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Oviasose: A Novel Oligosaccharide from the Milk of Gaddi Sheep (*Ovis aries*)

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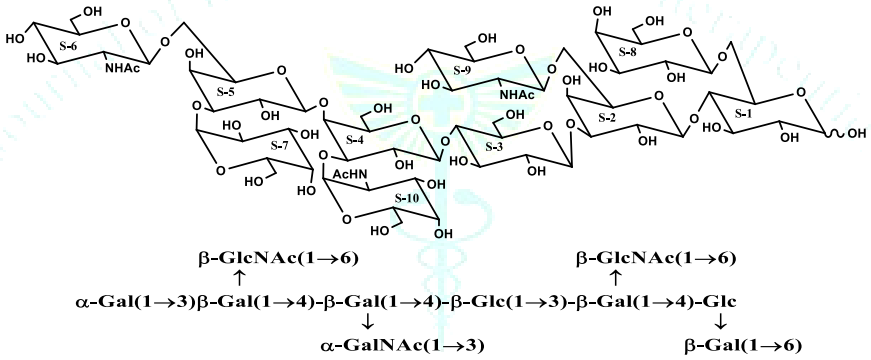
ABSTRACT

Oligosaccharides are the most biologically diverse and important carbohydrate among biological system, present as natural constituents in fruits, vegetables, milk, blood, bacteria fungus etc. Oligosaccharides present in milk protect infants by reducing the number of pathogen infections and promoting the development of the intestinal epithelium. Oligosaccharides isolated from sheep milk strongly stimulate the immune system and are used for treatment of immune system related disorders. Keeping above mentioned biological activities of Gaddi sheep's (Gaddi is a breed of sheep found at higher altitude of Himalayan region) milk oligosaccharides in mind we have isolated a novel oligosaccharide namely **Oviasose** from sheep's milk and elucidated its structure by chemical degradation & spectroscopic techniques (like ^{1}H NMR, ^{13}C NMR, COSY, TOCSY, HSQC and HMBC) and Mass Spectrometry. The structure of Oviasose was established by comparing the chemical shift (^{1}H NMR and ^{13}C NMR) of anomeric signals and other important signals of isolated milk oligosaccharide with the chemical shifts of known milk oligosaccharides, 2D-NMR and mass of Oviasose and structure was elucidated as follows-



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INTRODUCTION

Milk is an excellent gift of nature to mankind that contains all necessary nutrients for growth and development of intestinal and immune system of any mammalian neonate[1] that provide nutritional and immunological protection to their infants[2]. Milk is made up of milk fat, protein, milk carbohydrates (lactose and oligosaccharides), water, vitamins and minerals (including calcium). Each component is present in specific amount and has specific function. One of the major components of milk is carbohydrate which contains lactose and oligosaccharides[3]. The enormous biological activity of oligosaccharides such as immunostimulant, anti-tumour, anti-cancer, anti-inflammatory[4], anti-complementary[5], antiviral, antimicrobial[6,7], antioxidant, hypoglycemic activity, lipid lowering[8] and regulation of mineral absorption are reported in medicinal literature[9]. Sheep's milk has the natural ability to moisturize and nourish the skin and is safe on even most delicate skin. According to 'Ayurveda and Unani' system of medicine, the sheep's milk has various medicinal importance like aggravates hiccup and dyspnoea, elevates pitta and kapha and decreases fat[10,11]. It is used against tuberculosis in folk medicine and also helps in the enhancement of platelets count during dengue[12]. Keeping these activities in mind Gaddi sheep's milk was collected and processed by Modified method of Kobata and Ginsburg[13] 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. Comparison between ^{1}H and ^{13}C signals of natural and acetylated Oviasose and data generated from 2D-NMR studies involving COSY, TOCSY and HSQC techniques along with mass spectrometry data confirmed the position of linkage in oligosaccharides.

EXPERIMENTAL

General Procedures

Optical rotations were measured with a PERKIN-ELMER 241 automatic polarimeter in 1cm tube. ^{1}H and ^{13}C NMR spectra of oligosaccharides were recorded in D_2O and the spectra of acetylated oligosaccharides were recorded in $CDCl_3$ at 25^0C on a Bruker AM 300 FT NMR spectrometer. The electrospray mass spectra were recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. The samples (dissolved in suitable solvents such as methanol/acetonitrile/water) were 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 30% aqueous H_2SO_4 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 ethylacetate-pyridine (2:1) saturated with H_2O. Sephadex G-25 (PHARMACIA) was used in gel permeation chromatography. Freeze drying of the compound was done with the help of CT 60e (HETO) lyophilized 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 diod array detector and G.P. 100 printer plotter. Authentic samples of glucosamine, galactosamine, galactose and glucose were purchased from Aldrich Chemicals.

Isolation of Sheep Milk Oligosaccharide

Sheep milk (10 L) was collected from a domestic sheep and was stored at -20^0C. The milk was processed by the method of Kobata and Ginsburg[13]. It was centrifuged for 15 min at 5000 rpm at -4^0C. 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 0^0C. The white precipitate formed, mainly of lactose and protein was removed by centrifugation and washed twice with 68% ethanol at 0^0C. 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 ml/min. Each fraction was analysed by phenol sulphuric acid reagent for the presence of neutral sugar.

Sheep Milk Oligosaccharide Mixture Chromatographed Over Sephadex G-25 (1.6 × 40 cm) Column

The repeated gel filtration was performed by Sephadex G-25 chromatography of crude sheep milk oligosaccharide mixture. The oligosaccharide mixture (21.5 g) was packed in a column (1.6 × 40 cm) (void volume = 25 mL) equilibrated with glass triple distilled water (TDW) and left for 10-12 h to settle down (17.2 g). The material was applied onto a Sephadex G-25 column and was eluted for separation of protein and glycoprotein from oligosaccharide mixture (low molecular weight component). Presence of neutral sugars was monitored in all eluted fractions by phenol-sulphuric acid test. The sephadex G-25 chromatography of sheep milk oligosaccharide mixture which was monitored by UV spectrophotometry showed five peaks i.e. I, II, III, IV and V. A substantial amount of proteins, glycoproteins and serum albumin were eluted in the void volume which was confirmed by positive colouration with p-dimethylaminobenzaldehyde reagent and phenol-sulphuric acid reagent. Fractions under peaks II, III and IV gave a positive phenol-sulphuric acid test which showed the presence of oligosaccharide mixture in sheep milk. They were pooled together and lyophilized.

Acetylation of Oligosaccharide Mixture

The pooled fraction (II, III and IV) (11.3 g) which gave positive phenol sulphuric acid test was acetylated with pyridine (11 mL) and acetic anhydride (11 mL) at 60^0C and the solution was stirred overnight. The mixture was evaporated under reduced pressure and the viscous residue was taken in $CHCl_3$ (250 mL) and washed in sequence with 2N HCl (1 × 25 mL), ice cold 2N $NaHCO_3$ (2 × 25 mL) and finally with H_2O (2 × 25 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding the acetylated mixture (12.1 g). The acetylation converted the oligosaccharides into their nonpolar acetyl derivatives which were resolved very nicely on TLC.

Purification of Acetylated Milk Oligosaccharide on Silica Gel Column

Separation of the acetylated products (12.1 g) was carried over silica gel using varying proportions of C_6H_{12}:$CHCl_3$, $CHCl_3$ and $CHCl_3$:CH_3OH mixture which was resolved into eleven fractions. Repeated column chromatography of fraction II led to the isolation of one chromatographically pure compound Oviasose (142 mg).

Deacetylation of Compound Oviasose

The Compound Oviasose (29.2 mg) obtained from column chromatography of acetylated oligosaccharide mixture was dissolved in acetone (2 mL) and NH_3 (2 mL) was added and left overnight in a stoppered hydrolysis flask. Ammonia was removed under reduced pressure and the product was washed with $CHCl_3$ (3 × 3 mL) (to remove acetamide) and was finally freeze dried giving the deacetylated oligosaccharide (23.4 mg).

Methylglycosidation/Acid Hydrolysis of Compound Oviasose

The Compound Oviasose (5mg) was ref1uxed 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 Oviasose in 1,4-dioxane (1ml), 0.1 N H_2SO_4 (1 ml) was added and the solution was warmed for 30 minutes at 50^0C and solution was left over night. The hydrolysis was complete after 24h. The hydrolysate were neutralized with freshly prepared $BaCO_3$ 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).

Kiliani Hydrolysis of Compound Oviasose

The Compound Oviasose (3 mg) was dissolved in 2ml Kiliani mixture (AcOH-H_2O-HCI, 7:11:2) and heated at 100^0C for 1 h followed by evaporation under reduced pressure. It was

dissolved in 2 ml of H_2O and extracted twice with 3ml $CHCl_3$. 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.

Description of Isolated Compound Oviasose

^{1}H NMR of Oviasose in D_2O

δ5.274 [d, 1H, J=3.6Hz, αGlc (S_1) & αGal (S_7), H-1], δ4.721 [d, 1H, J=7.8Hz, βGlc (S_1'), H-1], δ5.276 [d, 1H, J=4.2Hz, αGalNAc (S_{10}), H-1], δ4.660 [d, 1H, J=8.4Hz, βGlc (S_3), H-1], δ4.606 [d, 1H, J=7.8Hz, βGlcNAc (S_9), H-1] δ4.542 [d, 1H, J=7.8 Hz, βGlcNAc (S_6), H-1] δ4.507 [d, 1H, J=6.9Hz, βGal (S_5), H-1], δ4.497 [d, 1H, J=7.8Hz, βGal (S_4), H-1] and δ4.445 [d, 1H, J=7.8Hz, βGal (S_2) & βGal (S_8), H-1], δ3.340 [t, 2H, J=6.1, βGlc (S_1') & βGlc (S_3), H-2], δ2.051 [s, 6H, βGlcNAc (S_6) & βGlcNAc (S_9), NHCO$\underline{CH_3}$], and δ1.965 [s, 3H, αGalNAc (S_{10}), NHCO$\underline{CH_3}$].

^{13}C NMR of Oviasose in D_2O

δ171.20 [βGlcNAc (S_6) NH$\underline{CO}CH_3$], δ169.10[βGlcNAc (S_9), NH$\underline{CO}CH_3$], δ168.20 [αGalNAc (S_{10}), NH$\underline{CO}CH_3$], δ101.65 [βGal (S_2), βGal (S_4), βGal (S_5) & βGal (S_8), C-1], δ100.50 [βGlcNAc (S_6) & βGlcNAc (S_9), C-1], δ95.10 [βGlc (S_3), C-1], δ90.60 [αGalNAc (S_{10}), C-1], δ89.50 [αGal (S_7), C-1], δ88.20 [βGlc (S'_1), C-1] and δ86.50 [αGlc (S_1), C-1], δ20.05 [αGalNAc (S_{10}), NHCO$\underline{CH_3}$] and δ20.01 [βGlcNAc (S_6) & βGlcNAc (S_9), NHCO$\underline{CH_3}$].

^{1}H NMR of Acetylated Oviasose in $CDCl_3$

δ6.225 [d, 1H, J=3.6Hz, αGlc (S_1), H-1], δ5.656 [d, 1H, J=7.8Hz, βGlc (S_1'), H-1], δ5.371 [αGal (S_7), H-1], δ5.311 [d, 1H, J=4.2Hz, αGalNAc (S_{10}), H-1], δ4.664 [d, 1H, J=8.4Hz, βGlc (S_3), H-1], δ4.621 [d, 1H, J=7.8Hz, βGlcNAc (S_9), H-1] δ4.583 [d, 1H, J=7.8 Hz, βGlcNAc (S_6), H-1], δ4.553 [βGal (S_8), H-1], δ4.528 [d, 1H, J=6.9Hz, βGal (S_5), H-1], δ4.498 [d, 1H, J=7.8Hz, βGal (S_4), H-1] and δ4.447 [d, 1H, J=7.8Hz, βGal (S_2), H-1], δ2.064 [s, 3H, βGlcNAc (S_9), NHCO$\underline{CH_3}$], δ2.057 [s, 3H, βGlcNAc (S_6), NHCO$\underline{CH_3}$] and δ1.987 [s, 3H, αGalNAc (S_{10}), NHCO$\underline{CH_3}$].

^{13}C NMR Acetylated Oviasose in $CDCl_3$

δ171.60 [βGlcNAc (S_6) NH$\underline{CO}CH_3$], δ171.20 [βGlcNAc (S_9), NH$\underline{CO}CH_3$], δ170.80 [αGalNAc (S_{10}), NH$\underline{CO}CH_3$],δ104.13 [βGal (S_2), βGal (S_4), βGal (S_5) & βGal (S_8), C-1], δ101.69 [βGlcNAc (S_6) & βGlcNAc (S_9), C-1], δ95.18 [βGlc (S_3), C-1], δ92.20 [αGalNAc (S_{10}), C-1], δ91.55 [αGal (S_7), C-1], δ90.14[βGlc (S'_1), C-1] and δ89.20 [αGlc (S_1), C-1], δ20.83 [βGlcNAc (S_6), NHCO$\underline{CH_3}$], δ20.68 [βGlcNAc (S_9), NHCO$\underline{CH_3}$] and δ20.59 [αGalNAc (S_{10}), NHCO$\underline{CH_3}$].

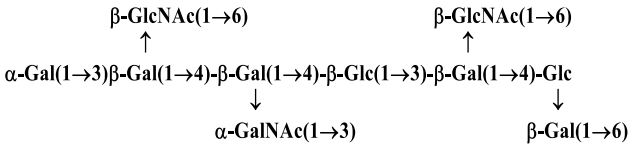
ES-Mass Spectral Fragments of Compound Oviasose

m/z 1823[M+Na+K]$^+$, m/z 1784[M+Na]$^+$, m/z 1761[M]$^+$, m/z 1712, m/z 1700, m/z 1694, 1662, m/z 1623, m/z 1606 , m/z 1599, m/z 1588, m/z 1556, m/z 1519, m/z 1506, m/z 1483, m/z 1448, m/z 1440, m/z 1396, m/z1358 , m/z 1300 , m/z 1271, m/z 1266 , m/z 1234, m/z 1231, m/z 1203, m/z 1185, m/z 1182, m/z 1161, m/z 1105, m/z 1093, m/z 1086, m/z 1075, m/z 1031, m/z 1015, m/z 979, m/z 995, m/z 948, m/z 927, m/z 908 , m/z 905, m/z 869, m/z 790, m/z 772, m/z 742, m/z 722, m/z 707, m/z 692, m/z 670, m/z 650, m/z 640, m/z 618, m/z 590, m/z 568, m/z 550, m/z 512, m/z 480 , m/z 465, 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

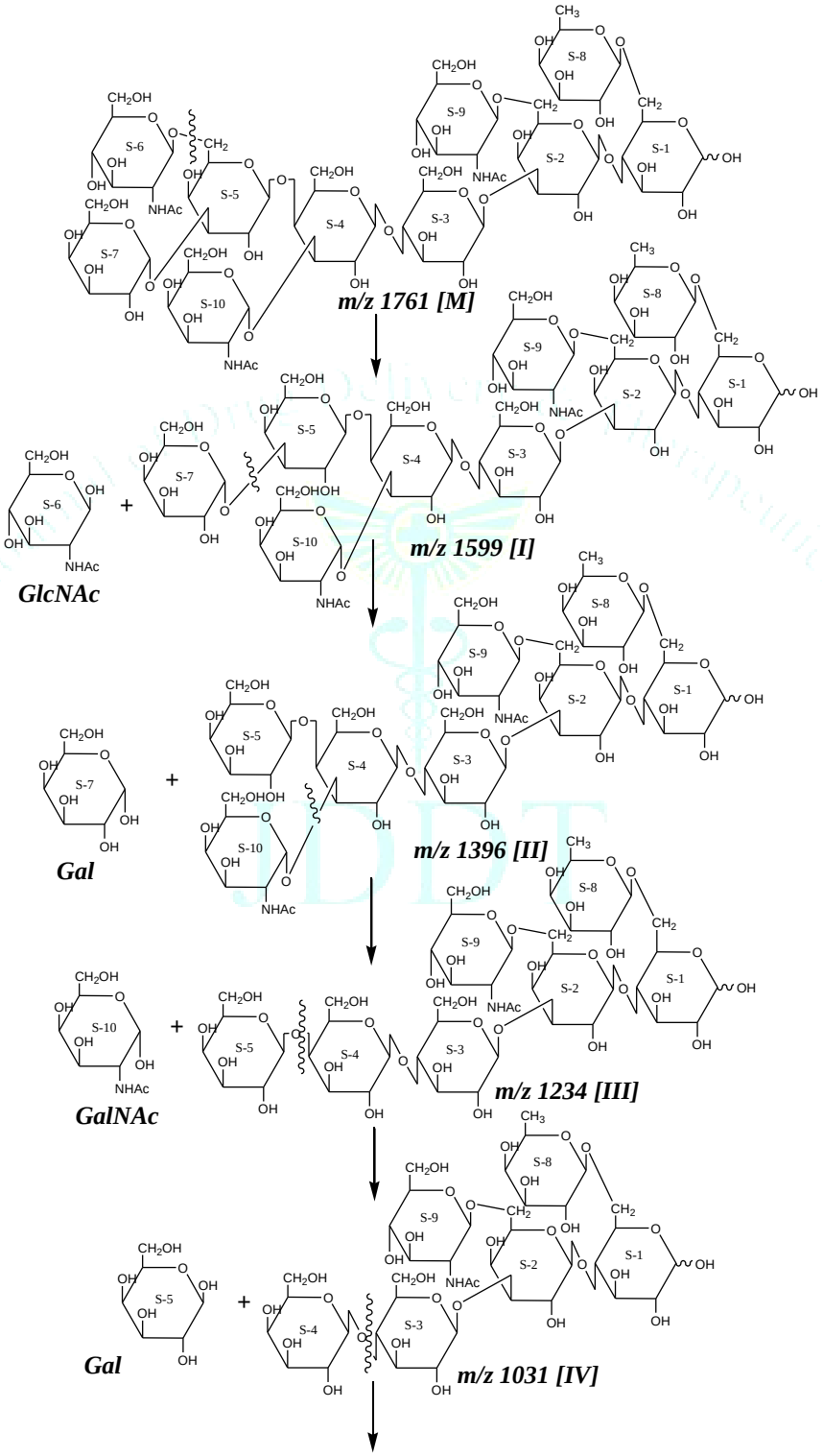
Compound Oviasose, $C_{66}H_{111}N_3O_{51}$, $[\alpha]_D$ +115.39, gave positive Phenol-sulphuric acid test[14], Feigl test[15] and Morgon-Elson test[16] showing the presence of normal and amino sugar(s) in the compound. The HSQC spectrum of acetylated compound at 300 MHz exhibited ten cross peaks for 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 x101.69, δ 4.583 x101.69, δ 4.553 x104.13, δ 4.528 x104.13, δ 4.498 x 104.13, andδ 4.447 x104.13 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 ^{1}H 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 5.371(1H), δ 5.311(1H), δ 4.664(1H), δ 4.621(1H), δ 4.583(1H), δ 4.553(1H), δ 4.528(1H), δ 4.498(1H) and δ 4.447(1H), in ^{1}H NMR, respectively. The ten monosaccharides present in compound have been designated as S_1, S_2, S_3, S_4, S_5, S_6, S_7, S_8, S_9 and S_{10} for convenience starting from reducing end. To confirm the monosaccharide constituents in compound, it was hydrolyzed under strong acidic conditions. In Kiliani hydrolysis under strong acid condition, it gave four monosaccharides i.e. glucose, galactose, N-acetylgalactosamine and N-acetyl-glucosamine, confirming that the decasaccharide 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 ^{1}H NMR of Oviasose in D_2O 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 (S_1') as a triplet at δ 3.3402, indicated that the equatorially oriented hydroxyl groups at C-4 of the reducing β-Glc (S_1') was substituted and involved in glycosidation, suggested the presence of a lactosyl moiety i.e β-Gal(1→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 (S_8) moiety. The splitting pattern of S_8 anomeric signal with J-value of 7.8Hz shows β-configuration of anomeric linkage between S_1→S_8. It was further confirmed by the chemical shift of β-Glc (S_1') at H-6 and C-6 at δ3.765 and δ77.21, respectively. The TOCSY spectrum of acetylated Oviasose showed two corresponding signals of anomeric signal of β-Glc (S1') at δ3.881 and δ3.765 suggested that the β-Glc (S_1') is glycosidically linked at two positions which were confirmed by ^{1}H COSY spectrum of Oviasose acetate, proposing that the position 4 and 6 of β-Glc (S_1') are substituted by two monosaccharide units. The next two anomeric proton signals, which appeared at δ4.660 and δ4.542 with amide signal at δ2.051, were due to presence of Glc (S_3) and GlcNAc (S_9) moieties, respectively. The position of anomeric proton values at δ4.660 and δ4.542 with downfield shifted value of H-4 of β-Gal(S_2) (SRG) suggested it may be (1→3) and (1→6) linked, respectively. The coupling constant of S_3 and S_9 anomeric signals with J values of 8.4Hz and 7.8Hz shows β-configuration of anomeric linkage between S_3→S_2 and S_9→S_2,

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 δ3.799 & δ72.66 respectively, with upfield shifted H-4 value of β-Gal(S_7) at δ4.108(SRG) in acetylated spectrum of Oviasose. Further the presence of next anomeric proton doublet at δ4.445 (J=7.8Hz) was due to the presence of Gal (S_4) moiety. The H-2 triplet of β-Glc (S_3) at δ3.3402(SRG) confirmed the (1→4) linkage between β-Gal (S_4) and β-Glc (S_3). The coupling constant of S_4 anomeric signal with J value of 7.8Hz shows β-configuration of anomeric linkage between S_4→S_3. It was further confirmed by the H-4 proton resonance of β-Glc(S_3), appeared at δ 3.813 in Oviasose acetate. Further the presence of another anomeric proton doublet at δ4.395 was due to the presence of Gal (S_5) moiety. The presence of anomeric proton of β-Gal (S_5) at δ4.445(SRG) confirmed the (1→4) linkage between β-Gal (S_4) and β-Gal (S_5), respectively. It was further confirmed by the H-4 & C-4 resonances of β Gal (S_4) at δ4.168 & δ75.62, respectively. Further the presence of next anomeric proton doublet at δ5.270 with amide signal at δ2.051 was due to the presence of GalNAc (S_{10}) moiety. The presence of anomeric proton of α-Gal (S_{10}) at δ5.270 (SRG) confirmed the (1→3) linkage between α-Gal (S_{10}) and β-Gal (S_4) and (1→3) linkage between β-Gal (S_9) and β-Gal (S_4), respectively. It was further confirmed by the H-3 and C-3 resonances of β Gal (S_4) at δ3.799 & δ79.20, respectively. The next two anomeric proton signal, which appeared at δ5.274(J=4.2) and δ4.606(J=7.8) along with signal of amide methyl group at δ1.965 proposed the presence of β-GlcNAc(S_6) and α-Gal(S_7) moieties, respectively. The position of anomeric proton values of β-GlcNAc(S_6) at δ4.606 & α-Gal (S_7) at δ5.274 with downfield shifted value of H-4 of β-Gal(S_5) (SRG) suggested it may be (1→6) and (1→3) linked, respectively. The coupling constant of anomeric signals with J value of δ7.8 Hz and δ4.2 Hz shows the β- and α-configuration of anomeric linkages between S_6→S_5 and S_7→S_5, respectively. It was further confirmed by the chemical shift of β-Gal (S_5) of H-3 & C-3 resonances at δ3.781 & δ72.49, respectively and H-6 & C-6 at δ4.012 & δ72.01 respectively, with upfield shifted H-4 value of β-Gal(S_7) at δ4.2.00(SRG) in acetylated spectrum of Oviasose. The ^{13}C 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 ^{13}C value of their respective monosaccharides, which also supports the derived structure. 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 ^{13}C NMR spectra by showing cross peaks of α-Glc (S_1) H-4 and C-4 at δ 3.920 x 73.14 showed (1→4) linkage of S_2 and S_1 and α-Glc (S_1) H-6 and C-6 at δ 3.920 x 73.14 showed (1→6) linkage of S_8 and S_1, respectively, β-Glc (S_1) H-4 and C-4 at (δ3.881 x 73.56) showed (1→4) linkage of S_2 and S_1 and β-Glc (S_1) H-6 and C-6 at (δ3.881 x 73.56) showed (1→6) linkage of S_8 and S_1, respectively, i.e. its 4 and 6-positions of Glc (S_1) was involved in linkage, β-Gal (S_2) H-3 and C-3, H-6 and C-6 at (δ3.966 x 74.82) and (δ3.799 x 72.66) showed (1→3) & (1→6) linkages of S_3 → S_2 and S_9→ S_2 respectively. β-Glc (S_3) H-4 and C-4 at (δ 3.813 x 74.57) showed (1→4) linkage of S_4 and S_3, β-Gal (S_4) H-3 and C-3, H-4 and C-4 at (δ3.799 x 79.20) and (δ4.108 x 75.62) showed (1→3) & (1→4) linkages of S_{10}→ S_4 and S_5 → S_4 , respectively, β-Gal (S_5) H-3 and C-3, H-6 and C-6 at (δ3.781 x 72.49) and (δ4.021 x 72.01) showed (1→3) and (1→6) linkages of S_6 →S_5 and S_7 →S_5, 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. Thus, based on the pattern of chemical shifts of ^1H NMR , ^{13}C NMR, HOMOCOSY, TOCSY and HSQC NMR experiments it was interpreted that the compound was a 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 ^{13}C 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). 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 S_6 sugar unit linked to S_5 of the oligosaccharide, which was supported by its respective fragment at m/z 203, this confirmed the presence of GlcNAc (S_6) 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 (S_7). 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 (S_{10}). This heptasaccharide mass ion on further fragmentation gave hexasaccharide segment (IV) at m/z 1031, by the loss of Gal (S_5). This hexasaccharide mass ion on further fragmentation gave pentasaccharide segment (V) at m/z 869, by the loss of Gal (S_4). This pentasaccharide mass ion on further fragmentation gave tetraccharide segment (VI) at m/z 707, by the loss of GlcNAc (S_9). This tetrasaccharide mass ion on further fragmentation gave trisaccharide segment (VII) at m/z 504, by the loss of Glc (S_3). This trisaccharide mass ion on further fragmentation gave disaccharide segment (VIII) at m/z 342, by the loss of Gal (S_8), which by further fragmentation gave monosaccharide (X) at m/z 180, by loss of Gal (S_2). The other mass fragments obtained which supports anchoring moieties in the oligosaccharide are at m/z 869 [M-S_4,S_5,S_6,S_7,S_{10}], m/z 707 [M-S_1,S_2,S_3,S_8,S_9,S_{10}], m/z 707 [M-S_3,S_4,S_5,S_6,S_7,S_{10}], m/z 707 [M-S_1,S_2,S_3,S_6,S_8,S_9], m/z 545 [M-S_1,S_2,S_3,S_4,S_8,S_9,S_{10}], m/z 531 [M-S_1,S_4,S_5,S_6,S_7,S_8,S_{10}], 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 Oviasose also showed other supporting mass ion peaks. The other supporting mass fragments obtained at m/z 1700 [M-HCHO,CH_2OH], m/z 1623[1700-OH,2HCHO], m/z 1588[1623-OH,H_2O], m/z 1556 [1588-CH_3OH], m/z 1712 [M-CH_2OH,H_2O], m/z 1694 [1712-H_2O], m/z 1662 [1694-CH_3OH], m/z 1606 [1662-$NHCOCH_3$,HCHO] and m/z 1599[1662-CH_3OH,CH_3O]. 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-CH_2CHO], m/z 1506 [1556-H_2O,CH_3OH], m/z 1519 [1556-H_2O,H_3O^+], m/z 1448 [1506-$NHCOCH_3$], m/z 1483 [1519-$2H_2O$], m/z 1440 [1483-CH_2CHO], m/z 1448 [1483-OH,H_2O] and m/z 1396 [1440-$CHCOCH_3$]. 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/z1358 [1396-H_2O,HCHO], m/z 1300 [1358-$NHCOCH_3$], m/z 1266 [1300-2OH], m/z 1271 [1358-2HCHO,$2H_2O$], m/z 1231 [1266-OH,H_2O], m/z 1182 [1231-CH_2OH,H_2O], m/z 1105 [1182-$CHCOCH_3$,OH,H_2O], m/z 1086 [1105-H_3O^+] and m/z 1234 [1266-CH_2OH]. 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-CH_2OH], m/z 1185 [1203-H_2O], m/z 1161 [1203-CH_2CHO], m/z 1093 [1161-2H_2O,CH_3OH], m/z 1075 [1093-H_2O], m/z 1015 [1075-2HCHO], m/z 1031 [1093-2CH_2OH], m/z 979 [1015-2H_2O] and m/z 927 [979-2OH,H_2O]. 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 [995-2H_2O,CH_2OH], m/z 908 [927-H_3O^+], m/z 948 [995-OH,HCHO], m/z 905 [948-CH_3CHO] and m/z 869 [948-OH,2HCHO].



Scheme I: ES-Mass Fragmentation of Oviasose

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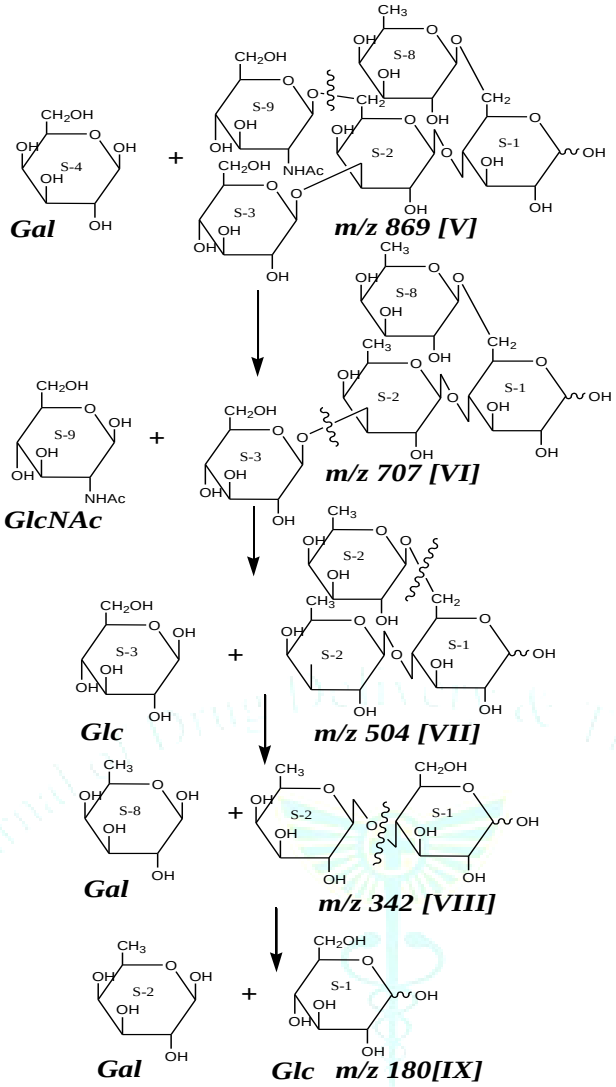
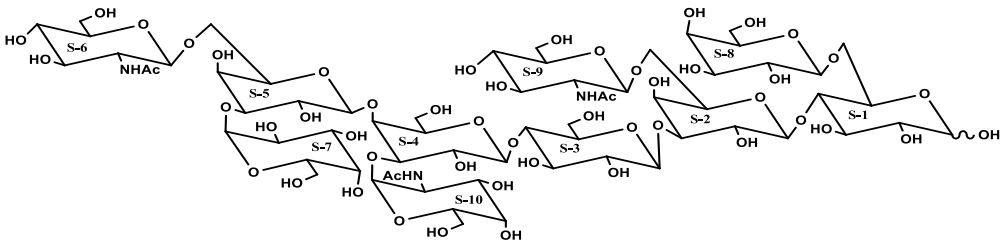


Scheme II: ES-Mass Fragmentation of Oviasose

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,CH_2OH,H_2O], m/z 772 [790-H_2O], m/z 722 [772-H_2O,CH_3OH], m/z 742 [772-HCHO], m/z 707 [742-H_2O,OH], m/z 692 [742-H_2O,CH_3OH], m/z 650 [692-CH_2CHO], m/z 618 [650-CH_3OH], m/z 550 [618-2H_2O,CH_3OH], m/z 480 [550-2HCHO], m/z 465 [480-CH_3] 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-H_2O,H_3O^+], m/z 640 [670-HCHO], m/z 618 [670-2OH,H_2O], m/z 590 [640-H_2O,CH_3OH], m/z 568 [618-H_2O,CH_3OH], m/z 512 [590-2HCHO,H_2O], m/z 512 [568-$CHCOCH_3$]and m/z 454 [512-$NHCOCH_3$]. 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-CH_3OH], m/z 406 [440-2OH], m/z 357 [406-H2O] and m/z 342 [406-2CH_3OH]. 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-CH_3OH,H_2O,CH_2OH] and m/z 202 [261-$NHCOCH_3$]. 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-H_2O) and m/z 144 (162-H_2O). Based on the results obtained from chemical degradation/acid hydrolysis, chemical transformation, Electro spray mass spectrometry and 1D-NMR viz. ^1H NMR, ^{13}C NMR and 2D-NMR viz. COSY, TOCSY and HSQC NMR spectra of Oviasose acetate and Oviasose, the structure and sequence of isolated novel decasaccharide Oviasose was deduced as-



CONCLUSION

From the above informations, we conclude the structure of isolated sheep milk oligosaccharide, **Oviasose**. This novel oligosaccharide was reported for the first time from any natural source or any milk and its structure was elucidated with the help of spectroscopic techniques like ^{1}H, ^{13}C, 2D-NMR (COSY, TOCSY and HSQC) spectroscopy and mass spectrometry.

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REFERENCES

1. Singh P., Maurya R.K., Rizvi A.H. and Deepak D. Famiose: A novel hexasaccharide from Donkey's milk. *J. Biol. Chem. Research*. 2017; 34(2):548-556.
2. Singh P., Srivastava A.K. and Deepak D. Isolation and structure elucidation of Caprose (novel oligosaccharide) from Goat milk. *J. Biol. Chem. Research.* 2017; 34(1):14-20.
3. Srivastava A.K., Singh P. and Deepak D. Isolation and NMR Studies of novel oligosaccharide from Goat milk. *J. Biol. Chem. Research.* 2016; 33(2):901-908.
4. Singh P., Gupta N.K. and Deepak D. Structure Elucidation of Novel Trisaccharide Isolated from Donkey's milk. *J. Biol. Chem. Research.* 2019; 36(1):157-163.
5. Saksena R., Deepak D., Khare A., Sahai R., Tripathi L.M. and Srivastava V.M.L.A novel pentasaccharide from immunostimulant oligosaccharide fraction of buffalo milk. *Biochemia et. Biophysica Acta.* 1999; 1428:433-445.
6. Yang B., Chuang H. and Chen R.F. Protection from viral infections by human milk oligosaccharides: direct blockade and indirect modulation of intestinal icology and immune reactions. *Open Glycoscience. 2012;* 5:19-25.
7. Singh P., Maurya R.K., Rizvi A.H. and Deepak D. Isolation and 2D-NMR Studies of Aliose- A Novel Hexasaccharide from Donkey's Milk. *J. Biol. Chem. Research.* 2018; 35(2):378-385.
8. Halas V. and Nochta I. Review: mannan oligosaccharides in nursery pig nutrition and their potential mode of action. 2012; 2:261-274.
9. Poeikhampha T. and Bunchasak C., Comparative effects of sodium gluconate, mannan oligosaccharides and potassium diformate on growth performances and small intestinal morphology of nursery pigs. 2011; 24:841-850.
10. Singh P., Shahi S. and Deepak D. Isolation and NMR Characterization of Riesose- A Novel Oligosaccharide from Gaddi Sheep's Milk. *J. Biol. Chem. Research.* 2018; 35(2):752-760.
11. Shahu S., Verma P., Sarkar J. and Deepak D. Isolation of a novel Sheep milk oligoasaccharide as biologically active component. *J. Biol. Chem. Research.* 2017; 34(2):583-596.
12. Shahi S., Gangwar L., Verma P. and Deepak D. Isolation, purification and NMR study of a novel nonasaccharide from Gaddi Sheep milk. *J. Biol. Chem. Research.* 2017; 34(2):569-582.
13. Kobata A., Ginsburg V. An enzymatic basis for Lewis blood types in man. *J. Biol. Chem.* 1970; 245:1484.
14. Dubois M., Gilles K.A., Hamilton J.K., Rebers P.A and Smith F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 1956; 28(3):350-356.
15. Fiegl. F. 1975. Spot test in organic analysis. Elsevier Publication, Amsterdam, pp. 337.
16. Partridge S.M. and Westall R.G. *Biochem. J.* 1948; 42(2):238-250.