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1. NMR and Mass Characterization of a Novel Oligosaccharide Isolated from Goat Milk

Dr. Pushpraj Singh

Assistant Professor, Department of Chemistry,
Govt. Girls Degree College, Chhibramau, Kannauj,
(Affiliated to CSJM University, Kanpur),
Uttar Pradesh, India.

Abstract:s

Milk Oligosaccharides are the third most abundant constituents present in milk with immuno-stimulant activities and are emerging as potent drugs against fatal diseases like cancer and AIDS. Recently a number of natural oligosaccharides have been isolated from milk of various origin and were examined in animal models of different human diseases. Numbers of biologically active oligosaccharides have been isolated from cow, camel, buffalo, human, sheep, mare, yak, elephant and goat milk. The medicinal property of Goat milk is well defined in our ancient literature, according to Ayurveda and Unani medicinal system, enormous biological activities such as anti-bacterial, immunological, anti-inflammatory, hypoallergenic and therapeutic properties have been reported. It is used against tuberculosis in folk medicine and also helps in the enhancement of platelets count during dengue fever. Goat milk Oligosaccharides have anti-inflammatory properties and are involved in the repairing process after a DSS-induced colitis. Keeping in mind the biological importance of goat milk and the role of oligosaccharides, in the present studies, goat milk was analyzed for its oligosaccharides contents which led to isolation of a novel tetrasaccharide, ***Rasose****, $C_{26}H_{45}O_{21}N$. The structure of the isolated oligosaccharide was elucidated by chemical transformation, chemical degradation, ^{1}H, ^{13}C, 2D-NMR (COSY, TOCSY, HMBC and HSQC) and mass spectrometry as under.*

β-Gal(1→3)-β-GalNAc(1→2)-β-Gal(1→4)Glc

The geometry of compound Rasose was optimized by B3LYP method and 6-31 G (d, p) basis set.

Keywords:

Goat milk, carbohydrates, oligosaccharides, tetrasaccharide, NMR, Rasose.

1.1 Introduction:

Milk is of gold standard for nourishment of infants which is due to presence of number of oligosaccharides present in it [1]. Oligosaccharides are the biologically diverse and important carbohydrate amongst the naturally occurring compounds present as natural constituents [2]. Numerous oligosaccharides have been isolated from milk or colostrum of

many mammalian species which are dynamic and structurally diverse [3,4] which play an essential role in various physiological, pathological and biological activities such as anti-complementary, anti-coagulant, anti-inflammatory, anti-viral, anti-oxidant and immunological activities [5,6]. Oligosaccharides play a critical role in preventing inflammatory processes, reducing diabetes, obesity and cardiovascular risks, modulating the gut flora and affecting different gastrointestinal activities [7-9]. The milk oligosaccharides recognize cancer associated antigens, used as antimicrobial agents; tumor associated antigens and has physiological significance in infants [10]. Furthermore, the structural similarities of milk oligosaccharides with cell surfaces glycans as soluble receptors, these oligosaccharides are able to prevent the attachment of pathogenic bacteria to intestinal epithelial cells [11]. Goat milk has been recommended as an ideal substitute for bovine milk, especially for those who suffer from cow milk allergy. Goat milk contain galacto-oligosaccharides which may be recommended to decrease infant allergy and diseases [12]. Goat milk oligosaccharides have anti-inflammatory effects in rats with trinitrobenzenesulfonic (T) acid induced colitis and may be useful in the management of inflammatory bowel disease [13]. Goat milk oligosaccharides play an important role in intestinal protection and repair after damage caused by DSS (Dextron sodium sulphate) induced colitis and their implication in human intestinal inflammation [14]. Milk oligosaccharides derived from goat milk possess antihypertensive and immune modulatory properties [15].

In view of above facts, we have chosen goat milk for its oligosaccharide constituents and their structure elucidation. For this purpose, goat milk was collected in bulk and was processed by the modified method of Kobata and Ginsburg involving deproteination, filtration, lyophilization followed by gel filtration HPLC and column chromatography. We are describing the structure elucidation of one novel goat milk oligosaccharide, Rasose and its DFT (Density Functional Theory) studies.

1.2 Theoretical Study:

The quantum chemical calculation have been performed on B3LYP functional and 6-31 G (d, p) basis set employing Density Functional Theory (DFT). Geometry of compound Rasose has been first optimized and the presence of positive wave numbers values for all the optimized geometry indicates stability of the compound. All computations were performed using the Gaussian 09 program package [16].

1.3 Experimental:

1.3.1 General Procedures:

The sugars were visualized on TLC with 50% aqueous H_2SO_4 reagent and on Paper Chromatography with acetyl acetone and p-dimethyl amino benzaldehyde reagents. The adsorbent 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 of toluene, butanol and water in varied proportions. Sephadex G-25 (PHARMACIA) was used in gel permeation chromatography. Freeze drying of the oligosaccharide mixture was done with the help of CT 60e (HETO) lyophylizer 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 N-acetylgalactosamine (GalNAc), galactose (Gal), glucose (Glc), were purchased from Aldrich Chemicals. Optical rotations were measured with an AA-5 series automatic polarimeter in 1cm tube. ^{1}H and ^{13}C NMR spectra of oligosaccharide was recorded in D_2O and the spectra of acetylated oligosaccharide was 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 5ul per min. The ESI capillary was set at 3.5 KV and the cone voltage was 40V. The spectra were collected in 6s scans and the printouts were averaged spectra of 6-8 scans. The C, H and N analysis were recorded on CARLO-ELBA 1108 an elemental analyzer.

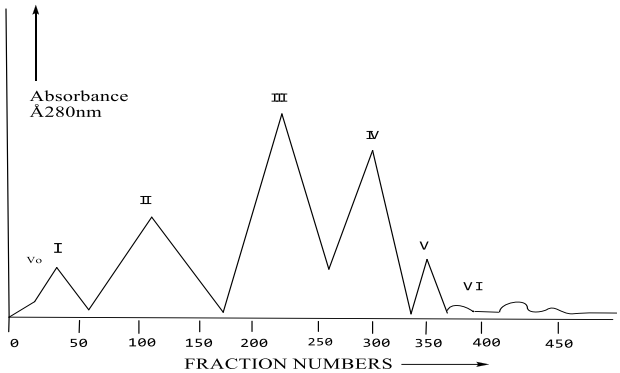
1.3.2 Isolation of Goat Milk Oligosaccharides by Modified Method Kobata and Ginsburg

11 litres of Goat milk was collected in 15 days with normal milking condition from a single domestic goat (Battisi) from Village- Nagla Seth, Post- Shamsabad, Dist- Farrukhabad, Uttar Pradesh, India. The milk was fixed by addition of equal amount of ethanol (11 litres), the preserved milk was taken to laboratory and then it was centrifuged for 15 min at 5000 rpm at 4^oC. The solidified lipid layer was removed by filtration through glass wool column under cold atmospheric condition. More ethanol was added to clear filtrate to a final concentration of 68% and the resulting solution was left over night at 0^oC. The white precipitate formed mainly of lactose and protein was removed by centrifugation and washed twice with 68% ethanol at 0^oC. The supernatant and washing were combined and filtered through a micro filter and lyophilization affording 215 gm of crude oligosaccharide mixture.

1.3.3 Sephadex G-25 Gel Filtration of Goat Milk Oligosaccharide Mixture:

13.20 gm of lyophilized material (mixture of oligosaccharides) of goat milk was purified on Sephadex G-25 column chromatography for separation of oligosaccharides from other constituent of milk by using glass triple distilled water as eluent at a flow rate of 3 ml/min.

Goat milk oligosaccharide mixture was packed in a column (1.6x40 cm) (void volume = 25 ml) equilibrated with glass triple distilled water (TDW) and it was left for 10-12 hrs to settle down. Presences of neutral sugars were monitored in all eluted fractions by phenol-sulphuric acid test. In this U.V. monitored Sephadex G-25 chromatography of goat milk oligosaccharide mixture showed seven peaks *i.e.* I, II, III, IV, V, VI and VII. A substantial amount of proteins, glycoproteins and serum albumin were eluted in void volume that was confirmed by positive colouration with p-dimethyl aminobenzaldehyde reagent [17] and phenol-sulphuric acid reagent [18]. Fractions under peaks II, III and IV gave a positive phenol-sulphuric acid test for sugars, which showed the presence of oligosaccharide mixture in goat milk. These fractions under peak II, III and IV were pooled and lyophilized together affording 8.6gm of oligosaccharide mixture.



Graph 1.1: Sephadex G-25 chromatography of Goat milk Oligosaccharides Detected by Phenol Sulphuric Acid Method. Elution was made with TDW

Table 1.1: Goat Milk Oligosaccharide Mixture (13.20 gm) Chromatographed over Sephadex G-25 Column Chromatography

Fraction No.	Solvent	Compound (in gm)	Phenol-H_2SO_4 Test for Sugar	Further Investigation
	Glass triple			
1-60	Distilled H_2O	0.73	-ve [I]	_
61-171	”	2.45	+ve [II]	Purified by
172-275	”	2.95	+++ve [III]	Column
276-343	”	2.86	+++ve [IV]	Chromatography
344-374	”	0.96	++ve [V]	-
375-389	”	0.51	-ve [VI]	_
				_

The amount of oligosaccharide mixture of pooled fractions (peaks II, III and IV) obtained from Sephadex G-25 column chromatography was 8.6 gm. This process was repeated further, which resulted into a total of 14.35 gm of oligosaccharide mixture.

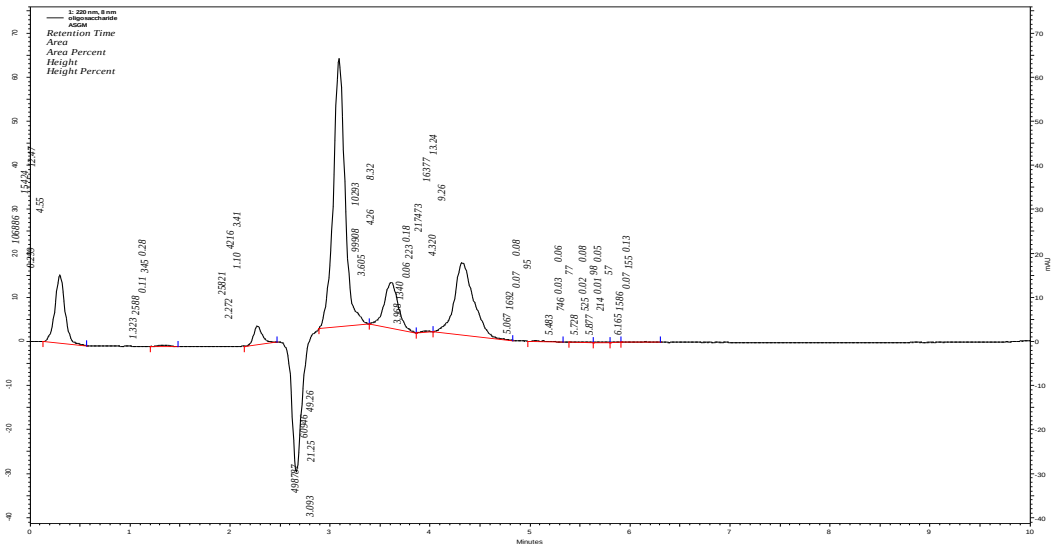
1.3.4 Confirmation of Homogeneity of Goat Milk Oligosaccharides by Reverse Phase HPLC

Pooled fractions (peaks II, III and IV) obtained from Sephadex G-25 column, containing oligosaccharide mixture were qualitatively analyzed by reverse phase HPLC. The HPLC system was equipped with Shimadzu CLASS-VP V6.13 solvent delivering system, 235-diode array detector and G.P. 100 printer plotter. The column used for this purpose was C18 Purosphere 25 cm × 0.4 cm × 5-µ m (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. Twelve peaks were noticed in the sample (pooled fraction II and III) at the varied retention times from 1.092 min to 20.675 in, for convenience the peaks were numbered in their increasing order of retention time *i.e.*[.299(R_1), 1.323(R_2), 2.272(R_3), 3.093(R_4), 3.605(R_5), 3.968(R_6), 4.320(R_7), 5.067(R_8), 5.483(R_9), 5.728(R_{10}), 5.877(R_{11}) and 6.165(R_{12})].

Table 1.2: HPLC Table of Crude Goat Milk Oligosaccharides

Sr. No.	Retention Time	Area	Area %	Height	Height %
1	0.299	106886	4.55	15424	12.47
2	1.323	2588	0.11	345	0.28
3	2.272	25821	1.10	4216	3.41
4	3.093	498787	21.25	60946	49.26
5	3.605	99908	4.26	10293	8.32
6	3.968	1340	0.06	223	0.18
7	4.320	217473	9.26	16377	13.24
8	5.067	1692	0.07	95	0.08
9	5.483	746	0.03	77	0.06
10	5.728	525	0.02	98	0.08
11	5.877	214	0.01	57	0.05
12	6.165	1586	0.07	155	0.13



Graph 1.2: Reverse Phase HPLC of Goat Milk Oligosaccharides

1.3.5 Acetylation of Oligosaccharide Mixture:

11 gm oligosaccharide mixture obtained from Sephadex chromatography was acetylated with pyridine (11 ml) and acetic anhydride (11 ml) at 60°C and solution was stirred overnight. Further in order to remove reagent, reaction mixture was evaporated under reduced pressure and viscous residue was taken in $CHCl_3$ (200 ml) and washed twice with ice cold water, evaporated to dryness yielding acetylated mixture (12.30 gm). The acetylation converted the free sugars into their non-polar acetyl derivatives which were resolved nicely on TLC giving 10 spots i.e. a, b, c, d, e, f, g, h, i and j. Detection of the spots on TLC was done by spraying with 50% H_2SO_4 in distilled water and heating. Varied proportions of $CHCl_3$: MeOH was used as chromatography solvent.

1.3.6 Purification of Acetylated Milk Oligosaccharide on Silica Gel Column:

Purification of acetylated oligosaccharide mixture (11.0 gm) was carried out over silica gel (500 gm) using varied proportion of Hex: $CHCl_3$, $CHCl_3$, $CHCl_3$: MeOH as eluents, collecting fraction of 500 ml each. All these fractions were checked on TLC and the fractions showing similar spots were collected together for further investigations. Repeated column chromatography of fractions I and II, led to the isolation of one chromatographically pure compound **'a' Rasose** (218 mg).

1.3.7 Deacetylation of Compound 'a':

Acetylated compound 'a' (50 mg) was dissolved in acetone (3 ml) and 3 ml of NH3 was added in it and was left overnight in a stoppered hydrolysis flask. After 24 hrs ammonia was removed under reduced pressure and the compound was washed with (3 x 5 ml) CHCl3 (to remove acetamide) and the water layer was finally freeze dried giving the natural oligosaccharide ‘A’ Rasose (40 mg).

1.3.8 Methylglycosidation/Acid Hydrolysis of Compound 'A' Rasose:

Compound 'A' Rasose (8 mg) was ref1uxed with absolute MeOH (2 ml) at 70°C for 18 hrs in the presence of cation exchange IR-120 (H) resin. The reaction mixture was filtered while hot and filtrate was concentrated giving methylglycoside of compound 'A'. In the solution of methylglycoside of compound 'A', 1, 4-dioxane (1 ml), and 0.1N H_2SO_4 (1 ml) was added and the solution was warmed for 30 min at 50°C. The hydrolysis was complete after 24 hrs. The hydrolysate was neutralized with freshly prepared $BaCO_3$, filtered and concentrated under reduced pressure to afford α-and β-methylglucosides along with the Gal and GalNAc. Their identification was confirmed by comparison with authentic samples of α-and β-methylglucosides, Gal and GalNAc (TLC, PC).

1.3.9 Kiliani Hydrolysis of Compound 'A' Rasose:

Compound 'A' Rasose (5 mg) was dissolved in 2 ml Kiliani mixture (AcOH-H_2O-HCl, 7:11:2) and heated at 100°C for 1 hr 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 and was evaporated under reduced pressure to afford Glc, Gal and GalNAc which were compared by authentic samples of Glc, Gal and GalNAc.

1.3.10 Description of Compound 'A' Rasose:

Acetylated Rasose 'a' (218 mg) was obtained from column chromatography. On deacetylation of 50 mg of compound Rasose Acetate 'a' with NH_3/acetone afforded compound **Rasose 'A'** (40 mg) $[\alpha]_D^{25}$ = -22° (*c* 1% H_2O).

For experimental analysis, this compound was dried over P_2O_5 at 100°C and 0.1 mm pressure for 8 hr. It gave positive Phenol-sulphuric acid test [18], Feigl test [19] and Morgan-Elson test [20].

$C_{26}H_{45}O_{21}N$	%C	%H	%N
Calculated	44.13	6.36	1.98
Found	44.12	6.36	1.98

^{1}H NMR of Acetylated Compound 'a', Rasose Acetate in $CDCl_3$ at 300 MHz

6.29 [d, 1H, J=3.0 Hz, *α*-Glc(S-1) H-1], 5.67 [d, 1H, J=8.1 Hz, β-Glc(S-1) H-1], 4.48 [d, 2H, J=8.1 Hz, β-Gal(S-2) H-1 & β-Gal(S-4) H-1], 4.45 [d, 1H, J=7.8 Hz, β-GalNAc(S-3)], 3.84 [m, 1H, β-Glc(S-1) H-4], 3.86 [m, 1H, β-Gal(S-2) H-2] and 3.85 [m, 1H, β-GalNAc(S-3) H-1].

^{13}C NMR of Acetylated Compound 'a', Rasose Acetate in $CDCl_3$ at 300 MHz

88.95 [1C, *α*-Glc(S-1) C-1], 91.52 [1C, β-Glc(S-1) C-1], 100.92 [2C, β-Gal (S-2) & β-Gal (S-4), C-1] and 101.17 [1C, β-GalNAc(S-3) C-1].

^{1}H NMR of Compound 'A', Rasose in D_2O at 300 MHz

5.58 [d, 1H, J=3.3 Hz, *α*-Glc(S-1) H-1], 4.51 [d, 1H, J=7.5 Hz, β-Glc(S-1) H-1], 4.36 [d, 2H, J=7.8 Hz, β-Gal(S-2) H-1 & β-Gal(S-4) H-1], 4.30 [d, 1H, J=7.2 Hz, β-GalNAc(S-3) H-1] and 1.94 [s, 3H, NHCO$\underline{CH_3}$, β-GalNAc (S-3)].

ES Mass of Compund 'A', Rasose

746[M+K]$^+$, 730[M+Na]$^+$, 707[M$^+$], 669[707-2H_3O^+], 651[669-H_2O], 567[651-2CH_2CO], 545[707-S_4], 487[545-NHCOCH_3], 465[545-CH_3OH-HCHO-H_2O], 448[465-OH], 438[487-CH_3OH-OH], 421[438-OH], 406[448-CH_2CO], 342[545-S_3], 304[342-2H_3O^+], 300[342-CH_2CO], 258[304-CHO-OH], 240[300-2HCHO], 222[258-2H_2O] and 180[342-S_2].

1.4 Result and Discussion:

1.4.1 Stability of Molecular Geometry of Isolated Compound 'A' Rasose:

Density functional theory (DFT), a computational method, was employed to evaluate the structure-activity relationship. In this study, the geometry of compound Rasose was optimized at B3LYP method and 6-31 G (d, p) basis set using Gaussian 09 program package [16]. The molecular geometries can be determined by the quantum mechanical behavior 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, color, magnetism, and biological activity. The compound 'A' was found to be highly polar in nature with the total dipole moment of **11.3750 Debye**. The total energy of compound was **-2652.2818 a. u.**, which represents the stability of molecule. All the rings were present in the most stable chair form. The compound 'A' Rasose possesses **C_1 symmetry**. The optimized structure of compound is given below (Figure 1):

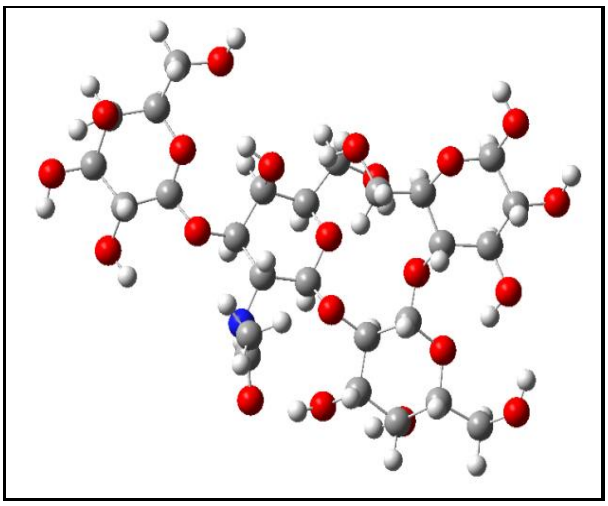


Figure 1.1: Optimized Geometry of Compound 'A' Rasose

1.4.2 Structure Elucidation of Isolated Goat Milk Oligosaccharide Rasose 'A'

A. NMR Spectroscopy:

The structure of novel milk oligosaccharide, compound 'A' Rasose was elucidated with the help of chemical degradation, chemical transformation, spectroscopic techniques like NMR (^{1}H, ^{13}C and 2D-NMR), structure reporter group theory and mass spectrometry. 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 oligosaccharides were made by comparing the ^{1}H NMR data of acetylated oligosaccharides and natural oligosaccharides. The ^{1}H NMR assignments were made by interpretation of data of COSY, TOCSY, HSQC and HMBC experiments.

Compound 'A' Rasose, $C_{26}H_{45}O_{21}N$ $[\alpha]_D^{25}$= -22°, isolated by repeated column chromatography of the acetylated oligosaccharide mixture, obtained from Goat milk, gave positive Phenol-sulphuric acid test, Feigl test, Morgon-Elson test showing the presence of normal and amino sugar(s) in the compound. The ^{1}H NMR spectrum of acetylated Rasose 'a' in $CDCl_3$ at 300 MHz exhibited four doublets for five anomeric protons at δ6.29(1H), δ5.67(1H), δ4.48(2H) and δ4.45(1H) indicating that Rasose 'A' may be a tetrasaccharide in its reducing form giving signals for α and β-anomers at the reducing end. The tetrasaccharide nature of Rasose acetate 'a' was further confirmed by the presence of four signals for five anomeric carbons at δ88.95(1C), δ91.52(1C), δ100.92 (2C) and δ101.17(1C) in ^{13}C NMR of acetylated Rasose 'a' at 300 MHz in $CDCl_3$. Moreover the tetrasaccharide nature of Rasose 'A' was supported by the presence of four anomeric proton doublets for five anomeric protons at δ5.58 (1H), δ4.51 (1H), δ4.36 (2H), and δ4.30 (1H) in ^{1}H NMR spectrum of compound Rasose 'A' in D_2O at 300 MHz. The reducing nature of compound Rasose 'A' was again confirmed by its methylglycosylation followed by its acid hydrolysis ($MeOH/H^+$), which led to the isolation of α and β-methylglucosides, along with Gal and GalNAc suggesting the presence of glucose at the reducing end and presence of Gal and GalNAc in the Rasose 'A'. The HSQC spectrum of acetylated Rasose 'a' at 300 MHz in $CDCl_3$ showed the presence of four cross peaks of five anomeric protons and carbons in their respective region at δ6.29x88.95, δ5.67x91.52, δ4.48x100.92 and δ4.45x101.17 suggesting that compound Rasose 'A' must be a tetrasaccharide in its reducing form. Thus ^{1}H and ^{13}C NMR spectra of acetylated Rasose 'a' justify the five anomeric signals for tetrasaccharide with total integral intensity of four anomeric protons/carbons. For convenience, starting from reducing end, the monosaccharides present in compound Rasose 'A' have been designated as S-1, S-2, S-3 and S-4. To confirm the monosaccharide constituents in compound Rasose 'A', it was hydrolyzed under strong acidic conditions of Kiliani hydrolysis which gave three monosaccharides i.e. Glc, Gal and GalNAc which were found identical with the authentic samples of Glc, Gal and GalNAc by co-chromatography (TLC and PC), confirming that the tetrasaccharide compound Rasose 'A' was consist of three types of monosaccharide units i.e. Glc, Gal and GalNAc.

The molecular formula $C_{26}H_{45}O_{21}N$ was in agreement with mass ion peak obtained from ES-MS spectrum of Compound Rasose 'A' which showed the highest mass ion peak at m/z 707 $[M]^+$ for a tetrasaccharide. The ^{1}H NMR spectrum of compound Rasose 'A' in D_2O at 300 MHz contain two anomeric proton doublets at δ5.58 (d, J=3.3 Hz) and δ4.51 (d, J= 7.5 Hz) for α and β-anomers of reducing monosaccharides (S-1) i.e. Glc. The ^{1}H NMR spectrum of Rasose acetate 'a' in $CDCl_3$ at 300 MHz contain two anomeric proton doublets at δ6.29 (d, J=3.0 Hz) and δ5.67 (d, J= 8.1 Hz) for α and β-anomers of reducing monosaccharides (S-1) i.e. Glc. The anomeric protons signal present at δ5.67 in TOCSY Spectrum of Rasose acetate 'a' assigned to β-Glc (S-1) gave three cross peaks at δ5.67x5.01, δ5.67x5.23 and δ5.67x3.79, which was later identified as H-2, H-3 and H-4 of reducing Glc respectively by COSY spectrum of acetylated Rasose 'a' at 300 MHz in $CDCl_3$. The chemical shift of H-4 of S-1 at δ3.79 suggested that H-4 of S-1 was available for glycosidic linkage by next monosaccharide unit. Further the ^{1}H signal present at δ3.79 assigned to H-4 of reducing Glc (S-1) gave a cross peak at δ3.79x100.92 in HMBC spectrum of Rasose acetate 'a' which was between H-4 of reducing Glc and C-1 of S-2, confirmed the (1→4) linkage between Glc (S-1) and S-2. The anomeric carbon of S-2 at δ100.92 gave its complimentary anomeric proton signal at δ4.48 (8.1 Hz) in the HSQC spectrum of Rasose acetate 'a'.

The chemical shift values of anomeric carbon at δ100.92 and anomeric proton at δ4.48 were having resemblance with literature value of anomeric chemical shift value of Gal hence S-2 was confirmed as Gal. Further the coupling constant of anomeric signal (S-2) at δ4.48 with larger J value of 8.1 Hz confirmed the β-configuration of the glycosidic linkage between (S2→S1) in Rasose acetate 'a'. Moreover the presence of β-Gal as next monosaccharide in Rasose 'A' was confirmed by appearance of anomeric proton signal at δ4.36 (7.8 Hz) in ^{1}H NMR spectrum of Rasose 'A' in D_2O at 300 MHz. Further, the anomeric proton signal at δ4.48 assigned to S-2 (β-Gal) showed three cross peaks at δ4.48x3.74, δ4.48x5.37 and δ4.48x4.97 in the TOCSY spectrum of Rasose acetate 'a' at 300 MHz which was later identified as H-2, H-3 and H-4 of β-Gal (S-2) respectively by COSY spectrum of acetylated Rasose 'a' in $CDCl_3$ at 300 MHz.

Table 1.3: Anomeric Proton Values of Compound 'A' Rasose in D_2O and Rasose Acetate 'a' in $CDCl_3$ at 300 MHz

Moieties	In D_2O		In $CDCl_3$	
	^{1}HNMR (δ)	**Coupling constant (J) Hz**	**^{1}HNMR (δ)**	**Coupling constant (J)Hz**
α-Glc(S-1)	5.58	3.3Hz	6.29	3.0Hz
β-Glc(S-1)	4.51	7.5Hz	5.67	8.1Hz
β-Gal(S-2)	4.36	7.8Hz	4.48	8.1Hz
β-GalNAc(S-3)	4.30	7.2Hz	4.45	7.8Hz
β-Gal(S-4)	4.36	7.8Hz	4.48	8.1Hz

The chemical shift of H-2 of S-2 at δ3.74, suggested that H-2 was available for glycosidic linkage by next monosaccharide unit i.e. S-3. Further the ^{1}H NMR signal present at δ3.74 assigned to H-2 of β-Gal (S-2) gave a cross peak at δ3.74x101.17 in HMBC spectra of Rasose acetate 'a' which was between H-2 of β-Gal (S-2) and C-1 of S-3 confirmed a 1→2 linkage between S-2 and S-3. The anomeric carbon of S-3 at δ101.17 gave its complimentary anomeric proton signal at δ4.45 in the HSQC spectrum of Rasose acetate 'a'. The chemical shift values of anomeric carbon at δ101.17 and anomeric proton at δ4.45 were having resemblance with literature value of anomeric chemical shift value of GalNAc, confirming that S-3 was GalNAc. Further the coupling constant of anomeric signal of S-3 at δ4.45 with larger J value of 7.8 Hz confirmed the β-configuration of the glycosidic linkage between (S3→S2) in Rasose acetate 'a'. Further the presence of β-GalNAc as next monosaccharide in Rasose 'A' was confirmed by appearance of anomeric proton doublet at δ4.30 (7.2 Hz) along with a singlet of three protons at δ1.94 in ^{1}H NMR spectrum of Rasose 'A' in D_2O at 300 MHz. Further the anomeric proton signal at δ4.45 (7.8 Hz) assigned for β-GalNAc (S-3) showed three cross peaks at δ4.45x4.04, δ4.45x3.79 and δ4.45x5.16 in the TOCSY spectrum of acetylated Rasose 'a' which was later identified as H-2, H-3 and H-4 of β-GalNAc (S-3) respectively by COSY spectrum of acetylated Rasose 'a' in $CDCl_3$ at 300 MHz. The chemical shift of the cross peak at δ4.04 was due to presence of $NHCOCH_3$ at C-2 of S-3 (β-GalNAc).

Moreover the chemical shift of H-3 of S-3 at δ3.79 showed the availability of -OH group for glyosidic linkage by next monosaccharide unit i.e. S-4. Further the HMBC spectrum of Rasose acetate 'a' at 300 MHz showed a cross peak signal of H-3 of β-GalNAc (S-3) and anomeric carbon of next monosaccharide C-1 of S-4 at δ3.79x100.92 confirmed a (1→3) linkage between S-4 and S-3. The anomeric carbon at δ100.92 gave its complimentary anomeric proton signal at δ4.48 in the HSQC spectrum of acetylated Rasose 'a'. The chemical shift values of anomeric carbon at δ100.92 and anomeric proton at δ4.48 were having resemblance with literature value of anomeric chemical shift value of Gal, confirming that S-4 was Gal. The coupling constant of anomeric signal (S-4) at δ4.48 with larger J value of Hz 8.1 Hz confirmed the β-configuration of the glycosidic linkage between (S4→S3) in Rasose acetate 'a'. Further the presence of β-Gal as next monosaccharide in Rasose 'A' was confirmed by appearance of anomeric proton signal at δ4.36 (7.8 Hz) in ^{1}H NMR spectrum of Rasose 'A' in D_2O. The anomeric proton signal at δ4.48 (8.1 Hz) assigned to β-Gal (S-4) gave three cross peaks at δ4.48x5.06, δ4.48x4.92 and δ4.48x5.32 in the TOCSY spectrum of Rasose acetate 'a' which was later identified as H-2, H-3 and H-4 of β-Gal (S-4) respectively by COSY spectrum of acetylated Rasose 'a' in $CDCl_3$ at 300 MHz. Since The chemical shift values of ring protons of S-4 at δ5.06, δ4.92 and δ5.32 does not reside in the linkage region and hence they did not show any cross peak in the linkage region i.e. δ3.5-4.2ppm confirming that β-Gal (S-4) was present at non-reducing end and none of its -OH group were available for glycosidic linkage, which was confirmed by the TOCSY and COSY spectra of acetylated Rasose 'a' in $CDCl_3$ at 300 MHz. All the ^{1}H NMR assignments for ring protons of monosaccharide units of Rasose 'A' were confirmed by COSY and TOCSY experiments. The positions of glycosidation in the oligosaccharide were confirmed by position of anomeric signals, Structure reporter groups (SRG) and comparing the signals in ^{1}H and ^{13}C NMR of acetylated and deacetylated oligosaccharide. The glycosidic linkages in Rasose 'A' were assigned by the cross peaks for glycosidically linked carbons with their protons in the HSQC and HMBC spectra of Rasose acetate 'a'. All signals obtained in ^{1}H and ^{13}C NMR of compound Rasose 'A' were in conformity with the assigned structure and their positions were confirmed by 2D NMR viz. COSY, TOCSY, HSQC and HMBC experiments of Rasose acetate 'a'. Thus based on the pattern of chemical shifts of ^{1}H NMR, ^{13}C NMR, COSY, TOCSY, HSQC and HMBC experiments, it was interpreted that the compound 'A' Rasose, was a tetrasaccharide having the following structure :

β-Gal(1→3)-β-GalNAc(1→2)-β-Gal(1→4)Glc

The Electron spray Mass Spectrometry data of compound 'A' Rasose not only confirmed the derived structure of Rasose 'A' but also supported the sequences of monosaccharides in this compound. The highest mass ion peak were recorded at m/z 746 and at m/z 730 which were due to [M+K]+ and [M+Na]+. It also contains the molecular ion peak at m/z 707 confirming the molecular weight of Rasose 'A' as 707[M]+ and was in agreement with its molecular formula C26H45O21N. 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 tetraasaccharide Rasose m/z 707(I) fragmented to give mass ion peak at m/z 545(II) [707-S4], this fragment was arised due to the loss of terminal β-Gal (S4) (162) moiety from tetrasaccharide indicating the presence of β-Gal (S4) at the non-reducing end. It was further fragmented to give mass ion peak at m/z 342(III) [545-S3] which was due to loss of β-GalNAc (S3) (203) moiety from trisaccharide.

This fragment of 342 was further fragmented to give mass ion peak at m/z 180(IV) [342-S2] which was due to loss of β-Gal (S2) (162) moiety from the disaccharide. The other fragmentation pathway in ES Mass spectrum of Rasose 'A', m/z 707 shows the mass ion peak at 669[707-2H3O+], 651[669-H2O], 567[651-2CH2CO], 545[707-S4], 487[545-NHCOCH3], 465[545-CH3OH-HCHO-H2O], 448[465-OH], 438[487-CH3OH-OH], 421[438-OH], 406[448-CH2CO], 342[545-S3], 304[342-2H3O+], 300[342-CH2CO], 258[304-CHO-OH], 240[300-2HCHO], 222[258-2H2O] and 180[342-S2].

Based on result obtained from chemical degradation/acid hydrolysis, chemical transformation, Electro spray mass spectrometry and 1D-NMR viz. ^{1}H NMR, ^{13}C NMR, Structure Reporter Groups (SRGs) and 2D-NMR viz. COSY, TOCSY, HMBC and HSQC spectra of Rasose acetate 'a' and Rasose 'A', the structure and sequence of isolated a novel oligosaccharide was deduced as:

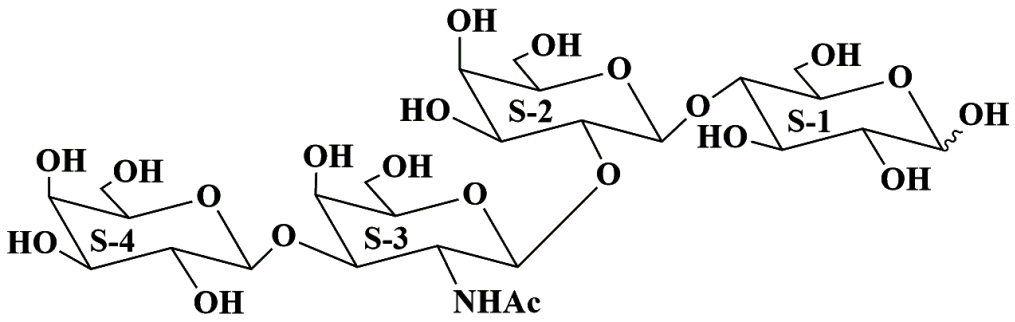


Figure 1.2: Compound 'A' Rasose

1.5 Conclusion:

In summary, we conclude that the structure of isolated goat milk oligosaccharide, compound 'A' Rasose was novel and reported for the first time from any natural source or any milk and elucidated with the help of ^{1}H, ^{13}C, 2D NMR spectroscopy and mass spectrometry. Using Gaussian 09 program at B3LYP method and 6-31 G basis set, the geometry of compound 'A' Rasose has been optimized.

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