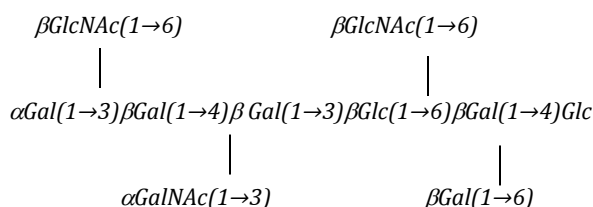


SEPARATION AND STRUCTURE ELUCIDATION OF NOVEL DECASACHHARIDE “OVIASOSE” FROM GADDI SHEEP’S MILK

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ABSTRACT: Milk is a rich source of nutrition. After water carbohydrate is main constituent of milk. It is primary source for infant nutrition. It protects them from various diseases like tuberculosis, microbial infections etc. It acts as anti-inflammatory, anti-coagulant, anti-cancer etc. Remember these things Gaddi Sheep's milk is collected from high altitude region and isolate oligosaccharides by Kobata and Ginsberg method. Isolated oligosaccharide named as Oviasose, it is a decasaccharide. Structure of Oviasose are confirmed by HPLC and chemical degradation which further supported by NMR (¹H, ¹³C, HSQC, HMBC, COSY, TOCSY etc.) and Mass Spectroscopy as-



KEYWORDS: Oligosaccharide, HPLC, ¹H, ¹³C and 2D NMR, Mass Spectroscopy

INTRODUCTION

Milk is a rich source of nutrition. It is a white liquid food produced by the mammary glands of mammals. It is the primary source of nutrition for infant mammals, before they are able to digest other types of food. Early-lactation milk contains colostrum, which carries the mother's antibodies to its young and can reduce the risk of many diseases. It contains many other substance like protein and lactose. Inter-species imbibing of milk is common, particularly among humans, many of whom suction the milk of other mammals. An accumulating body of evidence suggests that milk oligosaccharides are anti-adhesive antimicrobials that serve as soluble decoy receptors, prevent pathogen attachment to infant mucosal surfaces and lower the risk for viral, bacterial and protozoan parasite infections. Milk oligosaccharides may modulate epithelial and immune cell responses, reduce excessive mucosal leukocyte infiltration and activation, lower the risk for necrotizing enterocolitis and provide the infant with sialic acid as a potentially essential nutrient for brain development and cognition. According to 'Ayurveda and Unani' system of medicine, the sheep's milk has various medicinal importance, aggravates hiccup and dyspnoea, elevates pitta and kapha and decreases fat. It is used against tuberculosis in folk medicine and also helps in the enhancement of platelets count during dengue. Remembered these biological activities, Gaddi sheep's milk was collected and processed by Modified method of Kobata and Ginsburg and then it was purified by Sephadex G-25 Gel column. Further the acetylation of oligosaccharides mixture followed by the silica gel chromatography led to isolation of a novel oligosaccharide Oviasose which gave positive chemical test for normal and amino sugars. From analysis of data, obtained by chemical analysis confirmed the position of linkage in oligosaccharide which is re-confirmed by different spectral methods (like NMR and Mass spectroscopy).

MATERIAL AND METHODS:

2.1. GENERAL PROCEDURE

2.12. Optical rotations were measured with a PERKIN-ELMER 241 automatic polarimeter in 1cm tube. ¹H and ¹³C NMR spectra of oligosaccharides were recorded in D₂O and the spectra of acetylated oligosaccharides were recorded in CDCl₃ at 250C on a Bruker AM 300 FT NMR spectrometer. The electrospray mass spectra were recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. The sample (dissolved in suitable solvents such as methanol/acetonitrile/water) was introduced into the ESI source through a syringe pump at the rate 5μl per min.

The ESI capillary was set at 3.5 KV and the cone voltage was 40 V. The spectra were collected in 6s scans and the print outs are averaged spectra of 6-8 scans. The C, H and N analysis were recorded on CARLO-ELBA 1108 an elemental analyzer. The sugars were visualized on TLC with 50% aqueous H₂SO₄ reagent and on Paper Chromatography with acetyl acetone and p-dimethyl amino benzaldehyde reagents. The absorbent for TLC was silica gel G (SRL) and CC silica gel (SRL, 60-120 mesh). PC was performed on Whatman No.1 filter paper using solvent system ethyl acetate-pyridine (2:1) saturated with H₂O. Sephadex G -25 (PHARMACIA) was used in gel permeation chromatography. Freeze drying of the compound was done with the help of CT 60e (HETO) lyophilizer and centrifuged by a cooling centrifuged Remi instruments C-23 JJRCI 763. To check the homogeneity of the compounds reverse phase HPLC system was used equipped with Perkin Elmer 250 solvent delivering system, 235 diode array detector and G.P. 100 printer plotter. Authentic samples of glucosamine, galactosamine, galactose and glucose were purchased from Aldrich Chemicals.

2.22. Isolation of Compounds by Kobata and Ginsburg Method- 10L Gaddi Sheep milk was collected from a sheep from high altitude region and was stored at -20°C. The milk was processed by the method of Kobata and Ginsburg. It was centrifuged for 15 min at 5000 rpm at -4°C. The solidified lipid layer was removed by filtration through glass wool column in cold. Ethanol was added to the clear filtrate to a final concentration of 68% and the resulting solution was left overnight at 0°C. The white precipitate formed, mainly of lactose and protein was removed by centrifugation and washed twice with 68% ethanol at 0°C. The supernatant and washings were combined and filtered through a micro filter (0.24 mm) (to remove remaining lactose) and lyophilized affording crude oligosaccharide mixture (162 g). This lyophilized material (mixture of oligosaccharide) was further purified by fractionating it on Sephadex G-25 chromatography using glass triple distilled water as eluent at a flow rate of 5 min/mm. Each fraction was analysed by phenol sulphuric acid reagent for the presence of neutral sugar.

2.3. Isolation and Purification of Oviasose

326.0 mg Oviasose obtained from column chromatography. On deacetylation of 29.2mg of acetylated compound k with NH₃/ acetone it afforded 23.4 mg of Oviasose obtained as a viscous mass, [α]_D+115.39° (c, 4, H₂O).

2.4. Deacetylation of Oviasose

Oviasose (29.2mg) obtained from column chromatography 2 of acetylated oligosaccharide mixture was dissolved in acetone (2ml) and 3 ml of NH₃ was added and left overnight in a stoppered hydrolysis flask. After 24 h ammonia was removed under reduced pressure and the compound was washed with (3 x 5ml) CHCl₃ (to remove acetamide) and the water layer was finally freeze dried giving the deacetylated oligosaccharide Oviasose (23.4mg).

2.41. Methylglycosidation/Acid hydrolysis of Oviasose-Oviasose (5mg) was refluxed with absolute MeOH (2 ml) at 70°C for 18h in the presence of cation exchange IR-120 (H) resin. The reaction mixture was filtered while hot and filtrate was concentrated. To a solution of methylglycoside of K in 1,4-dioxane (1ml), 0.1 N H₂SO₄ (1 ml) was added and the solution was warmed for 30 minutes at 50°C and solution was left over night. The hydrolysis was complete after 24h. The hydrolysate were neutralized with freshly prepared BaCO₃ filtered and concentrated under reduced pressure to afford α- and β-methylglucosides along with the Glc, Gal, GalNAc and GlcNAc. Their identification was confirmed by comparison with authentic samples (TLC, PC).

2.42. Killani hydrolysis of Oviasose- Oviasose (3 mg) was dissolved in 2ml Kiliani mixture (AcOH-H₂O-HCl, 7:11:2) and heated at 100°C for 1 h followed by evaporation under reduced pressure. It was dissolved in 2 ml of

H₂O and extracted twice with 3ml CHCl₃. The aqueous residual solution was made neutral by addition of 1-2 drops of 2N NaOH, to it and was evaporated under reduced pressure to afford glucose, galactose, GalNAc and GlcNAc on comparison with authentic samples of glucose, galactose, GalNAc and GlcNAc.

2.5. Description of Oviasose

Oviasose (326.00mg) obtained from column chromatography. On deacetylation of 29.2mg of acetylated Oviasose with NH₃/ acetone it afforded 23.4 mg Oviasose as a viscous mass, [α]_D+115.39⁰ (c, 4, H₂O).

For experimental analysis, this compound was dried over P₂O₅ at 100⁰ C and 0.1 mm pressure for 8 hr.

C₆₆H₁₁₁N₃O₅₁	%C	%H	%N	%O
Calculated	44.97	6.35	2.38	46.29
Practically observed	44.96	6.34	2.36	46.28

It gave positive Phenol-sulphuric acid test, Feigl test and Morgon-Elson test.

The presence of specific sugar unit in Oviasose is further confirmed by NMR and Mass spectroscopy.

2.41. In D₂O: ¹H NMR values of Oviasose: δ 5.274 [d, 1H, J=3.6Hz, α Glc (S₁) & α Gal (S₇), H-1], δ 4.721 [d, 1H, J=7.8Hz, β Glc (S₁'), H-1], δ 5.276 [d, 1H, J=4.2Hz, α GalNAc (S₁₀), H-1], δ 4.664 [d, 1H, J=8.4Hz, β Glc (S₃), H-1], δ 4.606 [d, 1H, J=7.8Hz, β GlcNAc (S₉), H-1] δ 4.542 [d, 1H, J=7.8 Hz, β GlcNAc (S₆), H-1] δ 4.507 [d, 1H, J=6.9Hz, β Gal (S₅), H-1], δ 4.497 [d, 1H, J=7.8Hz, β Gal (S₄), H-1] and δ 4.445 [d, 1H, J=7.8Hz, β Gal (S₂) & β Gal (S₈), H-1].

2.42. In D₂O: ¹³C NMR values of Oviasose: δ 171.20 [β GlcNAc (S₆) NHC(O)CH₃], δ 169.10 [β GlcNAc (S₉) NHC(O)CH₃], δ 168.20 [β GalNAc (S₁₀) NHC(O)CH₃], δ 101.65 [β Gal (S₄), β Gal (S₅) & β Gal (S₈) C-1], δ 100.50 [β GlcNAc (S₆) β GlcNAc (S₉) C-1], δ 95.10 [β Glc (S₃) C-1], δ 90.60 [α GalNAc (S₁₀) C-1], δ 89.50 [α Gal (S₇) C-1], δ 88.20 [β Glc (S₁') C-1], δ 86.50 [α Glc (S₁) C-1], δ 20.05 [α GalNAc (S₁₀) NHC(O)CH₃] and δ 20.01 [β GlcNAc (S₉) NHC(O)CH₃].

2.43. In CDCl₃: ¹H NMR values of Acetylated Oviasose: δ 6.225 [d, 1H, J=3.6Hz, α Glc (S₁) H-1], δ 5.656 [d, 1H, J=7.8Hz, β Glc (S₁'), H-1], δ 5.371 [α Glc (S₇) H-1], δ 5.311 [d, 1H, J=4.2Hz, α GalNAc (S₁₀), H-1], δ 4.664 [d, 1H, J=8.4Hz, β Glc (S₃), H-1], δ 4.621 [d, 1H, J=7.8Hz, β GlcNAc (S₉), H-1], δ 4.583 [d, 1H, J=7.8 Hz, β GlcNAc (S₆), H-1] δ 4.553 [β Gal (S₈), H-1], δ 4.528 [d, 1H, J=6.9Hz, β Gal (S₅), H-1], δ 4.498 [d, 1H, J=7.8Hz, β Gal (S₄), H-1] and δ 4.447 [d, 1H, J=7.8Hz, β Gal (S₂) H-1], δ 2.064 [s, 3H, β GlcNAc (S₉), NHC(O)CH₃], δ 2.057 [s, 3H, β GlcNAc (S₆), NHC(O)CH₃] and δ 1.987 [s, 3H, α GalNAc (S₁₀), NHC(O)CH₃]

2.44. In CDCl₃: ¹³C NMR values of Acetylated Oviasose: δ 171.60 [β GlcNAc (S₆) NHC(O)CH₃], δ 171.20 [β GlcNAc (S₉) NHC(O)CH₃], δ 170.80 [α GalNAc (S₁₀) NHC(O)CH₃], δ 104.13 [β Gal (S₂), β Gal (S₄), β Gal (S₅) & β Gal (S₈) C-1], δ 101.69 [β GlcNAc (S₆) & β GlcNAc (S₉) C-1], δ 95.18 [β Glc (S₃) C-1], δ 92.60 [α GalNAc (S₁₀) C-1], δ 91.55 [α Gal (S₇) C-1], δ 90.14 [β Glc (S₁') C-1], δ 89.20 [α Glc (S₁) C-1], δ 20.83 [β GlcNAc (S₆) NHC(O)CH₃] δ 20.68 [β GlcNAc (S₉) NHC(O)CH₃] and δ 20.59 [α GalNAc (S₁₀) NHC(O)CH₃]

2.45. ES mass of Oviasose: m/z 1823, m/z 1784, m/z 1761, m/z 1700, m/z 1623, m/z 1588, m/z 1556, m/z 1712, m/z 1694, m/z 1662, m/z 1606, m/z 1599, m/z 1599, m/z 1556, m/z 1506, m/z 1519, m/z 1448, m/z 1483, m/z 1440, m/z 1448, m/z 1396, m/z 1358, m/z 1300, m/z 1266, m/z 1271, m/z 1231, m/z 1182, m/z 1105, m/z 1086, m/z 1234, m/z 1203, m/z 1185, m/z 1161, m/z 1093, m/z 1075, m/z 1015, m/z 1031, m/z 979, m/z 927, m/z 995, m/z 927, m/z 908, m/z 948, m/z 905, m/z 869, m/z 790, m/z 772, m/z 722, m/z 742, m/z 707, m/z 692, m/z 650, m/z 618, m/z 550, m/z 480, m/z 465, m/z 406, m/z 670, m/z 640, m/z 618, m/z 590, m/z 568, m/z 512, m/z 512, m/z 454, m/z 440, m/z 406, m/z 357, m/z 342, m/z 261, m/z 202, m/z 162 and m/z 144.

RESULT AND DISCUSSION

Oviasose C₆₆H₁₁₁N₃O₅₁, [α]_D+115.39, gave positive Phenol-sulphuric acid test, Feigl test, Morgon-Elson test showing the presence of normal and amino sugar(s) in the compound. The HSQC spectrum of acetylated compound at 300 MHz exhibited ten cross peaks for ten anomeric proton signals at δ 6.225 x 89.20, δ 5.656 x 90.14, δ 5.371 x 91.55, δ 5.311 x 92.20, δ 4.664 x 95.18, δ 4.621 x 101.69, δ 4.583 x 101.69, δ 4.553 x 104.13, δ 4.528 x 104.13, δ 4.498 x 104.13, and δ 4.447 x 104.13 indicating that the Oviasose may be a decasaccharide in its reducing form giving signals for α and β anomers of glucose in its reducing end. Methylglycosidation of Oviasose by MeOH/H⁺ followed by its acid hydrolysis led to isolation of α and β - methyl glucoside, which confirmed the presence of glucose at the reducing end of the oligosaccharide. It was also confirmed by the

presence of two anomeric proton signals at δ 5.274 and δ 4.721 for α - and β -Glc in ^1H NMR of Oviasose. The decasaccharide nature of acetylated compound Oviasose was further confirmed by the presence of eleven anomeric carbon and proton at δ 89.20(1C), δ 90.14(1C), δ 91.55(1C), δ 92.20(1C), δ 95.18(1C), δ 101.69(2C), and δ 104.13(4C) in ^{13}C NMR and δ 6.225(1H), δ 5.656(1H), δ 4.447(1H), δ 4.498(1H), δ 4.528(1H), δ 4.553(1H), δ 4.583(1H), δ 4.621(1H), δ 4.664(1H), δ 5.311(1H), and δ 5.371(1H), respectively. The ten monosaccharides present in compound have been designated as S₁, S₂, S₃, S₄, S₅, S₆, S₇, S₈, S₉ and S₁₀ for convenience starting from reducing end. To confirm the monosaccharide constituents in compound, it was hydrolyzed under strong acidic conditions. In Killiani hydrolysis under strong acid condition, it gave four monosaccharides i.e. glucose, galactose, N-acetylgalactosamine and N-acetyl-glucosamine, confirming that the nonasaccharide is consist of four types of monosaccharide units i.e. glucose, galactose, N-acetylgalactosamine and N-acetyl-glucosamine. Since the glucose was present in its reducing form which was supported by ^1H NMR of Oviasose in D₂O which contains two anomeric proton signals for α - and β -Glc at δ 5.274(J=3.6) and at δ 4.721(J=7.8). Further the presence of another anomeric proton doublet signal at 4.445 was due to presence of β -Gal moiety in the Oviasose. Since it showed H-2 signal of β -Glc (S₁) as a triplet at δ 3.3402, indicated that the equatorially oriented hydroxyl groups at C-4 of the reducing β -Glc (S₁) was substituted and involved in glycosidation, suggested the presence of a lactosyl moiety i.e β -Gal(1 \rightarrow 4)Glc. It was also supported by the presence of β -Glc H-4 proton resonance at δ 3.881 in acetylated derivative of Oviasose. Another anomeric proton signal, which appeared at δ 4.497, was due to presence of Gal (S₈) moiety. The splitting pattern of S₈ anomeric signal with J value of 7.8Hz shows β -configuration of anomeric linkage between S₁ \rightarrow S₈. It was further confirmed by the chemical shift of β -Glc (S₁') at H-6 and C-6 at δ 3.765 and δ 77.21, respectively. The TOCSY spectrum of acetylated Oviasose showed two corresponding signals of anomeric signal of β -Glc (S₁') at δ 3.881 and δ 3.765 suggested that the β -Glc (S₁') is glycosidically linked at two positions which were confirmed by ^1H COSY spectrum of Oviasose acetate, proposing that the position 4 and 6 of β -Glc (S₁') are substituted by two monosaccharide units. The next two anomeric proton signals, which appeared at δ 4.663 and δ 4.542 with amide signal at δ 2.051, were due to presence of Glc (S₃) and GlcNAc (S₉) moieties, respectively. The position of anomeric proton values at δ 4.663 and δ 4.542 with downfield shifted value of H-4 of β -Gal(S₅) (SRG) suggested it may be (1 \rightarrow 6) and (1 \rightarrow 3) linked, respectively. The coupling constant of S₃ and S₉ anomeric signals with J values of 8.4Hz and 7.8Hz shows β -configuration of anomeric linkage between S₃ \rightarrow S₂ and S₉ \rightarrow S₂, respectively. It was further confirmed by the chemical shift of upfield shifted values of β -Gal at H-3 and C-3 at δ 3.966 and δ 74.82, respectively and H-6 & C-6 at δ 3.799 & δ 72.66 respectively, with upfield shifted H-4 value of β -Gal(S₇) at δ 4.108(SRG) in acetylated spectrum of Oviasose. Further the presence of next anomeric proton doublet at δ 4.445 (J=7.8Hz) was due to the presence of Gal (S₄) moiety. The H-2 triplet of β -Glc (S₃) at δ 3.3402(SRG) confirmed the (1 \rightarrow 4) linkage between β -Gal (S₄) and β -Glc (S₃). The coupling constant of S₄ anomeric signal with J value of 7.8Hz shows β -configuration of anomeric linkage between S₄ \rightarrow S₃. It was further confirmed by the H-4 proton resonance of β -Glc(S₃), appeared at δ 3.813 in Oviasose acetate. Further the presence of another anomeric proton doublet at δ 4.395 was due to the presence of Gal (S₅) moiety. The presence of anomeric proton of β -Gal (S₅) at δ 4.445(SRG) confirmed the (1 \rightarrow 4) linkage between β -Gal (S₄) and β -Gal (S₅), respectively. It was further confirmed by the H-4 & C-4 resonances of β Gal (S₄) at δ 4.168 & δ 75.62, respectively. Further the presence of next anomeric proton doublet at δ 5.270 with amide signal at δ 2.051 was due to the presence of GalNAc (S₁₀) moiety. The presence of anomeric proton of α -Gal (S₁₀) at δ 5.270 (SRG) confirmed the (1 \rightarrow 3) linkage between α -Gal (S₁₀) and β -Gal (S₄) and (1 \rightarrow 3) linkage between β -Gal (S₉) and β -Gal (S₄), respectively. It was further confirmed by the H-3 and C-3 resonances of β Gal (S₄) at δ 3.799 & δ 79.20, respectively. The next two anomeric proton signal, which appeared at δ 5.274(J=4.2) and δ 4.606(J=7.8) along with signal of amide methyl group at δ 1.965 proposed the presence of β -GlcNAc(S₆) and α -Gal(S₇) moieties,

Table 1: - ^1H and ^{13}C NMR values of Oviasose in D₂O

Moieties	^1H NMR	Coupling Constt.(J)	^{13}C NMR
α -Glc (S ₁)	5.274	3.6	86.50
β -Glc (S ₁ ')	4.721	7.8	88.20
β -Gal (S ₂)	4.445	7.8	101.65

β -Glc (S ₃)	4.664	8.4	95.10
β -Gal (S ₄)	4.445	7.8	101.65
β -Gal (S ₅)	4.507	6.9	101.65
β -GlcNAc (S ₆)	4.542	7.8	100.50
α -Gal (S ₇)	5.274	4.2	89.50
β -Gal (S ₈)	4.497	7.8	101.65
β -GlcNAc (S ₉)	4.606	7.8	100.50
α -GalNAc (S ₁₀)	5.270	3.0	90.60

respectively. The position of anomeric proton values of β -GlcNAc(S₆) at δ 4.606 & α -Gal (S₇) at δ 5.274 with downfield shifted value of H-4 of β -Gal(S₅) (SRG) suggested it may be (1 \rightarrow 6) and (1 \rightarrow 3) linked, respectively. The coupling constant of anomeric signals with J value of δ 7.8 Hz and δ 4.2 Hz shows the β - and α -configuration of anomeric linkages between S₆ \rightarrow S₅ and S₇ \rightarrow S₅, respectively. It was further confirmed by the chemical shift of β -Gal (S₅) of H-3 & C-3 resonances at δ 3.781 & δ 72.49, respectively and H-6 & C-6 at δ 4.012 & δ 72.01 respectively, with upfield shifted H-4 value of β -Gal(S₇) at δ 4.2.00(SRG) in acetylated spectrum of Oviasose.

Table 2: ¹H NMR values of acetylated Oviasose in CDCl₃

Moieties	H-1	H-2	H-3	H-4	H-5	H-6	-CH ₃
α - Glc (S ₁)	6.225	5.103	4.718	3.920	5.316	3.707	
β - Glc (S' ₁)	5.656	5.111	5.367	3.881	4.720	3.765	
β - Gal (S ₂)	4.447	5.332	3.966	4.163	4.993	3.799	
β - Glc (S ₃)	4.664	5.077	4.441	3.813			
β - Gal (S ₄)	4.498	5.092	3.799	4.108			
β - Gal (S ₅)	4.528	5.077	3.781	4.200	5.103	4.012	
β - GlcNAc (S ₆)	4.583	4.108	5.015	4.718			2.057
α - Gal (S ₇)	5.371	4.909	5.203	5.217	4.090		
β -Gal (S ₈)	4.553	4.468	5.133	4.720			
β - GlcNAc(S ₉)	4.621	4.175	4.718	4.863			2.064
α - GalNAc(S ₁₀)	5.311	3.765	4.645	5.474			1.987

The ¹³C NMR values of anomeric carbons and ring carbons of Oviasose are given in the above table. The various values of ring carbons are in accordance with ¹³C value of their respective monosaccharides, which also supports the derived structure.

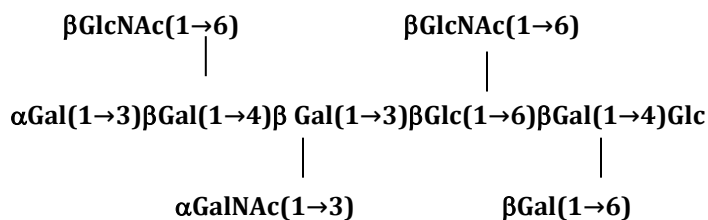
Table 3: ¹³C NMR values of acetylated Oviasose in CDCl₃

Moieties	C-1	C-2	C-3	C-4	C-5	C-6	CONH ₂	-CH ₃
α - Glc (S ₁)	89.20	61.80	62.65	73.14				
β - Glc (S' ₁)	90.14	66.78	60.85	73.56				
β - Gal (S ₂)	104.13	63.20	74.82	62.00	62.90	72.66		
β - Glc (S ₃)	95.18	67.03	62.29	74.57				
β - Gal (S ₄)	104.13	68.21	79.20	75.62				

β - Gal (S ₅)	104.13	68.12	72.49	68.85	62.60	72.01		
β - GlcNAc (S ₆)	101.69	62.26	69.26	68.43			171.60	20.83
α - Gal (S ₇)	91.55	67.10	62.80	62.65				
β -Gal (S ₈)	104.13	62.16	63.10	68.10				
β - GlcNAc(S ₉)	101.69	62.32	69.29	67.23			171.20	20.68
α - GalNAc(S ₁₀)	92.20	62.20	66.00	63.13			170.80	20.59

The decasaccharide nature of compound was further confirmed by the spectral studies of acetylated derivative of compound. The heteronuclear single quantum coherence (HSQC) spectrum of acetylated compound confirmed linkages in ¹H and ¹³C NMR spectra by showing cross peaks of α -Glc (S₁) H-4 and C-4 at δ 3.920 x 73.14 showed (1 \rightarrow 4) linkage of S₂ and S₁ and α -Glc (S₁) H-6 and C-6 at δ 3.920 x 73.14 showed (1 \rightarrow 6) linkage of S₈ and S₁, respectively, β -Glc (S₁) H-4 and C-4 at (δ 3.881 x 73.56) showed (1 \rightarrow 4) linkage of S₂ and S₁ and β -Glc (S₁) H-6 and C-6 at (δ 3.881 x 73.56) showed (1 \rightarrow 6) linkage of S₈ and S₁, respectively, i.e. its 4 and 6-positions of Glc (S₁) was involved in linkage, β -Gal (S₂) H-3 and C-3, H-6 and C-6 at (δ 3.966 x 74.82) and (δ 3.799 x 72.66) showed (1 \rightarrow 3) & (1 \rightarrow 6) linkages of S₃ \rightarrow S₂ and S₉ \rightarrow S₂ respectively. β -Glc (S₃) H-4 and C-4 at (δ 3.813 x 74.57) showed (1 \rightarrow 4) linkage of S₄ and S₃, β -Gal (S₄) H-3 and C-3, H-4 and C-4 at (δ 3.799 x 79.20) and (δ 4.108 x 75.62) showed (1 \rightarrow 3) & (1 \rightarrow 4) linkages of S₁₀ \rightarrow S₄ and S₅ \rightarrow S₄, respectively, β -Gal (S₅) H-3 and C-3, H-6 and C-6 at (δ 3.781 x 72.49) and (δ 4.021 x 72.01) showed (1 \rightarrow 3) and (1 \rightarrow 6) linkages of S₆ \rightarrow S₅ and S₇ \rightarrow S₅, respectively showing in the same chemical region in acetylated and deacetylated spectra. It was further confirmed by the presence of same peaks in COSY and TOCSY spectrum.

Based on the pattern of chemical shifts of ¹H, ¹³C, HOMOCOSY, TOCSY and HSQC NMR experiments it was interpreted that the compound was decasaccharide having structure as-



The result obtained from the ES mass spectrum further substantiated the structure of Oviasose which was derived by its ¹H and ¹³C NMR spectra. The highest mass ion peak were recorded m/z 1823 which was due to [M+Na+K]⁺. Other mass ion peak recorded at m/z 1784 and m/z 1761 was due to [M+Na]⁺ and [M]⁺ respectively, confirmed that the molecular weight of compound was 1761. Further the mass fragments were formed by repeated H transfer in the oligosaccharide and was accompanied by the elimination of terminal sugar less water. The fragmentation pathway confirmed the sequence of monosaccharide units in the decasaccharide (scheme: 1, 2, 3& 4). The decasaccharide fragment mass ion peak at m/z 1761 (M) on further fragmentation gave the nonasaccharide mass ion peak at m/z 1599(I) which was obtained by the loss of S₆ sugar unit linked to S₅ of the oligosaccharide, which was supported by its respective fragment at m/z 203, this confirmed the presence of GlcNAc (S₆) at the non-reducing end. The mass ion peak at 1599 further fragmented to give mass ion fragment for octasaccharide moiety which was arose by loss of sugar Gal (S₇). It was accounted for the mass ion fragment at m/z 1396 (II). Further the octasaccharide mass ion fragmented to gave heptasaccharide (III) fragment at m/z 1234, by loss of GalNAc (S₁₀). This heptasaccharide mass ion on further fragmentation gave hexasaccharide segment (IV) at m/z 1031, by the loss of Gal (S₅).

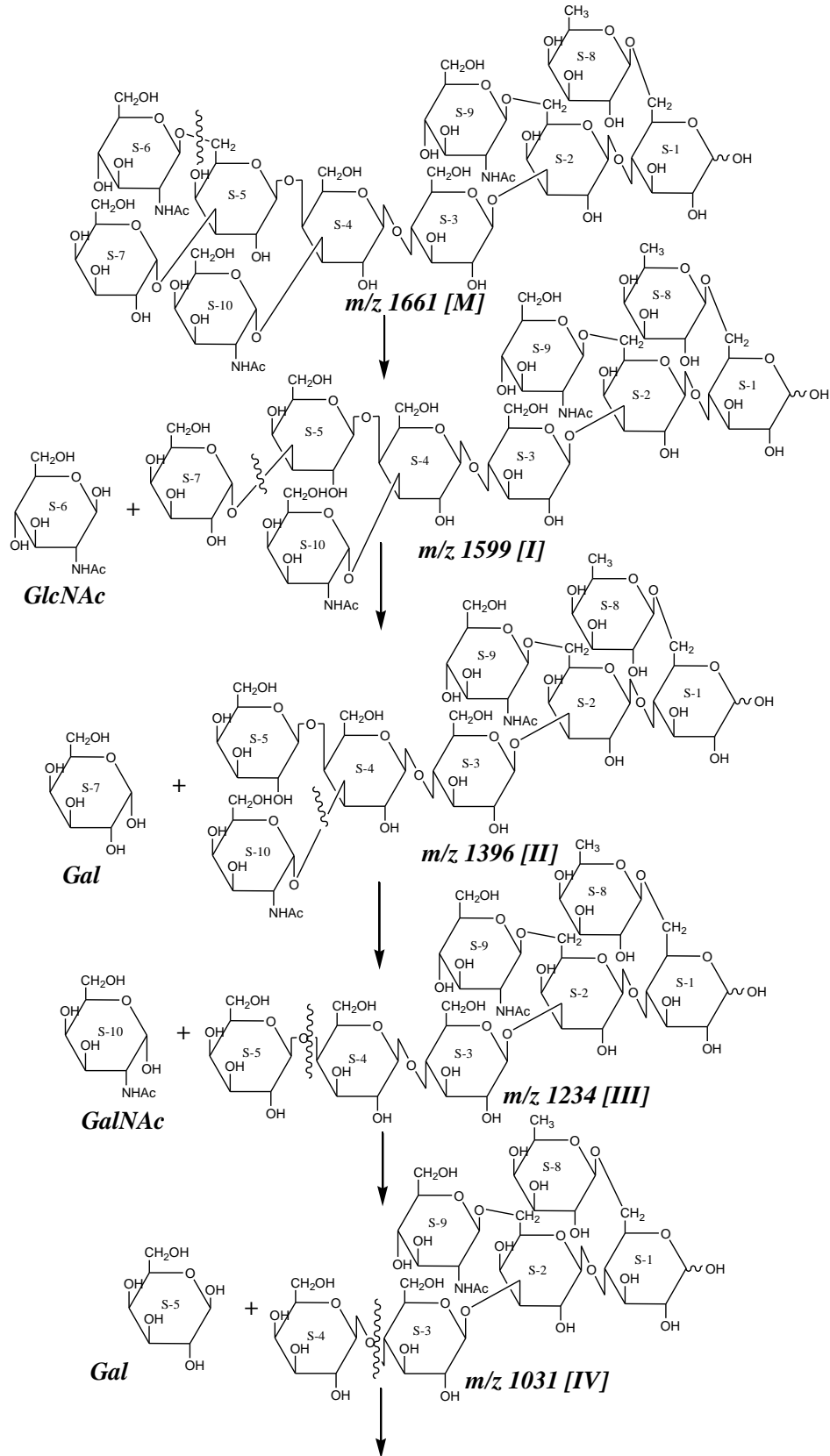
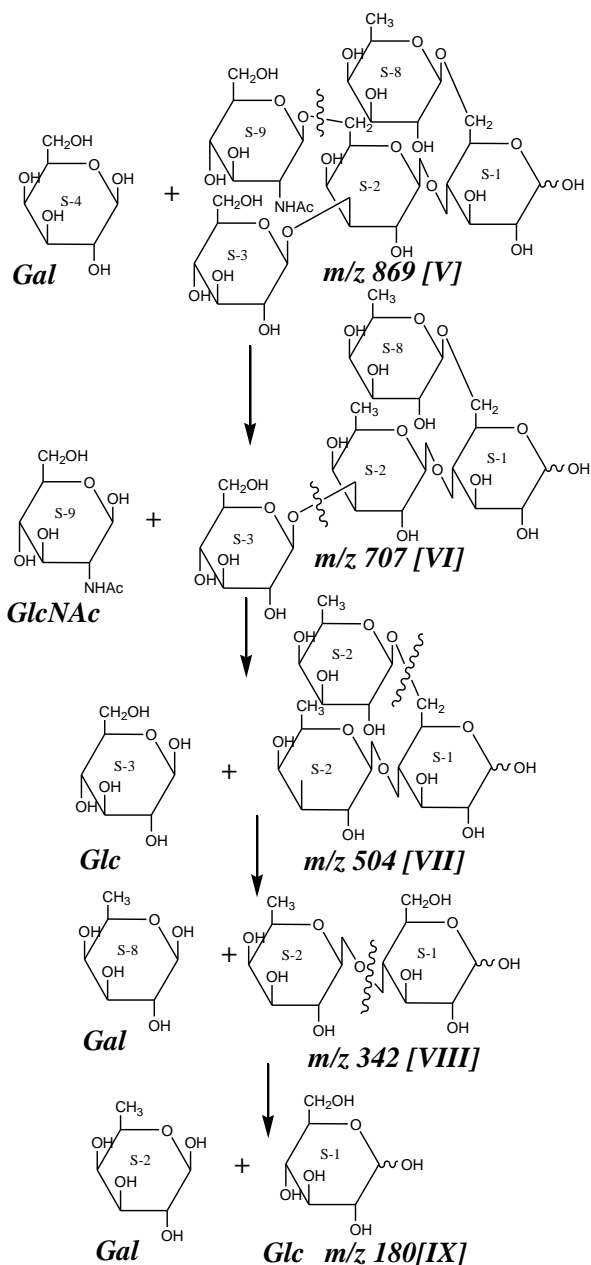


Fig 1: ES-Mass Fragmentation of Oviasose (Compound K)

Cont...



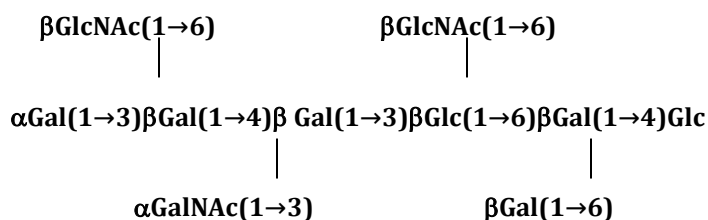
Scheme: ES-Mass Fragmentation of Oviasose (Compound K)

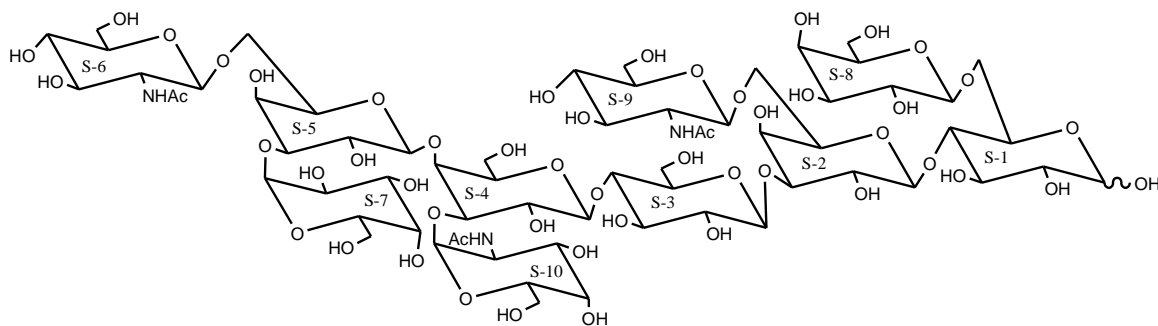
This hexasaccharide mass ion on further fragmentation gave pentasaccharide segment (V) at m/z 869, by the loss of Gal (S₄). This pentasaccharide mass ion on further fragmentation gave tetracosaccharide segment (VI) at m/z 707, by the loss of GlcNAc (S₉). This tetrasaccharide mass ion on further fragmentation gave trisaccharide segment (VII) at m/z 504, by the loss of Glc (S₃). This trisaccharide mass ion on further fragmentation gave disaccharide segment (VIII) at m/z 342, by the loss of Gal (S₈), which by further fragmentation gave monosaccharide (X) at m/z 180, by loss of Gal (S₂).

The other mass fragments obtained which supports anchoring moieties in the oligosaccharide are at m/z 869 [M-S₄,S₅,S₆,S₇,S₁₀], m/z 707 [M-S₁,S₂,S₃,S₈,S₉,S₁₀], m/z 707 [M-S₃,S₄,S₅,S₆,S₇,S₁₀], m/z 707 [M-S₁,S₂,S₃,S₆,S₈,S₉], m/z 545 [M-S₁,S₂,S₃,S₄,S₈,S₉,S₁₀], m/z 531 [M-S₁,S₄,S₅,S₆,S₇,S₈,S₁₀], m/z 504[M-

$S_3, S_4, S_5, S_6, S_7, S_9, S_{10}$] and m/z 504 [M- $S_1, S_2, S_3, S_6, S_8, S_9, S_{10}$]. The decasaccharide mass ion peak at m/z 1761 in the spectrum of compound M also showed other supporting mass ion peaks which are shown in scheme 3 & 4. The other supporting mass fragments obtained at m/z 1700 [M-HCHO, CH₂OH], m/z 1623 [1700-OH, 2HCHO], m/z 1588 [1623-OH, H₂O], m/z 1556 [1588-CH₃OH], m/z 1712 [M-CH₂OH, H₂O], m/z 1694 [1712-H₂O], m/z 1662 [1694-CH₃OH], m/z 1606 [1662-NHCOCH₃, HCHO] and m/z 1599 [1662-CH₃OH, CH₃O]. The decasaccharide m/z 1761 on fragmentation gave nonasaccharide m/z 1599 (1761- S_6), which was further confirmed by its other fragments ions at m/z 1556 [1599-CH₂CHO], m/z 1506 [1556-H₂O, CH₃OH], m/z 1519 [1556-H₂O, H₃O⁺], m/z 1448 [1506-NHCOCH₃], m/z 1483 [1519-2H₂O], m/z 1440 [1483-CH₂CHO], m/z 1448 [1483-OH, H₂O] and m/z 1396 [1440-CHCOCH₃]. The nonasaccharide m/z 1396 on fragmentation gave octasaccharide m/z 1234 (1396- S_7), which was further confirmed by its other fragments ions at m/z 1358 [1396-H₂O, HCHO], m/z 1300 [1358-NHCOCH₃], m/z 1266 [1300-2OH], m/z 1271 [1358-2HCHO, 2H₂O], m/z 1231 [1266-OH, H₂O], m/z 1182 [1231-CH₂OH, H₂O], m/z 1105 [1182-CHCOCH₃, OH, H₂O], m/z 1086 [1105-H₃O⁺] and m/z 1234 [1266-CH₂OH]. The octasaccharide m/z 1396 on fragmentation gave heptasaccharide m/z 1234 (1396- S_{10}), which was further confirmed by its other fragments ions at m/z 1203 [1234-CH₂OH], m/z 1185 [1203-H₂O], m/z 1161 [1203-CH₂CHO], m/z 1093 [1161-2H₂O, CH₃OH], m/z 1075 [1093-H₂O], m/z 1015 [1075-2HCHO], m/z 1031 [1093-2CH₂OH], m/z 979 [1015-2H₂O] and m/z 927 [979-2OH, H₂O]. The heptasaccharide m/z 1234 on fragmentation gave hexasaccharide m/z 1031 (1234- S_5), which was further confirmed by its other fragments ions at m/z 995 [1031-2H₂O], m/z 927 [995-2H₂O, CH₂OH], m/z 908 [927-H₃O⁺], m/z 948 [995-OH, HCHO], m/z 905 [948-CH₃CHO] and m/z 869 [948-OH, 2HCHO]. The hexasaccharide m/z 1031 on fragmentation gave pentasaccharide m/z 869 (1031- S_4), which was further confirmed by its other fragments ions at m/z 790 [869-HCHO, CH₂OH, H₂O], m/z 772 [790-H₂O], m/z 722 [772-H₂O, CH₃OH], m/z 742 [772-HCHO], m/z 707 [742-H₂O, OH], m/z 692 [742-H₂O, CH₃OH], m/z 650 [692-CH₂CHO], m/z 618 [650-CH₃OH], m/z 550 [618-2H₂O, CH₃OH], m/z 480 [550-2HCHO], m/z 465 [480-CH₃] and m/z 406 [465-CH₂CHO, OH]. The pentasaccharide m/z 869 on fragmentation gave tetrasaccharide m/z 707 (869- S_9), which was further confirmed by its other fragments ions at m/z 670 [707-H₂O, H₃O⁺], m/z 640 [670-HCHO], m/z 618 [670-2OH, H₂O], m/z 590 [640-H₂O, CH₃OH], m/z 568 [618-H₂O, CH₃OH], m/z 512 [590-2HCHO, H₂O], m/z 512 [568-CHCOCH₃] and m/z 454 [512-NHCOCH₃]. The tetrasaccharide m/z 666 on fragmentation gave trisaccharide m/z 504 (707- S_3), which was further confirmed by its other fragments ions at m/z 440 [504-CH₃OH], m/z 406 [440-2OH], m/z 357 [406-H₂O] and m/z 342 [406-2CH₃OH]. The trisaccharide m/z 504 on fragmentation gave disaccharide m/z 342 (504- S_8), which was further confirmed by its other fragments ions at m/z 261 [342-CH₃OH, H₂O, CH₂OH] and m/z 202 [261-NHCOCH₃]. The disaccharide m/z 342 on fragmentation gave monosaccharide m/z 180 (342- S_2), which was further confirmed by its other fragments ions at m/z 162 (180-H₂O) and m/z 144 (162-H₂O).

Based on the results obtained from chemical degradation chemical transformation, mass spectrometry and ¹H, ¹³C, HOMOCOSY, TOCSY, HSQC NMR, the structure of the isolated novel decasaccharide, Oviasose was deduced as-





CONCLUSION

From the above discussion, we conclude that the structure of isolated from Gaddi sheep milk is novel oligosaccharide named as Oviasose (deca-saccharide). This oligosaccharide was reported for the first time from any natural source or any milk and elucidated with the help of spectroscopic technique like ^1H , ^{13}C , 2DNMR (COSY, TOCSY and HSQC) spectroscopy and mass spectroscopy.

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