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NMR, Mass and DFT Studies of Ariose: A Novel Oligosaccharide from Donkey *(Equus asinus)* Milk

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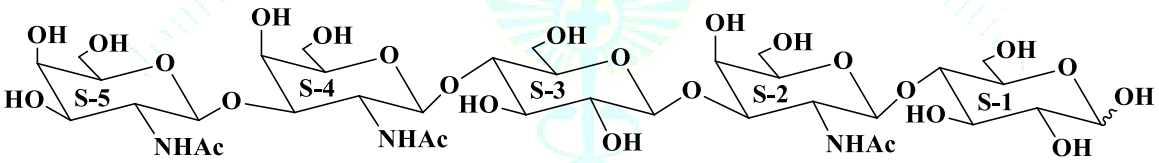
ABSTRACT

Oligosaccharides play a key role in various physiological, pathological and biological activities such as biological recognition, anti-complementary, anti-coagulant, anti-inflammatory, anti-viral, anti-bacterial, anti-tumour, anti-oxidant, lipid lowering, immunological activities, prebiotic activity and hypoglycemic activity. In our endeavour to find biologically active novel oligosaccharides, donkey milk was taken, which is a rich source of oligosaccahrides and its milk is used as anti-hypertensive, anti-oxidant and heart strengthening agent in folk medicine. For this purpose donkey milk was processed by modified method of Kobata and Ginsburg followed by Gel filtration HPLC and Column Chromatography (CC) which resulted in the isolation of one novel milk oligosaccharide namely Ariose. The structure of purified milk oligosaccharide was determined with the help of chemical degradation, chemical transformation, spectroscopic techniques like 1D-NMR (^{1}H and ^{13}C), 2D-NMR (COSY, TOCSY, HSQC and HMBC), Structure Reporter Group (SRG) theory and Mass spectrometry (ESI-MS). The geometry optimization of compound was done by using B3LYP method at 6-31G (d, p) basis set employing Density Functional Theory (DFT). The structure is elucidated as-



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1. INTRODUCTION

Milk is a primary dynamic biological fluid responsible for development of mammalian neonates[1]. Besides the other regular constituents (protein, lipid, fat, minerals, vitamins and calcium) it have oligosaccharides (complex carbohydrates) in it which are responsible for antitumor, anticancer, antigenic and immunostimulant activities[2]. These complex carbohydrates (oligosaccharides) are known to be responsible for the beneficial effects of breast feeded newly borns and perform a number of bioactive functions including prebiotic enrichment of a protective micro biota, limiting the virulence of several pathogens and increasing postnatal neural development[3] and also inhibit the adhesion of pathogenic micro-organism to the intestinal and urinary tract by acting receptor analogues to preventing gastric and urinary infections[4]. So these oligosaccharide exhibits varied biological activity such as anti-inflammatory[5], anti-tumour, antithrombotic, immunostimulant[6], anti-cancer, antiviral, antimicrobial and cardio-protective activities[7]. Moreover, these oligosaccharides have been isolated from various mammalian milk of different origin e.g. cow, buffalo, mare, yak, sheep, equine, caprine, elephant, donkey, goat, camel and human etc[8]. Donkey milk is a rich source of simple as well as complex oligosaccharides. Donkey milk oligosaccharides have ability to stimulate non-specific and specific immunological resistance and prevention of athereosclerosis[9]. The oligosaccharide mixture of Donkey's milk has shown significant stimulation of antibody, delayed type hypersensitivity response to sheep red blood cells in BALB/c mice[10]. In the present study, the structure of one novel donkey milk oligosaccharide (Ariose) was elucidated with the help of spectroscopic techniques (^{1}H NMR, ^{13}C NMR, COSY, TOCSY, HSQC and HMBC) and other techniques

like deacetylation, hydrolysis, chemical degradation and ESI-MS (mass spectrometry).

2. THEORETICAL STUDY

The quantum chemical calculations have been performed on B3LYP functional and 6-31 G (d, p) basis set employing Density Functional Theory (DFT). The theoretical calculations have been performed using Guassian09W package. The optimized geometry is visualized by Gauss View 5.0.9 utility software[11].

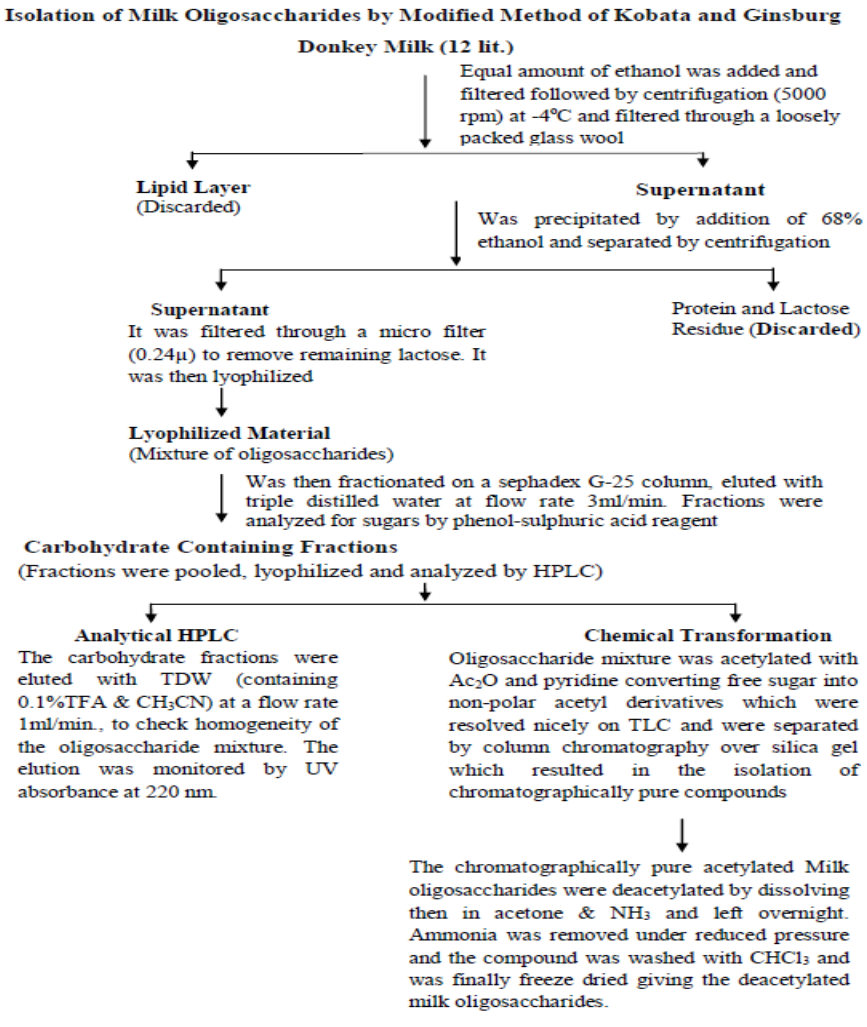
2.1. Experimental

General Procedures

For evaporation of alcohol from crude extract of milk oligosaccharides, Buchi Rotary evaporator was used. Freeze drying of the compounds was done with the help of CT 60e (HETO) lyophilizer and centrifuged by a cooling centrifuge Remi instruments C-23 JJRCI 763. Optical rotations were measured with a Buchi automatic Polarimeter in 1.2 cm tube. The C, H and N analyses were recorded on CARLO-ELBA 1108 elemental analyzer. All melting points were recorded on BOETIUS micro-melting point apparatus and are uncorrected. ^{1}H and ^{13}C NMR and 2D-NMR experiments were recorded in solvent $CDCl_3$ and D_2O at 25° on a Bruker AM 300 MHz FT NMR spectrometer. The Electrospray mass spectra were recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. The milk oligosaccharide sample (dissolved in water as solvent) was introduced into the ESI source through a syringe pump at the rate of 5ul per min. The ESI capillary was set at 3.5 kV and the cone voltage was 40 V. The spectra were collected in 6 s scans and the print outs are averaged spectra of 6-8 scans. Spectrum recorded in higher mass scale is computerized deconvoluted. Authentic samples of glucosamine, galactosamine, galactose, glucose, fucose and sialic acid were purchased from Aldrich Chemicals.

2.2. Isolation of Donkey Milk Oligosaccharides by Modified Kobata and Ginsburg Method[12]

Donkey milk (12 lit) was collected from a domestic donkey and was stored at -20°C. 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 clear the 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 by 68% ethanol at 0°C. The supernatant and washing were combined and filtered through a microfilter (0.24 μ m) (to remove remaining lactose) and lyophilized affording crude oligosaccharide mixture (178 gm). The lyophilized material responded positively to Morgon-Elson test and Thiobarbituric acid test suggesting the presence of N-acetyl sugars and sialic acid in the oligosaccharide mixture. This lyophilized material (mixture of oligosaccharides) was further purified by fractionating it on Sephadex G-25 column using glass double distilled water as eluant at a flow rate of 3 ml/min. Each fraction was analyzed for sugars by phenol-sulphuric acid reagent for presence of neutral sugars.



2.3. Sephadex G-25 Gel Filtration of Donkey Milk Oligosaccharide Mixture

The repeated gel filtration was performed by Sephadex G-25 chromatography of crude donkey milk oligosaccharide mixture. Donkey milk oligosaccharide mixture was packed in a column (1.6 x 40 cm, void volume = 25 ml) equilibrated with glass triple distilled water (TDW) and it was left for 10-12 hours to settle down. The material was applied on a Sephadex G-25 column and was eluted for separation of protein and glycoprotein from oligosaccharide (low molecular weight components). Presence of neutral sugars was monitored in all eluted fractions by phenol-sulphuric acid test. In this U.V. monitored Sephadex G-25 chromatography of donkey milk oligosaccharide mixture showed four peaks i.e. I, II, III, IV. A substantial amount of proteins, glycoproteins and serum albumin were eluted in the void volume which was confirmed by positive coloration with p-dimethylaminobenzaldehyde reagent and phenol-sulphuric acid reagent. Fractions under peaks II and III gave a positive phenol-sulphuric acid test for sugars which showed the presence of oligosaccharide mixture in donkey milk. These fractions (peak II and III) were pooled and lyophilized together.

2.4. Confirmation of Homogeneity of Donkey Milk Oligosaccharide by Reverse Phase HPLC

Pooled fractions (peak II and III) obtained from Sephadex G-25 column, containing oligosaccharide mixture were qualitatively analyzed by reverse phase HPLC. The HPLC system was equipped with Perkin-Elmer 250 solvent delivering system, 235 diode array detector, and G.P. 100 printer plotter. The column used for this purpose was C_{18} Purosphear 25 cm x 0.4 cm x 5 µm (from E. Merck). A binary gradient system of acetonitrile: 0.5% trifluoro acetic acid (5:95) in triple distilled water (TDW) to CH_3CN: 0.5% TFA (60:40) within 25 min at a flow rate of 1 ml/min was used. The eluents were detected at 220 nm. Eighteen peaks were noticed in the sample (pooled fractions II and III) at the varied retention times from 3.50 to 20.32 min, for convenience the peaks were numbered in their increasing order of retention time in minute i.e. .083(R_1), .658(R_2), 1.933(R_3), 2.633(R_4), 3.25(R_5), 4.467(R_6), 6.85(R_7), 8.4(R_8), 9.333(R_9), 10.475(R_{10}), 11.475(R_{11}), 13.00(R_{12}), 14.425(R_{13}), 15.067(R_{14}), 17.058(R_{15}), 19.85(R_{16}), 20.683(R_{17}), and 22.45(R_{18}).

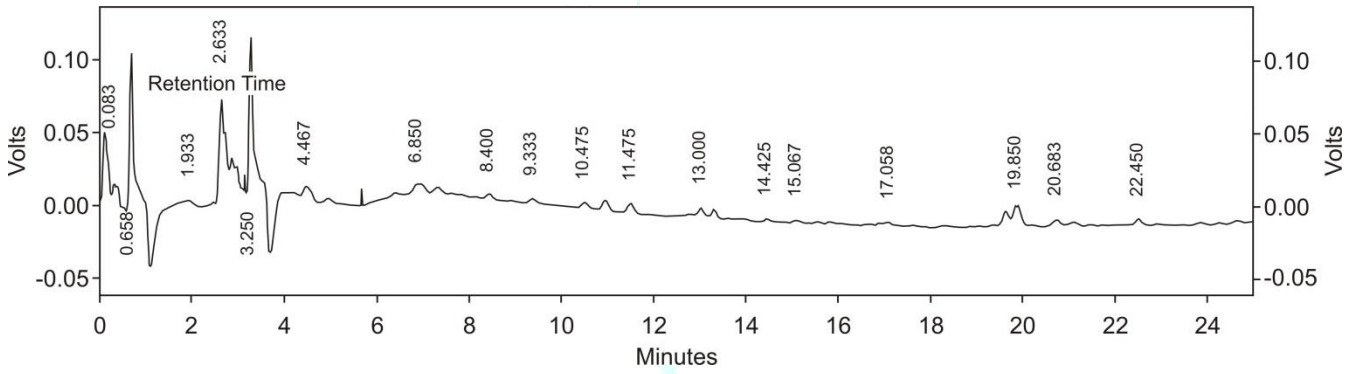


Fig. 1: Reverse Phase HPLC of Pooled Fractions (Peak II and Peak III) of Sephadex G-25 Chromatography Sample

Table 1: HPLC of Crude Donkey Milk Oligosaccharide Mixture

Detector A - 1 (220nm)

Pk #	Retention Time	Area	Area %	Height	Height %
1	0.083	133288	0.394	24837	3.158
2	0.658	1305836	3.857	117768	14.973
3	1.933	2364985	6.985	44050	5.600
4	2.633	3480989	10.281	111947	14.233
5	3.250	2151175	6.353	154406	19.631
6	4.467	4736840	13.990	50165	6.378
7	6.850	6513570	19.237	49136	6.247
8	8.400	1942181	5.736	40291	5.122
9	9.333	2192451	6.475	36058	4.584
10	10.475	1657578	4.895	31927	4.059
11	11.475	1397645	4.128	29962	3.809
12	13.000	2186863	6.459	24974	3.175
13	14.425	528146	1.560	15438	1.963
14	15.067	1055227	3.116	14087	1.791
15	17.058	731217	2.160	10137	1.289
16	19.850	961026	2.838	17807	2.264
17	20.683	239379	0.707	7335	0.933
18	22.450	281495	0.831	6227	0.792
Totals		33859891	100.000	786552	100.000

2.5. Acetylation of Oligosaccharide Mixture

4.49 gm of pooled fractions (peak II and III) which gave positive phenol-sulphuric acid test were acetylated with pyridine and acetic anhydride at 60^{0}C 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 (5.73 gm).

2.6. Purification of Acetylated Milk Oligosaccharide Mixture on Silica Gel Column

Separation of the acetylated products (5.73 gm) 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 Ariose (193 mg).

2.7. Deacetylation of Compound Ariose

Compound Ariose (38 mg) was dissolved in acetone (2 ml) and 3 ml of NH_3 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 5 ml) $CHCl_3$ (to remove acetamide) and the water layer was finally freeze dried giving the deacetylated oligosaccharide M (31 mg).

2.8. Methylglycosidation/Acid Hydrolysis of Compound Ariose

Compound Ariose (5 mg) was refluxed with absolute MeOH (2 ml) at 70^{0}C for 18 h 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 compound Ariose in 1,4-dioxane (1 ml), 0.1 N H_2SO_4 (1 ml) was added and the solution was warmed for 30 minutes at 50^{o}C. The hydrolysis was completed after 22 h. 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 and GalNAc. Their identification was confirmed by comparison with authentic samples (TLC, PC).

2.9. Kiliani Hydrolysis of Compound Ariose

Compound Ariose (5 mg) was dissolved in 2 ml Kiliani mixture (AcOH-H_2O-HCl, 7:11:2) and heated at 100^{0}C for 1 h followed by evaporation under reduced pressure. It was dissolved in 2 ml of H_2O and extracted twice with 3 ml $CHCl_3$. 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 and GalcNAc on comparison with authentic samples (TLC, PC).

2.10. Mannich Hydrolysis of Compound Ariose

To a solution of compound Ariose (5 mg) in acetone (2 ml), conc. HCl (0.05 ml) was added. The solution was kept under carbon dioxide in dark room at room temperature. After three days, the reaction mixture exhibited one new spot on TLC which was identical in mobility to GalNAc. After ten days, the reaction mixture exhibited one new spot on TLC which was identical in mobility to glucose. Hydrolysis was complete in 18 days showing only two spots on TLC which were found identical with glucose, GalNAc (PC, TLC). The hydrolysate was neutralized with freshly prepared Ag_2CO_3 and filtered. The filtrate was cooled in ice, H_2S was passed and the solution was again filtered (to remove Ag^+ ions as Ag_2S). The filtrate was concentrated under reduced pressure affording glucose and GalNAc identified by comparison with the authentic samples (TLC, PC).

2.11. Methylation/ Acid Hydrolysis of Compound Ariose

NaH (1 mg) was added to compound Ariose (5 mg) in THF (1ml). The mixture was stirred at room temperature for 1 h and then cooled to 0^{o}C. MeI (0.02 ml) was added and the reaction mixture was allowed to reach at room temperature over a period of 3 h. Excess NaH was destroyed by the addition of methanol, the solvents were evaporated and the residue was taken in chloroform. The chloroform solution was washed twice with aqueous NaCl and once with water, dried, filtered and then concentrated. To a solution of methylated compound Ariose in 1,4-dioxane (1 ml), 0.1 N H_2SO_4 (1 ml) was added and the solution was warmed for 30 minutes at 50^{o}C. The hydrolysis was complete after 22 h exhibiting eight spots on TLC. In the hydrolysate one of the compounds was identified as 6-O-methyl-2-deoxy-2-N-acetyl-glucopyranose on comparison with synthetically prepared authentic sample of 6-O-methyl-2-deoxy-2-N-acetyl-glucopyranose (TLC, PC).

2.12. Description of Isolated Compound Ariose

Elemental Analysis

For elemental analysis, this compound was dried over P_2O_5 at 100°C and 0.1 mm pressure for 8 hr. The molecular formula of compound was **$C_{36}H_{61}O_{26}N_3$**.

Elemental Analysis:

Calculated: % C 45.43, % H 6.41, % N 4.42

Found: % C 45.41, % H 6.40, % N 4.43

It gave positive Phenol-sulphuric acid test[13], Feigl test[14] and Morgon-Elson test[15].

^{1}H NMR of Ariose (Acetylaed) in $CDCl_3$

δ1.95 [s, 3H, $NHCOCH_3$], 2.03 [s, 3H, $NHCOCH_3$], 2.05 [s, 3H, $NHCOCH_3$], 3.32 [t, 1H, J=8.4 Hz, β-Glc(S_1), H-2], 3.87 [t, 2H, β-Glc(S_1,S_5), H-4], 3.82 [t, 3H, β-GalNAc (S_2, S_4, S_5), H-3], 4.49 [d, 3H, J=8.1 Hz, β-GalNAc (S_2, S_4, S_5), H-1], 5.67 [d, 2H, J=8.1 Hz, β-Glc (S_1, S_3), H-1], 6.25 [d, 1H, J=3.3 Hz, α-Glc (S_1), H-1]

^{1}H NMR of Compound Ariose in D_2O

δ1.95 [s, 3H, $NHCOCH_3$], 2.03 [s, 3H, $NHCOCH_3$], 2.05 [s, 3H, $NHCOCH_3$], 3.32 [t, 1H, J=8.4 Hz, β-Glc(S_1), H-2], 3.87 [t, 2H, J=9.0 Hz, β-Glc(S_5), H-4], 4.09 [t, 1H, J=5.1 Hz, β-GalNAc (S_5), H-4], 4.356 [d, 1H, J=8.7 Hz, β-GalNAc (S_5), H-1], 4.493 [d, 1H, J=7.8 Hz, β-GalNAc (S_4), H-1], 4.499 [d, 1H, J=7.5 Hz, β-GalNAc (S_2), H-1], 4.562 [d, 1H, J=7.5 Hz, β-Glc (S_3), H-1], 4.705 [d, 1H, J=8.1 Hz, β-Glc (S_1), H-1], 5.264 [d, 1H, J=3.0 Hz, α-Glc(S_1), H-1]

^{13}CNMR of Compound Ariose in D_2O

δ 23.28, 25.01, 56.64, 59.87, 60.10, 60.52, 61.03, 62.08, 69.25, 70.97, 71.07, 71.71, 72.54, 73.82, 74.37, 74.80, 74.98, 75.36, 76.24, 78.13, 78.35, 78.71, 91.82, 95.76, 95.90, 102.85,173.64, 175.98, 177.37

Mass Spectral Fragments of Compound Ariose

m/z 974, 952, 938, 915, 891, 873, 866, 855, 849, 837, 835, 819, 815, 807, 797, 795, 757, 753, 717, 681, 675, 659, 646, 617, 615, 748, 485, 467, 449, 383, 365, 307, 289, 180

3. RESULT AND DISCUSSION

3.1. Stability of Molecular Geometry of Isolated Compound Ariose

As we know that molecular geometry for determining the structure-activity relationship, conformational analysis plays a very important role. The geometry of compound Ariose has been optimized at B3LYP method and 6-31 G (d, p) basis set employing Density Functional Theory (DFT). The molecular geometry can be determined by the quantum mechanical behaviour of the electrons and computed by *ab-initio* quantum chemistry methods to high accuracy. Molecular geometry represents the three-dimensional arrangement of the atoms that determines several properties of a substance including its reactivity, polarity, phase of matter, colour, magnetism and biological activity. The optimized geometry of compound shows positive wave-number values indicated the stability of the compound Ariose. The optimized molecular geometry of compound Ariose is given below:

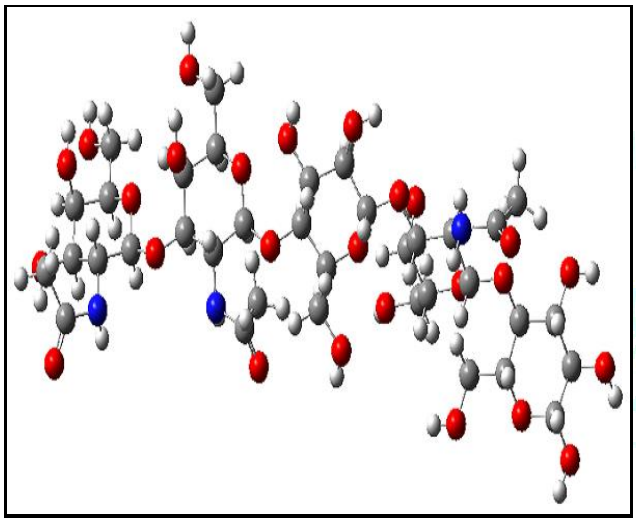


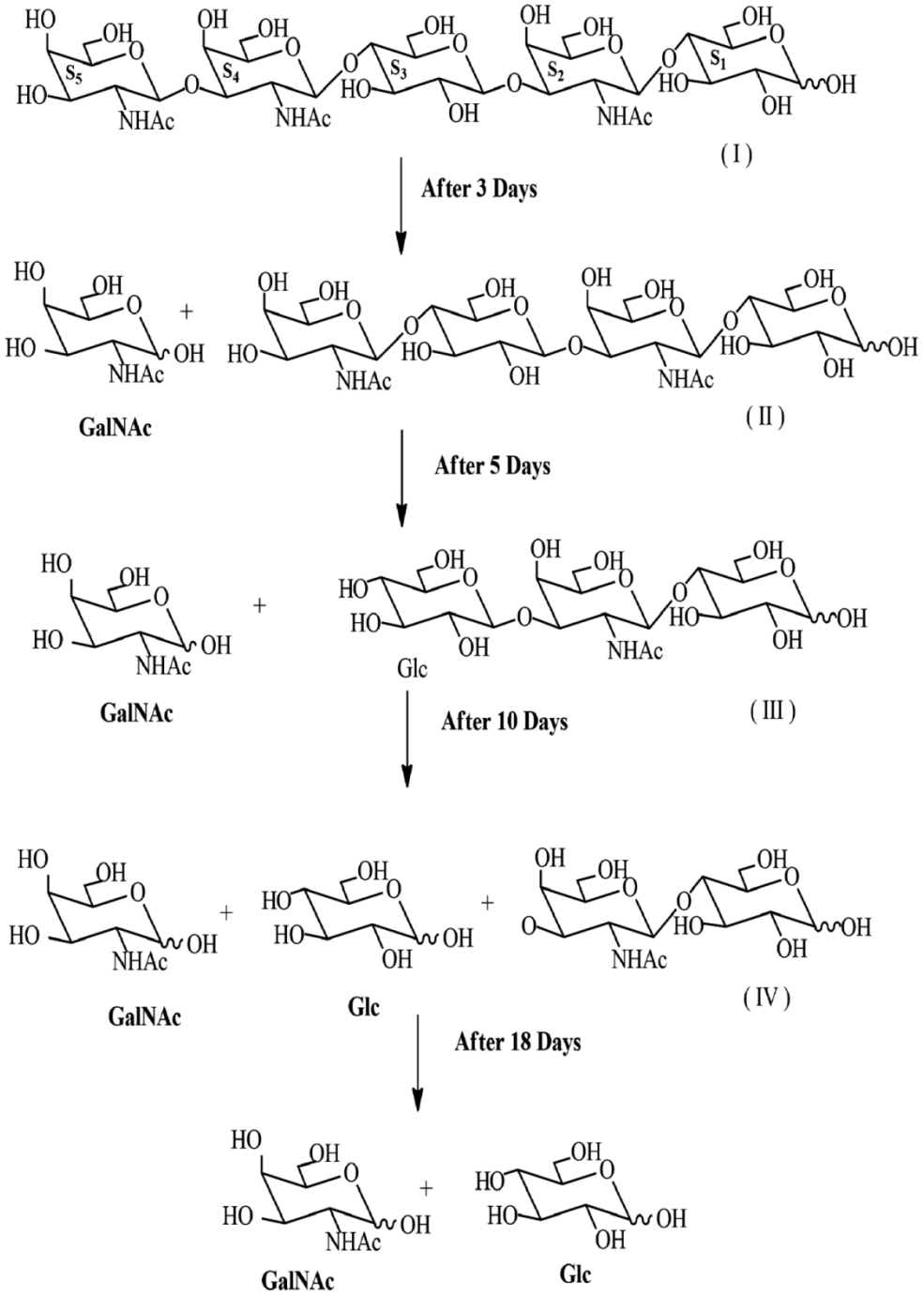
Fig: Optimized Geometry of Compound Ariose

3.2. Structure Elucidation of Isolated Donkey Milk Oligosaccharide

3.2.1. NMR Spectroscopy Studies

The isolated compound has been identified and its structure was elucidated with the help of ^{1}H, ^{13}C NMR and 2D-NMR, mass spectrometry, chemical degradation and chemical transformation. In the present study, analogies between chemical shift of certain 'structural reporter group resonances' were used to make proton resonance assignments as well as structural assignments of the oligosaccharide.

Compound Ariose, $[\alpha]_D + 31^0$ (c, 0.42, H_2O), $C_{36}H_{61}O_{26}N_3$ gave positive Phenol-sulphuric acid test[13], Feigl test[14] and Morgon-Elson test[15] indicating the presence of normal and amino sugar(s) in the moiety. The ^{1}H NMR spectrum of Ariose at 300 MHz exhibited six signals in the anomeric proton region as doublets at δ 5.264 (1H), 4.705 (1H), 4.562 (1H), 4.499 (1H), 4.493 (1H) and 4.356 (1H) for six protons indicating that it is a pentasaccharide in its reducing form. It was further supported by the appearance of four signals for six anomeric carbons at δ 102.855 (3C), 95.925 (1C), 95.76 (1C), 91.825 (1C) in the ^{13}C NMR spectrum of Ariose. The ES mass spectrum of Ariose showed the highest mass ion peak at m/z 974 $[M+Na]^+$ and m/z 952 $[M+H]^+$, which was in agreement of derived molecular formula $C_{36}H_{61}O_{26}N_3$ with the molecular ion at m/z 951 for a pentasaccharide. The reducing nature of compound Ariose was confirmed by methylglycosidation by $MeOH/H^+$ followed by its acid hydrolysis which led to the isolation of α and β-methyl glucoside confirming the presence of glucose at the reducing end in the oligosaccharide. The five monosaccharide units present in Ariose have been designated as S_1, S_2, S_3, S_4, and S_5 for convenience starting from the reducing end. To confirm the monosaccharide constituents and their sequence in Ariose, it was hydrolyzed under strong acidic conditions (Kiliani hydrolysis) and mild acidic conditions (Mannich-Siewert method) respectively. In Kiliani hydrolysis the reducing pentasaccharide gives only two spots of Glc and GalNAc, which were identified by the comparison with authentic samples of Glc and GalNAc suggesting that the pentasaccharide is comprised of two monosaccharide units i.e. Glc and GalNAc. Hydrolysis of compound Ariose under mild acidic conditions gave three spots after three days two of which was identical in mobility to GalNAc and un-reacted pentasaccharide (I) while the other may be tetrasaccharide (II) showing that the GalNAc (S_5) was the terminal monosaccharide in the pentasaccharide. After 5 days the aliquot showed four spots which were identified as unreacted pentasaccharide (I), the earlier spots of tetrasaccharide (II), GalNAc and another spot of intermediate mobility between tetrasaccharide and GalNAc which could be the trisaccharide (III) suggesting that the last two monosaccharide present in pentasaccharide were GalNac (S_5 and S_4). Further after ten days the reaction mixture showed two new spots which were identical as Gal (PC) and the disaccharide (III). After fourteen days the partially completed hydrolysis showed six spots which were comparable in mobility with un-reacted pentasaccharide (I), tetrasaccharide (II), trisaccharide (III), disaccharide (IV), GalNAc and Glucose. The reaction was completed in eighteen days showing two spots which were identified as GalNAc and Glc. The results obtained from acid hydrolysis confirmed that the pentasaccharide was made up of GalNAc and Glc in the sequence GalNAc-GalNAc-Glc-GalNAc-Glc.



Mannich Hydrolysis of Ariose under Mild Acidic Conditions

The chemical shifts of anomeric carbons observed in ^{13}C NMR spectrum and of anomeric protons observed in ^{1}H NMR spectrum of Ariose are also in agreement with the reported values of ^{1}H and ^{13}C anomeric chemical shifts of Glc and GalNAc. The presence of only two monosaccharide units i.e. Glc and GalNAc in compound Ariose and its molecular formula $C_{36}H_{61}O_{26}N_3$ suggested that there may be two Glc and three GalNAc units in Ariose. The presence of glucose at the reducing end was further supported by presence of two anomeric proton signals as doublets at δ5.264 (1H) and δ4.7052 (1H) and their coupling constants as 3.0 Hz and 8.1 Hz, for α and β Glc respectively. Further, the ^{1}H NMR spectrum of Ariose which showed another anomeric proton appearing as a doublet at δ4.499 (1H, J = 7.5 Hz) along with a singlet of three protons of NHAc at δ2.0366 for GalNAc, which was present as second monosaccharide (S_2) in Ariose and was linked to H-4 of the reducing glucose unit which gave a triplet at δ3.872 (J = 9.0 Hz) for H-4 of glucose (S_1) as compared to the ^{1}H NMR spectrum of acetate of ariose showing no downfield shifting in the chemical shift of H-4 of Glc (S_1). Thus the second monosaccharide in the pentasaccharide sequence was GalNAc which resembles with the pattern of lactose (Galβ 1→4 Glc) with an extra signal for NHAc of GalNAc. This pattern was also confirmed by the characteristic signal of H-2 of β-Glc which appeared as a triplet at δ3.32 (J = 8.4 Hz). The third anomeric proton which appeared at δ4.562 (J = 7.5 Hz) was due to presence of another β-Glc present in reducing pentasaccharide. The downfield shifted signal of H-4 of β-GalNAc (S_2) at δ4.10 (J = 5.1 Hz) suggested that the equatorially oriented hydroxyl group of C-3 of β-GalNAc (S_2) was substituted and it was involved in glycosidation with β-Glc (S_3). The fourth sugar in the pentasaccharide was identified as β-GalNAc which was supported by the presence of an anomeric proton doublet at δ4.493 (J = 7.8 Hz) and its linkage to S_3 i.e. β-Glc by a (1→4) linkage was

confirmed by the presence of another H-4 methine proton triplet of β-Glc (S_3) at δ3.872 (J = 9.0 Hz) in ^{1}H NMR spectrum of acetate of Ariose. The NHAc group of second GalNAc (S_4) appeared at δ1.95 as a singlet. These observations confirmed that the ariose was made up of β-GalNAc (1→4) Glc repeating units. The tetrasaccharide unit comprising of GalNAc-β-(1→4)-Glc-β-(1→3)-GalNAc-β-(1→4)Glc was reported earlier in our laboratory and all the anomeric and ring protons assignment were comparable. The ^{1}H NMR spectrum of Ariose gave an anomeric proton doublet at δ4.356 (J=8.7 Hz) which was identified as β-GalNAc (S_5). The downfield shifted signal of H-4 of β-GalNAc (S_4) at δ4.10 (J = 5.1 Hz) suggested that the equatorially oriented hydroxyl group of C-3 of β-GalNAc (S_4) was substituted and it was involved in glycosidation with β-GalNAc (S_5). The NHAc group of third GalNAc (S_5) appeared at δ2.05 as a singlet. All the ^{1}H NMR assignments for ring protons of monosaccharide units of Ariose were confirmed by HOMOCOSY and TOCSY experiments. The chemical shifts of the anomeric carbons of Ariose at δ91.825 (1C, α-Glc, S_1), 95.76 (1C, β-Glc, S_1), 95.92 (1C, β-Glc, S_3) and δ102.9 (3C, 3 β-GalNAc, S_2, S_3 and S_5) present in the ^{13}C NMR spectrum are in accordance with the anomeric carbon values of Glc and GalNAc.

Table: ^{13}C NMR Values of Compound Ariose

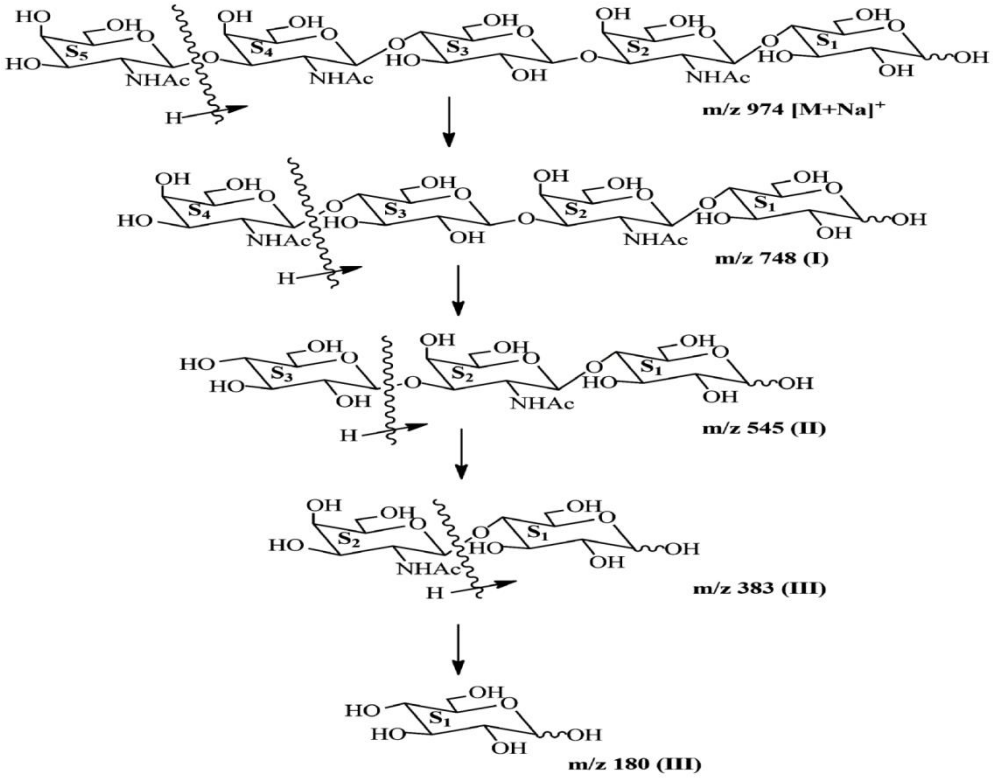
Moieties	C-1	C-2	C-3	C-4	C-5	C-6	-CO	-CH_3
α-Glc(S_1)	91.82	70.85	72.51	75.68	70.63	61.39		
β-Glc(S_1)	95.76	76.57	76.90	75.52	77.19	61.66		
β-GalNAc (S_2)	102.85	60.74	73.39	70.63	77.42	60.77	170.20	29.55
β-Glc(S_3)	95.90	76.57	76.90	75.52	77.19	61.66		
β-GalNAc (S_4)	102.85	60.74	73.39	70.63	77.42	60.77	170.20	29.55
β-GalNAc (S_5)	102.85	60.74	72.51	70.85	77.42	60.77	170.02	29.55

The Heteronuclear Single Quantum Coherence (HSQC) spectrum of acetate of compound Ariose confirmed anomeric assignments in ^{1}H and ^{13}C NMR spectra by showing the ^{1}H and ^{13}C cross peaks of α-Glc (δ6.25 × δ 88.8) and β-Glc (δ5.67 × δ 91.43). It also contains other cross peak of three α-GalNAc at δ4.49 x δ100.81 (2C) and δ4.49 x δ101.07 (1C). The cross peaks of carbon atoms involved in glycosidation were also present in HSQC spectrum at δ3.80 x 75.52 (Glc (S_1 and S_3) C_4 x H_4 showing 1→4 linkage), and δ3.78 x δ 73.39 (GalNAc (S_2, S_4, and S_5) C_3 x H_3 showing 1→3 linkage). The values of chemical shifts of ring carbons of tetrasaccharide also support the derived structure (Table 1). Based on the pattern of chemical shift of ^{1}H, ^{13}C, HOMOCOSY, TOCSY and HSQC NMR experiments, it was interpreted that the compound Ariose was a pentasaccharide having two Glc and three GalNAc moieties having the following structure:

GalNAc-β-(1→3)-GalNAc-β-(1→ 4)-Glc-β-(1→ 3)-GalNAc-β-(1→4) Glc

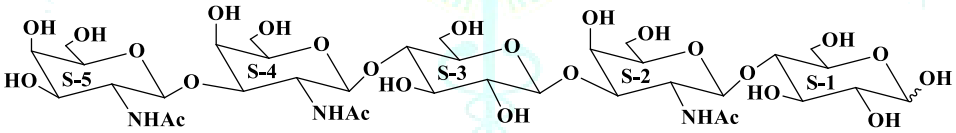
3.2.2. Mass Spectrometry Studies

The derived structure of Ariose as GalNAc-β-(1→3)-GalNAc-β-(1→ 4)-Glc-β-(1→ 3)-GalNAc-β-(1→4) Glc was further supported by its ES-Mass spectrum. The highest mass ion peaks were recorded at m/z 974 and 952 which were due to $[M+Na]^+$ and $[M+H]^+$ respectively, confirming the molecular weight of Ariose as 951. The fragment ion at m/z 952 further fragmented to give mass ion fragment at m/z 748, which was assigned to the tetrasaccharide unit (I). This fragmentation corresponded to the loss of terminal GalNAc moiety from pentasaccharide [M-S_5], indicating the presence of GalNAc (S_5) at the non-reducing end. The Electron Spray mass spectrum of Ariose also contained other mass ion peaks [974-2H_2O], 915[M-2H_2O], 891[M-CH_2OHCHO], 873[915-CH_2=C=O], 866[974-2NHCOCH_3], 855[891-2H_2O], 849[891-CH_2=C=O], 837 [873-2H_2O], 835[M-2NHCOCH_3], 819[855-2H_2O], 815[837-H_2O], 807[849-CH_2=C=O], 797[855-NHCOCH_3], 795[855-CH_2OHCHO], 757[873-2NHCOCH_3], 753[795-CH_2=C=O], 748[797-CH_2OH, H_2O], which were obtained from the molecular ion. The mass ion fragment at m/z 748 was also supported by its respective fragments at m/z 717[748-CH_2OH], 681[717-2H_2O], 675[717-CH_2=C=O], 659[717-NHCOCH_3], 646[681-H_2O-OH], 617[659-CH_2=C=O], 615[646-CH_2OH] or 615[675-CH_2OHCHO]. The mass ion fragment at m/z 748 (I) further fragmented to give mass ion peak at m/z 545 (II) assigned to the loss of GalNAc from fragment I was not detected in the mass spectrum but the other supporting peaks due to this trisaccharide fragment (II) at m/z 485 (545-CH_2OH CHO), 467 (485-H_2O), and 449 (485-2H_2O) were present in the spectrum confirming that the GalNAc was the second sugar in sequence from nonreducing end. On further fragmentation, the trisaccharide fragment (II) at m/z 545gave a disaccharide fragment (III) at m/z 383 attributed to the loss of Glc (S_3), confirming the presence of glucose as a third sugar in sequence from non-reducing end. The mass ion fragment at m/z 383 was also supported by its respective fragments at m/z 365 (383-H_2O) 307 (365-NHCOCH_3), and 289 (307-H_2O) confirming that the Glc was the third sugar in sequence from non-reducing end. On further fragmentation, the disaccharide fragment (III) at m/z 383 gave a peak at m/z 180 attributed to the loss of GalNAc (S_2), confirming the presence of GalNAc as a fourth sugar in sequence from non-reducing end. These mass ion fragments not only confirmed the derived structure but also fixed the sequence of the sugars **GalNAc—GalNAc—Glc—GalNAc—Glc.**



SCHEME : MASS FRAGMENTATION OF COMPOUND ARIOSE

Based on the results obtained from chemical degradation/acid hydrolysis, chemical transformation, Electro spray mass spectrometry and 1D-NMR viz. ^{1}H NMR, ^{13}C NMR and 2D-NMR viz. COSY, TOCSY and HSQC NMR spectra of Ariose acetate and Ariose, the structure and sequence of isolated novel hexasaccharide Ariose was determined as:



4. CONCLUSION

From the above informations, we conclude the structure of isolated Donkey milk oligosaccharide, **Ariose**. This 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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6. REFERENCES

1. 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.
2. 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.
3. Daniela B. and Angela M.Z. Oligosaccharides for improving Human Health. American society for Nutrition. *Adv. Nutr.* 2011; 2: 284-289.
4. 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.
5. 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.
6. 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.
7. Ehresmann D.W., Dieg E.F. and Hatch M.T. Antiviral Properties of Algal Polysaccharide and Related Compound. *Marine in Algae Pharmaceutical Science*. 1979; 58: 293-302.
8. Singh P., Gupta N.K. and Desh Deepak. NMR and Mass Characterization of Novel Oligosaccharide (Gariose) from Donkey Milk. *Universal Review*. 2019; VIII(V): 361-374.
9. 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.
10. 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.
11. Frisch M. J. *Gaussian 09*, Gaussian, Inc., Pittsburgh. (2009).
12. Kobata A. and Ginsburg V. An Enzymatic basis for Lewis Blood Types in Man. *J. Biol. Chem.* 1970; 245: 1484.
13. 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.
14. Fiegl F. Spot test in organic analysis. Elsevier Publication, Amsterdam, 1975; pp. 337.
15. Partridge S.M. and Westall R.G. Filter-paper Partition Chromatography of Sugars (I), General Description and Application to the Qualitative Analysis of Sugars in Apple Juice, Egg White and Fetal Blood of Sheep. *J. Biochem.* 1948; 42: 238-250.