.775	n. d.	3.456
3MeGal-II	4.474	3.535	3.324	4.242	3.835	4.045	3.878	3.427
3MeGal-III	4.440	3.542	3.318	4.208	3.671	3.888	n. d.	3.448

^a Values may have to be interchanged.

Table 2. ^1H chemical shifts of the glyceraldehyde 3'-hydroxypropylene diacetal part of S2, together with a reference dioxolane compound, of which the most stable *cis* form has been taken, although it makes essentially no difference for this comparison [35].

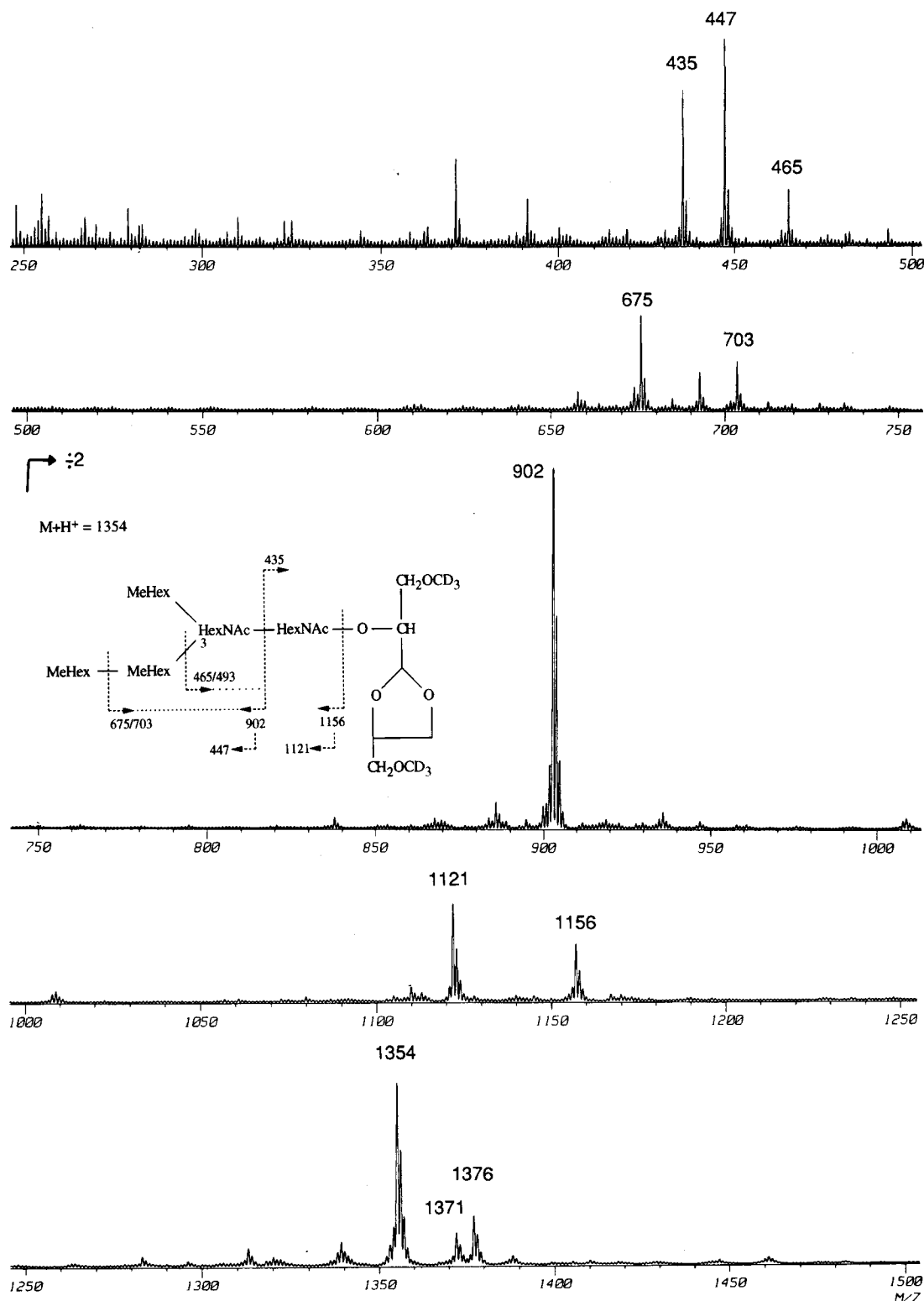
Structure	Proton	(J)	δ (J)				
			S2	R1			
	S2	H-1	(J _{1,2})	4.994	(4.1)	4.89	—
		H-2	(J _{2,3a})	3.905	(3.5)	—	—
		H-3 a	(J _{3a,3b})	3.780	(−12.3)	—	—
		H-3 b	(J _{2,3b})	3.710	(7.0)	—	—
		H-1'a	(J _{1'a,2'})	4.008	(7.0)	3.73	(7.2)
		H-1'b	(J _{1'a,1'b})	3.730	(−7.8)	3.68	(−8.0)
		H-2'	(J _{1'b,2'})	4.276	(6.6)	4.03	(5.6)
		H-3'a	(J _{2',3'a})	3.714	(2.9)	3.52	(4.0)
		H-3'b	(J _{2',3'b})	3.599	(5.2)	3.15	(9.6)
	R1		(J _{3'a,3'b})		(−12.1)		(−11.4)

Methylation analysis of the partially trideuteromethylated alditol acetates [24, 25], generated from the pertrideuteromethylated fraction S2, allowed the identification of 1,5-di-*O*-acetyl-3-*O*-methyl-2,4,6-tri-*O*-trideuteromethyl-[1- ^2H]hexitol, 1,5,6-tri-*O*-acetyl-3-*O*-methyl-2,4-di-*O*-trideuteromethyl-[1- ^2H]hexitol, 1,4,5-tri-*O*-acetyl-2-deoxy-2-*N*-methylacetamido-3,6-di-*O*-trideuteromethyl-[1- ^2H]hexitol, and 1,3,5,6-tetra-*O*-acetyl-2-deoxy-2-*N*-methylacetamido-4-*O*-trideuteromethyl-[1- ^2H]hexitol in the approximate molar ratio 2:1:1:1, consistent with the presence of two terminal 3MeHex residues, a 6-substituted 3MeHex residue, a 3,6-disubstituted HexNAc residue, and a 4-substituted HexNAc residue.

The mass of the reducing-terminal substituent X in S2 (see above; Fig. 3) is consistent with a pertrideuteromethylated 2-

linked glyceraldehyde 3'-hydroxypropylene diacetal, which could be established by ^1H -NMR analysis, as will be shown below. Such an aglycon has been described previously as a by-product of the Smith degradation of α -linked Man [34].

The ^1H -NMR spectrum of S2 is depicted in Fig. 4A, and the NMR data are summarized in Table 1 (oligosaccharide part) and in Table 2 (glyceraldehyde 3'-hydroxypropylene diacetal fragment). Using 2D HOHAHA and 2D ROESY experiments, most of the skeleton protons of the five monosaccharide residues were assigned in a similar way to that described for fraction S3. The five β -anomeric signals at δ 4.760 ($J_{1,2}$ 8.1 Hz), 4.591 ($J_{1,2}$ 8.5 Hz), 4.496 ($J_{1,2}$ 7.9 Hz), 4.474 ($J_{1,2}$ 7.8 Hz), and 4.440 ($J_{1,2}$ 7.9 Hz) correspond to GlcNAc, GalNAc, 3MeGal-I, 3MeGal-II, and 3MeGal-III, respectively (Table 1). The presence of the 2-



linked glyceraldehyde 3'-hydroxypropylene diacetal could be demonstrated as follows. The downfield signals at δ 4.994 (doublet, $J_{1,2}$ 4.1 Hz) and δ 4.276 (multiplet) are attributed to the dioxolane ring protons glyceraldehyde H-1 and 3'-hydroxypropylene H-2', respectively (Fig. 4A, Table 2). On the glyceraldehyde H-1 HOHAHA track (Fig. 4B) cross-peaks are detected

with H-2 (δ 3.905), H-3a (δ 3.780), and H-3b (δ 3.710). At the 3'-hydroxypropylene H-2' HOHAHA track (Fig. 4C) connectivities are observed with H-1'a (δ 4.008), H-1'b (δ 3.730), H-3'a (δ 3.714), and H-3'b (δ 3.599). A distinction between H-1' and H-3' is possible on the basis of the comparison of the $^2J_{1'a,1'b}$ (-7.8 Hz) and $^2J_{3'a,3'b}$ (-12.1 Hz) values of S2 with

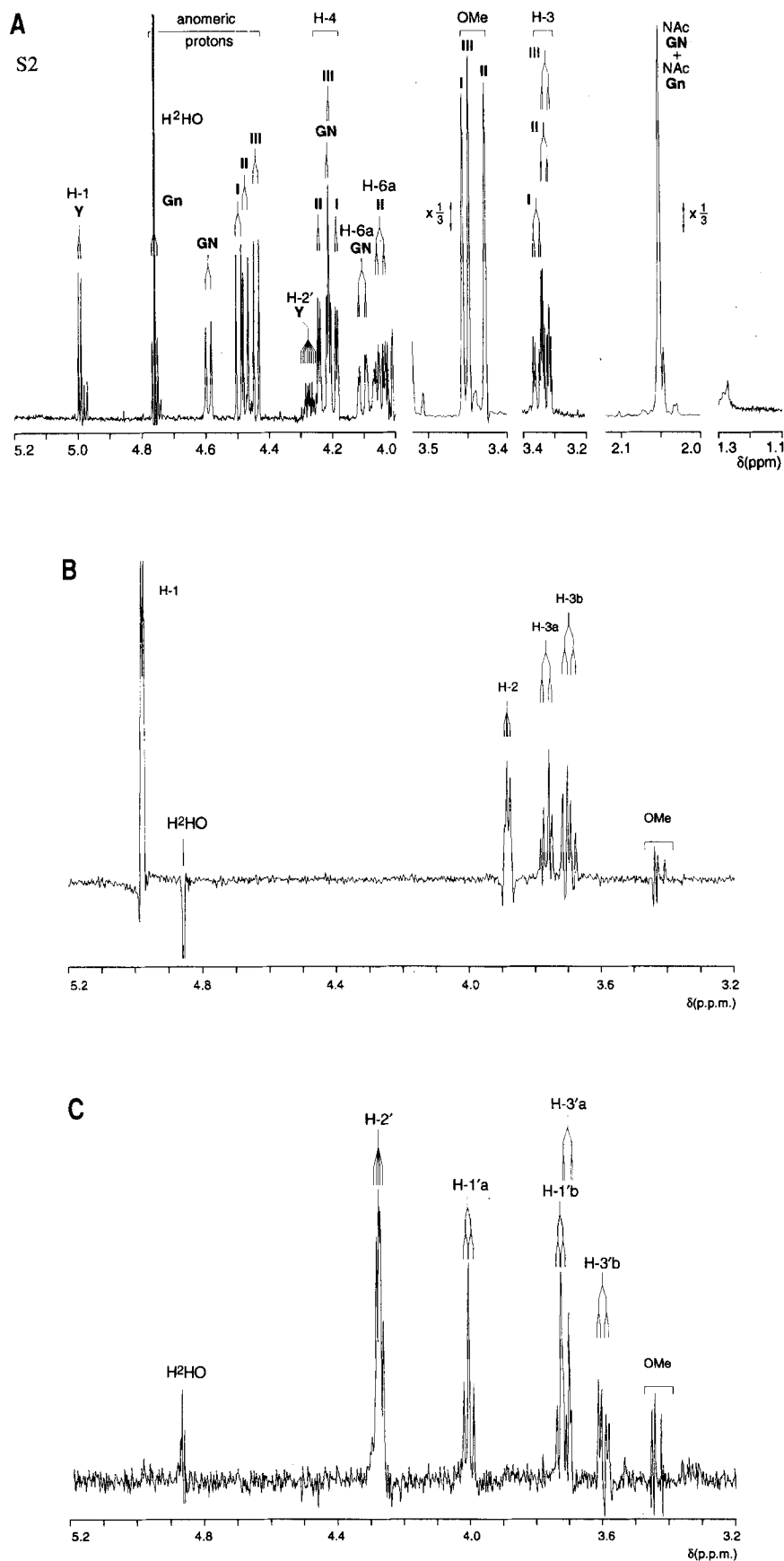


Fig. 4. 1D 500-MHz ^1H -NMR spectrum of compound S2, generated by Smith degradation of a pronase digest of α_D -Hc of *H. pomatia*, recorded in $^2\text{H}_2\text{O}$ at 300 K (A), the glyceraldehyde H-1 track of its HOHAHA spectrum (mixing time, 100 ms) at δ 4.994, recorded in $^2\text{H}_2\text{O}$ at 290 K (B), and the 3'-hydroxypropylene diacetal H-2' track of its HOHAHA spectrum (mixing time, 100 ms), at δ 4.276, recorded in $^2\text{H}_2\text{O}$ at 290 K (C). In (A) Y denotes the glyceraldehyde 3'-hydroxypropylene part of the molecule (see text). For the additional abbreviated notations used, see Fig. 2. In (B, C) the OMe signals indicated are the result of t_1 -noise in the spectra from the 3MeGal residues of S2.

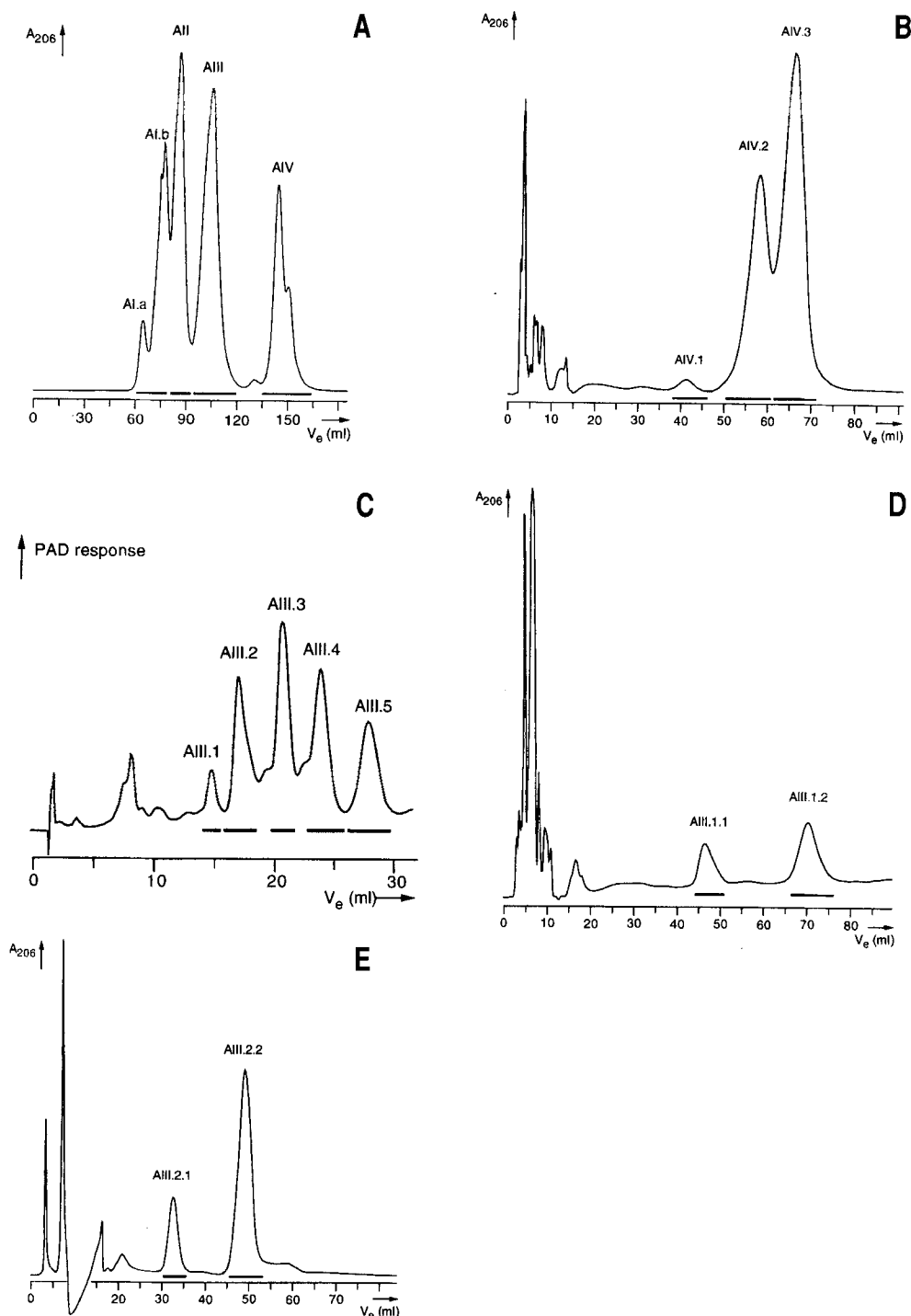


Fig. 5. Fractionation patterns of PNGase-F-released oligosaccharides from *H. pomatia* α_D -Hc. (A) Fractionation pattern of the mixture of PNGase-F-released intact oligosaccharides on a Bio-Gel P-4 column. The column was eluted with water at a flow rate of 8.8 ml/h, and monitored at 206 nm. (B) Subfractionation pattern of Bio-Gel P-4 fraction AIV on a Lichrosorb-NH₂ column, isocratically eluted with a mixture of acetonitrile and water (76:24, by vol.) at a flow rate of 1.5 ml/min, and monitored at 206 nm. (C) Subfractionation pattern of Bio-Gel P-4 fraction AIII on a CarboPac PA-1 column, isocratically eluted with 50 mM NaOH at a flow rate of 1 ml/min, and monitored with PAD. (D) Subfractionation of HPAEC fraction AIII.1 on a Lichrosorb-NH₂ column, isocratically eluted with a mixture of acetonitrile and water (68:32, by vol.) at a flow rate of 1.5 ml/min, and monitored at 206 nm. (E) Subfractionation of HPAEC fraction AIII.2 on a Lichrosorb-NH₂ column, isocratically eluted with a mixture of acetonitrile and water (67:33, by vol.) at a flow rate of 1.5 ml/min, and monitored at 206 nm.

the corresponding values of a reference compound (Table 2). The ¹H-NMR data of the 3'-hydroxypropylene fragment were compared with the results of a computer simulation of the ¹H-NMR spectrum and shown to be identical.

Summarizing the various data described above, the structure of S2 is

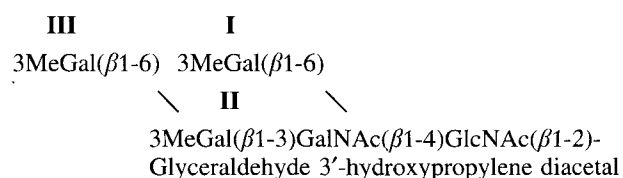


Table 3. ^1H chemical shifts of structural-reporter-group protons of the constituent monosaccharides of core structures of *H. pomatia* of $\alpha_D\text{-Hc}$. Chemical shifts are given relative to internal acetone (δ 2.225) in $^2\text{H}_2\text{O}$ at 300 K and p^{H} 7 [28]. Compounds are represented by shorthand symbolic notation: (●) GlcNAc; (◆) Man; (◻) Xyl; (□) Fuc. For numbering of the monosaccharide residues, see text. n.d., not determined. The subscripts α and β stand for the anomeric configuration of GlcNAc-1.

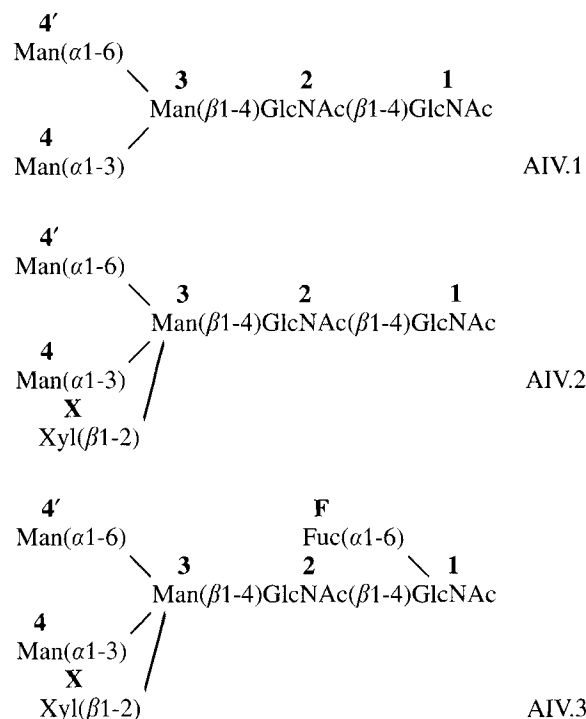
Residue	Reporter group	Chemical shift in		
		AIV.1	AIV.2	AIV.3
		ppm		
GlcNAc-1	H-1 α	5.186	5.190	5.181
	H-1 β	n. d.	4.696	4.693
	NAc	2.039	2.039	2.039
GlcNAc-2	H-1 α	n. d.	4.609	4.666
	H-1 β	n. d.	4.601	4.673
	NAc	2.077	2.075	2.088
Man-3	H-1	n. d.	4.871	4.873
	H-2	4.254	4.266	4.266
Man-4	H-1	5.101	5.121	5.121
	H-2	4.067	4.038	4.039
Man-4'	H-1	4.916	4.912	4.913
	H-2	3.973	3.983	3.982
Xyl-X	H-1		4.449	4.450
	H-2		3.375	3.375
	H-3		3.440	3.456
	H-5 α		3.248	3.252
Fuc-F	H-1 α			4.890
	H-1 β			4.898
	H-5 α			4.099
	H-5 β			4.135
	Me α			1.209
	Me β			1.222

Isolation and characterization of oligosaccharides generated by PNGase-F digestion. Taking into account the absence of an α -1,3-fucosylated core structure, as demonstrated by ^1H -NMR analysis of the $\alpha_D\text{-Hc}$ glycopeptide mixture which was used as the starting material for the Smith degradation (data not shown), PNGase-F was applied to release the N-linked glycans from copper-free $\alpha_D\text{-Hc}$. The Con-A binding assay demonstrated that the various digestions resulted in 81–96% liberation of the carbohydrate chains, depending on the protein concentration and the quantity of PNGase-F used. The mixture of oligosaccharides was separated from the remaining protein material on Bio-Gel P-100. In view of the earlier observation that less than 2% of the carbohydrate chains had an acidic character [11], the carbohydrate fraction was further purified on a combined anion- and cation-exchange column, and then fractionated on Bio-Gel P-4. This yielded four main fractions, denoted AI–AIV (Fig. 5A).

HPLC subfractionation of Bio-Gel P-4 fraction AIV on Lichrosorb-NH₂ yielded two major fractions AIV.2 and AIV.3, and one minor fraction AIV.1 (Fig. 5B). HPAEC subfractionation of Bio-Gel P-4 fraction AIII on CarboPac PA-1 resulted in the carbohydrate-containing fractions AIII.1–AIII.5 (Fig. 5C). Subfractions AIII.1 and AIII.2 were further fractionated on Lichrosorb-NH₂, affording fractions AIII.1.1 and AIII.1.2

(Fig. 5D), and AIII.2.1 and AIII.2.2 (Fig. 5E), respectively. All fractions belonging to the AIII and AIV series were analyzed by ^1H -NMR spectroscopy, and the structural determination of the various compounds will be presented in an order which is convenient for the discussion of the ^1H -NMR data. The structures all have an N,N' -diacetylchitobiose or an α -1,6-fucosylated N,N' -diacetylchitobiose unit in common, as is evident from the relevant ^1H -NMR structural-reporter-group signals (Tables 3–5) [27, 28].

The fractions AIV.1–AIV.3 contain the low-molecular-mass oligosaccharides of $\alpha_D\text{-Hc}$, for which the relevant ^1H -NMR data are listed in Table 3.

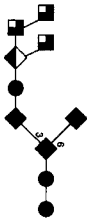
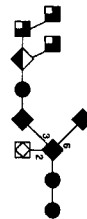
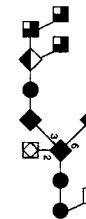


Compounds AIV.2 and AIV.3 have already been identified as their corresponding alditols [11]. The structural-reporter-group signals for AIV.1 fit those of the trimannosyl- N,N' -diacetylchitobiose core [36], whereas the structural-reporter-group data for AIV.2 match those reported for the same compound from ascorbic acid oxidase [37].

The ^1H -NMR spectrum of fraction AIII.4 is depicted in Fig. 6. Inspection of the structural-reporter-group signals (Table 4) indicates that the core structure of AIII.4 is identical with AIV.3 (Table 3). In comparison to AIV.3, the downfield shifts of Man-4 H-1 ($\Delta\delta$ +0.019) and H-2 ($\Delta\delta$ +0.108), and the upfield shifts of Man-3 H-2 ($\Delta\delta$ –0.016) and Xyl H-1 ($\Delta\delta$ –0.010) reflect an extension at O-2 of Man-4 [13]. The positions of the H-1 signals at δ 4.498, 4.476, and 4.440, and of the O-methyl signals at δ 3.451, 3.446, and 3.426 indicate the presence of three 3MeGal residues in the same structural environment as in S3 (Table 1). The N -acetyl groups of the additional GalNAc(β 1-4)GlcNAc element both resonate at δ 2.046. The GalNAc H-1 signals in S3 and AIII.4 are at the same position (δ 4.582).

Conclusive evidence for the antenna on O-2 of Man-4 was obtained from 2D HOHAHA and 2D ROESY experiments. In Table 5 a survey of the protons identified as belonging to the monosaccharide constituents of the antenna is presented. As observed for S3 (Table 1), the 3MeGal-II H-5 signal (δ 3.810) has a relatively large downfield chemical shift, compared with the 3MeGal-I and 3MeGal-III H-5 signals (δ 3.655 and 3.665, respectively; see also H-5 of the β -anomer of 3MeGal at δ 3.672 [13]). The same holds comparing the H-5 signals of GalNAc(β 1-

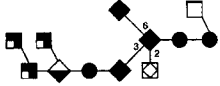
Table 4. ^1H chemical shifts of structural-reporter-group protons of the constituent monosaccharides of monoantennary carbohydrate chains of *H. pomatia* α_0 -Hc. Chemical shifts are given relative to internal acetone (δ 2.225) in $^2\text{H}_2\text{O}$ at 300 K and $p^2\text{H}$ 7 [28]. Compounds are represented by short-hand symbolic notation: (●) GlcNAc; (◆) Man; (◻) Xyl; (□) Fuc; (◐) GalNAc; (■) 3MeGal. For numbering of the monosaccharide residues, see text. n.d., not determined. The subscripts α and β stand for the anomeric configuration of GlcNAc-1. $\Rightarrow 4$, $\Rightarrow 4'$ indicate values for the antenna linked to O-2 of Man-4 and Man-4', respectively.

Residue	Reporter group	Chemical shift in					
							
		AIII.2.1	AIII.5	AIII.4	AIII.1.1	AIII.3	AIII.2.2
		ppm					
GlcNAc-1	H-1 α	5.188	5.188	5.180	5.188	5.182	5.182
	H-1 β	n.d.	n.d.	n.d.	n.d.	n.d.	4.694
	NAc	2.037	2.039	2.038	2.037	2.041	2.040
GlcNAc-2	H-1 α	4.610	4.609	4.664	4.610	4.667	4.667
	H-1 β	4.602	4.601	4.671	4.602	4.673	4.673
	NAc	2.077	2.075	2.088	2.077	2.095	2.095
Man-3	H-1	n.d.	4.869	4.869	n.d.	4.874	4.873
	H-2	4.245	4.251	4.250	4.248	4.261	4.265
Man-4	H-1	5.115	5.141	5.140	5.115	5.124	4.124
	H-2	4.181	4.148	4.147	4.183	4.037	4.036
Man-4'	H-1	4.917	4.915	4.914	4.918	4.899	4.898
	H-2	3.972	3.979	3.983	n.d.	4.093	4.094
Xyl-X	H-1		4.439	4.440		4.447	4.448
	H-2		3.370	3.368		3.374	3.375
	H-5 α		3.249	3.250		3.251	3.251
Fuc-F	H-1 α			4.897		4.895	4.894
	H-1 β			4.890		4.902	4.901
	Me α			1.209		1.212	1.211
	Me β			1.220		1.223	1.222
		$\Rightarrow 4$	$\Rightarrow 4$	$\Rightarrow 4$	$\Rightarrow 4$	$\Rightarrow 4'$	$\Rightarrow 4'$
GlcNAc-5/5'	H-1	4.573	4.537	4.537	4.569	4.572	4.569
	NAc	2.049	2.048	2.046	2.049	2.048	2.047
GalNAc-GN/GN'	H-1	4.584	4.581	4.582	4.581	4.588	4.584
	H-4	4.212	4.212	4.214	4.195	4.214	4.198
	NAc	2.049	2.048	2.046	2.049	2.055	2.055
3MeGal-I/I'	H-1	4.500	4.499	4.498	4.524	4.501	4.523
	H-3	3.344	3.344	3.343	3.353	3.348	3.356
	H-4	4.181	4.181	4.181	4.234	4.182	4.233
	OMe	3.453	3.452	3.451	3.450	3.454	3.451
3MeGal-II/II'	H-1	4.473	4.474	4.476	4.475	4.475	4.476
	H-3	3.321	3.325	3.323	3.323	3.328	3.321
	H-4	4.240	4.241	4.241	4.248	4.242	4.247
	OMe	3.426	3.426	3.426	3.427	3.428	3.428
3MeGal-III/III'	H-1	4.439	4.439	4.440	4.439	4.440	4.440
	H-3	3.319	3.319	3.319	3.320	3.322	3.321
	H-4	4.207	4.206	4.207	4.208	4.208	4.208
	OMe	3.447	3.447	3.446	3.444	3.448	3.445
3MeGal-IV/IV'	H-1				4.462		4.462
	H-3				3.357		3.358
	H-4				4.213		4.214
	OMe				3.450		3.451

O)Me (δ 3.32 [30]) and GalNAc-GN in S3 (δ 3.987) or in AIII.4 (δ 3.962). Taking into account the ^1H -NMR data of oligosaccharide S3, the ROE cross-peaks at δ 3.922 and 3.873 represent the interaction between 3MeGal-I H-1 and GalNAc-GN H-6b, and between 3MeGal-III H-1 and 3MeGal-II H-6b, respectively. Combining the findings for the H-5 signals and the ROE connectivities supports the assignment of 1,6-linkages, as established for S3. In a similar way, the detection of the 3MeGal-II H-1, GalNAc-GN H-3 and GalNAc-GN H-1, GlcNAc-5 H-4 interresidual ROE cross-peaks is in agreement with the 1,3- and 1,4-linkages in S3. The observa-

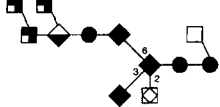
Table 5. ^1H chemical shifts of antennary constituent monosaccharides of the monoantennary compounds AIII.4, AIII.3 and AIII.2.2 of *H. pomatia* α_D -Hc. Chemical shifts are given relative to internal acetone (δ 2.225) in $^2\text{H}_2\text{O}$ at 300 K and p^2H 7 [28]. Compounds are represented by short-hand symbolic notation: (●) GlcNAc; (◆) Man; (◻) Xyl; (□) Fuc; (◈) GalNAc; (■) 3MeGal. For numbering of the monosaccharide residues, see text. n.d., not determined.

Compound/constituent	Chemical shift of							
	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	OMe/NAc



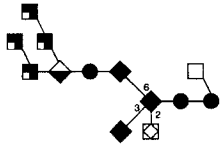
AIII.4

ppm								
GlcNAc-5	4.537	3.752	n.d.	n.d.	n.d.	n.d.	n.d.	2.046
GalNAc-GN	4.582	4.067	3.923	4.214	3.962	4.097	3.922	2.046
3MeGal-I	4.498	3.526	3.343	4.181	3.655	3.767	n.d.	3.451
3MeGal-II	4.476	3.537	3.323	4.241	3.810	4.047	3.873	3.426
3MeGal-III	4.440	3.538	3.319	4.207	3.665	3.782	n.d.	3.446



AIII.3

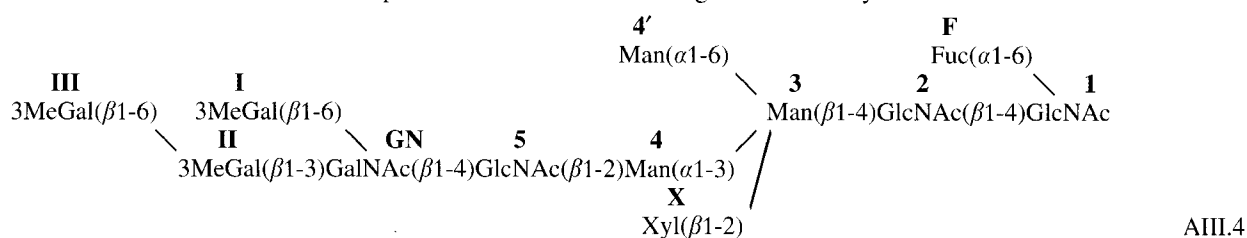
GlcNAc-5'	4.572	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.048
GalNAc-GN'	4.588	4.026	3.932	4.214	3.985	n.d.	n.d.	2.055
3MeGal-I'	4.501	3.531	3.348	4.182	3.663	3.773	n.d.	3.454
3MeGal-II'	4.475	3.543	3.328	4.242	3.836	n.d.	n.d.	3.428
3MeGal-III'	4.440	3.544	3.322	4.208	3.666	3.789	n.d.	3.448



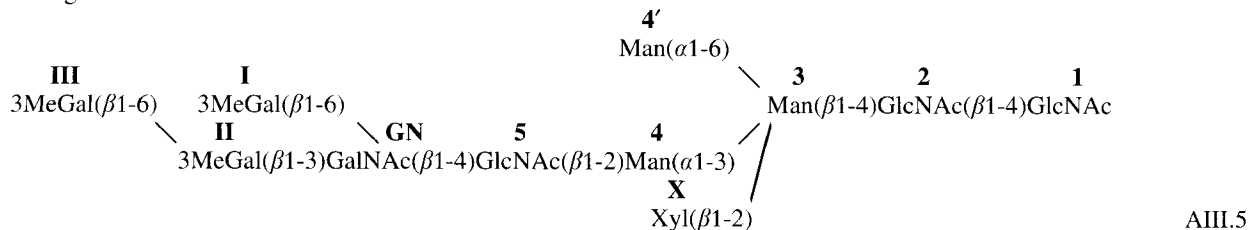
AIII.2.2

GlcNAc-5'	4.569	n.d.	n.d.	3.657	n.d.	n.d.	n.d.	2.047
GalNAc-GN'	4.584	4.067	3.928	4.198	3.972	4.081	3.912	2.055
3MeGal-I'	4.523	3.529	3.356	4.233	3.853	4.050	n.d.	3.451
3MeGal-II'	4.476	3.536	3.321	4.247	3.826	4.045	n.d.	3.428
3MeGal-III'	4.440	3.549	3.321	4.208	3.675	3.778	n.d.	3.445
3MeGal-IV'	4.462	3.558	3.358	4.214	3.682	3.778	n.d.	3.451

tion of the interresidual connectivities GlcNAc-5 H-1,Man-4 H-2 and GlcNAc-5 H-1,Man-4 H-1 demonstrate the attachment of GlcNAc-5 at O-2 of Man-4. In conclusion, compound AIII.4 has the following monoantennary structure:



Comparison of the ^1H -NMR data of AIII.5 (Table 4) with those of AIII.4 shows that AIII.5 (major component) is the non-fucosylated analog of AIII.4.



From the ^1H -NMR spectrum of fraction AIII.3 (Fig. 7, Tables 4 and 5), the structure of the monoantennary isomer of AIII.4 can be deduced. In this case, compared with the values of the Man-4' H-1,2 signals of AIV.3, the typical shifts of both Man 4' H-1 ($\Delta\delta$ -0.014) and H-2 ($\Delta\delta$ +0.111) point to an extension on O-2 of Man-4' [13]. In a similar way to that reported for AIII.4, the 2D HOHAHA and 2D ROESY measurements of AIII.3, in combination with the ^1H -NMR data of S3, further support the assignment of a pentasaccharide fragment in AIII.3 to be present on O-2 of Man-4' (ROE contacts GlcNAc-5' H-1,Man-4' H-2 and GlcNAc-

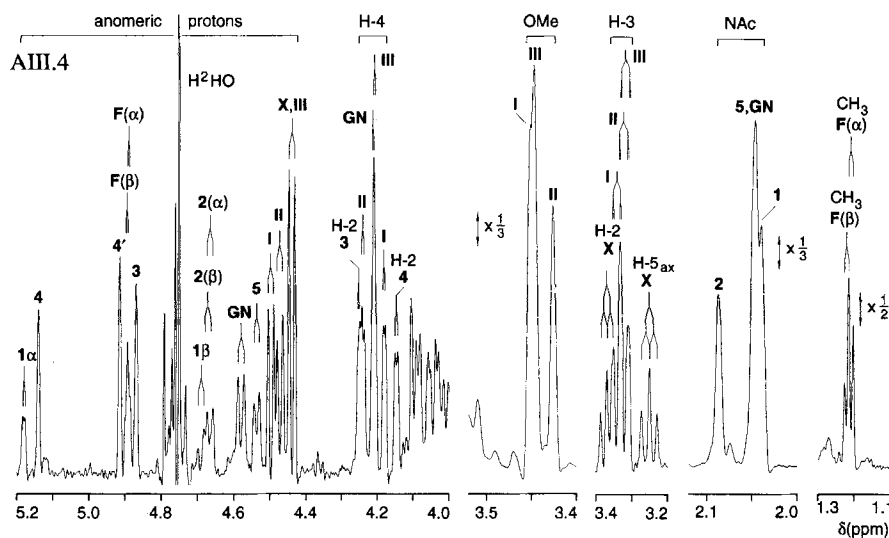


Fig. 6. Structural-reporter-group-signal regions of the resolution-enhanced 500-MHz ^1H -NMR spectrum of compound AIII.4 of *H. pomatia* $\alpha_{\text{D}}\text{-Hc}$, recorded in $^2\text{H}_2\text{O}$ at 300 K. The relative scale of the OMe, NAc and CH_3 protons regions differs from that in the rest of the spectrum. For the abbreviated notations for the monosaccharides, see text.

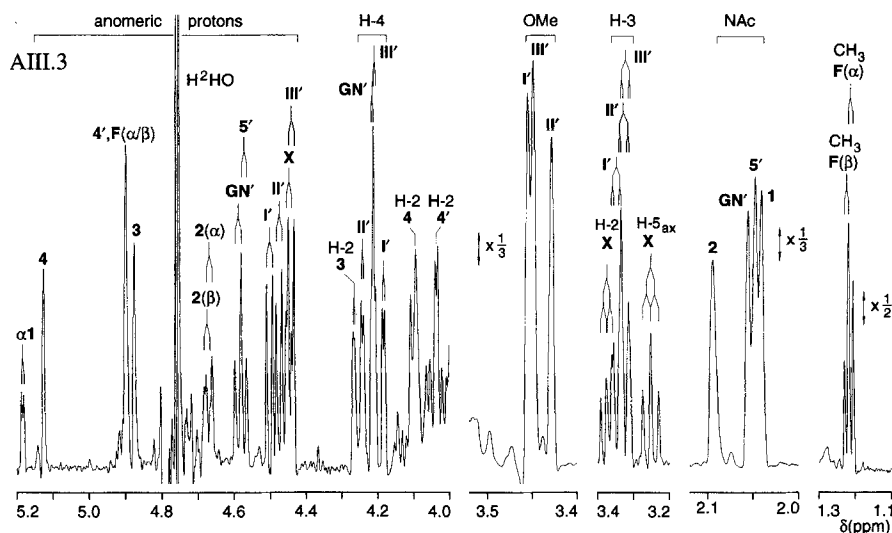
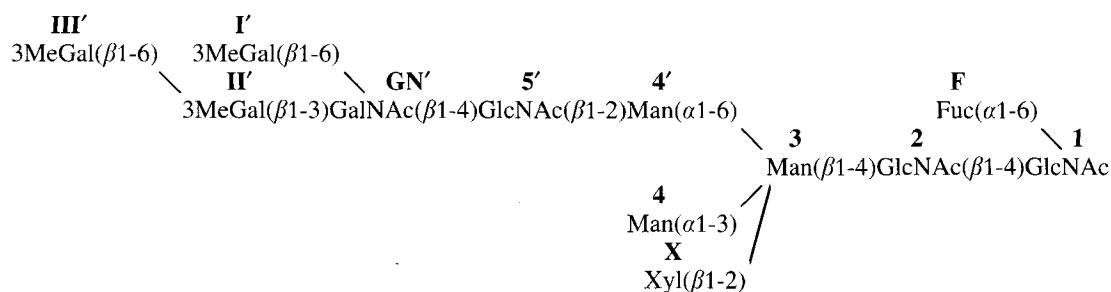


Fig. 7. Structural-reporter-group-signal regions of the resolution-enhanced 500-MHz ^1H -NMR spectrum of compound AIII.3 of *H. pomatia* α_{D} -Hc, recorded in $^2\text{H}_2\text{O}$ at 300 K. The relative scale for the OMe, NAc and CH_3 proton regions differs from that in the rest of the spectrum. For the abbreviated notations for the monosaccharides, see text.

5' H-1, Man-4' H-1). It has to be noted that, on comparison with the data for AIV.3, the signal for GlcNAc-2 NAc is also shifted downfield ($\Delta\delta$ +0.007). Based on earlier studies dealing with comparable carbohydrate chains [13], the NAc-value of 2.055 ppm can be assigned to GalNAc-GN' and that of 2.048 ppm to GlcNAc-5'. The finding that a GalNAc-GN' NAc signal resonates at lower field (AIII.3) than the GalNAc-GN NAc signal (AIII.4) fits the observations made in [13]. Based on these results, AIII.3 has the following primary structure.



Analysis of the ^1H -NMR data of fraction AIII.2.1 (Table 4) showed that AIII.2.1 is a non-xylosylated variant of AIII.5. Compared with the Man-4, H-1,2 values in AIV.1, the typical downfield shifts of Man-4 H-1 ($\Delta\delta +0.014$) and H-2 ($\Delta\delta +0.114$) provide

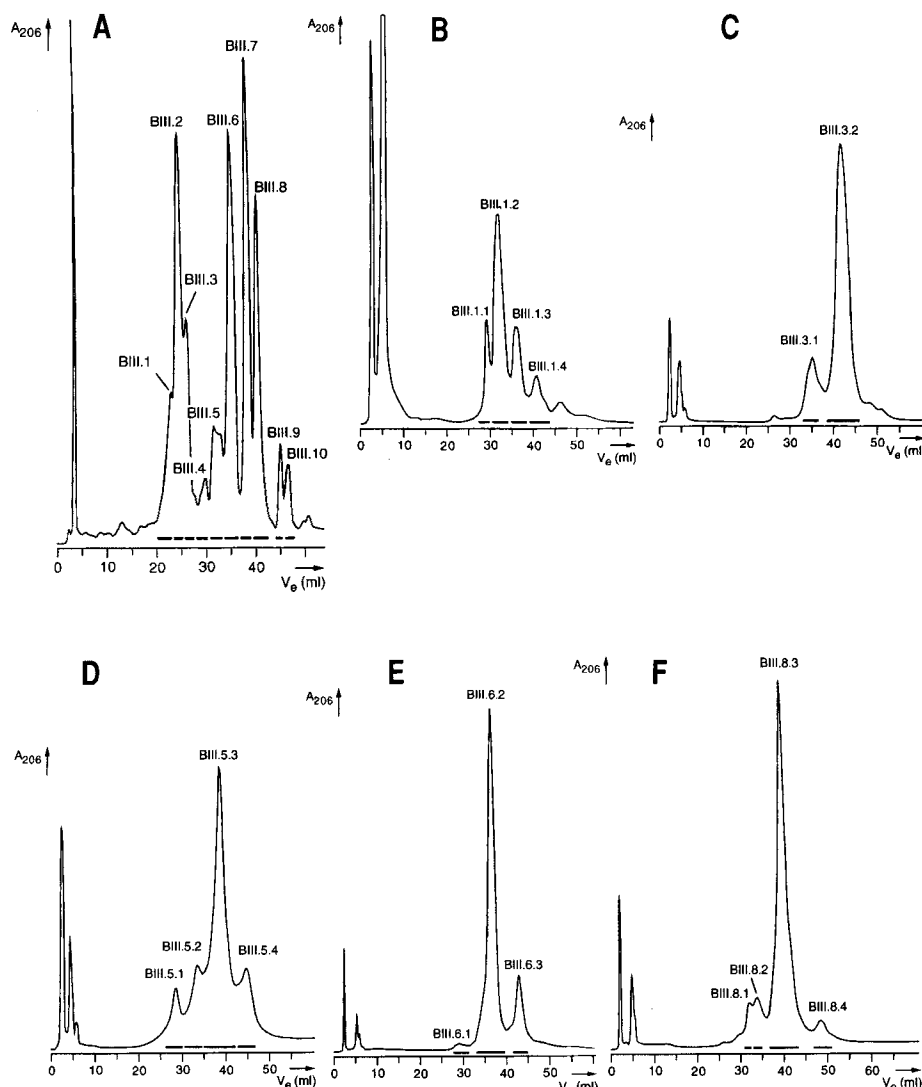
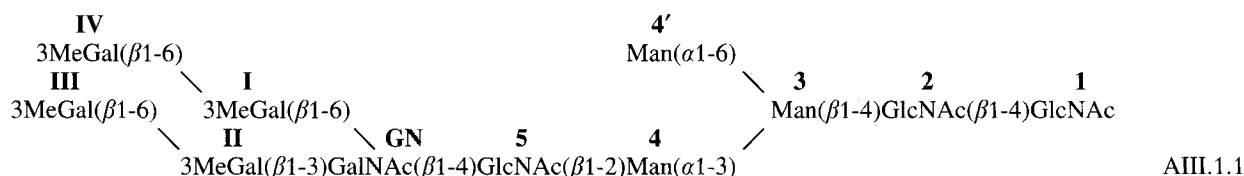


Fig. 9. Fractionation patterns of PNGase-F-released and NaB₂H₄-reduced oligosaccharides from Bio-Gel P-4 fraction AIII (→BIII) of *H. pomatia* α_D-Hc. (A) Fractionation pattern of fraction BIII on a reverse-phase Lichrosphere-C₁₈ column. The column was eluted with 2.5% (by vol.) aqueous methanol for 5 min, followed by a linear concentration gradient to 14.4% (by vol.) aqueous methanol in 60 min, at a flow rate of 1.5 ml/min, and monitored at 206 nm. (B–F) Subfractionation patterns of the C₁₈ fractions BIII.1, BIII.3, BIII.5, BIII.6 and BIII.8, respectively, on a Lichrosphere-NH₂ column. The column was eluted with a linear concentration gradient from 30.0% to 34.0% (by vol.) water in acetonitrile in 60 min at a flow rate of 1.5 ml/min, and monitored at 206 nm.

having the 3MeGal-I residue substituted at O-6 with an additional 3MeGal-IV residue, as in the antenna linked to O-2 of Man-4' of AIII.2.2 with the 3MeGal-IV'(β1-6)3MeGal-I' fragment.



In comparison with AIII.2.1, typical $\Delta\delta$ values are found for GalNAc-GN' H-4 ($\Delta\delta$ -0.017), 3MeGal-I H-1 ($\Delta\delta$ +0.024), H-3 ($\Delta\delta$ +0.009), and H-4 ($\Delta\delta$ +0.053), in accordance with the 6-substituted 3MeGal-I residue (see AIII.2.2). Furthermore, the new set of δ values for 3MeGal-IV (H-1, δ 4.462; H-3, δ 3.357; H-4, δ 4.213; OMe, δ 3.450) points to its terminal position.

Fractionation and characterization of oligosaccharide-alditols generated by PNGase-F digestion and reduction. In view of the low resolution of the sequentially applied CarboPac PA-1 and Lichrosorb-NH₂ fractionation procedures for Bio-Gel P-4 fraction AII, probably related to the hydrophobicity of the N-linked carbohydrate chains, another fractionation strategy based on initial reverse-phase chromatography of oligosaccharide-alditols was investigated. For comparison, the latter approach was also applied to Bio-Gel P-4 fraction AIII. Fractions AII and AIII were treated with NaB₂H₄, affording fractions BII and BIII, respectively. HPLC subfractionation of fraction BIII on a C₁₈ column yielded ten fractions, BIII.1–BIII.10 (Fig. 9A). Further fractionations were carried out on Lichrosphere-NH₂ (Fig. 9B–F) for fractions BIII.1 (BIII.1.1–BIII.1.4), BIII.3 (BIII.3.1–BIII.3.2), BIII.5 (BIII.5.1–

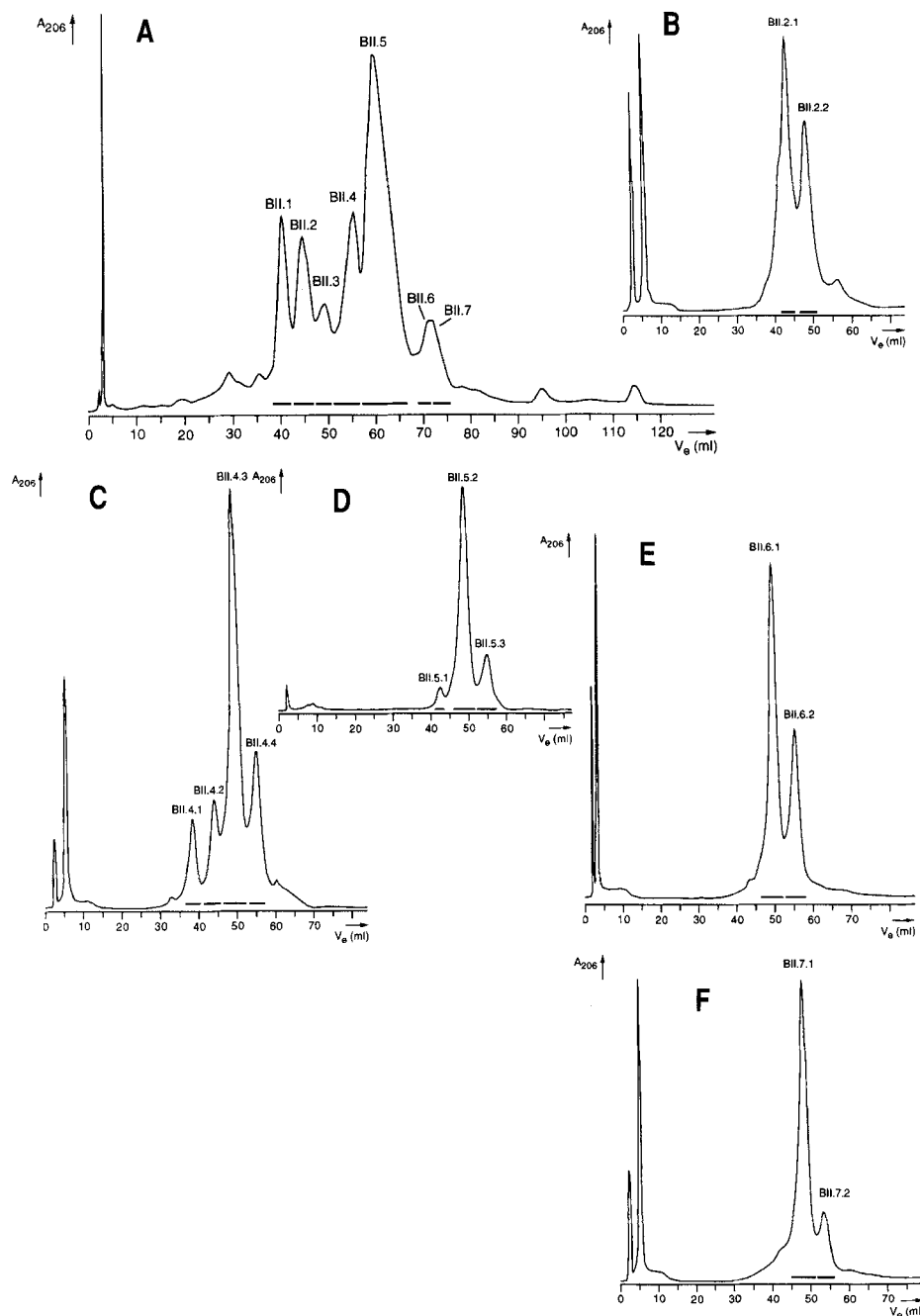
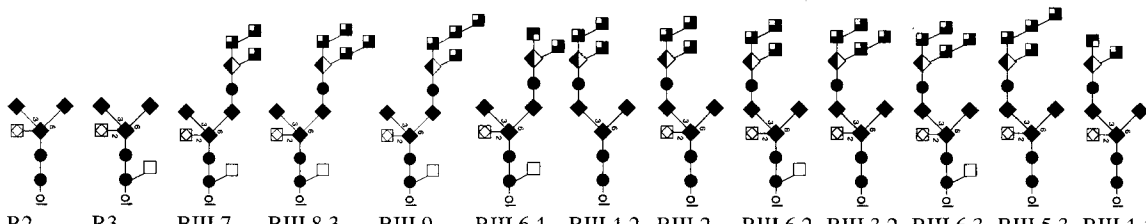


Fig. 10. Fractionation patterns of PNGase-F-released and NaBH₄-reduced oligosaccharides from Bio-Gel P-4 fraction AII (→BII) of *H. pomatia* α_D-Hc. (A) Fractionation pattern of fraction BII on a reverse-phase Lichrosphere-C₁₈ column. The column was eluted with 5.0% (by vol.) aqueous methanol for 5 min, followed by a linear concentration gradient to 9.0% (by vol.) aqueous methanol in 60 min, at a flow rate of 1.5 ml/min, and monitored at 206 nm. (B–F) Subfractionation patterns of the C₁₈ fractions BII.2, BII.4, BII.5, BII.6 and BII.7, respectively, on a Lichrosphere-NH₂ column. The column was eluted with a linear concentration gradient from 34.0% to 38.0% (by vol.) water in acetonitrile in 60 min at a flow rate of 1.5 ml/min, and monitored at 206 nm.

BIII.5.4), BIII.6 (BIII.6.1–BIII.6.3) and BIII.8 (BIII.8.1–BIII.8.4). HPLC subfractionation of fraction BII on a C₁₈ column yielded seven fractions, BII.1–BII.7 (Fig. 10A). Further fractionations were carried out on Lichrosphere-NH₂ (Fig. 10B–F) for fractions BII.2 (BII.2.1–BII.2.2), BII.4 (BII.4.1–BII.4.4), BII.5 (BII.5.1–BII.5.3), BII.6 (BII.6.1–BII.6.2) and BII.7 (BII.7.1–BII.7.2). All fractions were analyzed using ¹H-NMR spectroscopy; fractions which are not discussed below did not contain enough material, or were too heterogeneous for structural determination by ¹H-NMR spectroscopy. In a similar way to that already noted for the non-reduced oligosaccharides, the oligosaccharide alditols all have a corresponding *N,N'*-diacetylchitobitol or an α-1,6-fucosylated *N,N'*-diacetylchitobitol unit in common, as is indicated by the specific ¹H-NMR structural-reporter-group signals (see Table 6 and [11]).

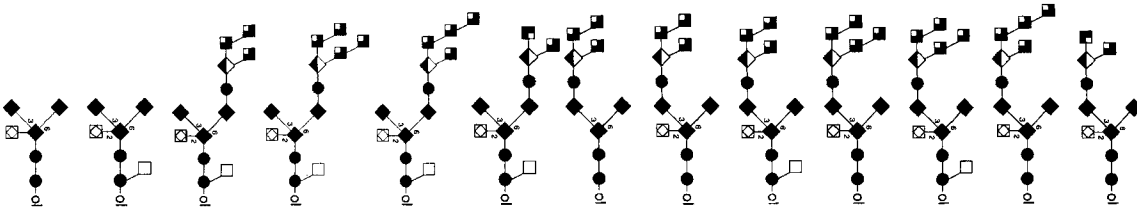
¹H-NMR analysis of the fractions BIII.6.2, BIII.2, BIII.1.2, BIII.7, and BIII.8.3 (Table 6) indicated the presence of the corresponding alditols of AIII.4, AIII.5, AIII.2.1, AIII.3 and AIII.2.2 (Table 4), respectively, and these data are not discussed further. Of

Table 6. ^1H chemical shifts of structural-reporter-group protons of the constituent monosaccharides of the monoantennary carbohydrate chains of *H. pomatia* $\alpha_n\text{-Hc}$ as oligosaccharide-alditols, together with data of the reference compounds R2 and R3 [11]. Chemical shifts are given relative to internal acetone (δ 2.225) in $^2\text{H}_2\text{O}$ at 300 K and $p^2\text{H}$ 7 [28]. Compounds are represented by short-hand symbolic notation: (●) GlcNAc; (◆) Man; (◻) Xyl; (◻) Fuc; (◻) GalNAc; (■) 3MeGal; (■) 4MeGal. For numbering of the monosaccharide residues, see text. n.d., not determined.

Residue	Re- porter group	Chemical shift in													
															
		ppm													
GlcNAc-ol-1	H-2	4.239	4.219	4.219	n. d.	n. d.	n. d.	n. d.	4.238	4.219	4.237	n. d.	4.236	4.237	
	NAc	2.057	2.058	2.058	2.058	2.058	2.058	2.055	2.056	2.056	2.056	2.056	2.055	2.055	
GlcNAc-2	H-1	4.634	4.718	4.718	4.719	4.718	n. d.	4.636	4.634	4.716	4.633	4.716	4.636	4.633	
	NAc	2.073	2.081	2.088	2.088	2.088	2.087	2.076	2.074	2.081	2.073	2.081	2.073	2.073	
Man-3	H-1	4.883	4.884	4.885	4.886	4.885	4.885	n. d.	4.879	4.880	4.879	4.880	4.879	4.877	
	H-2	4.270	4.270	4.269	4.269	4.268	4.269	4.253	4.257	4.256	4.256	4.256	4.256	4.255	
Man-4	H-1	5.122	5.124	5.127	5.127	5.127	5.127	5.119	5.142	5.142	5.141	5.142	5.142	5.140	
	H-2	4.039	4.040	n. d.	n. d.	4.098	n. d.	4.182	4.150	4.149	4.148	4.146	4.148	4.148	
Man-4'	H-1	4.913	4.914	4.899	4.899	4.899	4.898	4.919	4.916	4.916	4.917	4.917	4.917	4.916	
	H-2	3.983	3.982	n. d.	n. d.	n. d.	n. d.	3.976	n. d.	n. d.	n. d.	n. d.	3.980	n. d.	
Xyl-X	H-1	4.449	4.449	4.447	4.447	4.446	4.447		4.440	4.439	4.439	4.439	4.439	4.438	
	H-2	3.377	3.379	3.378	3.378	3.378	3.377		3.375	3.373	3.372	3.373	3.373	3.373	
	H-5 _{ax}	3.250	3.253	3.251	3.251	3.251	3.251		3.249	3.251	3.248	3.251	3.249	3.248	
Fuc-F	H-1		4.898	4.902	4.902	4.902	4.902			4.898		4.898			
	Me		1.225	1.227	1.227	1.227	1.226			1.224		1.224			
GlcNAc-5'	H-1			4.572	4.568	4.572	4.572								
	NAc			2.047	2.047	2.048	2.047								
GalNAc-GN'	H-1			4.587	4.585	4.590	4.584								
	H-4			4.214	4.197	4.220	4.205								
	NAc			2.054	2.054	2.058	2.052								
3MeGal-I'	H-1			4.501	4.525	4.502	4.487								
	H-3			3.347	3.355	3.348	3.343								
	H-4			4.182	4.233	4.181	4.182								
	OMe			3.454	3.450 ^a	3.455	3.452								
3MeGal-II'	H-1			4.473	4.476	4.470									
	H-3			3.325	3.321	3.333									
	H-4			4.241	4.247	4.233									
	OMe			3.427	3.428	3.428									
3MeGal-III'	H-1			4.440	4.440	4.457									
	H-3			3.320	3.321	3.325									
	H-4			4.208	4.208	4.248									
	OMe			3.448	3.445	3.440									
3MeGal-IV'	H-1				4.462										
	H-3				3.358										
	H-4				4.214										
	OMe				3.453 ^a										
3MeGal-V'	H-1					4.470									
	H-3					3.357									
	H-4					4.214									
	OMe					3.447									
4MeGal-II*'	H-1						4.416								
	OMe						3.510								

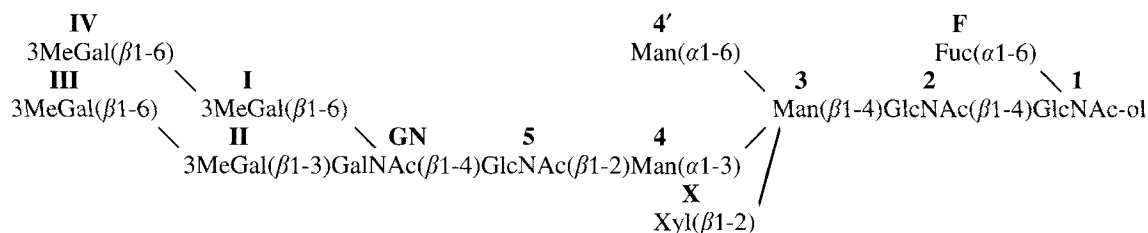
^{a-d} Values may have to be interchanged.

Table 6. (continued).

Residue	Re- porter group	Chemical shift in								
										
		ppm								
GlcNAc-5	H-1		4.575	4.539	4.538	4.533	4.533	4.538	4.538	
	NAc		2.050 ^b	2.048 ^c	2.047 ^d	2.048	2.049	2.049	2.047	
GalNAc-GN	H-1		4.584	4.582	4.581	4.579	4.579	4.584	4.577	
	H-4		4.214	4.213	4.212	4.196	4.196	4.214	4.203	
	NAc		2.055 ^b	2.050 ^c	2.049 ^d	2.048	2.049	2.049	2.047	
3MeGal-I	H-1		4.502	4.500	4.499	4.524	4.524	4.500	4.485	
	H-3		3.346	3.344	3.344	3.352	3.352	3.344	3.343	
	H-4		4.182	4.182	4.181	4.233	4.233	4.181	4.182	
	OMe		3.454	3.454	3.453	3.450	3.450	3.454	3.451	
3MeGal-II	H-1		4.473	4.473	4.472	4.475	4.475	4.470		
	H-3		3.322	3.323	3.322	3.321	3.321	3.331		
	H-4		4.241	4.241	4.240	4.246	4.246	4.248		
	OMe		3.427	3.427	3.426	3.427	3.427	3.428		
3MeGal-III	H-1		4.440	4.440	4.439	4.439	4.439	4.457		
	H-3		3.320	3.320	3.319	3.321	3.321	3.325		
	H-4		4.208	4.208	4.207	4.208	4.208	4.250		
	OMe		3.448	3.448	3.447	3.444	3.444	3.439		
3MeGal-IV	H-1					4.462	4.462			
	H-3					3.358	3.358			
	H-4					4.213	4.213			
	OMe					3.452	3.450			
3MeGal-V	H-1							4.470		
	H-3							3.358		
	H-4							4.214		
	OMe							3.446		
4MeGal-II*	H-1								4.415	
	OMe								3.509	

the oligosaccharide-alditols present in the remaining BIII subfractions, the corresponding non-reduced oligosaccharides have not been characterized from fraction AIII.

The ¹H-NMR spectrum of fraction BIII.6.3 is presented in Fig. 11, and the relevant data in Table 6. Comparison of the ¹H-NMR data for BIII.6.3 with those for BIII.6.2 and BIII.8.3 shows that compound BIII.6.3 is an analog of BIII.6.2 with one additional 3MeGal-IV residue. The terminal Man-4' residue is indicated by the H-1 resonance at δ 4.917 (supported by Xyl and GlcNAc-5 H-1 signals at δ 4.439 and 5.533, respectively). The substitution at O-6 of 3MeGal-I with 3MeGal-IV causes downfield shifts for 3MeGal-I H-1 ($\Delta\delta$ +0.025), H-3 ($\Delta\delta$ +0.008), and H-4 ($\Delta\delta$ +0.052), and an upfield shift of GalNAc-GN H-4 ($\Delta\delta$ -0.016), compared with the data for BIII.6.2. Identical chemical shift differences were found when going from BIII.7 to BIII.8.3, BIII.8.3 having the antenna linked to O-2 of Man-4' (see also AIII.3 going to AIII.2.2).



BIII.6.3

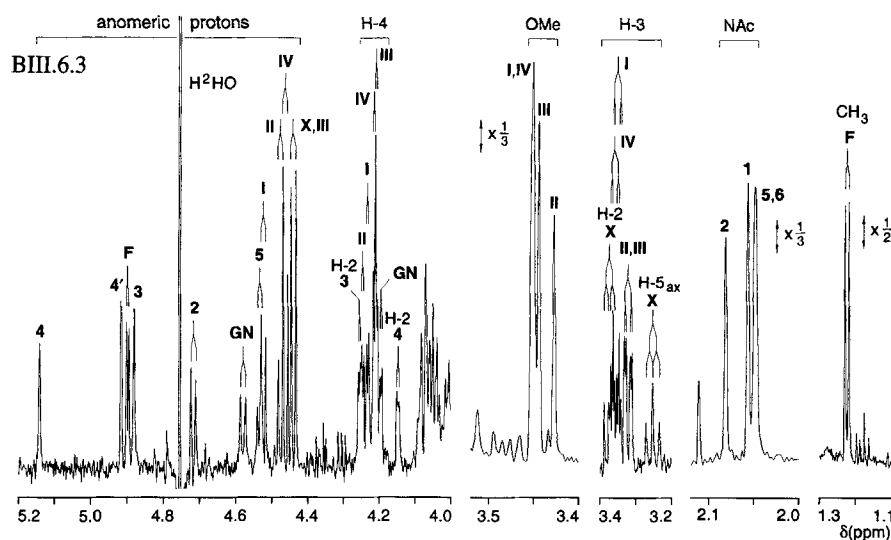


Fig. 11. Structural-reporter-group-signal regions of the resolution-enhanced 600-MHz ^1H -NMR spectrum of compound BIII.6.3 of *H. pomatia* α_D -Hc, recorded in $^2\text{H}_2\text{O}$ at 300 K. The relative scale for the OMe, NAc and CH_3 proton regions differs from that in the rest of the spectrum. For the abbreviated notations for the monosaccharides, see text.

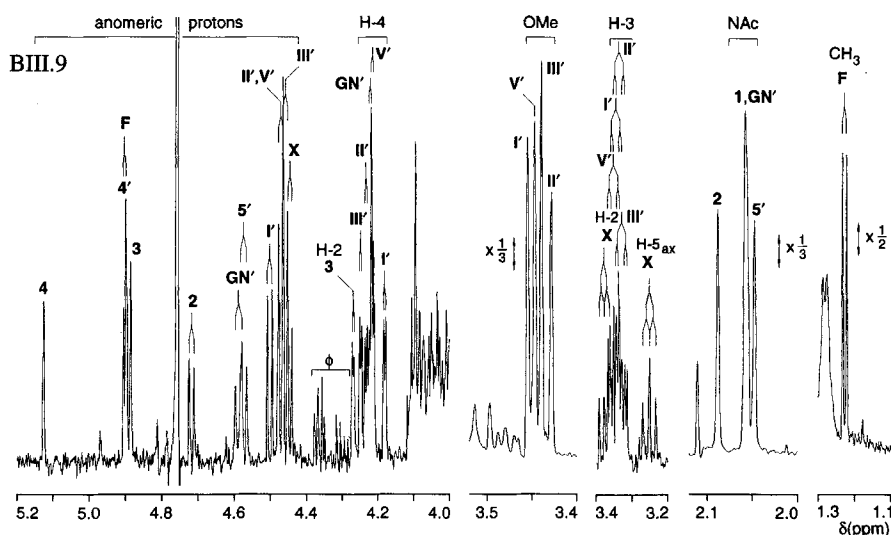
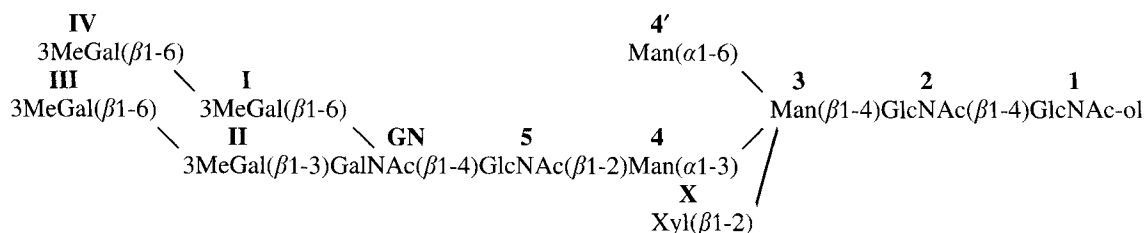


Fig. 12. Structural-reporter-group-signal regions of the resolution-enhanced 600-MHz ^1H -NMR spectrum of compound BIII.9 of *H. pomatia* α_D -Hc, recorded in $^2\text{H}_2\text{O}$ at 300 K. The relative scale for the OMe, NAc and CH_3 proton regions differs from that in the rest of the spectrum. For the abbreviated notations for the monosaccharides, see text.

Compound BIII.3.2 (Table 6) is the non-fucosylated variant of BIII.6.3, as inferred from the identical values for the structural reporters, except for those of the core element.



The ^1H -NMR spectrum of fraction BIII.9 is depicted in Fig. 12. As will be deduced in the following, compound BIII.9 is a structural isomer of BIII.8.3 with a terminal 3MeGal-I' residue and a 3MeGal-III' residue, extended at O-6 by an additional 3MeGal unit, given the code 3MeGal-V'. Supporting evidence was obtained from 2D HOHAHA and 2D ROESY measurements. The terminal position of Man-4 in the xylosylated trimannosyl-N,N'-diacetylchitobitol core element is clear from the Man-4 H-1 signal at δ 5.127. In the antenna linked to O-2 of Man-4' the presence of four 3MeGal residues is indicated by the typical H-1,3,4,5 and

Table 7. ^1H chemical shifts of antennary constituent monosaccharides of the monoantennary compounds BIII.9 of *H. pomatia* α_0 -Hc. Chemical shifts are given relative to internal acetone (δ 2.225) in $^2\text{H}_2\text{O}$ at 300 K and p^{H} 7 [28]. The compound is represented by short-hand symbolic notation: (●) GlcNAc; (◆) Man; (◻) Xyl; (□) Fuc; (◊) GalNAc; (■) 3MeGal. For numbering of the monosaccharide residues, see text. n.d., not determined.

Constituent of BIII.9	Chemical shift of					
	H-1	H-2	H-3	H-4	H-5	OMe/NAc
ppm						
GlcNAc-5'	4.572	n. d.	n. d.	n. d.	n. d.	2.048
GalNAc-GN'	4.590	4.068	3.920	4.220	3.963	2.058
3MeGal-I'	4.502	3.351	3.348	4.181	3.664	3.455
3MeGal-II'	4.470	3.535	3.333	4.233	3.823	3.428
3MeGal-III'	4.457	3.538	3.325	4.248	3.863	3.440
3MeGal-V'	4.470	3.552	3.357	4.214	3.680	3.447

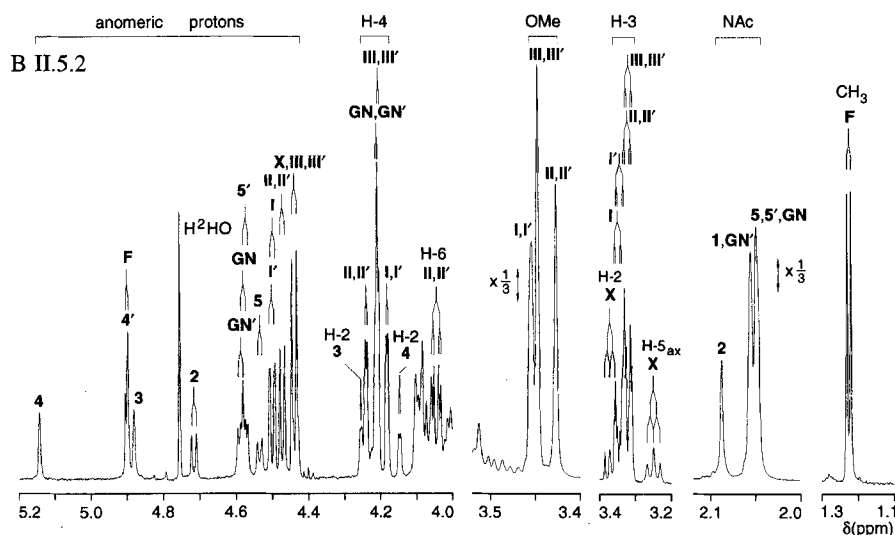
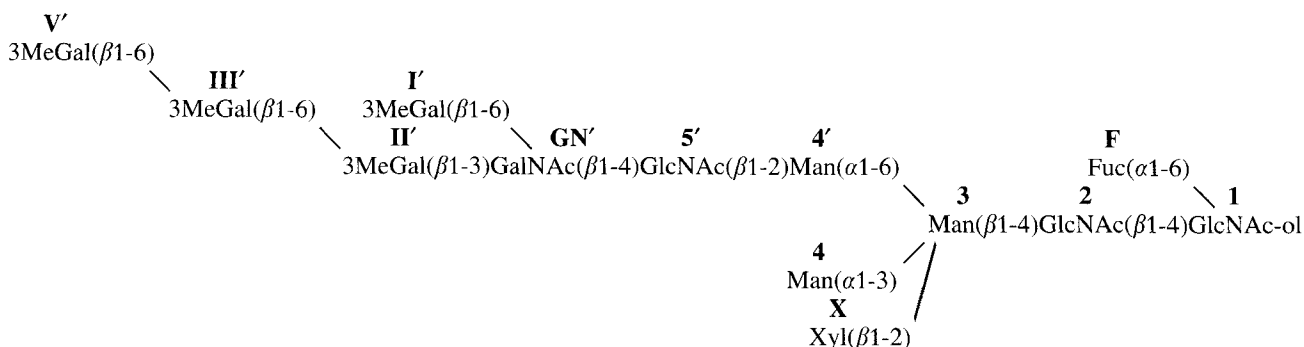
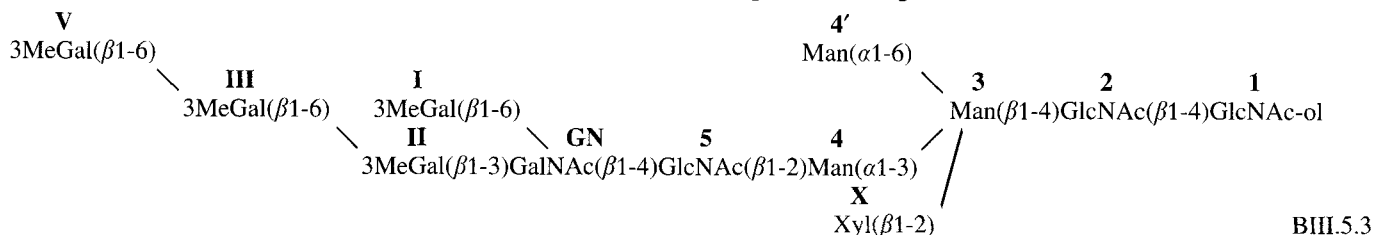


Fig. 13. Structural-reporter-group-signal regions of the resolution-enhanced 600-MHz ^1H -NMR spectrum of compound BII.5.2 of *H. pomatia* α_0 -Hc, recorded in $^2\text{H}_2\text{O}$ at 300 K. The relative scale for the OMe and NAc proton regions differs from that in the rest of the spectrum. For the abbreviated notations for the monosaccharides, see text.

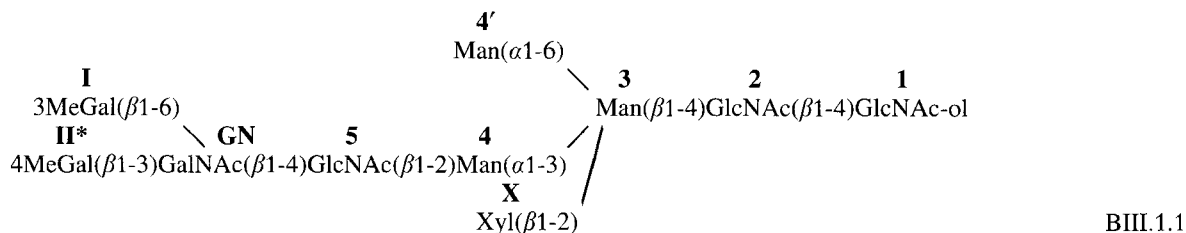
OMe signal positions (Table 7), of which two (3MeGal-I' and 3MeGal-V') are terminal (H-5, δ 3.66–3.68), and two (3MeGal-II' and 3MeGal-III') occur as 6-substituted residues (H-5, δ 3.82–3.86) (see e.g. AIII.2.2). Comparison with the ^1H -NMR data for BIII.7 supports the assignment of the 3MeGal-III' residue substituted on O-6 (H-1, $\Delta\delta$ +0.017; H-4, $\Delta\delta$ +0.040; Table 6), and the 3MeGal-I' residue being terminal (Table 6). The occurrence of the GalNAc and GlcNAc units is reflected by their H-1 and NAc signals (Table 6). The GalNAc-GN' H-5 resonance is found at δ 3.97 (Table 7), a position in agreement with a 6-substituted GalNAc residue (see also Table 5). Finally, the ROESY spectrum has a clear cross-peak between 3MeGal-II' H-1 and GalNAc-GN' H-3. Taking all data together, the structure for BIII.9 is proposed to be:



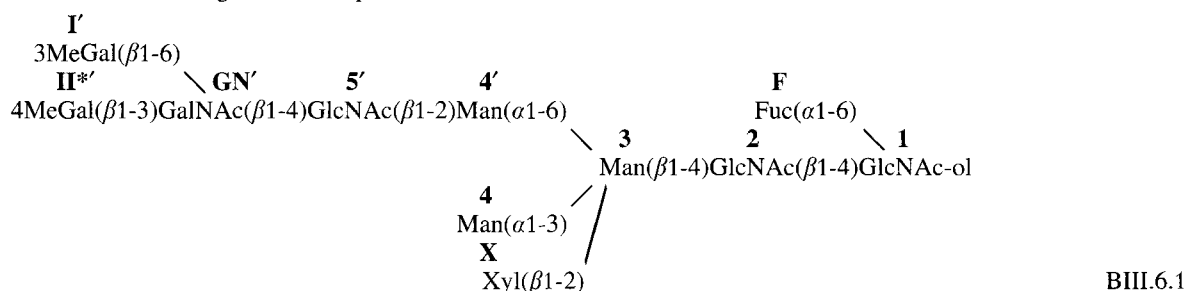
In a similar way to that discussed for BIII.9, comparing the structural-reporter-group data with those of BIII.7 (BIII.9 without 3MeGal-V') and BIII.8.3 (BIII.9 with 3MeGal-IV' and without 3MeGal-V'), the structural data for fraction BIII.5.3 (Table 6) could be interpreted by analogy with the data of BIII.2 and BIII.3.2, affording the following structure for BIII.5.3:



¹H-NMR analysis of fraction BIII.1.1 (Table 6) shows the presence of a non-fucosylated, xylosylated trimannosyl-*N,N'*-diacetylchitobiose core structure with Man-4' in a terminal position (H-1, δ 4.916) and a GalNAc(β1-4)GlcNAc element linked to O-2 of Man-4 (compare with BIII.2). The presence of two MeGal residues is reflected by the H-1 doublets at δ 4.485 and 4.415, and the OMe singlets at δ 3.451 and 3.509. Taking into account the NMR data for 3Me-β-Gal (H-1, δ 4.583; H-2, δ 3.501; H-3, δ 3.335; H-4, δ 4.204; OMe, δ 3.439 [13]) and 4Me-β-Gal (H-1, δ 4.535; H-2, δ 3.426; H-3, δ 3.685; H-4, δ 3.630; OMe, δ 3.515; determined in this study), and the various NMR data for 3MeGal in the oligosaccharides and their alditols, discussed so far (Tables 4–7), it is likely that the set of signals at δ 4.415 and 3.509 belongs to a 4MeGal residue. The 3MeGal H-1,3,4,OMe set is comparable to that for terminal 3MeGal-I in BIII.2, with only an upfield shift for the H-1 signal ($\Delta\delta$ -0.015). The set of structural reporters for GalNAc-GN in BIII.2 and BIII.1.1 is in the same region, with an upfield shift for the H-4 signal ($\Delta\delta$ -0.010). Based on these data in combination with the more detailed study on fraction BII.5.1 (see below), the following structure is suggested for BIII.1.1:



Inspection of the ¹H-NMR data of fraction BIII.6.1 (Table 6) indicates the occurrence of a fucosylated, xylosylated trimannosyl-*N,N'*-diacetylchitobiose core structure with a terminal Man-4' residue (H-1, δ 5.127) and a GalNAc(β1-4)GlcNAc element linked to O-2 of Man-4' (compare with BIII.7). The presence of two MeGal residues is shown by the H-1 signals at δ 4.487 and 4.416, and the OMe resonances at δ 3.452 and 3.510. In a similar way to that discussed for BIII.1.1, it is suggested that the set of signals at δ 4.416 and 3.510 belongs to a 4MeGal residue. The 3MeGal H-1,3,4,OMe set is comparable to that for terminal 3MeGal-I' in BIII.7, with an upfield shift of the H-1 signal ($\Delta\delta$ -0.014). The sets of structural reporters for GalNAc-GN' in BIII.6.1 and BIII.7 are in the same region, with an upfield shift for the H-4 signal ($\Delta\delta$ -0.009). Based on these data in combination with the more detailed study of fraction BII.5.1, the following structure represents BIII.6.1:



Analysis of fraction BII resulted in the determination of a series of novel diantennary oligosaccharide-alditols. In all cases the trimannosyl-*N,N'*-diacetylchitobiose element was xylosylated, whereas both α-1,6-fucosylated and non-fucosylated cores were identified (Table 8). The diantennary character is indicated by the typical set of Man structural-reporter-group signals: Man-3 H-1,2 (δ 4.881–4.882, 4.252–4.260), Man-4 H-1,2 (δ 5.142–5.143, 4.146–4.149) and Man-4' H-1 (δ 4.897–4.898) (see also [13]).

The ¹H-NMR spectrum of fraction BII.5.2 is depicted in Fig. 13. Inspection of the ¹H-NMR data for the antennae, and comparison with those of compounds BIII.6.2 and BIII.7, indicates the following structure for the major diantennary compound (approximately 30%) BII.5.2:

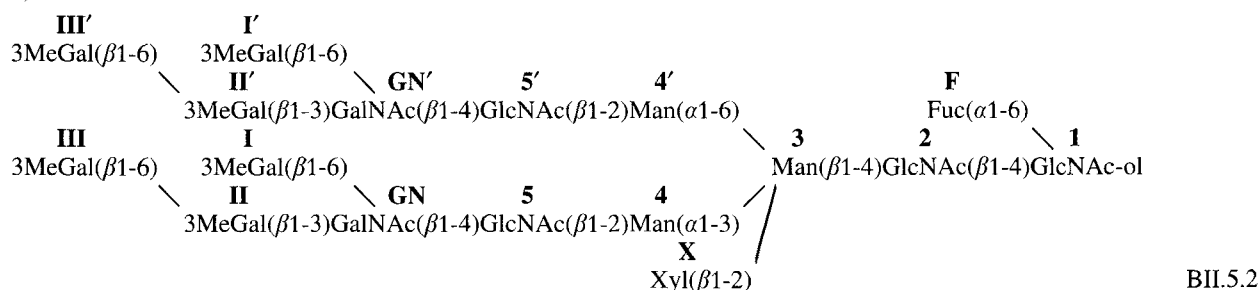


Table 8. (continued).

Residue	Reporter group	Chemical shift in									
ppm											
3MeGal-III/III'	H-1	4.440	4.440	4.440	4.457	4.440	4.440	4.440	4.440	4.440	
	H-3	3.320	3.320	3.321	3.325	3.320	n.d.	n.d.	3.320	3.321	
	H-4	4.208	4.208	4.208	4.248	4.208	4.208	4.208	4.208	4.207	
	OMe	3.447	3.447	3.450	3.439	3.448	3.446	3.446	3.447	3.447	
3MeGal-IV	H-1			4.461							
	H-3			3.358							
	H-4			4.214							
	OMe			3.450							
3MeGal-V	H-1				4.469						
	H-3				3.355						
	H-4				4.214						
	OMe				3.447						
4MeGal-II *	H-1					4.416					
	OMe					3.509					
3MeGal-IV'	H-1						4.462	4.463			
	H-3						n.d.	n.d.			
	H-4						4.213	4.214			
	OMe						3.452	3.451 ^a			
3MeGal-V'	H-1								4.470		
	H-3								3.356		
	H-4								4.214		
	OMe								3.447		
4MeGal-II *	H-1									4.419	
	OMe									3.510	

^a Values may have to be interchanged.

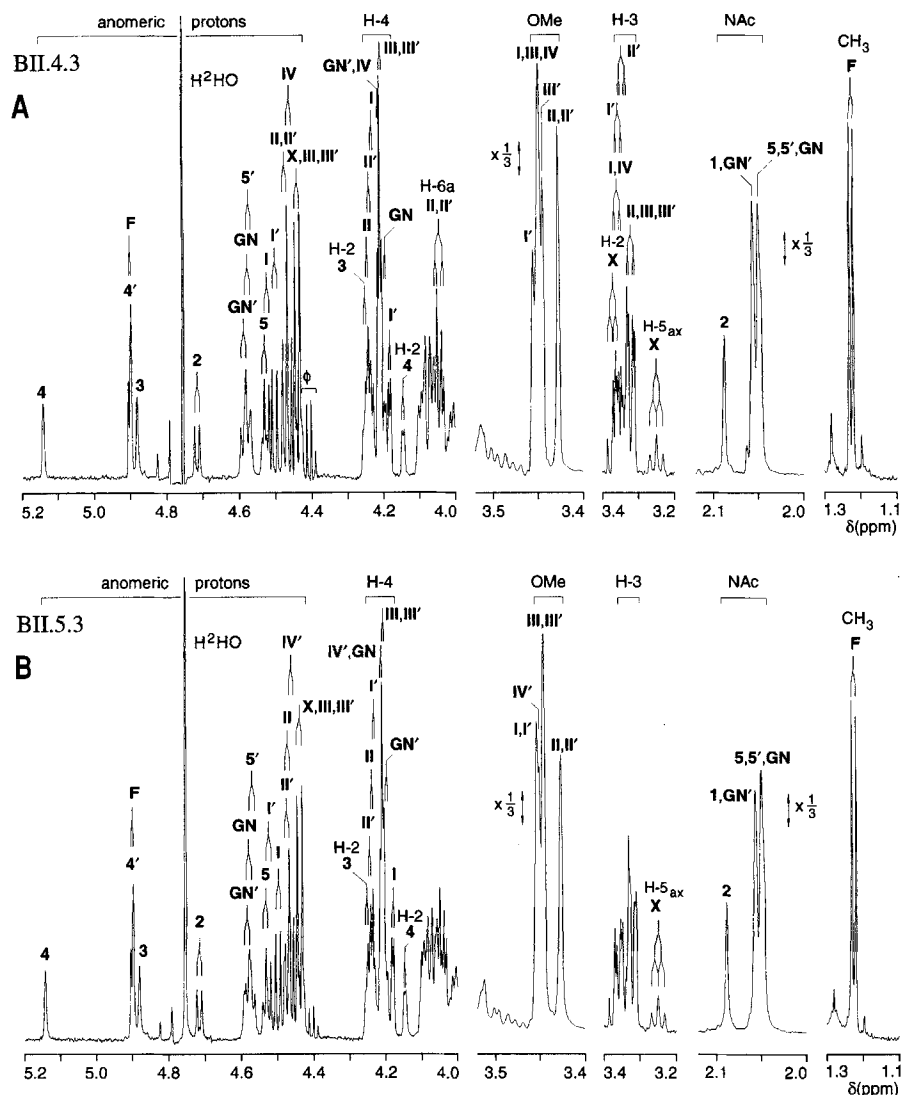
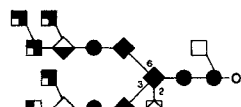


Fig. 14. Structural-reporter-group-signal regions of the resolution-enhanced 600-MHz ^1H -NMR spectra of compounds BII.4.3 (A) and BII.5.3 (B) of *H. pomatia* α_5 -Hc, recorded in $^2\text{H}_2\text{O}$ at 300 K. The relative scale for the OMe and NAc proton regions differs from that in the rest of the spectrum. For the abbreviated notations for the monosaccharides, see text.

Table 9. ^1H chemical shifts of antennary constituent monosaccharides of the diantennary compound BII.5.1 of *H. pomatia* α_5 -Hc. Chemical shifts are given relative to internal acetone (δ 2.225) in $^2\text{H}_2\text{O}$ at 300 K and $p^3\text{H}$ 7 [28]. The compound is represented by short-hand symbolic notation: (●) GlcNAc; (◆) Man; (◻) Xyl; (◻) Fuc; (◊) GalNAc; (■) 3MeGal; (■) 4MeGal. For numbering of the monosaccharide residues, see text. n.d., not determined.

Constituent of	Chemical shift of				
	H-1	H-2	H-3	H-4	OMe/NAc
					
BII.5.1					
	ppm				
GalNAc-GN	4.575	4.05 ^a	3.89 ^a	4.208	2.047
3MeGal-I	4.486	3.528	3.343	4.183	3.448
4MeGal-II*	4.416	3.445	3.668	3.619	3.509
GalNAc-GN'	4.589	4.05 ^a	3.90 ^a	4.214	2.056
3MeGal-I'	4.503	3.528	3.349	4.183	3.456
3MeGal-II'	4.474	3.541	3.326	4.242	3.427
3MeGal-III'	4.441	3.541	3.320	4.208	3.448

^a Values given in two decimals because of spectral overlap.

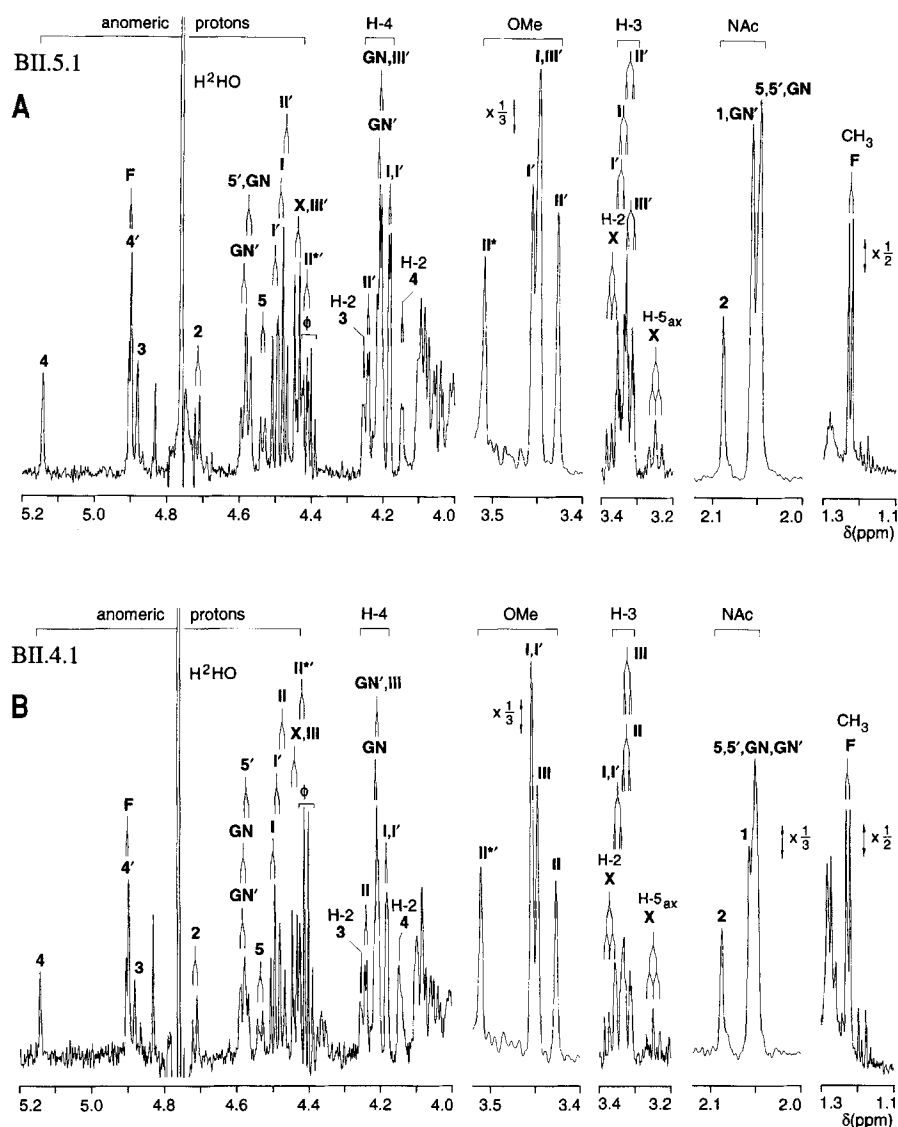
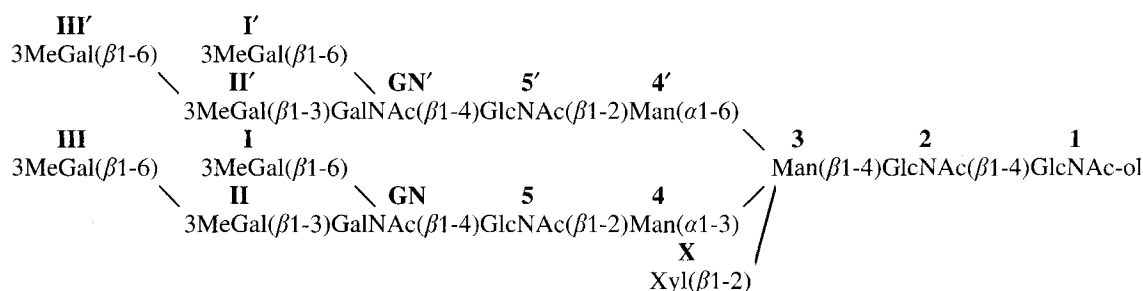


Fig. 15. Structural-reporter-group-signal regions of the resolution-enhanced 600-MHz ¹H-NMR spectra of compounds BII.5.1 (A) and BII.4.1 (B) of *H. pomatia* α_6 -Hc, recorded in ²H₂O at 300 K. The relative scale for the OMe and NAc proton regions differs from that in the rest of the spectrum. For the abbreviated notations for the monosaccharides, see text.

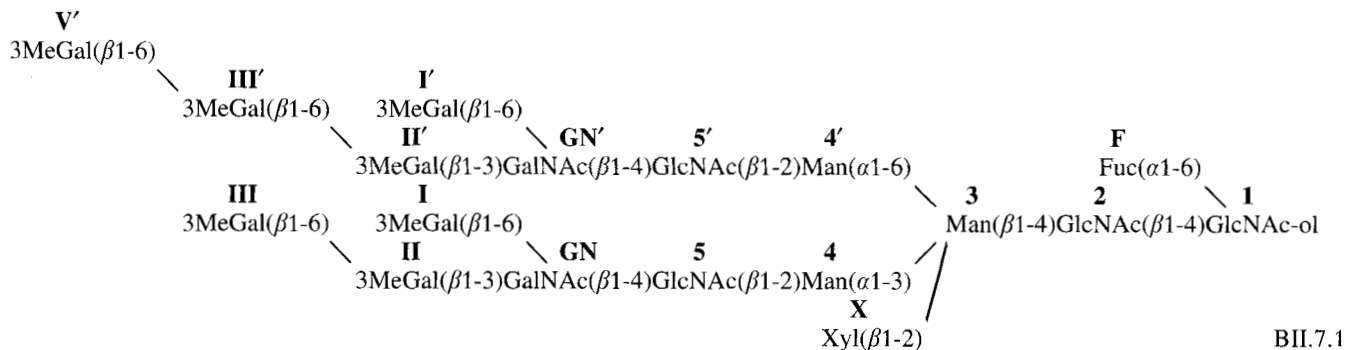
It should be noted that the GlcNAc-5, GlcNAc-5', GalNAc-GN and GalNAc-GN' H-1 atoms resonate at four distinct positions, with GlcNAc-5, H-1 typically at higher field than the other three signals (Table 8). Furthermore, the chemical shift value for the *N*-acetyl singlet of GalNAc-GN' clearly differs from those for the other three antennary HexNAc residues. Distinction between the 3MeGal-I and 3MeGal-I' residues, based on the H-1 and OMe signals, is only possible at high resolution.

Comparison of the ¹H-NMR data of fraction BII.2.1 (Table 8) with those of BII.5.2 shows that BII.2.1 is the non-fucosylated variant of BII.5.2.



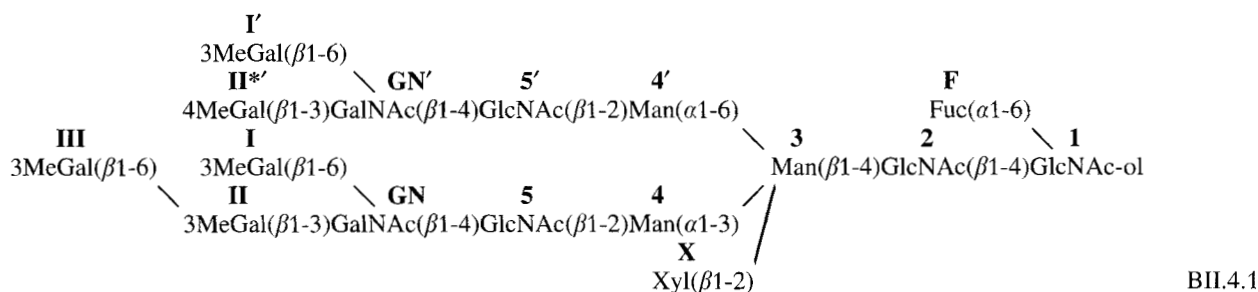
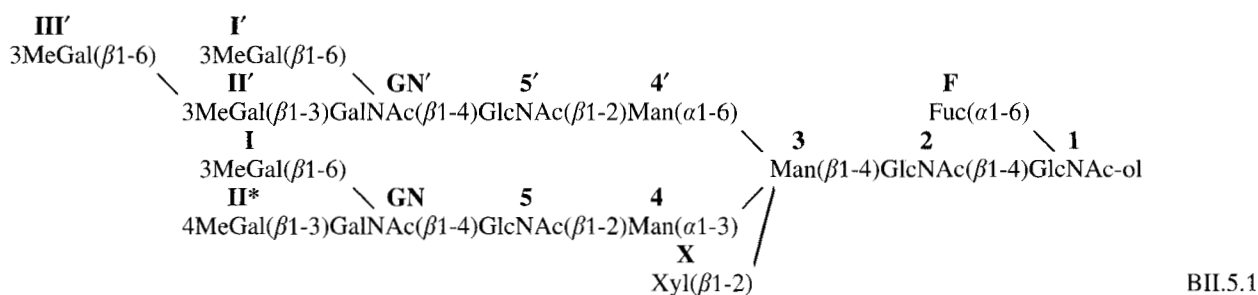
BII.2.1

The ¹H-NMR spectra of fractions BII.4.3 (Fig. 14A) and BII.5.3 (Fig. 14B) are very similar. In both cases seven 3MeGal residues are present (see e.g. *O*-methyl signal regions). Making use only of the trends in chemical shift differences ($\Delta\delta$), when comparing the GlcNAc, GalNAc and 3MeGal-I/I' H-1 signals of BIII.7 and BIII.8.3 (going from three 3MeGal to four 3MeGal residues in the upper chain), and of BIII.6.2 and BIII.6.3 (going from three 3MeGal to four 3MeGal residues in the lower chain)



Going from BIII.7 (containing three 3MeGal residues) to BIII.9 (four 3MeGal residues), the H-1 resonances of GalNAc-GN', 3MeGal-II' and 3MeGal-III' show $\Delta\delta$ values of +0.003, -0.003 and +0.017, respectively. Going from BIII.6.2 (three 3MeGal residues) to BIII.5.3 (four 3MeGal residues), the anomeric resonances of GalNAc-GN, 3MeGal-II and 3MeGal-III give rise to $\Delta\delta$ values of +0.003, -0.002 and +0.018, respectively. To conclude, going from BII.5.2 to BII.6.1, these trends can be detected for the antenna linked to O-2 of Man-4 whereas going from BII.5.2 to BII.7.1, these trends can be found for the antenna linked to O-2 of Man-4'.

The ^1H -NMR spectra of BII.5.1 and BII.4.1 are very similar (Fig. 15A and B). Both compounds contain one 4MeGal residue (H-1, δ 4.42; 4OMe, δ 3.51), four 3MeGal residues (H-1, δ 4.44–4.50; 3OMe, δ 3.43–3.46), two GalNAc and two GlcNAc residues (H-1, δ 4.53–4.59; NAc, δ 2.05–2.06). Trends in $\Delta\delta$ of GalNAc H-1, GalNAc H-4 and 3MeGal-1/ H by comparison of BIII.1.1 with BIII.2 and BII.6.1 with BIII.7, make the assignments of the sequences containing one 4MeGal and one 3MeGal residue or containing three 3MeGal residues possible. These results point to the following primary structures for BII.5.1 and BII.4.1:



Going from BIII.2 (three 3MeGal residues) to BIII.1.1 (one 4MeGal and one 3MeGal), the $\Delta\delta$ values of GalNAc-GN H-1, GalNAc-GN H-4 and 3MeGal-I H-1 are -0.005 , -0.010 and -0.015 , respectively, and going from BIII.7 to BIII.6.1 GalNAc-GN' H-1, GalNAc-GN' H-4 and 3MeGal-I' H-1 reveal $\Delta\delta$ values of -0.003 , -0.009 and -0.014 , respectively. Going from BII.5.2 to BII.5.1, these trends are observed for the antenna linked to O-2 of Man-4 and going from BII.5.2 to BII.4.1, they are detected for the antenna linked to O-2 of Man-4'.

Evidence for the presence of terminal 4MeGal was obtained from a HOHAHA experiment carried out on BII.5.1 (Table 9). The HOHAHA H-1 track with δ 4.416 indicates the *galacto*-configuration for the monosaccharide (H-2, δ 3.445; H-3, δ 3.668; H-4, δ 3.619). Combined with the *O*-methyl singlet at δ 3.509 and compared with the ^1H -NMR data for 3Me- β -Gal and 4Me- β -Gal (see above), a terminal 4MeGal residue is indicated. Furthermore, the HOHAHA H-1 track with δ 4.486 reveals the chemical shift values for H-2,3,4 of 3MeGal-I in the 4MeGal-containing antenna. These values are similar to those of 3MeGal-I in AIII.4 (Table 5), demonstrating its terminal position in the oligosaccharide chain as well as its 1,6-linkage to GalNAc-GN.

Concluding remarks. The structural analysis of complex high-molecular-mass carbohydrate chains from α_D -Hc of *H. pomatia*, released by PNGase-F, has resulted in the elucidation of 12 novel monoantennary N-glycans and 9 novel diantennary N-glycans. In combination with the three low-molecular-mass core structures, an almost complete picture of the glycosylation pattern of α_D -Hc can now be given. It should be noted that not all core structures contain xylose, and when xylose is absent, fucose is absent too. So far, no reliable information is available with respect to the structures present in fractions A1a (B1a) and A1b (B1b).

