Indian Journal of Novel Drug Delivery

IJNDD

An Official Publication of Karnataka Education and Scientific Society

Research Article

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

PUSHPRAJ SINGH[1]*, NITIN KUMAR GUPTA[2], DESH DEEPAK[2]

[1] Department of Chemistry, Govt. Girls Degree College, Chhibramau, Kannauj-209721, U.P., India

[2] Department of Chemistry, University of Lucknow, Lucknow-226007

ARTICLE DETAILS

Article history:
Received on 23 March 2020
Modified on 7 September 2020
Accepted on 21 September 2020

Keywords:
Donkey Milk,
Oligosaccharides,
Quinose.

ABSTRACT

Oligosaccharides have been found effective in gastrointestinal normal flora proliferation and pathogen suppression, dental caries prevention, enhancement of immunity, facilitation of mineral absorption, source of antioxidant, antibiotic alternative, regulators of blood glucose in diabetics and serum lipids in hyperlipidemics. The enormous biological activities of oligosaccharides such as immunostimulant, anti-tumour, anti-cancer, anti-inflammatory, anti-complementary, anti-viral, anti-microbial, anti-oxidant, hypoglycemic activity, lipid lowering and regulation of mineral absorption are well reported. In our endeavor to find biologically active novel oligosaccharides, donkey milk was taken, which is a rich source of oligosaccharides and its milk is used as anti-hypertensive, anti-oxidative 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 Quinose. 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 in Fig. 4.

INTRODUCTION

As per literature, most of the oligosaccharides are very active against several microorganisms [1] and such compounds have been dragging the eyes of chemist from a long time. Many oligosaccharides have been reported to exhibit an outstanding range of biological activities [2]. Oligosaccharides are a class of bioactive macromolecule found in mammalian milk that are receiving a lot of commercial attention. These complex carbohydrates (oligosaccharides) are known to be responsible for the beneficial effects of breast fed 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

Author for Correspondence:
Email: pushpraj1509@gmail.com

structure of one novel donkey milk oligosaccharide (Quinose) 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).

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].

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 $CDCI_3$ 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 5µl 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.

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 µm) (to remove remaining lactose) and lyophilized affording crude oligosaccharide mixture (178gm). 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 eluent at a flow rate of 3 mL/min. Each fraction was analyzed for sugars by phenol-sulphuric acid reagent for presence of neutral sugars.

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.

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}), Shown in Table 1, Fig. 2.

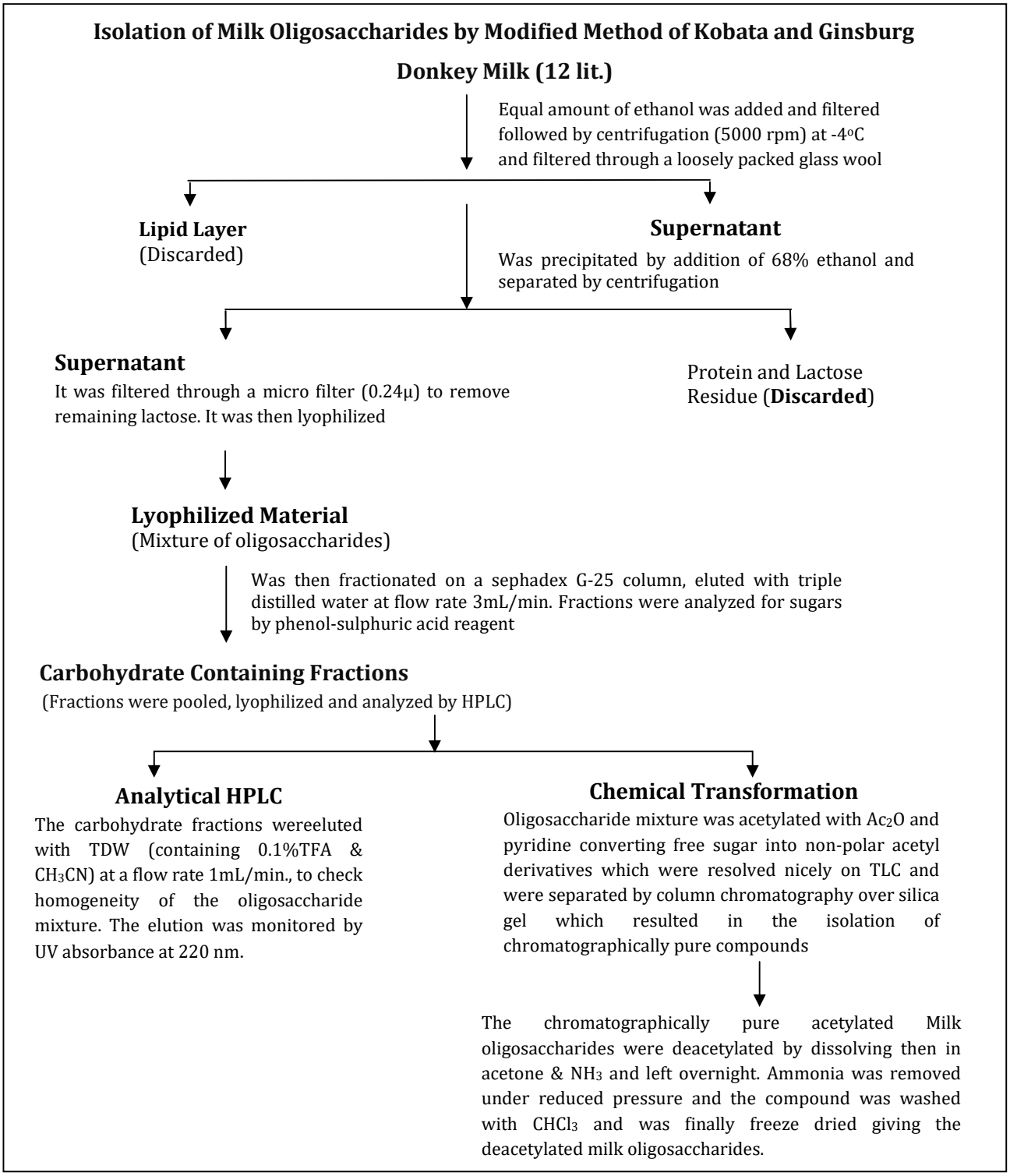


Figure 1: Isolation of Milk Oligosaccharides by Modified Method of Kobata and Ginsburg

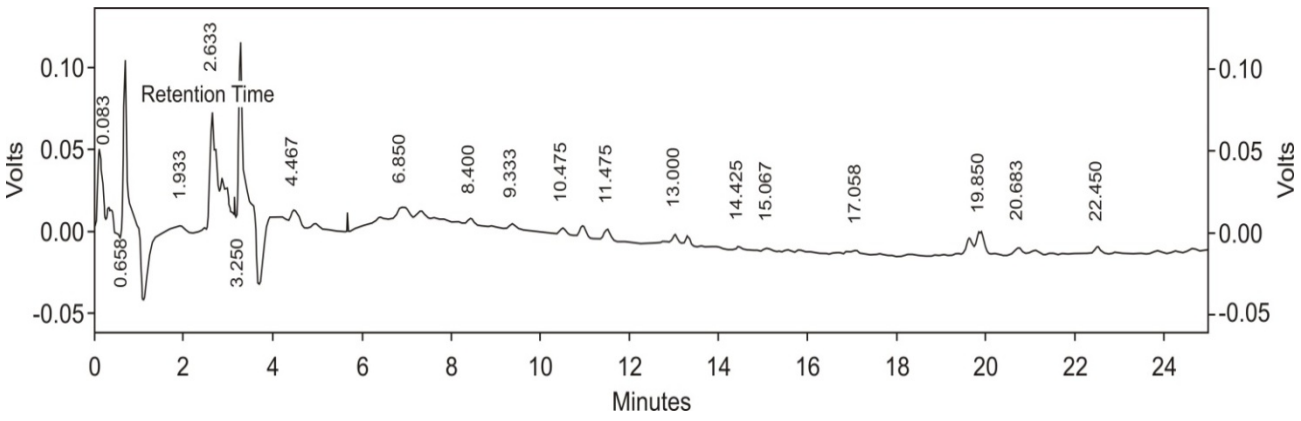


Figure 2: Reverse Phase HPLC of Pooled Fractions (Peak II and Peak III) of Sephadex G-25 Chromatography Sample.

Table 1: HPLC of Crude Donkey Milk Oligosaccharide Mixture

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

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°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).

Purification of Acetylated Milk Oligosaccharide Mixture on Silica Gel Column

Separation of the acetylated products (5.73gm) 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 Quinose (173 mg).

Deacetylation of Compound Quinose

Compound Quinose (32 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 (22 mg).

Methyl Glycosidation/Acid Hydrolysis of Compound Quinose

Compound Quinose (5 mg) was refluxed with absolute MeOH (2 mL) at 70^0C 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 methyl glycoside of compound Quinosein 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°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 Gal, GlcNAc, and Fuc. Their identification was confirmed by comparison with authentic samples (TLC, PC).

Kiliani Hydrolysis of Compound Quinose

Compound Quinose (5 mg) was dissolved in 2 mL Kiliani mixture (AcOH-H_2O-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 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, galactose and GlcNAc on comparison with authentic samples (TLC, PC).

Mannich Hydrolysis of Compound Quinose

To a solution of compound Quinose (5 mg) in acetone (2 mL), conc. HCl (0.02 mL) was added. The solution was kept under carbon dioxide in dark room at room temperature. After three days, the reaction mixture exhibited a new spot on TLC, which was identical with fucose. After seven days, one more spot became visible on TLC which was identical with GlcNAc. After ten days, one more spot became visible on TLC which was identical with Gal. After twenty days, one more spot became visible on TLC which was identical with Glc. Hydrolysis was completed in 27 days showing four spots on TLC which were found identical by comparison with the authentic samples with glucose, galactose, GlcNAc, and fucose (PC, TLC).

Methylation/ Acid Hydrolysis of Compound Quinose

NaH (1 mg) was added to compound Quinose (5 mg) in THF (1mL). The mixture was stirred at room temperature for 1 h and then cooled to 0°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 Quinose 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^oC. 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).

Description of Isolated Compound Quinose

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 $\mathbf{C_{46}H_{78}O_{34}N_2}$.

Elemental Analysis:

Calculated: % C 45.92, % H 6.49, % N 2.33

Found: % C 45.90, % H 6.50, % N 2.32

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

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

δ 1.21 (d, J= 6.6 Hz, 6H, α-fuc (S_2 and S_3) CH_3), 2.05 (s, 3H, $NHCOCH_3$), 2.11 (s, 3H, $NHCOCH_3$), 3.85 (t, 3H, β-Glc (S_1), H-3, β-Gal (S_3, S_5), H-3), 5.0 (t, 1H, β-Glc(S_1), H-2), 4.006 (t, 1H, β-GalNAc(S_4 and S_6), H-3), 6.24 (d, 1H, J= 3.6 Hz, α-Glc (S_1) H-1), 5.68(d, 1H, J= 8.1 Hz, β-Glc (S_1) H-1), 5.46 (d, 2H, J= 3.3 Hz, α-Fuc (S_2 and S_7) H-1), 4.61(d, 2H, J= 8.7 Hz, β-GlcNAc (S_4 and S_6) H-1), 4.46 (d, 1H, J= 7.8 Hz, β-Gal (S_2) H-1), 4.44 (d, 1H, J= 7.8 Hz, β-Gal (S_5) H-1)

^{1}H NMR of Compound Quinose in D_2O

δ 1.25 (d, 6H, J= 6.9 Hz, α-fuc (S_2 and S_7) CH_3), 2.05 (s, 3H, $NHCOCH_3$), 2.11 (s, 3H, $NHCOCH_3$), 3.44 (t, 1H, J=7.5 Hz, β-Glc (S_1), H-3), 4.01 (t, 1H, J = 7.5 Hz, β-Glc (S_1), H-2), 4.006 (t, 2H, J=5.7 Hz, β-GlcNAc (S_4, S_6), H-3), 4.11 (d, 2H, J = 2.4 Hz,

β-Gal(S_3, S_5), H-2), 4.25 (d, 2H, J = 2.4 Hz, β-GalNAc(S_4 and S_6), H-4), 4.313 (d, 1H, J= 7.8 Hz, β-Glc (S_1) H-1), 4.379(d, 2H, J= 7.8 Hz, β-Gal (S_2 and S_5) H-1), 4.49 (d, 2H, J= 8.1 Hz, β-GlcNAc (S_4 and S_6) H-1), 5.15(d, 2H, J= 3.9 Hz, α-Fuc (S_2 and S_7) H-1), 5.303(d, 1H, J= 3.9 Hz, α-Glc (S_1) H-1)

^{13}CNMR of Compound Quinose in D_2O

δ 15.0, 20.7, 20.9, 61.4, 61.6, 61.7, 61.9, 62.3, 68.5, 68.8, 69.0, 69.2, 70.2, 70.6, 70.8, 70.9, 71.1, 71.3, 71.8, 72.0, 72.6, 72.7, 73.1, 75.5, 76.6, 77.0, 77.4, 90.6, 91.3, 91.9, 95.4, 95.9, 100.7,100.9, 170.9, 171.0

Mass Spectral Fragments of Compound Quinose

m/z 1241, 1225, 1202, 1184, 1144, 1124, 1088, 1084, 1066, 1038, 1030, 1020, 995, 989, 972, 980,962, 941, 902, 873, 844, 835, 815, 808, 804, 800, 786, 777, 773, 740, 728, 698, 668, 667, 626, 545, 510, 503, 452, 434, 392, 387, 351, 342, 332, 324, 288, 284, 280, 180.

RESULT AND DISCUSSION

Stability of Molecular Geometry of Isolated Compound Quinose

As we know that molecular geometry for determining the structure-activity relationship, conformational analysis plays a very important role. The geometry of compound Quinose, 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.

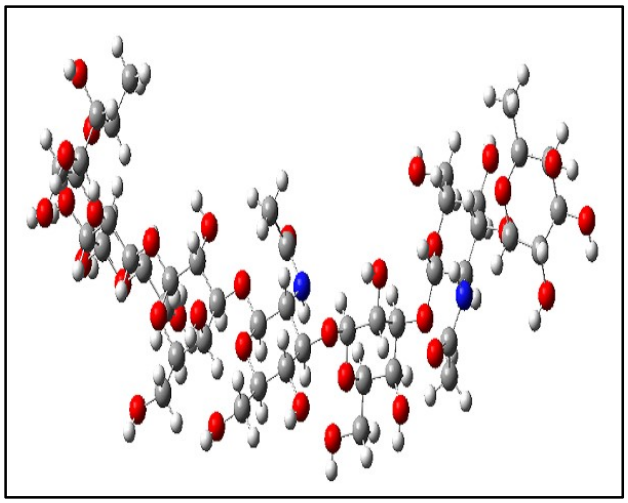


Figure 3: Optimized Geometry of Compound Quinose

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 Quinose. The optimized molecular geometry of compound Quinose is given in Fig. 3.

Structure Elucidation of Isolated Donkey Milk Oligosaccharide

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.

The compound Quinose, $[\alpha]_D$ + 42.7°(c, 0.11, H_2O), $C_{46}H_{78}O_{34}N_2$, gave positive Phenol-sulphuric acid test [13], Feigl test [14] and Morgon-Elson test [15] showing the presence of normal and amino sugar(s) in the moiety. The ^{1}H NMR spectrum of Quinose, at 300 MHz exhibited five signals in the anomeric proton region as doublets at δ 4.313 (1H), 4.379 (2H), 4.49 (2H), 5.15 (2H), 5.303(1H) for eight anomeric protons. These eight anomeric protons could be interpreted for presence of a heptasaccharide in its reducing form, giving signals for α and β anomers of glucose at its reducing end. The heptasaccharide nature of Quinose was further supported by seven anomeric carbon signals for eight anomeric carbon at δ 90.6 (2C), 91.3 (1C), 91.9 (1C), 95.4 (1C), 95.9 (1C), 100.7 (1C), 100.9 (1C) in the ^{13}C NMR spectrum of acetylated product of Quinose. The ES mass spectrum of Quinose showed the highest mass ion peak at m/z 1241 assigned to $[M+K]^+$ and m/z 1225 due to $[M+Na]^+$, which was in agreement of derived composition $C_{46}H_{78}O_{34}N_2$ with the molecular ion expected at m/z 1202. The reducing nature of compound Quinose was confirmed by methyl glycosidation by $MeOH/H^+$ followed by its acid hydrolysis which led to the isolation of α and β-methyl glucoside leading to the presence of glucose at the reducing end in the oligosaccharide. The seven monosaccharides present in Quinose have been designated as S_1, S_2, S_3, S_4, S_5, S_6 and S_7 for convenience from the reducing end. To confirm the monosaccharide

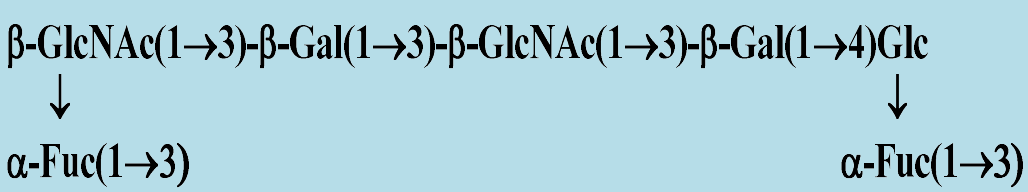
constituents in Quinose, it was hydrolyzed under strong acidic conditions (Kiliani hydrolysis). In Kiliani hydrolysis the reducing heptasaccharide gave four spots on paper chromatography, which were later identified as Glc, GlcNAc, Fuc and Gal by co-chromatography with authentic samples (paper chromatography) suggesting that the reducing heptasaccharide is made up of these monosaccharide units. The chemical shifts of anomeric carbons observed in ^{13}C NMR spectrum and of anomeric protons observed in ^{1}H NMR spectrum of Quinose are also in agreement with the reported values of ^{1}H and ^{13}C anomeric chemical shifts of Glc, Gal, Fuc and GlcNAc. The presence of a lactosyl moiety i.e. Gal β(1→4) Glc in compound Quinose was confirmed by two doublets of anomeric protons present at δ 4.379 (2H) J = 7.8 Hz and δ 4.313 (1H) J = 7.8 Hz for β-Gal (S_3) and β-Glc (S_1) residues respectively present in the lactosyl moiety. The reducing and free anomeric nature of S_1 (Glc) was supported by the doublet of α-Glc at δ 5.303 (1H) J = 3.9 Hz. The down field shifted triplet of H-2 of β-Glc as a triplet at δ 4.01 (J= 7.5 Hz) in comparison to the H-4 of Glc as observed at 3.18-3.31 and H-3 at δ 3.44 (J= 7.5 Hz) indicated that both the equatorially oriented hydroxyl groups at C-3 and C-4 of the reducing β-Glc (S_1) were substituted and probably involved in glycosidation. Since galactose of lactose is attached to C-4 of glucose (S_1), hence C-3 should be attached to the third monosaccharide moiety. The third monosaccharide unit present in Quinose was identified as α-Fuc (S_2) by a doublet for anomeric proton at δ 5.15 (J = 3.9 Hz) complemented by a doublet of secondary methyl protons at δ 1.25 (J = 6.9 Hz) and was probably attached at C-3 of S_1 by a (1→3) linkage between S_1 and fucose (henceforth assumed to be S_2). This was further confirmed on the basis of ^{1}H NMR spectrum of acetylated product of Quinose, which did not show the downfield shifting of H-3 methine proton of Glc (S_1). The fourth anomeric proton which appeared as a doublet at δ 4.49 (2H, J= 8.1 Hz) was due to the presence of β-GlcNAc in quinose, which was further supported by a singlet of amide methyl of N-acetyl glucosamine at δ 2.05. The glycosidic linkage β-GlcNAc (S_4) (1→3) β-Gal (S_3) was indicated by the downfield chemical shift of H-4 methine proton (structural reporter group) resonance of β-Gal (S_3) at δ 4.11 (J= 2.4 Hz). Another anomeric proton appearing as a doublet at δ 4.379 (2H) with a coupling constant of 7.8 Hz was assigned for β-Gal (S_5). The downfield signal of H-4 methine proton (structural reporter group) as a doublet at δ 4.25 and upfield shifted signal of H-3 of β-GlcNAc at δ 4.006 (J = 5.7 Hz) suggested that the equatorially oriented hydroxyl group at C-3 of β-GlcNAc (S_4) was substituted and it was involved in glycosidation with H-1 of β-Gal (S_5). Another anomeric proton which appeared as a doublet at δ 4.49 (2H, J= 8.1 Hz) was assigned to the presence of another β-GlcNAc (S_6) in quinose, which was supported by a singlet of amide methyl of N-acetyl glucosamine at δ 2.11. The glycosidic linkage β-GlcNAc (S_6) (1→3) β-Gal (S_5) was assigned on the basis of the downfield chemical shift of H-4 methine proton (structural reporter group) resonance of β-Gal (S_5) at δ 4.11 (J= 2.4 Hz). The seventh sugar in the heptasaccharide was identified as α-Fuc (S_7) by the presence of a doublet for anomeric proton at δ 5.151 (J = 3.9 Hz) supported by a doublet of secondary methyl protons at δ 1.25 (J = 6.9 Hz). The downfield signal of H-4 methine proton (structural reporter group) as a doublet at δ 4.25 and upfield shifted signal of H-3 of β-GlcNAc at δ 4.006 (J = 5.7 Hz) suggested that the equatorially oriented hydroxyl group at C-3 of β-GlcNAc (S_6) was substituted and it was involved in glycosidation with H-1 of α-Fuc (S_7). All the ^{1}H NMR assignments for ring protons of monosaccharide units of Quinose were confirmed by HOMOCOSY and TOCSY experiments. The chemical shifts of the anomeric carbons of compound M at δ 90.6 (2C, 2 x α-fuc), 91.3 (1C, α-Glc), 91.9 (1C, β-Glc), 95.4 (1C, β-GalNAc), δ 95.9(1C, β-GalNAc), δ 100.7 (1C, β-Gal) and δ 100.9 (1C, β-Gal) present in the ^{13}C NMR spectrum were in accordance with the reported anomeric carbon chemical shifts of Glc, GlcNAc, Gal and Fuc. The values of chemical shifts of ring carbons of hexasaccharide also supported the derived structure.

The heteronuclear single quantum-coherence (HSQC) spectrum of acetate of Quinose confirmed anomeric assignments in ^{1}H and ^{13}C NMR spectra by showing the ^{1}H and ^{13}C cross peaks of α-Glc (δ 6.243 ×δ 91.3) and β-Glc (δ 5.688 ×δ 91.9). It also exhibited other cross peaks of two α-Fuc (S_2 and S_7) at δ 5.461 x δ 90.6, two β-GlcNAc (S_4 and S_6) moieties at δ 4.613 ×δ 95.4 and δ 4.613 ×δ 95.9 and two β-Gal (S_3 and S_5) units at δ 4.462 x δ 100.7 and δ 4.441 x δ 100.9.

Table 2: ^{13}C NMR Values of Compound Quinose

Moieties	C-1	C-2	C-3	C-4	C-5	C-6	-CO	-CH_3
α-Glc(S_1)	91.3	75.52	70.9	70.4	71.8	62.3		
β-Glc(S_1)	91.9	72.52	69.2	70.2	75.1	61.9		
α-Fuc (S_2)	90.6	71.1	72.6	70.9	71.3	15.0		
β-Gal (S_3)	100.7	73.1	72.0	71.1	68.5	61.9		
β-GlcNAc (S_4)	95.4	61.4	76.6	77.4	77.0	61.9	170.9	20.7
β-Gal (S_5)	100.9	73.1	72.0	72.7	68.8	61.7		
β-GlcNAc (S_6)	95.9	61.6	76.6	77.4	77.0	61.9	171.0	20.9
α-Fuc (S_7)	90.6	71.1	72.6	70.9	71.3	15.0		

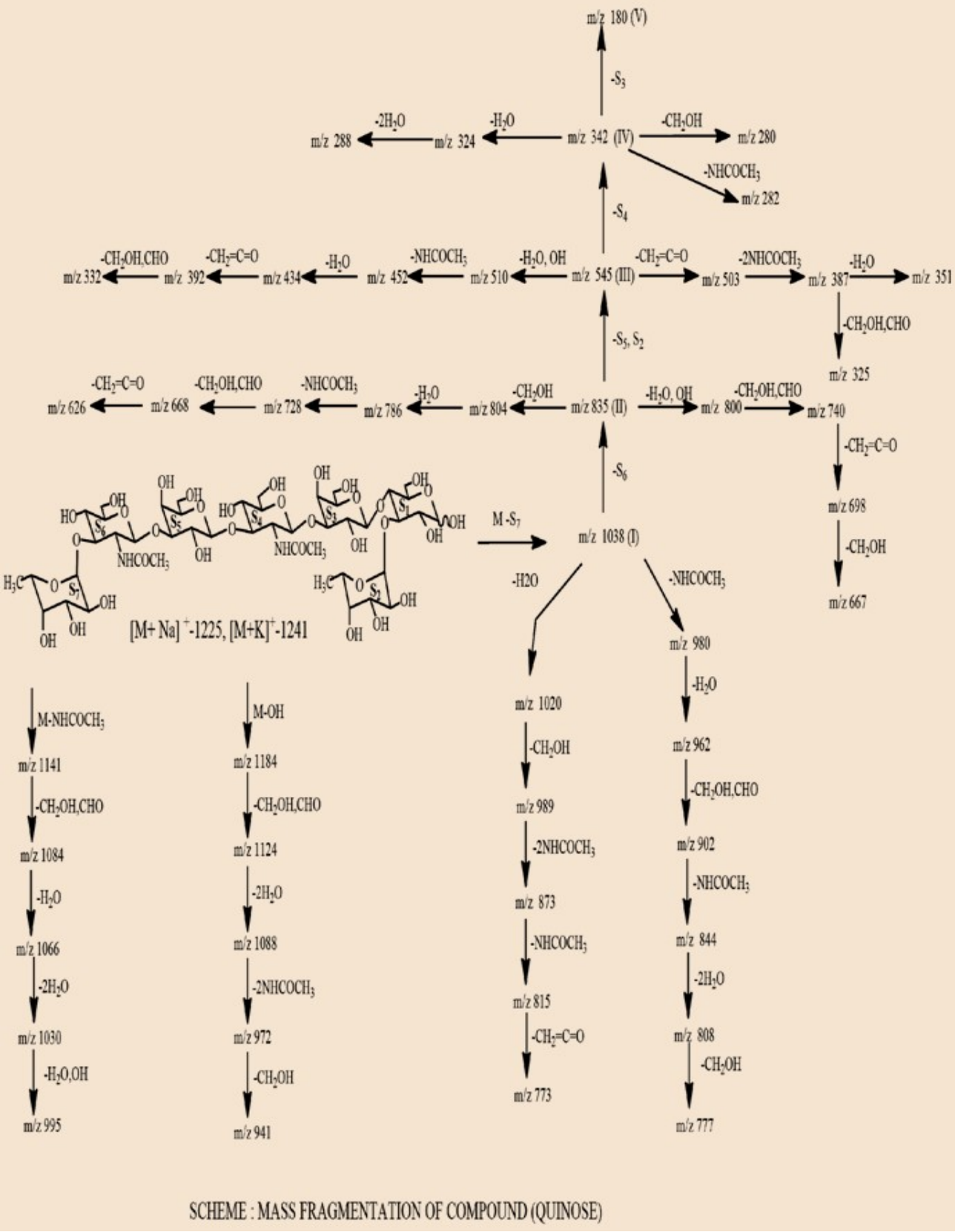
The cross peaks of carbon atoms involved in glycosidation were also present in HSQC spectrum at δ 3.85 x 70.4 (Glc (S_1) C_4 x H_4 showing 1→4 linkage), 5.0 x 70.9 (Glc (S_1) C_3 x H_3 showing 1→3 linkage), 3.85 x 73.1 (β Gal (S_3) C_3 x H_3 showing 1→3 linkage), 3.50 x 76.6 (β GlcNAc (S_4 and S_6) C_3 x H_3 showing 1→3 linkage), and 3.85 x 72.7 (β Gal (S_5) C_3 x H_3 showing 1→3 linkage). Based on the pattern of chemical shift of ^1H, ^{13}C, HOMOCOSY, HSQC and TOCSY NMR experiments, it was interpreted that the compound Quinose was a heptasaccharide comprised of one Glc, two GlcNAc, two Fuc and two Gal moieties having the structure:



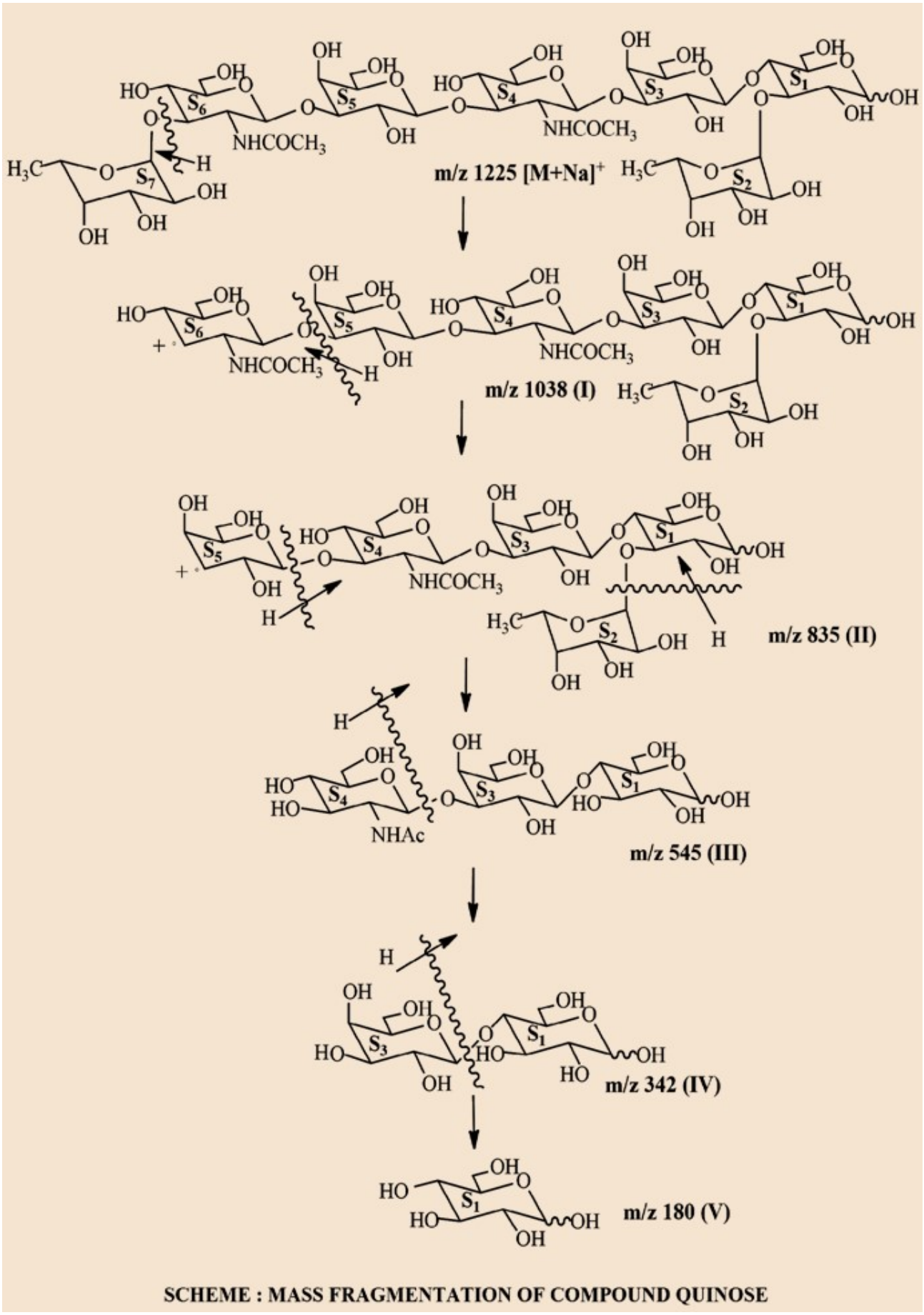
Mass Spectrosmetry Studies

The above derived structure of Quinose was further supported by its ES Mass spectrum. The highest mass ion peaks were recorded at m/z 1241 and 1225 which were due to $[M+K]^+$ and $[M+Na]^+$ confirming the molecular weight of compound M as 1202. The fragment ion at m/z 1202 further fragmented to give mass ion fragment at m/z 1038, which was assigned to the hexasaccharide unit (I). This fragmentation corresponded to the loss of terminal Fuc moiety from heptasaccharide [M-S_7], indicating the presence of Fuc (S_7) at the non-reducing end. The ES mass spectrum of compound M also contained other mass ion peaks at m/z 1184 [M-H_2O], 1144 [M-$NHCOCH_3$], 1124 [1184-CH_2OH, CHO], 1088 [1124-$2H_2O$], 1084 [1144- CH_2OH, CHO], 1066 [1084- H_2O], 1030 [1066-$2H_2O$], 995 [1030-H_2O, OH], 972 [1088- $2NHCOCH_3$], 941 [972- CH_2OH], which were obtained from the molecular ion. The mass ion fragment at m/z 1038 (I) further fragmented to give mass ion peak at m/z 835 (II) assigned to the loss of GlcNAc from fragment I confirming the presence of GlcNAc as a second sugar in sequence from non-reducing end. The pentasaccharide fragment ion (II) at m/z 835 on further fragmentation gave a trisaccharide fragment ion (III) at m/z 545 by the loss of Gal (S_5) and Fuc (S_2). The fragment ion III confirms the presence of Gal and Fuc moieties in the reducing heptasaccharide as the S_5 and S_2 units. On further fragmentation, the trisaccharide fragment ion (III) at m/z 545gave a disaccharide fragment ion (IV) at m/z 342 attributed to the loss of GlcNAc (S_4), confirming the presence of GlcNAc as a fifth sugar in sequence from non-reducing end. The mass ion fragment at m/z 342 also confirms the presence of lactosyl moiety in the reducing heptasaccharide. On further fragmentation, the disaccharide fragment ion (IV) at m/z 342 gave a peak at m/z 180 attributed to the loss of Gal (S_3), confirming the presence of Gal as a sixth sugar in sequence from non-reducing end. The mass ion fragment at m/z 1038 was also supported by its respective fragments at m/z 1020[1038- H_2O], 989 [1020- CH_2OH], 980 [1038- $NHCOCH_3$], 962 [980- H_2O], 902 [962- CH_2OH, CHO], 873 [989- $2NHCOCH_3$], 844 [902- $NHCOCH_3$], 815 [873- $NHCOCH_3$], 808 [844- $2H_2O$], 777 [808- CH_2OH] and 773 [815- CH_2=C=O]. The mass ion fragment at m/z 835 was further supported by its respective fragments at m/z 804 [835- CH_2OH], 800 [835- H_2O, OH], 786 [804- H_2O], 740 [800- CH_2OH, CHO], 728 [786-$NHCOCH_3$], 698 [740- CH_2=C=O], 668 [728- CH_2OH, CHO], 667 [698- CH_2OH], 626

[668- CH_2=C=O]. The mass ion fragment at m/z 545 was again supported by its respective fragments at m/z 510 [545- H_2O, OH], 503 [545- CH_2=C=O], 452 [510- $NHCOCH_3$], 434 [452- H_2O], 392 [434- CH_2=C=O], 387 [503- $2NHCOCH_3$], 351 [387- $2H_2O$], 332 [392- CH_2OH, CHO]. The mass ion fragment at m/z 342 was also supported by its respective fragments at m/z 324 [342- H_2O], 288 [324-$2H_2O$], 284 [342- $NHCOCH_3$], 280 [342- 2 CH_2OH].



Scheme 1a: Mass Fragmentation of Quinose.



Scheme 1b: Mass Fragmentation of Quinose

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 Quinose acetate and Quinose, the structure and sequence of isolated novel hexasaccharide Quinose was determined as:

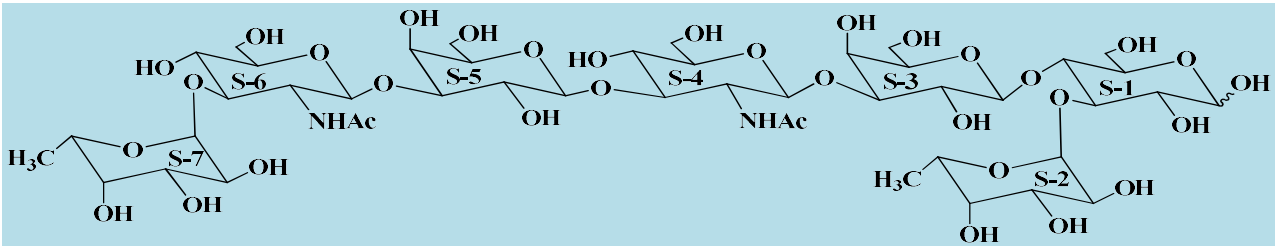


Figure 4: The structure and sequence of isolated novel hexasaccharide Quinose.

CONCLUSION

From the above informations, we conclude the structure of isolated Donkey milk oligosaccharide, **Quinose**. 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.

ACKNOWLEDGEMENT

Authors are thankful to Head, Department of Chemistry, University of Lucknow, Lucknow for providing lab facilities. We are also thankful to Prof. Raja Roy, CBMR-SGPGI Lucknow for providing NMR Spectroscopy facilities.

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. *Biochemiaet. BiophysicaActa.*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.