Carbohydrate Research, 203 (1990) 91–101
Elsevier Science Publishers B.V., Amsterdam

Purification, by high-performance liquid chromatography, and characterization, by high-field ^{1}H-n.m.r. spectroscopy, of two fucose-containing pentasaccharides of goat's milk

Prasoon Chaturvedi and Chandra B. Sharma*
Department of Biosciences and Biotechnology, University of Roorkee, Roorkee – 247 667 (INDIA)

(Received August 4th, 1989. Accepted, in revised form, December 18th, 1989)

ABSTRACT

Two fucose-containing pentasaccharides were isolated from goat's milk using a Bio-Gel P-4 column, followed by reverse-phase C-18 high-performance liquid chromatography. The structures of the pentasaccharides as characterized by high-field ^{1}H-n.m.r. spectroscopy and enzymatic digestion were found to be

$$\beta\text{-D-Gal}p\text{-}(1\rightarrow4)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow6)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow4)\text{-D-Glc}p$$
$$\uparrow$$
$$\alpha\text{-L-Fuc}p\text{-}(1\rightarrow3)$$

and

$$\beta\text{-D-Gal}p\text{-}(1\rightarrow3)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow6)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow4)\text{-D-Glc}p$$
$$\uparrow$$
$$\alpha\text{-L-Fuc}p\text{-}(1\rightarrow3)$$

INTRODUCTION

Fucose-containing oligosaccharides are reported to occur in a variety of animal tissue fluids[1,2]. Although no specific biological function has been ascribed to these oligosaccharides, they are an important source of substrates for studies of acceptor specificities of glycosyl transferases and substrate specificities of glycosidases, as well as important antigenic determinants for production as well as characterization of polyclonal and monoclonal antibodies[3–5]. Recently, Bush and co-workers have isolated various fucose-containing neutral oligosaccharides from human milk by high-performance liquid chromatography (h.p.l.c.) and determined their structures by ^{1}H- and ^{13}C-n.m.r. spectroscopy[6–8]. Egge and co-workers have also isolated some fucose-containing oligosaccharides from human milk as the reduced and per-*O*-acetyl derivatives using h.p.l.c. and characterized them by mass spectrometry and high-field ^{1}H-n.m.r. spectroscopy[2,9,10]. However, oligosaccharides of goat's milk, which is normally preferred for feeding of infants because of its ease of digestibility and low fat content as

* To whom correspondence should be addressed

0008-6215/90/$ 03.50

compared to cow's or buffalo's milk, have not been characterized. In the absence of such studies, it is not possible to compare the structures of fucose-containing oligosaccharides of human milk with those of goat's milk. We have earlier reported the isolation and characterization of three goat-milk oligosaccharides by h.p.l.c. and high-resolution ^{1}H-n.m.r. spectroscopy[11].

In this paper, we report the isolation and purification of two fucose-containing pentasaccharides of goat's milk by h.p.l.c. and their characterization by high-field ^{1}H-n.m.r. spectroscopy. These oligosaccharides show markedly different structures from those of human milk. The structures of these oligosaccharides have been further confirmed by enzymic degradation, followed by h.p.l.c. analysis of the resulting mixture of fragments.

EXPERIMENTAL

Materials. — Fresh goat's milk was procured from a local dairy and kept in ice until processed. Bio-Gel P-4 was purchased from Bio-Rad Laboratories (U.S.A.). High purity D_2O (99.98%) was obtained from Merck, Sharp, and Dohme (U.S.A.). All other chemicals were reagent grade from standard commercial sources.

Isolation of oligosaccharides. — Goat's milk was processed as described by Kobata and Ginsburg[4]. Briefly, the milk was centrifuged at 4°, and the lipid layer was removed by filtration through a loosely packed glass-wool column in the cold. Lactose and proteins were precipitated by the addition of ethanol up to a final concentration of 68%, and the supernatant was lyophilized. The lyophilizate was fractionated on a Bio-Gel P-4 column using glass double-distilled water as eluent. Fractions (0.75 mL) were collected at a flow rate of 0.25 mL.min^{-1}, and aliquots (50 μL) from every fraction were analysed for sugar by the phenol–sulphuric acid method[12]. Carbohydrate-containing fractions under various peaks were pooled, lyophilized, dissolved in glass double-distilled water (15 mg.mL^{-1}) and further analysed by h.p.l.c.

Purification of oligosaccharides by h.p.l.c. — The oligosaccharide fractions obtained from the Bio-Gel P-4 column were further purified by reverse-phase h.p.l.c.[1,6] using a Shimadzu Model LC-4A Liquid Chromatograph fitted with a Du-Pont Zorbax C-18 column (4.6 × 250 mm), u.v. detector, and a Shimadzu Chromatopac Model C-R 2AX data processing system. Oligosaccharides were eluted with h.p.l.c.-grade distilled water at a flow rate of 1 mL.min^{-1}. The effluent was monitored by u.v. absorbance at 202 nm. Multiple runs were made on the same column to collect sufficient amounts of each oligosaccharide peak, and fractions were collected manually at the detector outlet. Each fraction was then repeatedly chromatographed on the reverse-phase (C-18) column until a purification of >95% was achieved. In addition, the purified fractions were checked for contamination by any cross peak by h.p.l.c. on the normal-phase column (NH_2, Zorbax column, 4.6 × 250 mm) using 6:4 CH_3CN–H_2O at a flow rate of 1 mL.min^{-1}. The purified fractions were assayed for carbohydrate content as above.

Carbohydrate analysis by h.p.l.c. — Carbohydrate analysis was accomplished by h.p.l.c. of the benzoylated methyl glycosides of oligosaccharides using Jentoft's meth-

od[13]. Methanolic HCl (0.2 mL) was added to each sugar (1 μmol) sample, and the mixture was heated for 8 h at 80°. The samples were then cooled to room temperature, *tert*-butanol in methanol (0.1 mL of 80% v/v) was added to each, and the samples were dried under a stream of N_2. The hexosamines were reacetylated by dissolving the sample in dry methanol (0.1 mL), followed by addition of pyridine (40 μL) and acetic anhydride (40μL) and incubating the sample for 1 h at room temperature. The samples were dried under a stream of N_2. Toluene (40 μL) was added, and the samples were redried and dissolved in 9:1 CH_3CN–H_2O (v/v). Samples (10 μL) were analysed on a Zorbax C-18 column (25 cm, 5μ), eluting with 9:1 acetonitrile–water at a flow rate of 0.5 mL.min^{-1}. The retention times of sugars in acid hydrolysate were compared with those of standard samples.

Structure determination by ^{1}H-n.m.r. spectroscopy. — For ^{1}H-n.m.r. spectroscopic analysis, samples were dissolved in D_2O and repeatedly exchanged with D_2O, followed by lyophilization. The samples were then dissolved in high-purity D_2O (0.3 mL). The observed chemical shifts were reported relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) using acetone as an internal standard (δ = 2.225 p.p.m.) downfield from DSS. The ^{1}H-n.m.r. spectra were recorded at 77° in order to see the α-Fuc H-5 resonances. The spin-difference decoupling experiments (SDDS) were carried out at 22°. The nuclear Overhauser enhancement experiments were carried out at 70° in order to overcome the effects of unfavourable rotational correlation times[14]. The pulse sequence for the n.O.e. experiments utilised a 3-s pre-irradiation, followed by 90° observation pulse and acquisition with the irradiation turned off. The observed chemical shifts are reported relative to internal DSS using acetone as an internal standard (δ = 2.225 p.p.m. downfield from DSS).

Enzymatic digestion. — Fucosidase digestion was used to assist in the oligosaccharide structure determination. The compound (2 mg) was dissolved in 1.0mM sodium acetate buffer (pH 5.0, 1 mL), and bovine kidney α-L-fucosidase (200 μL, equivalent to 1 unit of enzyme, Sigma) was added. The mixture was incubated for 16 h at 37°, at the end of which time additional α-L-fucosidase (1 unit) was added, and the mixture was incubated for a further 16 h at 37°. The products were assayed by C-18 reverse-phase h.p.l.c. using water as eluent at a flow rate of 1 mL. min^{-1}.

RESULTS AND DISCUSSION

Fig. 1 shows the elution profile of the goat-milk oligosaccharides from the Bio-Gel P-4 column. Four distinct carbohydrate-containing peaks, designated as A, B, C, and D, were obtained. Of these oligosaccharide peaks, D was mostly lactose, whereas peaks C, B, and A contained a mixture of oligosaccharides of varying sizes. Fractions under these peaks were pooled separately and lyophilized. Fraction C, when further analysed by h.p.l.c. on the reverse-phase C-18 column using water as eluent, was resolved into five distinct oligosaccharide peaks, P-I, P-II, P-III, P-IV, and P-V (Fig. 2). The failure to resolve the oligosaccharides into their α- and β-anomeric pairs on the reverse phase C-18 column might be a consequence of poor resolution of anomers and

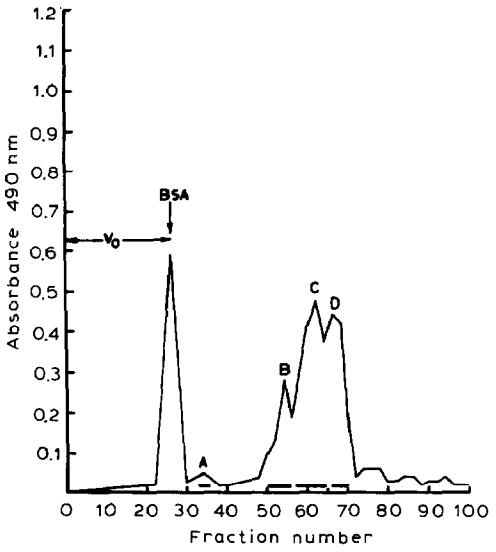

Fig. 1. Bio-Gel P-4 chromatography of goat-milk oligosaccharides. A sample (1 mL, equal to 250 mg of D-glucose) was loaded on the Bio-Gel column (1.5 × 60 cm), and the oligosaccharides were eluted with water at a flow rate of 0.25 mL. min^{-1} as described in the Experimental section. Fractions indicated by a bar (–) in each peak were pooled.

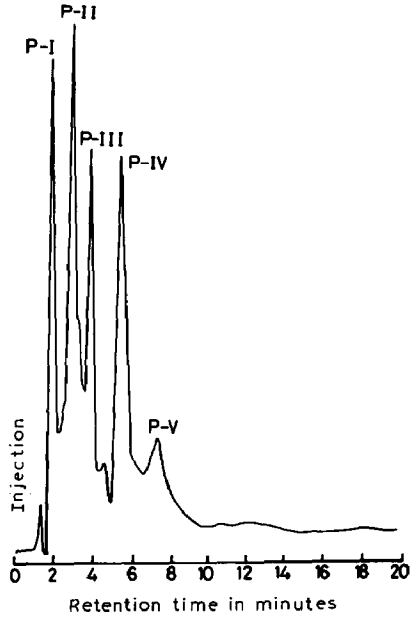

Fig. 2. Reverse-phase h.p.l.c. of the Bio-Gel P-4 fraction C. A sample (10 μL) was injected on a Zorbax C-18 column and eluted with water at a flow rate of 1 mL. min^{-1}. The elution was monitored by u.v. adsorption at 202 nm. Each fraction was manually collected.

peak broadening which occurs for cases in which the mutarotation time is comparable to the chromatographic time[15]. This possibility is further supported by the finding that a partial resolution into the anomeric pairs was possible when the chromatographic analysis was carried out at lower temperature (7–8°). Sufficient amounts of the individual oligosaccharide fractions eluting under different peaks were collected and purified by repeated chromatography on the reverse-phase column as described above. The purified oligosaccharide fractions showed no contamination of cross-peaks on the normal-phase amino column (Figs. 3 and 4), and, therefore, were found suitable for ^{1}H-n.m.r. studies.

Carbohydrate composition of oligosaccharides P-III and P-IV by h.p.l.c. — Table I shows the carbohydrate analysis data obtained by h.p.l.c. analysis of the benzoylated methyl glycosides using Jentoft's method[13]. The result showed that both compound P-III, as well as P-IV, were fucose-containing pentasaccharides having one Glc, one GlcNAc, two Gal, and one Fuc residues each.

Structure determination by high-field ^{1}H-n.m.r. spectroscopy. — The results of high-field ^{1}H-n.m.r. spectroscopy at 400 MHz of the oligosaccharide fractions P-III (Fig. 5) and fraction P-IV (Fig. 6) recorded at 77° showed that these were fucose-containing oligosaccharides. The assignment of their full structure was accomplished as described in the paragraphs which follow.

Integration of the anomeric region of the ^{1}H-n.m.r. spectrum of compound P-III (Fig. 5) shows it to be a pentasaccharide containing two β-galactose (Gal), one β-*N*-acetylglucosamine (GlcNAc), one α-Fucose (Fuc), and one glucose (Glc) residues. Comparison of its spectrum to that of compound P-II [β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-β-D-Gal-(1→4)-β-D-Glc*p*] isolated from the same goat-milk fraction[11] shows that it has an identical tetrasaccharide core structure with an additional α-Fuc residue indicated by its characteristic H-1, H-5, and H-6 resonances at δ 5.225 (*J* 3.1 Hz) and 4.861 and 1.292 (*J* 6.3 Hz) p.p.m. respectively[16]. The assignment of α-Fuc H-5 at 4.861 p.p.m. was made by recording the spectrum at a high temperature (77°) when the H-5

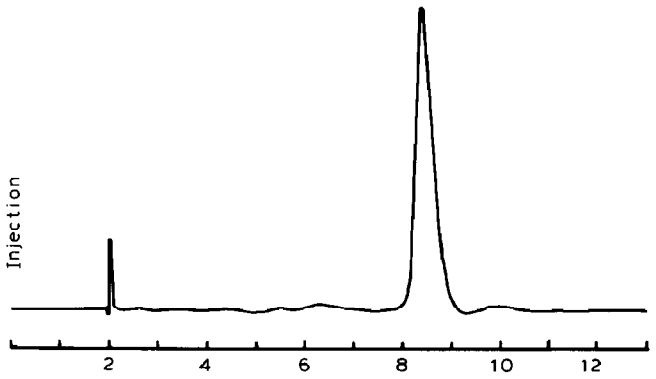

Fig. 3. Rechromatography of the oligosaccharide P-III (purified by reverse-phase C-18 chromatography) on normal-phase NH_2 column (Zorbax) eluted with 6:4 CH_3CN–H_2O at a flow rate of 1 mL. min^{-1}. The elution was monitored by u.v. absorption at 202 nm.

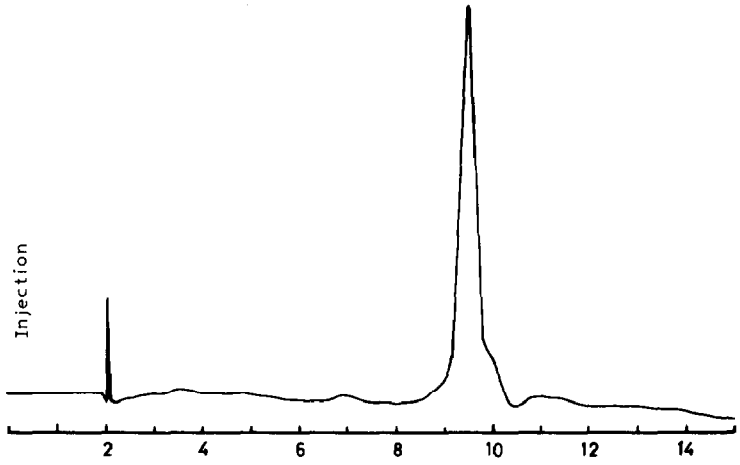

Fig. 4. Rechromatography of the oligosaccharide P-IV (purified by reverse-phase C-18 chromatography) on normal-phase NH_2 column (Zorbax) eluted with 6:4 CH_3CN–H_2O at a flow rate of 1 mL. min^{-1}. The elution was monitored by u.v. absorption at 202 nm.

clearly shows its characteristic shape, as the HOD peak shifts upfield at this temperature[1]. The α-Fuc H-5 position was further confirmed by decoupling at α-Fuc H-6. The absence of an α-Fuc H-5 resonance in the region of δ 4.2–4.3 suggests that fucose is not linked *via* a 1→2 linkage[1]. The chemical shifts analogies of the α-Fuc H-1 resonance (5.225 p.p.m.) of monofuco lacto-*N*-hexaose and difuco lacto-*N*-hexaose[16] indicated that fucose is linked through a 1→3 linkage and not by a 1→4 linkage[16]. Since in the tetrasaccharide core structure (compound P-II), the C-4 position of β-GlcNAc is occupied by a β-Gal, α-Fuc can only be 1→3 linked to β-GlcNAc, a deduction which supports our interpretation that the α-Fuc is in fact 1→3 linked. The remaining resonances were assigned as in the case of compound P-II[11]. A comparison of the spectrum to those of lactose[17], lacto-*N*-tetraose (LNT), and lacto-*N*-neotetraose (LNne-

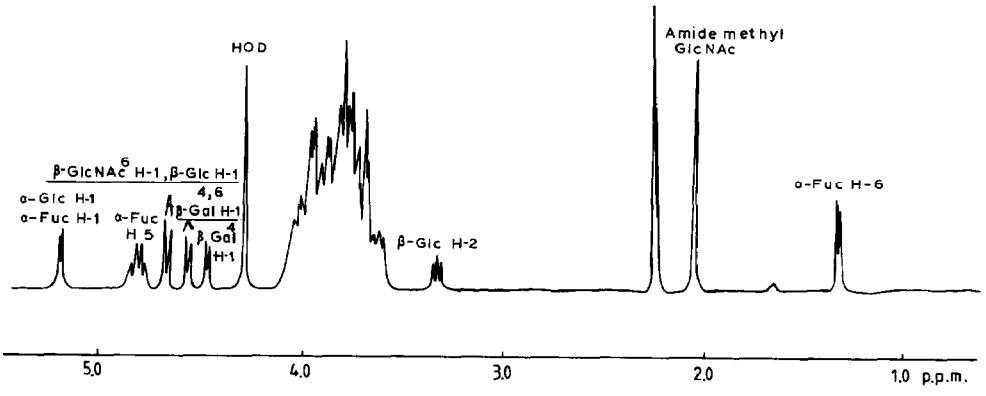

Fig. 5. ^{1}H-n.m.r. spectrum (400 MHz) of oligosaccharide P-III in D_2O at 77°.

TABLE I

Carbohydrate analysis of goat-milk oligosaccharides by reverse-phase h.p.l.c.

Oligosaccharide	*Residues*			
	Glc	*GlcNAc*	*Gal*	*Fuc*
P-III	1	1.04	1.9	0.9
P-IV	1	1.09	2.2	1.1

oT)[6] shows the presence of a lactosyl residue [β-D-Gal*p*-(1→4)-β-D-Glc*p*] at the reducing end. The doublets at δ 4.555 and 4.45 are assigned to H-1 of the terminal Gal[11,16] (linked 1→4 to GlcNAc) and the internal, 6-substituted GAl[6], respectively. The ^{1}H-signal (1.5 H by integration) at δ 4.663 is assigned to H-1 of β-GlcNAc and β-Glc. As there is no downfield shift of H-4 of β-Gal, none of the β-Gal residues is substituted at C-3 position[6]. This fact implies that the β-GlcNAc may be 1→6 linked to Gal which is assigned by SDDS and chemical shift analogies of β-GlcNAc[6] as reported by Dua *et al.*[16] Similar conclusions have been arrived at by Hanisch *et al.*[18], based on the chemical shifts of H-2, H-3, and H-4 protons of C-6 glycosylated Gal[4]. Since these shifts are practically identical in both P-III as well as P-IV compounds, these protons must be located at hydroxylated carbons, which means that C-6 must be glycosylated. The compound can thus be assigned the following structure:

β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-β-D-Gal*p*-(1→4)-D-Glc*p*
↑
α-D-Fuc*p*-(1→3)

This structure was futher confirmed by SDDS experiments which gave the resonances of the remaining protons (Table II). The assigned structure of compound P-III was further confirmed by enzymatic digestion with bovine kidney (1→3)-α-L-fucosidase (Sigma). Fig. 7 shows the chromatogram of compound P-III obtained after 12 h of digestion with (1→3)-α-L-fucosidase. The figure shows two peaks, one of the compound P-III with a retention time of 4.0 min, and a second peak, which is the product of fucosidase digestion, having a retention time of 3.0 min. The retention time of the product is identical to that of the parent tetrasaccharide, *i.e.*, compound P-II, thus further confirming the structure. Approximately 70% of P-III was shown to be converted into P-II at this stage.

The pentasaccharide P-III differs from the pentasaccharide LNF-III,

β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc*p*
↑
α-L-Fuc*p*-(1→3)

isolated from human milk[6] in having a 1→6 linkage between D-Glc*p*NAc and the Gal of the lactosyl residue at the reducing end. Further, the comparison of its structure to that of LNF-II[6],

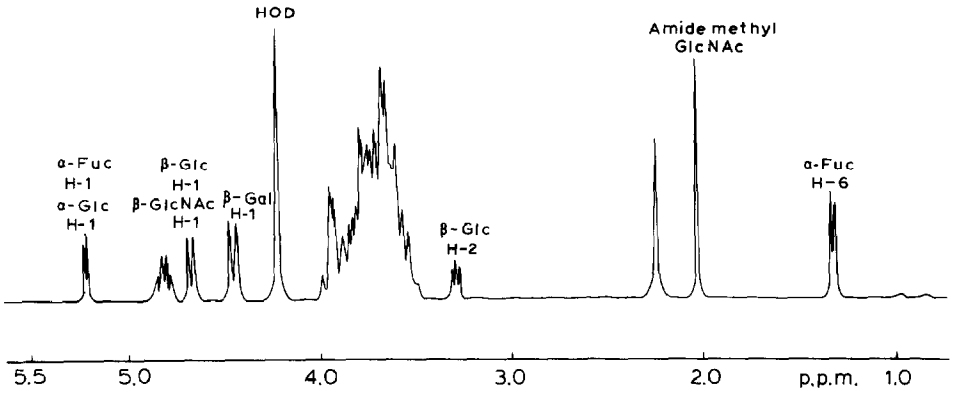

Fig. 6. ^{1}H-n.m.r. spectrum (400 MHz) of oligosaccharide P-IV in D_2O at 77°.

TABLE II

^{1}H-n.m.r. chemical shift data of goat-milk oligosaccharides[a]

Residue[b]	*Proton*	*Oligosaccharide*	
		P-III (δ)	*P-IV* (δ)
α-Glc	H-1	5.225	5.221
	H-2	3.583	3.582
β-Glc	H-1	4.663	4.658
	H-2	3.295	3.283
	H-3	3.632	3.709
β-Gal-(1→4)-D-Glc	H-1	4.451	4.453
	H-2	3.581	3.580
	H-3	3.714	3.691
	H-4	3.913	3.916
β-Gal-(1→4)-D-GlcNAc	H-1	4.555	—
	H-2	3.524	—
β-Gal-(1→3)-D-GlcNAc	H-1	—	4.453
	H-2	—	3.517
β-GlcNAc-(1→6)	H-1	4.663	4.658
	H-2	3.880	3.875
	N-Ac	2.059	2.053
α-Fuc-(1→3)	H-1	5.225	5.221
	H-2	3.682	3.688
	H-4	3.913	3.907
	H-5	4.861	4.865
	H-6	1.292	1.294

[a] The chemical shifts are reported relative to internal (DSS) using acetone as the internal standard (δ = 2.225 p.p.m. downfield from DSS). [b] Assignments refer te residue in bold type.

$$\beta\text{-D-Gal}p\text{-}(1\rightarrow3)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow3)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow4)\text{-D-Glc}p$$
$$\uparrow$$
$$\alpha\text{-L-Fuc}p\text{-}(1\rightarrow4)$$

shows three differences: (*i*) It has a 1→6 linkage between β-GlcNAc and β-Gal as compared to a 1→3 linkage in LNF-II, (*ii*) It has a 1→4 linked β-Gal at the nonreducing terminal, whereas LNF-II has a 1→3 linked β-Gal at the same position. (*iii*) It has a 1→3 linkage between α-Fuc and β-GlcNAc compared to a 1→4 linkage in LNF-II.

The integration of the anomeric region of the ^{1}H-n.m.r. spectrum of the compound P-IV (Fig. 8) indicates that the compound is a pentasaccharide having two Gal, one GlcNAc, one Glc, and one α-Fuc residues. A comparison of its spectrum to that of compound P-III shows the absence of the downfield-shifted H-1 resonance of the terminal (linked 1→4 to GlcNAc) Gal. Integration of Gal H-1 resonance at δ 4.453 indicates the presence of two β-Gal residues. The absence of the downfield-shifted resonance of Gal H-4, together with the chemical shift analogies of P-III, allows one to predict that the GlcNAc in this case is also 1→6 linked to Gal of the lactosyl residue. The absence of the terminal Gal as stated above, coupled with chemical shift analogies[16] of a linked 1→3 Gal, indicate that the second Gal residue is indeed 1→3 linked to GlcNAc. The linkage of α-Fuc as 1→3 was determined as in case of P-III. However, in this case the C-3 position of GlcNAc is occupied by β-Gal. Therefore, the α-Fuc residue can only be linked either to β-Gal at the nonreducing end or to Glc at the reducing end. Decoupling of the β-Glc H-2 resonance identified its corresponding H-3 resonance at δ 3.709. This downfield shift of β-Glc H-3 resonance, as compared to the position of β-Glc

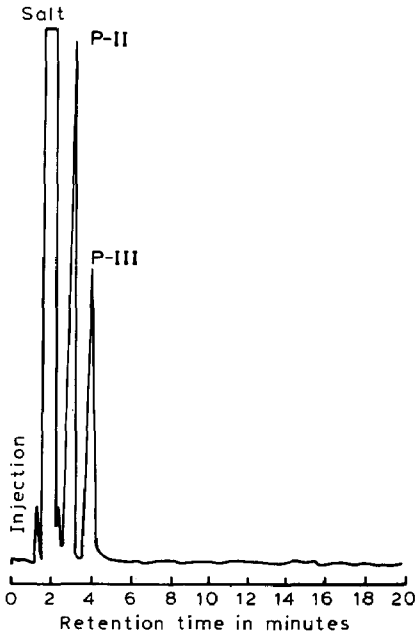

Fig. 7. Reverse-phase chromatogram of compound P-III obtained after 12 h. of enzymatic digestion with (1→3)-α-L-fucosidase.

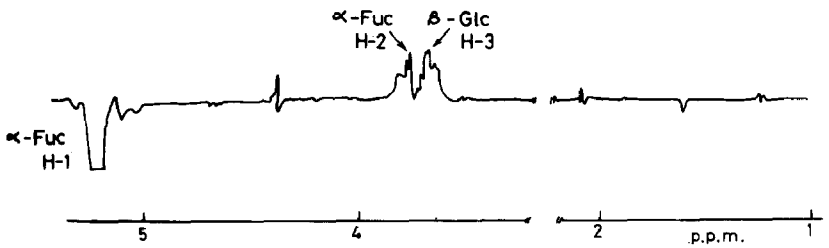

Fig. 8. N.O.e.s obtained at 70° upon saturation of the α-Fuc H-1 resonance in compound P-IV.

H-3 resonance at δ 3.632 p.p.m. of the compound P-III, suggests that Glc may be substituted at the C-3 position. Hence α-Fuc could be 1→3 linked to Glc at the reducing end. The position of α-Glc H-3 could not be confirmed as its corresponding H-2 (3.582 p.p.m.) resonance lies in the crowded region of the spectra. Therefore, our assumptions are based on the position of the α-Glc H-3 resonance, only. Based on the above interpretation, the compound P-IV may be assigned the following structure:

β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→6)-β-D-Gal*p*-(1→4)-D-Glc*p*
↑
β-L-Fuc*p*-(1→3)

The remaining proton resonances were obtained by SDDS experiments (Table II). The site of linkage of Fuc was confirmed by an n.O.e. experiment. The n.O.es obtained at 70° on saturation of α-Fuc H-1 are shown in Fig. 8. Positive n.O.es were observed at both the α-Fuc H-2 and the β-Glc H-3 resonance, confirming that α-Fuc was linked to Glc at the reducing terminus.

Enzymatic digestion of compound P-IV with (1→3)-α-L-fucosidase yielded a compound with a retention time of 3.4 min, which is different from that of tetrasaccharide P-II. This further confirmed the presence of a (1→3)-α-L-linked Fuc residue in this pentasaccharide.

The pentasaccharide, P-IV differs from LNF-I [α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc*p*][6] in two respects. Firstly, P-IV has a 1→6 linkage between Glc*p*NAc and the β-Gal of the lactosyl residue as compared to a 1→3 linkage in the case of LNF-I. Secondly, it has an α-Fuc, which is 1→3 linked to Glc at the reducing end as compared to LNF-I, where a α-Fuc is 1→2 linked to β-Gal at the nonreducing end.

The relatively early elution time of compound P-III in relation to that of P-IV may be attributed to the difference in the position of the α-Fuc which is attached to β-GlcNAc in the middle of the chain in the former, whereas it is linked to Glc at the reducing terminal in the latter. Moreover, the difference in the retention times of P-II and P-IV gives further evidence that the separation on a reverse-phase column is dependent on stereochemical differences. These structures should be useful in characterization of related structures isolated from glycolipids or glycoproteins, as well as in studies of the conformation of the oligosaccharides.

ACKNOWLEDGMENTS

We are grateful to Dr. V. K. Dua, Malaria Research Centre, Delhi for helpful discussions, and to the Department of Science and Technology, Government of India, for providing financial support. We also thank R. S. I. C., Central Drug Research Institute, Lucknow, and S. I. F., Indian Institute of Science, Bangalore, India for providing the n.m.r. spectroscopy facility.

REFERENCES

1 V. K. Dua, V. E. Dube, and C. A. Bush, *Biochim. Biophys. Acta.*, 802 (1984) 29–40.
2 H. Egge, A. Dell, and H. Van Nicolai, *Arch. Biochem. Biophys.*, 224 (1983) 235–253.
3 R. Kornfeld and S. Kornfeld, in W. J. Lennarz (Ed.), *The Biochemistry of Glycoprotein and Proteoglycans*, Plenum, New York, (1980) pp. 1–34.
4 A. Kobata and V. Ginsburg, *J. Biol. Chem.*, 245 (1970) 148–160.
5 A. Kobata and V. Ginsburg, *J. Biol. Chem.*, 247 (1972) 1525–1529.
6 V. K. Dua and C. A. Bush, *Anal. Biochem.*, 133 (1983) 1–8.
7 N. W. H. Cheetam and V. E. Dube, *J. Chromatogr.*, 262 (1983) 426–430.
8 C. A. Bush, M. M. Panitch, V. K. Dua, and T. E. Rohr, *Anal. Biochem.*, 145 (1985) 124–136.
9 U. Dabrowski, H. Egge, and J. Dabrowski, *Arch. Biochem. Biophys.*, 224 (1983) 254–260.
10 J. Dabrowski, H. Egge, U. Dabrowski, and Y. Peter-Katalinic, *Symp. on Glycoconj.*, Lund, Ronnby, Univ. of Lund, (1983) p. 132.
11 P. Chaturvedi and C. B. Sharma, *Biochim. Biophys. Acta*, 967 (1988) 115–121.
12 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, 2 (1956) 350–356.
13 N. Jentoft, *Anal. Biochem.*, 148 (1985) 424–433.
14 V. K. Dua, B. M. Narsinga Rao, Wu Shing-Shing, V. E. Dube, and C. A. Bush, *J. Biol. Chem.*, 261 (1986) 1599–1608.
15 K. Blumberg, L. Pustilink, F. Liniere, and C. A. Bush, *Anal. Biochem.*, 119 (1982) 407–412.
16 V. K. Dua, K. Goso, V. E. Dube, and C. A. Bush, *J. Chromatogr.*, 328 (1985) 259–269.
17 J. H. Bradbury and J. G. Collins, *Carbohydr. Res.* 71 (1979) 15–23.
18 F. G. Hanisch, G. Uhlenbruck, J. Peter-Katalinic, H. Egge, J. Dabrowski, and U. Dabrowski, *J. Biol. Chem.*, 264 (1989) 872–883.