Structure of the Asn-Linked Oligosaccharides of Apolipophorin III from the Insect *Locusta migratoria*. Carbohydrate-Linked 2-Aminoethylphosphonate as a Constituent of a Glycoprotein†

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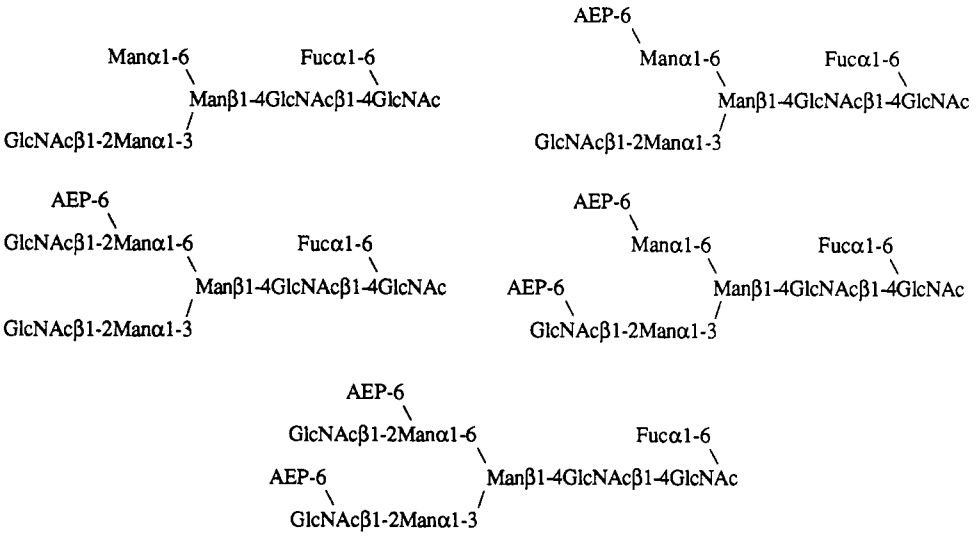
ABSTRACT: The primary structures of the N-linked carbohydrate chains of apolipophorin III from the insect *Locusta migratoria* have been determined. The glycoprotein was completely deglycosylated with peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F. Released oligosaccharides were separated from the remaining protein using gel-permeation chromatography on Bio-Gel P-100. Purification of the carbohydrate chains was achieved by a combination of FPLC anion-exchange chromatography on Mono-Q and amine adsorption HPLC on Lichrosorb-NH_2. The structures of the carbohydrate chains were deduced with a combination of fast atom bombardment mass spectrometry, ^{1}H- and ^{31}P-NMR spectroscopy, and methylation analysis. The majority of the carbohydrate chains contains 2-aminoethylphosphonate (AEP), which is linked to the 6-position of Man and/or GlcNAc. *L. migratoria* apolipophorin III is the first example of a glycoprotein containing carbohydrate-linked 2-aminoethylphosphonate. The structures of the major oligosaccharides were established to be the following:



In those insects which use lipids as fuel for flight, such as the migratory locust *Locusta migratoria* and the moth *Manduca sexta*, these lipids are transported to the flight muscles in the form of low-density lipoproteins [for reviews, see Chino (1985), Beenakkers et al. (1985), Shapiro et al. (1988), Van der Horst (1990), and Ryan (1990)]. During flight, diacylglycerol is released from triacylglycerol stores in the fat body and loaded onto high-density lipophorin (HDLp)[1] in the hemolymph. At the same time, several molecules of an additional abundant low-molecular-mass hemolymph protein, apolipophorin III (apoLp-III, molecular mass 18–20 kDa), associate with the HDLp particle, transforming HDLp into low-density lipophorin (LDLp). Hydrolysis of LDLp-carried diacylglycerol by flight muscle lipoprotein lipase results in regeneration of both HDLp and free apoLp-III, thus providing an efficient reutilizable lipid shuttle system during flight activity [for reviews, see Beenakkers et al. (1985), Shapiro et al. (1988), Van der Horst (1990), and Ryan (1990)].

The physicochemical properties of apoLp-III, the major function of which may be to stabilize the expanding hydro-

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[1] Abbreviations: AEP, 2-aminoethylphosphonate; apoLp, apolipophorin; COSY, correlation spectroscopy; deoxyhex, deoxyhexose; FAB, fast atom bombardment; GlcNAc, *N*-acetylglucosamine; HDLp, high-density lipophorin; Hex, hexose; HexNAc, *N*-acetylhexosamine; HOHAHA, homonuclear Hartmann–Hahn; LDLp, low-density lipophorin; Man, mannose; MLEV, composite pulse devised by M. Levitt; MS, mass spectrometry; MS–MS, tandem mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PMAA, partially methylated alditol acetate; PNGase-F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F; RESED, relayed spin-echo difference spectroscopy; 1D, one-dimensional; 2D, two-dimensional.

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phobic lipid surface area of the lipophorin particle resulting from diacylglycerol uptake (Shapiro et al., 1988; Kawooya et al., 1986; Wells et al., 1987; Demel et al., 1992), have been the focus of considerable interest. Although apoLp-IIIs have been isolated from several evolutionarily divergent insect species [for reviews, see Beenakkers et al. (1985), Shapiro et al. (1988), Van der Horst (1990), and Ryan (1990)], the amino acid sequences have only been reported for apoLp-III from *L. migratoria* and *M. sexta* (Cole et al., 1987; Kanost et al., 1988), revealing an overall sequence identity of only 29% (Kanost et al., 1988). Even though there is very little sequence identity between these two apoLp-IIIs, they are functionally equivalent in the production of LDLp particles in an in vitro lipid loading system, employing locust HDLp and the fat body (Van der Horst et al., 1988). This may relate to the presence of amphipathic helical segments in both apolipoproteins (Cole et al., 1987; Kanost et al., 1988; Breiter et al., 1991).

An important difference between apoLp-IIIs from different species is the degree of glycosylation. ApoLp-III from *M. sexta* contains no carbohydrate or other posttranslational modifications (Kawooya et al., 1984, 1986), whereas apoLp-III from *L. migratoria* is a glycoprotein (Van der Horst et al., 1984; Chino & Yazawa, 1986; Ryan et al., 1990). Asn 16 and Asn 83 are glycosylated, containing mannose, fucose, and either glucosamine (Chino & Yazawa, 1986) or *N*-acetylglucosamine (Ryan et al., 1990) as monosaccharide constituents. The biological role of apoLp-III glycosylation is not yet clear (Van der Horst et al., 1988; Demel et al., 1992). The three-dimensional structure of apoLp-III has been determined by X-ray analysis and shows that the glycoprotein can be classified into the general category of up-and-down α-helical bundles (Breiter et al., 1991). From the X-ray diffraction data one *N*-acetylglucosamine residue could be modeled into the electron density at each glycosylation site, but the structure of the rest of the carbohydrate chains remained obscure.

Recently, two isoforms of apoLp-III from *L. migratoria*, denoted apoLp-IIIa and apoLp-IIIb, have been identified. They occur in the hemolymph in a molar ratio of 5:9 and have similar molecular masses but different isoelectric points (Van der Horst et al., 1991). The N-terminal amino acid sequence of apoLp-IIIa is identical to that of apoLp-IIIb, except for an additional Arg-Pro segment at the N-terminus of apoLp-IIIa. In this report, the structures of the N-linked oligosaccharides of both isoforms of *L. migratoria* apoLp-III are presented, which include 2-aminoethylphosphonate as a novel non-carbohydrate substituent in a glycoprotein.

EXPERIMENTAL PROCEDURES

Isolation of ApoLp-III. Pooled samples (5–10 mL) of hemolymph, obtained from adult male *L. migratoria* (12 days after the imaginal ecdysis), were fractionated by gel-permeation chromatography on Ultrogel AcA 44 (IBF, Villeneuve la Garenne, France), and the fractions containing the free apoLp-III monomers were subjected to DEAE-Sephacel (Pharmacia) anion-exchange chromatography using a gradient of 20–150 mM ammonium acetate, pH 6.5, yielding the individual apoLp-III isoforms (Van der Horst et al., 1991). The fractions containing apoLp-IIIa or -IIIb were pooled, concentrated in an Amicon concentrator using a YM 2 membrane, lyophilized, and checked for purity by both native polyacrylamide gel electrophoresis (PAGE) (gel gradient 10–30%) and sodium dodecyl sulfate (SDS)–PAGE (gel gradient 10–30%).

Monosaccharide Analysis. Monosaccharide analysis was carried out by gas–liquid chromatography on a capillary SE-30 fused silica column (0.32 mm × 25 m, Pierce) using a Varian Aerograph 3700 gas chromatograph. The trimethylsilylated (methyl ester) methyl glycosides were prepared by methanolysis, *N*-(re)acetylation, and trimethylsilylation (Kamerling & Vliegenthart, 1989).

Liberation of the Carbohydrate Chains. Lyophilized samples of locust apoLp-IIIa or -IIIb (approximately 40 mg each) were dissolved in 2.0 mL of 50 mM Tris-HCl buffer, pH 8.4, containing 10 mM EDTA. N-Linked carbohydrate chains were released from the protein moiety with 10 units of PNGase-F added in a volume of 50 μL and incubated for 24 h at room temperature, essentially as described (Hård et al., 1990). The degree of deglycosylation of the glycoproteins was followed by SDS–PAGE (gel gradient 10–30%) 8, 16, and 24 h after the addition of PNGase-F. For both isoforms of apoLp-III, no further reduction in molecular mass was observed after 16 h of PNGase-F treatment. Additional proof for the completeness of the deglycosylation was obtained by SDS–PAGE followed by blotting onto nitrocellulose (Schleicher and Schüll) and probing the blot using the Boehringer Mannheim glycan detection kit. Staining of digoxigenin-labeled glycoconjugates was performed with an antibody/alkaline phosphatase conjugate in an enzyme immunoassay according to the manufacturer's protocol.

In each case, the liberated carbohydrate chains were separated from the deglycosylated apoLp-III samples on a Bio-Gel P-100 column (1.6 cm × 60 cm, 100–200-μm mesh, Bio-Rad) in 100 mM sodium bicarbonate, pH 7.5, containing 0.1% SDS. After lyophilization, the carbohydrate-containing fraction (orcinol/H_2SO_4 assay) was desalted on a Bio-Gel P-2 column (1.6 cm × 30 cm, 200–400-μm mesh, Bio-Rad) using water as eluent and monitoring at 206 nm. Fractions were assayed for carbohydrate according to Dubois et al. (1956), and positive fractions were pooled and lyophilized.

FPLC. Elimination of anionic non-carbohydrate contaminants from the carbohydrate-containing Bio-Gel P-100 pool was carried out by anion-exchange chromatography using a Mono Q HR 5/5 column (Pharmacia FPLC system). For both apoLp-IIIa and -IIIb the oligosaccharides failed to bind to the Mono-Q column in either water or 50 mM Tris-HCl, pH 8.0, and were consequently recovered in the void volume. After desalting on a Bio-Gel P-2 column as described above, the carbohydrate fractions were lyophilized.

HPLC Fractionation. Fractionation of the carbohydrate fractions obtained after FPLC was achieved on a Kratos Spectroflow 400 HPLC system (Kratos Analytical) using a Lichrosorb-NH_2 10-μm column (0.46 cm × 25 cm, Chrompack). Elutions were carried out with a mixture of acetonitrile/water (65:35 by volume) at a flow rate of 2 mL/min, collecting 0.5-mL fractions. Runs were monitored at 205 nm. The corresponding fractions from 10 runs of either apoLp-IIIa- or apoLp-IIIb-derived oligosaccharides were pooled, desalted on Bio-Gel P-2, and lyophilized.

NMR Spectroscopy. Prior to NMR spectroscopic analysis, the samples were exchanged twice in 99.8 atom % ^2H_2O (MSD Isotopes). Finally, samples were dissolved in 99.96 atom % ^2H_2O (MSD Isotopes) and transferred to NMR tubes. Spectra at 500 MHz were recorded on a Bruker AM-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University), and spectra at 600 MHz were recorded on a Bruker AM-600 spectrometer (NSR Center, University of Nijmegen, The Netherlands). The probe temperature was 27 °C, unless indicated otherwise. Chemical shifts are

expressed relative to internal acetone (2.225 ppm in ^2H_2O at 27 °C; Vliegenthart et al., 1983). One-dimensional (1D) and two-dimensional (2D) HOHAHA (Bax & Davis, 1985) ^{1}H-NMR spectra were recorded essentially as described (Hård et al., 1992). One-dimensional relayed spin-echo difference (RESED) spectra were recorded with a 5-mm inverse broadband probe according to the pulse sequence described by De Waard and Vliegenthart (1989). The duration of the MLEV-17 mixing time was varied between 10 and 120 ms. ^{31}P-NMR spectra were measured at 27 °C at a spectrometer frequency of 202 MHz. Phosphorus chemical shifts are given relative to external orthophosphoric acid (0.0 ppm).

Chemical Modifications. N-Acetylation, permethylation, and the preparation of partially methylated alditol acetates (PMAA) were carried out essentially as described (Lederkremer et al., 1991). Replacement of the AEP group with a deuteromethyl group was achieved by carrying out the permethylation procedure on the N-acetylated permethylated derivative using C^2H_3I instead of C^1H_3I, while all other conditions and procedures were kept the same.

Mass Spectrometry. Positive-ion fast atom bombardment (FAB) mass spectra were obtained using MS1 of a JEOL JMS-SX/SX102A tandem mass spectrometer (Bijvoet Center, Department of Mass Spectrometry, Utrecht University) with an 8-kV accelerating voltage. The FAB gun was operated at an emission current of 10 mA with 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 recorded and processed on a Hewlett Packard HP9000 data system. Tandem mass (MS–MS) spectra were obtained on the same instrument, using 10-kV accelerating voltage, with helium as the collision gas at a pressure sufficient to reduce the parent ion to one-third of its original intensity.

Gas–liquid chromatography–mass spectrometry (GLC–MS) of the PMAA derivatives was carried out using a JEOL JMS-AX505W mass spectrometer fitted with a Hewlett Packard 5890 gas chromatograph (Bijvoet Center, Department of Mass Spectrometry, Utrecht University) using an on-column injector and helium as the carrier gas at a flow rate of 2 mL/min. The monosaccharide derivatives were separated on a CP-Sil 5CB column (0.32 mm × 25 m, Chrompack) with the following temperature program: 90 °C for 2 min, gradient of 30 °C/min to 140 °C, gradient of 4 °C/min to 230 °C, and finally holding the temperature at 230 °C for 5 min. Mass spectra were obtained under electron impact conditions and were recorded using linear scanning from m/z 50–500 at an accelerating voltage of 3 kV.

Other Analytical Methods. Carbohydrate-containing HPLC fractions were analyzed for aminoethylphosphonate after hydrolysis with 4 M HCl at 100 °C for 8 h according to Araki et al. (1986), using an LKB Model 4151 Alpha plus analyzer and lithium citrate buffers. 2-Aminoethylphosphonic acid (Sigma) was used as a standard.

Native PAGE was performed in gradient gels (10–30%) with a constant current of 30 mA, and SDS–PAGE was run on gradient gels (10–30%) as described (Van der Horst et al., 1991). Gels were stained with Amido Black 10B.

RESULTS

Monosaccharide analysis of apoLp-IIIa from *L. migratoria*, having a total carbohydrate content of approximately 10% (by mass) [see also Van der Horst et al. (1984)], revealed the presence of Fuc, Man, GlcNAc, and GalNAc in a molar ratio of 1.3:3.0:3.3:0.2. Release of the N-linked carbohydrate chains from apoLp-IIIa and -IIIb with PNGase-F resulted in each

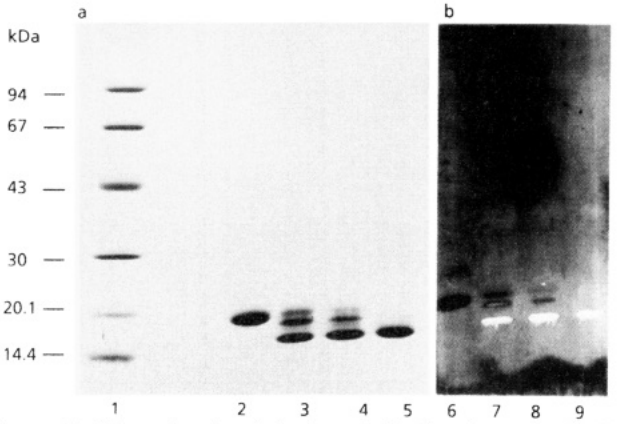


FIGURE 1: Liberation of carbohydrate chains from locust apoLp-III by incubation with PNGase-F. (A) SDS–PAGE (gradient 10–30%): lane 1, molecular mass markers; lane 2, untreated apoLp-III; lane 3, after incubation with PNGase-F for 8 h; lane 4, 16 h; lane 5, 24 h. The gel was stained with Amido Black 10B. (B) Nitrocellulose blot of the gel in (A) with subsequent staining for carbohydrate using a glycan detection kit (Boehringer Mannheim). Lanes 6–9 correspond to lanes 2–5, respectively.

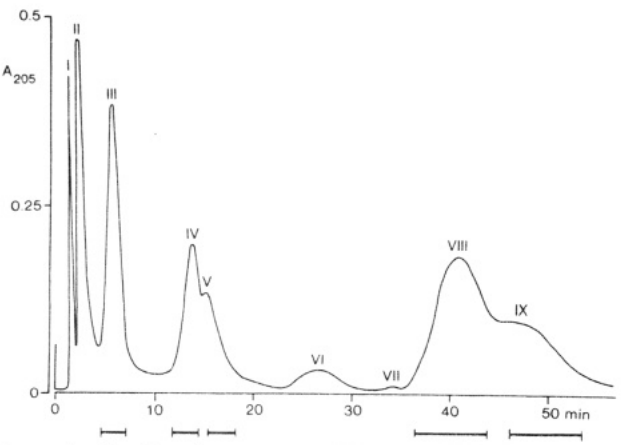


FIGURE 2: Fractionation pattern at 205 nm on a HPLC Lichrosorb-NH_2 column of PNGase-F-released oligosaccharides obtained from locust apoLp-IIIb. The column was eluted isocratically with a mixture of acetonitrile/water (65:35 by volume) at a flow rate of 2 mL/min. Fractions were pooled as indicated.

case in a decrease in apparent molecular mass of the glycoproteins from 18.3 to 16.0 kDa, as judged from SDS–PAGE (Figure 1A). The negative results obtained with the glycan detection kit after 24 h of PNGase-F treatment (Figure 1B) indicate complete cleavage of all N-linked chains and the absence of O-linked carbohydrate chains in both apoLp-III isoforms. From the number of new bands appearing with time during PNGase-F treatment (Figure 1A), it can be concluded that both isoforms of apoLp-III contain two glycosylated Asn residues [see also Kanost et al. (1988)].

The released oligosaccharides in the carbohydrate-containing Bio-Gel P-100 pool did not bind to an FPLC anion-exchange Mono Q column and were eluted in the void volume (data not shown). Separation of the carbohydrate-containing FPLC fraction by HPLC on Lichrosorb-NH_2 resulted in nine major fractions, denoted I–IX (Figure 2). Carbohydrate staining (Dubois et al., 1956) revealed that fractions I, II, VI, and VII did not contain carbohydrate material. Fractions III, IV, V, VIII, and IX were analyzed by 500- or 600-MHz ^{1}H-NMR spectroscopy, and except for fraction III, by fast atom bombardment mass spectrometry (FAB-MS), tandem mass spectrometry (MS–MS), and methylation analysis. Relevant ^{1}H-chemical shifts are compiled in Table I and data from the MS–MS study are in Table II. Since the monosac-

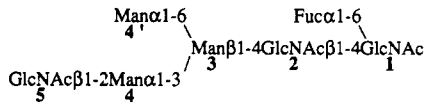
Table I: ^{1}H Chemical Shifts of Structural-Reporter-Group Protons of the Constituent Monosaccharides of N-Linked Oligosaccharides Derived from ApoLp-III of *L. migratoria*[a]

reporter group	residue	chemical shift (ppm) in III	IV.1	V.1	VIII.1	IX.1
H-1	GlcNAc-**1**α	5.180	5.180	5.180	5.179	5.179
	GlcNAc-**1**β	4.693	4.694	4.694	4.694	4.694
	GlcNAc-**2**α	4.664	4.666	4.667	4.666	4.666
	GlcNAc-**2**β	4.666	4.672	4.672	4.671	4.671
	Man-**4**	5.117	5.117	5.117	5.116	5.116
	Man-**4**′	4.917	4.915	4.912	4.914	4.913
	GlcNAc-**5**	4.551	4.558	4.562	4.584	4.584
	GlcNAc-**5**′			4.569		4.570
	Fucα	4.885	4.888	4.889	4.887	4.888
	Fucβ	4.893	4.895	4.896	4.894	4.895
H-2	Man-**3**	4.250	4.251	4.252	4.250	4.250
	Man-**4**	4.189	4.192	4.192	4.192	4.193
	Man-**4**′	3.973	3.986	4.126	3.986	4.127
H-5	Fucα	4.101	4.096	n.d.	4.10	n.d.
	Fucβ	4.131	4.13	n.d.	4.13	n.d.
NAc	GlcNAc-**1**	2.038	2.039	2.039	2.038	2.038
	GlcNAc-**2**α	2.093	2.095	2.096	2.095	2.096
	GlcNAc-**2**β	2.093	2.092	2.096	2.092	2.093
	GlcNAc-**5**	2.052	2.053	2.053	2.056	2.056
	GlcNAc-**5**′			2.069		2.069
CH_3	Fucα	1.208	1.209	1.209	1.208	1.208
	Fucβ	1.218	1.219	1.219	1.219	1.218

[a] Chemical shifts are given at 27 °C and were measured at 500 MHz (except for VIII.1, which was measured at 600 MHz) in ^2H_2O relative to internal acetone (2.225 ppm; Vliegenthart et al., 1983). For numbering of the monosaccharide residues, see the text. α and β refer to the anomeric configuration of GlcNAc-**1**.

charide analysis data, the HPLC profiles of the released carbohydrate chains, and the structure of the oligosaccharides obtained from both apoLp-IIIa and -IIIb turned out to be identical, only the results related to apoLp-IIIa will be discussed.

Fraction III. The ^{1}H-NMR spectrum of fraction III (not shown) indicates as the main constituent the presence of the following fucosylated heptasaccharide.



The ^{1}H-NMR data fit those previously published for an identical oligosaccharide structure [cf. compound {0,2+F} in Brockhausen et al. (1988)]. The presence of the α1–6-fucosylated *N*,*N*′-diacetylchitobiose unit is inferred from the H-1 signals of GlcNAc-**1**α at 5.180 ppm, GlcNAc-**1**β at 4.693 ppm, GlcNAc-**2** at 4.66–4.67 ppm, and Fuc at 4.88–4.89 ppm, together with the *N*-acetyl (NAc) methyl signals of GlcNAc-**1** at 2.038 ppm and GlcNAc-**2** at 2.093 ppm, as well as from the Fuc CH_3 signals at 1.208 ppm (GlcNAc-**1**α) and 1.218 ppm (GlcNAc-**1**β) (De Waard et al., 1991).

Fraction IV. Positive-ion FAB-MS analysis of fraction IV (data not shown) revealed the presence of one major (IV.1) and two minor carbohydrate-containing components. The major pseudomolecular ion cluster was observed at *m/z* 1389 $[(M + Na)^+]$, *m/z* 1411 $[(M - H + 2Na)^+]$, and *m/z* 1427 $[(M - H + Na + K)^+]$ corresponding to a composition of Hex_3,$HexNAc_3$,$deoxyhex_1$ with an additional substituent with a mass of 107 amu, which corresponds to the mass of an aminoethylphosphonate group. Following a ninhydrin-positive spot test, acid hydrolysis of fraction IV combined with amino acid analysis confirmed the substituent to be 2-aminoethylphosphonate [$NH_2CH_2CH_2P(O)(OH)O^-$; AEP].

Minor components observed on FAB-MS analysis correspond to species related to IV.1, the first of which lacks a deoxyhex residue {*m/z* 1243 $[(M + Na)^+]$, *m/z* 1265 $[(M - H + 2Na)^+]$, *m/z* 1281 $[(M - H + Na + K)^+]$}, while the second bears an extra HexNAc residue {*m/z* 1592 $[(M + Na)^+]$, *m/z* 1614 $[(M - H + 2Na)^+]$, and *m/z* 1630 $[(M - H + Na + K)^+]$}.

In order to determine the monosaccharide sequence and substitution pattern, an aliquot of fraction IV was subjected to N-acetylation, followed by permethylation, and the product was analysed using positive-ion FAB-MS. The FAB mass spectrum (data not shown) contains $(M + H)^+$ pseudomolecular ions for the fully methylated derivatives of the three components described above (*m/z* 1557, 1731, and 1976). Abundant fragment ions are also present in the spectrum, but since the fraction contains several molecular species, these fragments ions cannot allow unambiguous structure assignment for the different components.

To identify the fragment ions produced from the major N-acetylated permethylated component IV.1, the $(M + H)^+$ pseudomolecular ion at *m/z* 1731 was examined using collision-activated dissociation tandem mass spectrometry (MS–MS) (Figure 3). Structurally informative A^+-type fragment ions (Dell, 1987) were observed corresponding to $HexNAc^+$ (*m/z* 260), $(AEP)Hex^+$ (*m/z* 382) HexNAc-Hex^+ (*m/z* 464), (AEP)Hex-(HexNAc-Hex-)Hex^+ (*m/z* 1035), (AEP)Hex-(HexNAc-Hex-)Hex-$HexNAc^+$ (*m/z* 1280), and (AEP)Hex-(HexNAc-Hex-)Hex-HexNAc-(deoxyhex-)$HexNAc^+$ (*m/z* 1699). Double cleavage ions [produced by a second, β-cleavage (Dell, 1987) of nonreducing sequences from A^+-type ions] are seen at *m/z* 817 (β-cleavage of HexNAc-Hex from *m/z* 1280), *m/z* 899 [β-cleavage of (AEP)Hex from *m/z* 1280], and *m/z* 1021 (β-cleavage of HexNAc from *m/z* 1280). β-Cleavage ions are frequently accompanied by satellite ions 28 amu higher (*m/z* 845, 927, and 1049, respectively), which arise by cross-ring cleavages in which carbon 1 and the ring oxygen from the residue on the nonreducing side of the cleaved linkage are retained (Dell, 1987). Ions produced by a second, β-elimination reaction from A^+-type ions (Dell, 1987) are observed at *m/z* 228 (*m/z* 260 – MeOH), *m/z* 350 (*m/z* 382 – MeOH), *m/z* 432 (*m/z* 464 – MeOH), and *m/z* 1248 (*m/z* 1280 – MeOH). All of the ions described are consistent with the major structure IV.1 (see below). However, two additional fragment ions [*m/z* 423, for terminal $(AEP)HexNAc^+$, and *m/z* 595 for the elimination of (AEP)HexNAc from the A^+-type ion at *m/z* 1035] are observed in the MS–MS spectrum, which suggest the presence of a minor amount of an isomeric species containing a terminal (AEP)HexNAc residue in place of an (AEP)Hex. These two ions would also be consistent with a terminal Hex-Hex element (because Hex-Hex and (AEP)HexNAc have the same mass). However, from the elution position on HPLC in combination with the NMR data the existence of a terminal Hex-Hex element can be ruled out.

The ^{1}H-NMR spectrum of fraction IV (Figure 4A) is highly similar to that of fraction III, but notable downfield shifts are observed for GlcNAc-**5** H-1 ($\Delta\delta$ = +0.007) and for Man-**4**′ H-2 ($\Delta\delta$ = +0.013). ^{1}H-decoupled ^{31}P-NMR spectroscopy revealed a single signal at 24.2 ppm, suggesting the presence of a phosphonodiester (Lederkremer et al., 1991). In addition, a RESED spectrum (De Waard & Vliegenthart, 1989) with a long (120-ms) mixing time allowed for magnetization transfer from the phosphorus atom to Man-**4**′ H-1 at 4.914 ppm, indicating that the phosphonate is linked to Man-**4**′. Two non-carbohydrate ^{1}H signals were observed in the RESED spectrum at 3.24 ppm and 2.04 ppm, respectively, resembling those of free 2-aminoethylphosphonic acid (3.22 ppm and 1.97 ppm). Using a ^{31}P-decoupled ^{1}H difference spectrum,

Table II: Assignments of Ions Observed on MS/MS Analysis of the Major Component from N-Acetylated, Permethylated Fractions V, VIII, and IX[a]

m/z	fraction V	fraction VIII	fraction IX
228	260 minus MeOH	260 minus MeOH	260 minus MeOH
260	$HexNAc^+$	$HexNAc^+$	$HexNAc^+$
350		382 minus MeOH	
382		$(AEP)Hex^+$	
391		423 minus MeOH	423 minus MeOH
423	$(AEP)HexNAc^+$	$(AEP)HexNAc^+$	$(AEP)HexNAc^+$
432	464 minus MeOH		
434†	Deoxyhex-HexNAc/H^+minus MeOH	Deoxyhex-HexNAc/H^+minus MeOH	Deoxyhex-HexNAc/H^+minus MeOH
464	$HexNAc\text{-}Hex^+$		
595	627 minus MeOH	627 minus MeOH	627 minus MeOH
627	$HexNAc\text{-}(AEP)Hex^+$	$(AEP)HexNAc\text{-}Hex^+$	$HexNAc\text{-}(AEP)Hex^+$ or $(AEP)HexNAc\text{-}Hex^+$
668			$AEP_1HexNAc_2^+$ (or $Hex_2HexNAc^+$)
679†	HexNAc-(Deoxyhex-)HexNAc/H^+ minus MeOH	HexNAc-(Deoxyhex-)HexNAc/H^+ minus MeOH	HexNAc-(Deoxyhex-)HexNAc/H^+ minus MeOH
785		817 minus MeOH	
817		ß-cleavage of (AEP)HexNAc-Hex from 1443	
845		817+28 (see text)	
899	ß-cleavage of HexNAc-(AEP)Hex from 1525		
927	899+28		
1021		ß-cleavage of (AEP)HexNAc from 1443	
1049		1021+28	
1062	ß-cleavage HexNAc-Hex from 1525	ß-cleavage (AEP)Hex from 1443	ß-cleavage of (AEP)HexNAc-Hex (or HexNAc-(AEP)Hex) from 1688
1090	1062+28	1062+28	1062+28
1198		(AEP)Hex, Hex+ / (AEP)HexNAc-Hex	
1234	1266 minus MeOH		1266 minus MeOH
1266	ß-cleavage of HexNAc from 1525		ß-cleavage of (AEP)Hex from 1688
1268		ß-cleavage of (AEP)HexNAc-Hex from $(M+H)^+$	
1280	HexNAc-(AEP)Hex, Hex+ / HexNAc-Hex		
1294	1266+28		1266+28
1296		1268+28	
1411		1443 minus MeOH	
1429			ß-cleavage of HexNAc from 1688
1443		(AEP)Hex, Hex-HexNAc+ / (AEP)HexNAc-Hex	HexNAc-(AEP)Hex, Hex+ / (AEP)HexNAc-Hex
1457			1429+28
1493	1525 minus MeOH		
1525	HexNAc-(AEP)Hex, Hex-HexNAc+ / HexNAc-Hex		
1656			1688 minus MeOH
1674		ß-cleavage of Deoxyhex from 1862	
1688			HexNAc-(AEP)Hex, Hex-HexNAc+ / (AEP)HexNAc-Hex
1706		ß-cleavage Deoxyhex from $(M+H)^+$	
1734		1706+28	
1862		(AEP)Hex, Deoxyhex, Hex-HexNAc-HexNAc+ / (AEP)HexNAc-Hex	
1894		parent $(M+H)^+$	
1944	HexNAc-(AEP)Hex, Deoxyhex, Hex-HexNAc-HexNAc+ / HexNAc-Hex		
1976	parent $(M+H)^+$		
2107			HexNAc-(AEP)Hex, Deoxyhex, Hex-HexNAc-HexNAc+ / (AEP)HexNAc-Hex
2139			parent $(M+H)^+$

[a] The dagger symbol (†) indicates ions formed by elimination reaction from $(M + H)^+$ molecular ion.

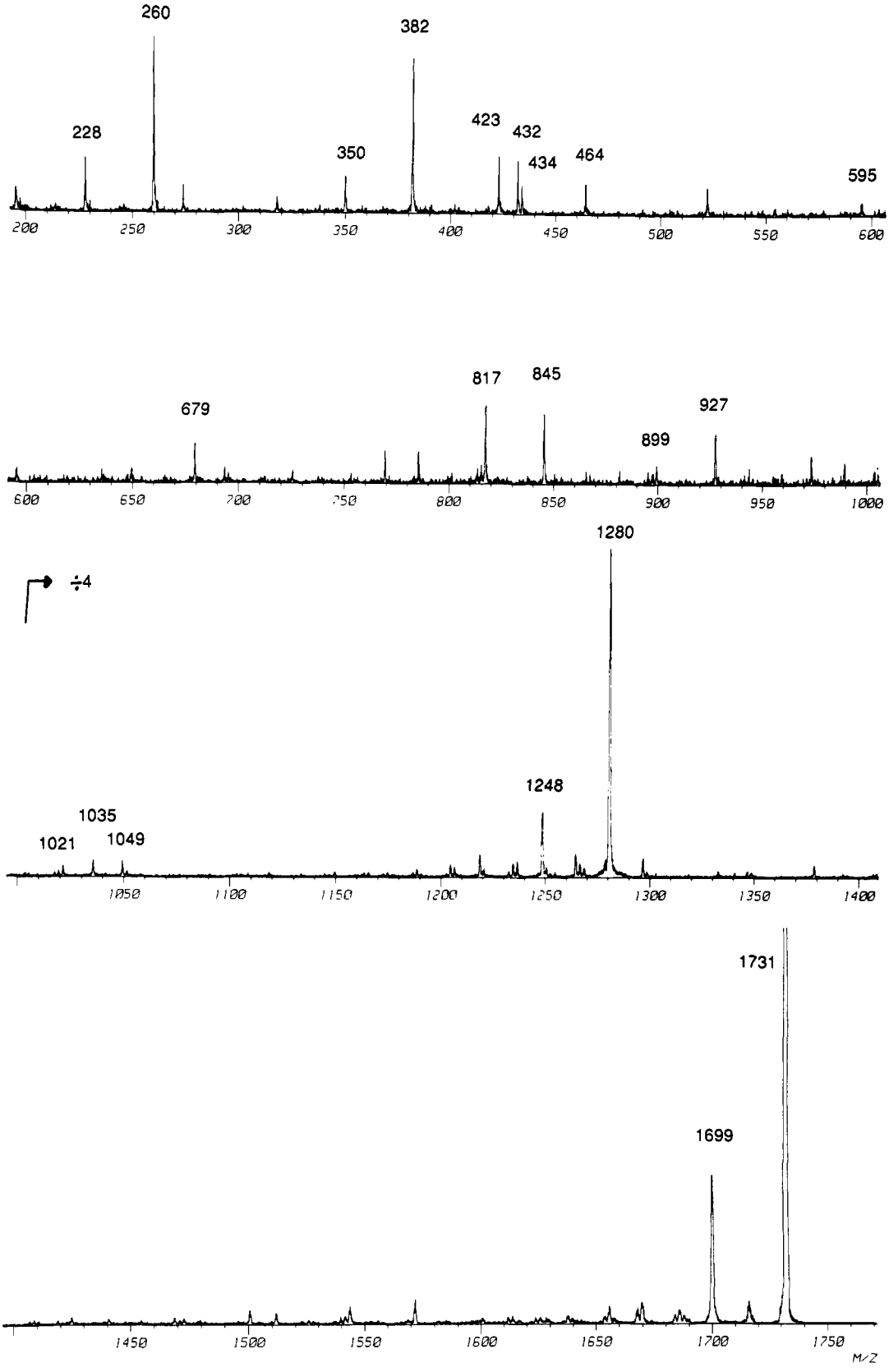


FIGURE 3: MS–MS spectrum of m/z 1731 [$(M + H)^+$ pseudomolecular ion] from N-acetylated permethylated derivative of fraction IV, obtained using thioglycerol as matrix. m/z values are quoted as nominal masses. Additional ions arising by an elimination event from the molecular species are present at m/z 434 [elimination of (AEP)Hex-(HexNAc-Hex-)Hex-HexNAc] and m/z 679 [elimination of (AEP)-Hex-(HexNAc-Hex-)Hex].

the signal of a phosphorus-coupled proton of the carbohydrate skeleton could be identified at 4.13 ppm, but due to severe resonance overlap with other protons, this signal could not be unambiguously assigned, even using a combination of 2D HOHAHA and DQF-COSY experiments. The presence of two minor components, identified by FAB-MS of underivatized fraction IV, was verified by ^{1}H-NMR spectroscopy. The compound lacking the α1–6-linked Fuc residue can be inferred from the minor GlcNAc-1 H-1α signal at 5.189 ppm and the GlcNAc-2 NAc methyl resonance at 2.080 ppm, while the

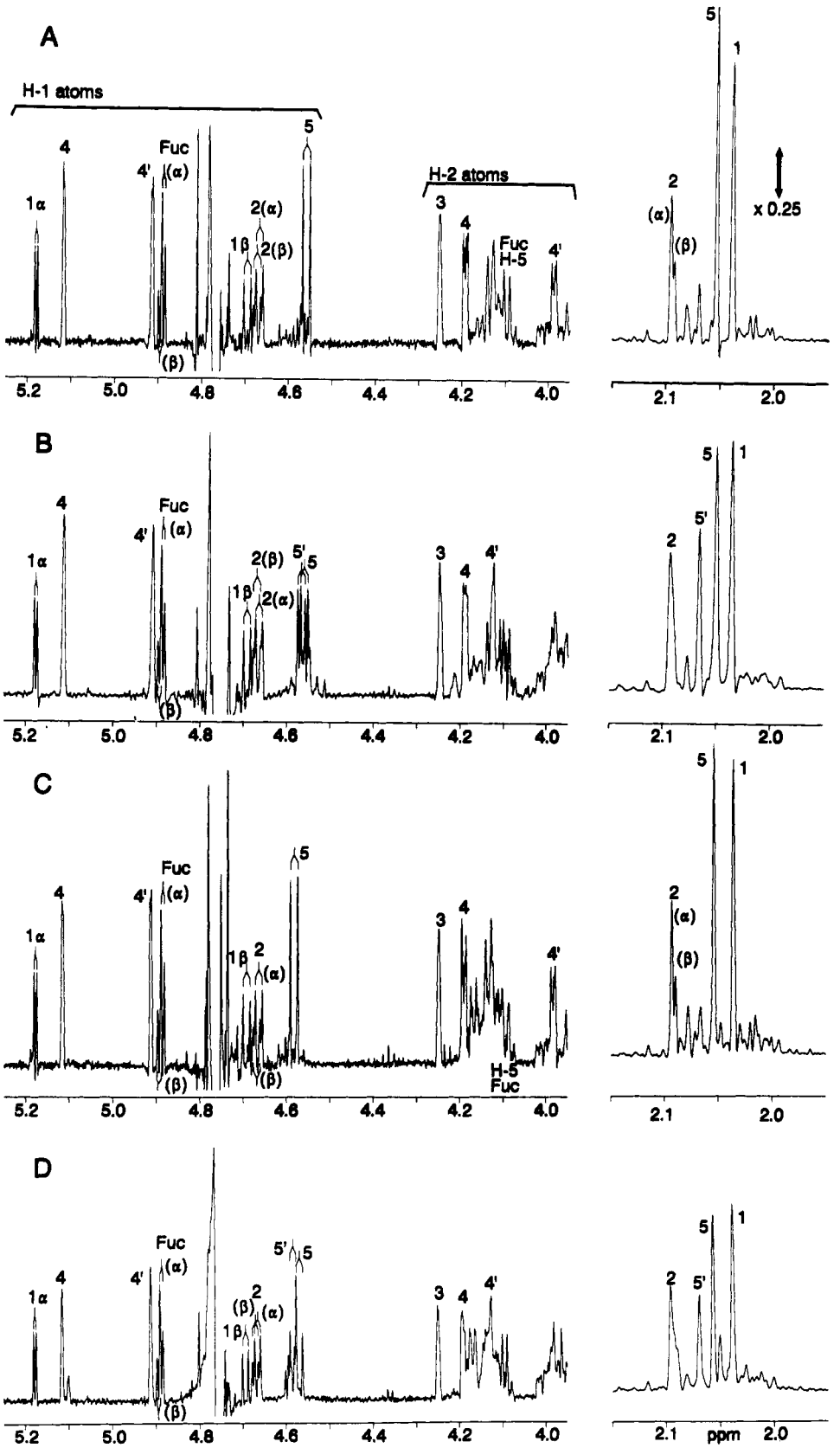


FIGURE 4: Structural-reporter-group regions of the resolution-enhanced ^{1}H-NMR spectra of 2-aminoethylphosphonate-containing N-linked oligosaccharides obtained from *L. migratoria* apoLp-III: (A) fraction IV; (B) fraction V; (C) fraction VIII; (D) fraction IX.

component carrying an additional GlcNAc-5′ residue is supported by a minor NAc methyl signal at 2.069 ppm (see also fraction V).

To determine the site of attachment of the 2-aminoethylphosphonate group on Man-4′, methylation analysis was carried out on an aliquot of N-acetylated permethylated fraction IV. GLC–MS of the PMAA derivatives allowed identification of the expected monosaccharide residues, with the exception of the AEP-bearing mannose. Proof for the substitution position of the AEP group on the mannose residue was obtained by exploiting a reaction which had been observed to displace AEP groups from permethylated derivatives

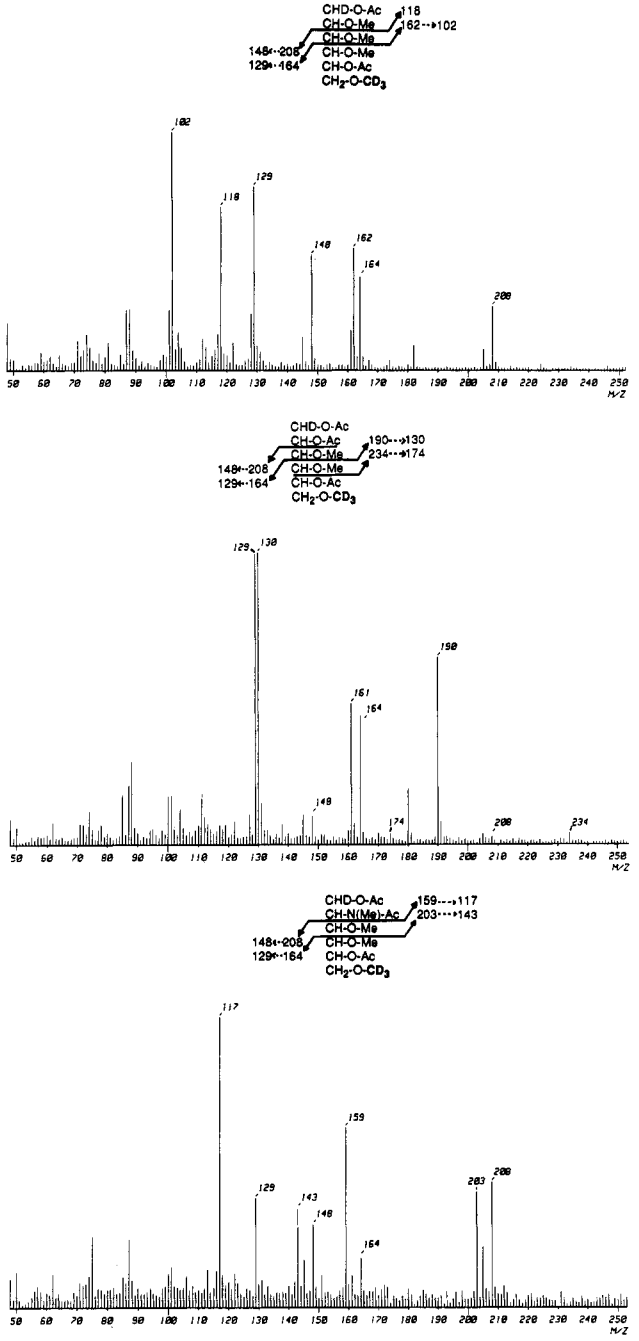
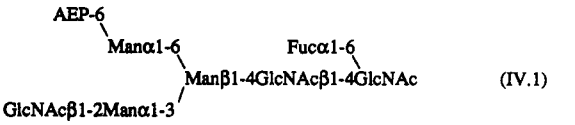


FIGURE 5: Electron impact mass spectra and fragmentation schemes (Lönngren & Svensson, 1974) for partially methylated alditol acetates obtained after partial replacement of AEP with CD_3. Each derivative contains a mixture of the deuterated with the nondeuterated species, since all fractions from which they were produced represent a mixture of different components (see text): (A, top) PMAA of terminal Hex residue bearing a 6-O-CD_3 substituent; (B, middle) PMAA of 2-substituted Hex residue bearing a 6-O-CD_3 substituent. The signal at m/z 180 corresponds to a closely eluting contaminant. (C, bottom) PMAA of terminal HexNAc residue bearing a 6-O-CD_3 substituent.

(Lederkremer et al., 1991): this involves the remethylation of a fully methylated derivative, when the AEP group is replaced by a methyl group. In this case an aliquot of the N-acetylated permethylated derivative was subjected to repeated methylation using ^{2}H-labeled methyl iodide, and the success of the AEP-elimination reaction was monitored by FAB-MS (data not shown). The resulting product bearing a ^{2}H-methyl group in place of the AEP substituent in approximately 50% of the molecules was subjected to methylation analysis. As well as the derivatives observed in the previous GLC–MS analysis, an additional PMAA derivative was identified corresponding to a terminal Hex residue bearing a ^{2}H-methyl group on C-6, demonstrating that the AEP substituent was attached to C-6 of a terminal Hex residue (Figure 5A).

In conclusion, the major oligosaccharide IV.1 present in fraction IV has the following structure:



(IV.1)

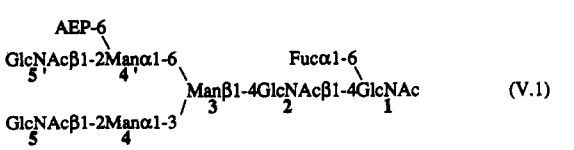
Fraction V. Positive-ion FAB-MS analysis of native fraction V demonstrated the presence of two major carbohydrate-containing components (data not shown), namely, one (V.1) with the composition AEP_1,Hex_3,$HexNAc_4$,$deoxyhex_1$ {pseudomolecular ion cluster at m/z 1592 [$(M + Na)^+$], m/z 1614 [$(M - H + 2Na)^+$], and m/z 1630 [$(M - H + Na + K)^+$], accounting for about 60% of the sample}, and one with the composition AEP_1,Hex_3,$HexNAc_3$,$deoxyhex_1$ {pseudomolecular ion cluster at m/z 1367 [$(M + H)^+$], m/z 1389 [$(M + Na)^+$], and m/z 1405 [$(M + K)^+$], accounting for about 40% of the sample}. The minor component stems from overlap of HPLC fractions IV and V.

Positive-ion FAB-MS analysis of fraction V after N-acetylation and permethylation yielded the $(M + H)^+$ pseudomolecular ion at m/z 1976, corresponding to the $HexNAc_4$-containing species, which was further examined using MS–MS. Fragment ions were observed (Table II) which are consistent with the structure V.1 (see below). As for fraction IV, a very small amount of an ion at m/z 423 was observed, corresponding to a minor isomeric component bearing terminal (AEP)HexNAc (as discussed for fraction IV).

The ^{1}H-NMR spectrum of fraction V is shown in Figure 4B. This spectrum, highly similar to that of fraction IV, reveals that the major component bears an additional GlcNAc-**5′** residue. This is manifested by the H-1 and NAc methyl signals at 4.569 ppm and 2.069 ppm, respectively. The Man-**4′** H-2 resonance at 4.126 ppm points to the penultimate position of this Man residue (Vliegenthart et al., 1983). In agreement with the FAB-MS data, fraction V contains a large amount (40%) of a second component, missing GlcNAc-**5′**, derived from overlap with fraction IV, which is evident from the GlcNAc-**5** H-1 signal at 4.558 ppm (Table I).

Methylation analysis after N-acetylation and permethylation of an aliquot of fraction V demonstrated the presence of PMAA derivatives for all the residues, except for the (AEP)-Man. The appearance of a 6-O-CD_3-bearing 2-substituted Hex residue on methylation analysis following displacement of the AEP with a CD_3 group demonstrates that the AEP was attached to C-6 of a 2-substituted Hex residue (Figure 5B).

Thus, the structure of the major component in fraction V is as follows:



(V.1)

Fraction VIII. Positive-ion FAB-MS analysis of underivatized fraction VIII produced a spectrum (data not shown) containing one major component (VIII.1) with pseudomolecular ions m/z 1474 [$(M + H)^+$] and m/z 1496 [$(M + Na)^+$], corresponding to a composition of AEP_2,Hex_3, $HexNAc_3$,$deoxyhex_1$. Two additional species are present, one corresponding to VIII.1 lacking a deoxyhex residue {pseudo-

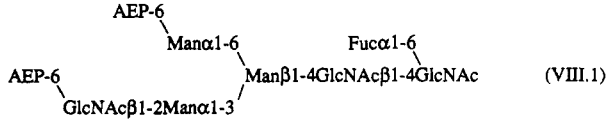
molecular ions at m/z 1328 [$(M + H)^+$] and m/z 1350 [$(M + Na)^+$]}, which accounts for approximately 30% of the sample, and a minor component containing one more HexNAc residue than VIII.1 {m/z 1677 [$(M + H)^+$] and m/z 1699 [$(M + Na)^+$], see fraction IX}.

Following N-acetylation and permethylation of fraction VIII, the $(M + H)^+$ pseudomolecular ion corresponding to the major component VIII.1 (m/z 1894) was examined by MS–MS. Fragment ions were observed (Table II) which are consistent with the structure VIII.1, as shown below. In addition there is a minor ion at m/z 260 which corresponds to $HexNAc^+$ (with no AEP) which suggests the presence of a small amount of an isomeric component bearing its second AEP on a different sugar residue.

The ^{1}H-NMR spectrum of fraction VIII is depicted in Figure 4C. When this spectrum is compared to that of fraction IV, notable differences are seen for the GlcNAc-**5** H-1 ($\Delta\delta$ = +0.026) and NAc methyl ($\Delta\delta$ = +0.003) signals, as well as in the increased intensity of the AEP protons at 3.2 ppm and 2.0 ppm. Furthermore, an additional signal is observed at 4.15 ppm, which from a 2D HOHAHA spectrum could be assigned to GlcNAc-**5** H-6. A RESED spectrum with a 120-ms mixing time revealed that both GlcNAc-**5** and Man-**4**′ bear a ^{31}P-containing substituent. The additional low intensity GlcNAc-**1** H-1α signal at 5.186 ppm indicates a small amount of an afucosyl derivative.

Methylation analysis produced PMAA derivatives for all the non-AEP-bearing monosaccharide residues. After replacement of the AEP residues with CD_3 groups, two new derivatives were observed in the methylation analysis, corresponding to 6-O-CD_3-substituted terminal HexNAc (Figure 5C) and 6-O-CD_3-substituted terminal Hex. These data are consistent with the presence of an AEP group on C-6 of both terminal Man and terminal GlcNAc.

From these data the structure of the major oligosaccharide VIII.1 in fraction VIII is as follows:



(VIII.1)

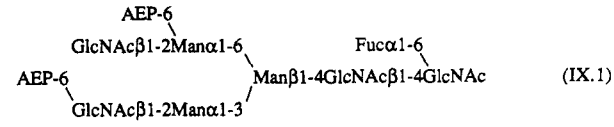
Fraction IX. Positive-ion FAB-MS analysis of fraction IX reveals the presence of two major and one minor carbohydrate-containing components. One of the major components which accounts for approximately 60% of the fraction {with pseudomolecular ions at m/z 1474 [$(M + H)^+$], 1496 [$(M + Na)^+$]} corresponds to VIII.1, due to overlap of HPLC fractions VIII and IX. The next most abundant species accounts for about 40% of the sample {with pseudomolecular ions at m/z 1677 [$(M + H)^+$] and 1699 [$(M + Na)^+$]} and has a composition of AEP_2,Hex_3,$HexNAc_4$,$deoxyhex_1$ (IX.1). In addition, there is a minor amount of a component corresponding to VIII.1 lacking a deoxyhex residue {pseudomolecular ions at m/z 1328 [$(M + H)^+$] and m/z 1350 [$(M + Na)^+$]}.

The ^{1}H-NMR spectrum of fraction IX is shown in Figure 4D. The presence of several components is evident from the unequal intensity of the H-1 signals around 4.55–4.60 ppm. All structural-reporter groups observed in the ^{1}H-NMR spectrum of fraction VIII (VIII.1) could be traced back in the spectrum of fraction IX. The occurrence of a component (IX.1) carrying an additional GlcNAc-**5**′ residue with respect to VIII.1 is deduced from the H-1 signal at 4.570 ppm, in combination with the NAc resonance at 2.069 ppm and the Man-**4**′ H-2 signal at 4.127 ppm.

The N-acetylated permethylated derivative was prepared, and the $(M + H)^+$ pseudomolecular ion at m/z 2139 (for the $HexNAc_4$-containing species IX.1) was analyzed by MS–MS (Table II). Fragment ions were observed which are consistent with structure IX.1 determined by NMR spectroscopy. The presence of an additional isomeric species is indicated by the ion at m/z 668, which corresponds either to AEP_1,$HexNAc_2^+$ or to Hex_2,$HexNAc^+$. However, the NMR data exclude the latter possibility. Since monosaccharide analysis of apoLp-III revealed the presence of GalNAc, and because the glycoprotein could be completely deglycosylated with PNGase-F, it is proposed that apoLp-III contains a small amount of a terminal AEP-containing GalNAcβ1-GlcNAc element. The β-configuration of the GalNAc is inferred indirectly from the absence of a characteristic α-anomeric GalNAc H-1 signal ($\delta > 4.8$, $^3J_{1,2}$ = 3–4 Hz) (Kamerling & Vliegenthart, 1992). The low intensity Man-**4** H-1 signal at 5.102 ppm suggests that this element resides in the Man-**4** branch (comparison made with unpublished data of Bergwerff et al.).

Methylation analysis of N-acetylated permethylated fraction IX produced PMAA derivatives for all non-AEP-bearing monosaccharide residues, while after replacement of the AEP moieties with CD_3 groups, two new derivatives appeared, corresponding to 6-O-CD_3-substituted 2-linked Hex and 6-O-CD_3-substituted terminal HexNAc (data not shown).

In conclusion, the structure of IX.1 is as follows:



(IX.1)

DISCUSSION

Thus far, only fragmentary data are available on the protein glycosylation capacity in insects. This study presents the primary structure of the carbohydrate chains of apolipophorin III from the insect *L. migratoria*. All carbohydrate chains could be split off the protein by PNGase-F, thereby confirming the presence of Asn-linked carbohydrates solely, and excluding the presence of O-glycans. In contrast to many other glycoproteins, SDS and mercaptoethanol appeared not to be required for efficient release of the carbohydrate chains by PNGase-F from locust apoLp-III, and concentrations of EDTA exceeding 10 mM appeared to inhibit the release of oligosaccharides from the polypeptide backbone.

In contrast to glycoproteins from some other orthopteran species, apoLp-III from *L. migratoria* does not bind to ConA (Ryan et al., 1990). This is most likely due to the presence of 2-aminoethylphosphonate (AEP) in the carbohydrate chains from *L. migratoria*. Although compound III, which lacks phosphonate, should possess ConA affinity, it seems to be present in too low of an amount to render the whole glycoprotein ConA-positive.

2-Aminoethylphosphonic acid was the first compound containing a carbon–phosphorus bond found in biological material, when it was detected in ciliated protozoa from the rumen of sheep (Horiguchi & Kandatsu, 1959). So far, carbohydrate-bound AEP has only been described in glycolipids of various water animals such as the sea hare (Araki et al., 1986), in the glycosylated inositolphosphoceramide of *Acanthamoeba castellani* (Dearborn et al., 1976), in the lipopeptidophosphoglycan from *Trypanosoma cruzi* epimastigotes (Previato et al., 1990; Lederkremer et al., 1991), and recently in a capsular polysaccharide from *Bacteroides fragilis*

(Baumann et al., 1992). N-Methylated AEP has been characterized in lipid-bound carbohydrates from several sea snail species (Hayashi, 1990). In a recent report (Kilby et al., 1991), 2-aminoethylphosphonic acid was identified as the main phosphorus compound in locust hemolymph. However, in sharp contrast to the present study, only free AEP could be found.

The main findings of the present report are the occurrence of 2-aminoethylphosphonate linked to C-6 of Man and GlcNAc, neither of which has been reported before. Thus apoLp-III from *L. migratoria* represents the first example of carbohydrate-bound AEP in a glycoprotein. It remains to be explored how the unique structural features of apoLp-III from *L. migratoria* presented here can be used in the battle against this harmful insect.

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