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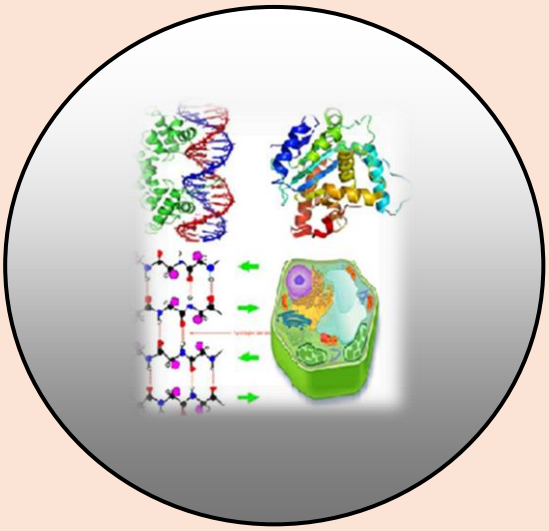

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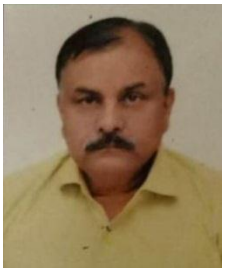

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Isolation and Structure Elucidation of a Novel Oligosaccharide (Gagriose) From Goat Milk by NMR and Mass Spectrometry

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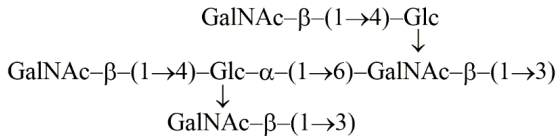
ABSTRACT

Goat milk play a very important role in traditional Indian medicinal system for treatment of intestinal disorder, coronary disease, premature infant nutrition, cystic fibrosis, infant allergy, and dissolving cholesterol deposits and is a rich source of protein, minerals, amino acid, calcium and vitamin A. Recently a number of natural oligosaccharides have been isolated from milk of various animal origin and were examined which shows a number of biological activities such as anti-tumor, anti-cancer, anti-viral, anticoagulant, antioxidant anti-bacterial, immunological, hypoallergenic and therapeutic properties. Among the different types of mammalian milk, goat milk shows great similarity with human milk and sometime it is used as breast milk for newborns. Goat milk oligosaccharide shows prebiotic activity in Bifido bacteria, anti-inflammatory effects in rats and also have anti-hypertensive, immunomodulatory properties. Some goat milk oligosaccharides isolated in our laboratory from different species of goat are Agriose, Boviose, Hircose, Gaurose, Apriose and Caprose. In the present work goat milk was collected in bulk and processed by modified method of Kobata and Ginsburg. The crude oligosaccharide mixture obtained by method of Kobata and Ginsburg was further purified by Sephadex chromatography, which was acetylated by Ac_2O/pyridine and purified by column chromatography which leads to isolation of another novel oligosaccharide Gagriose into its acetylated form which was further deacetylated by NH_4OH/Acetone to get the natural oligosaccharide Gagriose. The structure of purified oligosaccharide was confirmed by chemical degradation / transformation along with 2-D NMR and Mass spectrometry. The structure of novel oligosaccharide was also confirmed by ES-MS.

On the basis of the result obtain from the above experiment structure of novel oligosaccharide Gagriose was established as:



Keywords: Goat milk oligosaccharide, Hexasaccharide and NMR studies.

INTRODUCTION

Milk is a mixture of essential nutrient and other physiologically active substances responsible for the growth and development of any mammalian neonate (Singh et al., 2016). Milk is a rich source of bioactive oligosaccharides which depends on the nature of their origin to which mammals they belongs (Miller et al, 1994) (Singh et al, 2017). Oligosaccharides are the third largest solute in milk after lactose and fat Coppa et al., 1993). Among the different types of mammalian milk, goat milk contains largest amount of oligosaccharide which shows significant similarities to human milk oligosaccharides from the structural point of view (Kiskini et al). Goat milk is an ideal substitute for bovine milk, especially for those who suffer from cow milk allergy. Goat milk contains galacto-oligosaccharides which may be recommended to decrease infant allergy and diseases (Singh et al., 2018). Prebiotic activities of Goat milk oligosaccharides promote the growth of Bifido bacteria in the intestine which is beneficial for enhancement of neonates immunity, protection against intestinal pathogens as well as brain development (Raynal et al., 2008). Goat milk oligosaccharides have anti-inflammatory effects in rats with trinitrobenzenesulfonic acid induced colitis and may be useful in the management of inflammatory bowel disease (Hakkarainen et al., 2005). It also have anti-hypertensive and immunomodulatory (Kumar et al., 2011) properties. Its oligosaccharides play important roles in intestinal protection and repair after damage caused by DSS (Dextron sodium sulphate) induced colitis and their implication in human intestinal inflammation (Federico et al., 2006). N-linked oligosaccharides (N-glycans) from goat milk were recently found to be antipathogenic (Haiyun et al., 2020). It is used against tuberculosis in folk medicine and also helps in the enhancement of platelets count during dengue fever. Since goat milk is an ideal source of oligosaccharide for supplementary and therapeutic application, we have chosen to investigate 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 leads to the isolation of a novel goat milk oligosaccharide. Its structure was elucidated with the help of chemical degradation, chemical transformation and spectroscopic method like ^{1}H NMR, ^{13}C NMR and 2D NMR (i.e. COSY, TOCSY, HSQC and HMBC) technique and mass spectrometry.

EXPERIMENTAL

The sugars were visualized on TLC with 50% aqueous H_2SO_4 reagent and on paper chromatography sugars were visualized with acetyl acetone and p-dimethyl amino benzaldehyde reagents. For evaporation of alcohol from crude extract of milk oligosaccharides, Buchi Rotary evaporator was used. Freeze drying of the compound was done with the help of CT 60e (HETO) Lyophylizer and centrifuged by a cooling centrifuge Remi instruments C-25 at 5500 rpm.

To check the homogeneity of the compounds reverse phase HPLC system was used equipped with Perkin Elmer 250 solvent delivering system, 235 diode array detector and G.P. 100 printer plotter. Authentic samples of glucosamine, galactosamine, galactose, glucose, fucose and sialic acid were purchased from Aldrich Chemicals. The ^{1}H and ^{13}C NMR spectra of oligosaccharides were recorded in $CDCl_3$ and the spectra of deacetylated oligosaccharides were recorded in D_2O at 25^{o}C on a Bruker AM 300 NMR spectrometer. The electron spray mass spectra were recorded on a MICROMASS QUATTRO II triple quadruple mass spectrometer. The optical rotations of oligosaccharides were measured with AA-5 series automatic polarimeter in 1 cm tube. For mass spectroscopy the sample was dissolved in suitable solvent such as methanol/acetonitrile /water and was introduced into the ESI source through a syringe pump at the rate 5µl per min. The ESI capillary was set at 3.5 KV and the cone voltage was 40 V.

Isolation of Goat milk oligosaccharides by modified method of Kobata and Ginsburg (Gunjan et al., 2019)

10 litter of Goat milk was collected in 25 days with normal milking condition from a single domestic goat. The milk was fixed by addition of equal amount of ethanol, the preserved milk was taken to laboratory and there it was centrifuge for 30 min at 5000 rpm at 4^{o}C. 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^{o}C. The white precipitate formed mainly of lactose and protein was removed by centrifugation and washed twice with 68% ethanol at 0^{o}C. The supernatant and washing were combined and filtered through a micro filter and lyophilized affording crude oligosaccharides mixture 205gm. Lyophilized material responded positively towards phenol-sulphuric acid test and Morgon-Elson test showing the presence of natural sugar.

Sephadex G-25 gel filtration of goat milk oligosaccharide mixture

12 gm of lyophilized material (mixture of oligosaccharides) of goat milk was purified on Sephadex G-25 column chromatography for separation of low molecular weight component (enzymes, nucleic acids, peptide and proteins) from oligosaccharide by using glass distilled water as eluent at a flow rate of 3 ml/min. In this U.V. monitored Sephadex G-25 chromatography of goat milk oligosaccharide mixture showed six peaks *i.e.* I, II, III, IV, V, and VI. Fractions under peaks II, III and IV gave a positive phenol-sulfuric acid test (Dubois et al., 1956) 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.

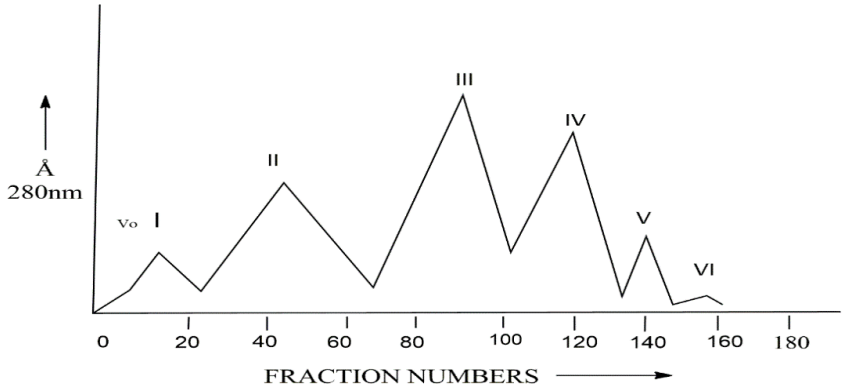


Table 1. Goat Milk Oligosaccharide Mixture (12 gm) Chromatographed over Sephadex G 25 Column Chromatography.

Fraction No.	Solvent	Compound (in gm)	Phenol-H_2SO_4 Test for Sugar
1-20	Glass (TDW)	0.29	-ve [I]
21-78	”	2.12	+ve [II]
79-105	”	3.95	+ve [III]
106-135	”	2.56	+ve [IV]
136-150	”	0.56	-ve [V]
151-160	”	0.15	-ve [VI]

Purified by column Chromatogarphy

The amount of oligosaccharide mixture of pooled fractions (peaks II, III and IV) obtained from Sephadex G-25 column chromatography was 8.63 gm. This process was repeated further, which resulted into a total of 15.56 gm of oligosaccharides mixture.

Acetylation of oligosaccharide mixture (Jain et al., 2000)

12.0 gm of crude oligosaccharide mixture was acetylated with pyridine (12.0 ml) and acetic anhydride (12.0 ml) at 60^0C and solution was left overnight. The mixture was evaporated under reduced pressure and the viscous residue was taken in $CHCl_3$ (100 ml) and washed in with ice cold water. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness yielding the acetylated mixture (10.5gm). By acetylation oligosaccharides were converted into their nonpolar acetyl derivatives which resolved nicely on TLC and gave well resolved seven spots on TLC i.e, a, b, c, d, e, f, and g.

Purification of acetylated Goat milk oligosaccharide on silica gel column

Purification of acetylated goat oligosaccharide mixture (10.0 gm) was carried out over silica gel (200 gm) using varying proportion of Hexane: $CHCl_3$, $CHCl_3$, $CHCl_3$: MeOH as eluants which was resolved into ten fractions namely I(248mg), II (47mg), III(1.986g), IV(1.563g), V(182mg), VI(834mg), VII(678mg) and VIII(2.323g) IX(427mg) and X(150mg). These fractions contain two to three compounds. Repeated column chromatography of fraction (III) led to the isolation of one chromatographically pure compound “a” (48mg).

Deacetylation of compound “a”

41 mg of compound “a” was dissolved in acetone (2 ml) and 3 ml of NH_4OH was added in it and was left overnight in a stoppered hydrolysis flask. After 24 hour ammonia was removed under reduced pressure and the compound was washed thrice with $CHCl_3$ (5 ml) (to remove acetamide) and water layer was finally freeze dried giving the deacetylated oligosaccharide “A” (32 mg).

Methyl glycosidation/Acid hydrolysis of compound A

Compound A (10 mg) was ref1uxed with absolute MeOH (2 ml) at 70°C for 18 hours in the presence of cation exchange IR-120 (H) resin. The reaction mixture was filtered while hot and filtrate was concentrated. To this solution 1, 4-dioxane (1 ml), and 0.1N H_2S0_4 (1 ml) was added and the solution was warmed for 30 minutes at 50^oC. 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-methyl glucosides along with the Glc and GalNAc. Their identification was confirmed by comparison with authentic samples (TLC, PC).

Killiani hydrolysis of compound A (Singh et al., 2016)

Compound A (5 mg) was dissolved in 2 ml Killiani mixture (AcOH-H_2O-HCl, 7:11:2) and heated at 100^oC for 1 hour 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 glucose and GalNAc on comparison with authentic samples of glucose and GalNAc.

Description of Compound 'A' Gagriose

Compound "a" (48 mg) was obtained from fraction 21-25 of column chromatography 2. On deacetylation of 41 mg of compound "a" with NH_4OH/acetone, it afforded compound "A" (32 mg) $[\alpha]_D^{25}$ = -6.9º (*c* 1% H_2O). For experimental analysis, this compound was dried over P_2O_5 at 100^oC and 0.1 mm pressure for 8 hr. It gave positive Phenol-sulphuric acid test, Feigl test and Morgan-Elson test.

$C_{44}H_{74}O_{31}N_4$		%C	%H	%N
	Calculated	45.75	6.41	4.85
	Found	45.74	6.40	4.83

^{1}H NMR of Compound-a Gagriose Acetate in $CDCl_3$ at 300 MHz

6.27[d, 1H, J=3.9 Hz, *α*-Glc(S-1) H-1], 5.71[d, 1H, J=3.6Hz, *α*-Glc(S-4) H-1], 5.67[d, 1H, J=8.7Hz, *β*-Glc(S-1) H-1], 4.47 [d, 1H, J=8.1Hz, β-GalNAc(S-6) H-1], 4.44[d, 2H, J=7.8Hz, *β*-GalNAc(S-2 & S-5), H-1], 4.38[d, 1H, J=7.5Hz, *β*-GalNAc(S-3), H-1], 3.83[m, 2H, *β*-Glc(S-1) & *α*-Glc(S-4)) H-3], 3.70[m, *β*-GalNAc(S-3) H-6], 3.53[t, 2H, *β*-Glc(S-1) & *α*-Glc(S-4) H-4].

^{13}C NMR of Compound-a Gagriose Acetate in $CDCl_3$ at 300 MHz

89.42[1C, *α*-Glc(S-1) C-1], 91.72[2C, *β*-Glc(S-1) & *α*-Glc(S-4) C-1], 103.73[1C, *β*-GalNAc(S-3) C-1], 104.43[1C, *β*-GalNAc(S-6) C-1], 104.58[2C, *β*-GalNAc(S-2 & S-5) C-1].

^{1}H NMR of Compound-A Gagriose in D_2O at 300 MHz

5.26[d, 1H, J=3.6Hz *α*-Glc(S-4) H-1], 5.21[d, 1H, J=3.6Hz, *α*-Glc(S-1) H-1], 4.67[d, 1H, J=8.1Hz, *β*-Glc(S-1) H-1], 4.52[d, 1H, J=7.5Hz, *β*-GalNAc(S-3) H-1], 4.47[d, 1H, J=7.5Hz, *β*-GalNAc(S-6) H-1], 4.46[d, 2H, J=7.5Hz, *β*-GalNAc(S-2 & S-5) H-1] 3.27[t, 1H, *β*-Glc(S-1) H-2], 2.03[s, 3H, ($NHCOCH_3$), *β*-GalNAc(S-6)], 2.00[s, 6H, ($NHCOCH_3$), *β*-GalNAc(S-3 & S-5)], 1.90[s, 3H, ($NHCOCH_3$), *β*-GalNAc (S-2)].

Table 2. ^{1}H NMR values of Compound C in D_2O and $CDCl_3$ at 300MHz.

Moieties	In D_2O		In $CDCl_3$	
	^{1}HNMR (δ)	Coupling constant (J)	^{1}HNMR (δ)	Coupling constant (J)
α-Glc(S-1)	5.21	3.9 Hz	6.27	3.9Hz
β-Glc(S-1)	4.67	8.1 Hz	5.67	8.7Hz
β-GalNAc(S-2)	4.46	7.5 Hz	4.44	7.8Hz
β-GalNAc(S-3)	4.52	7.5 Hz	4.38	7.5Hz
α-Glc(S-4)	5.26	3.6 Hz	5.71	3.6Hz
β-GalNAc(S-5)	4.46	7.5 Hz	4.44	7.8Hz
β-GalNAc(S-6)	4.47	7.5 Hz	4.47	8.1Hz

ES Mass Spectometry

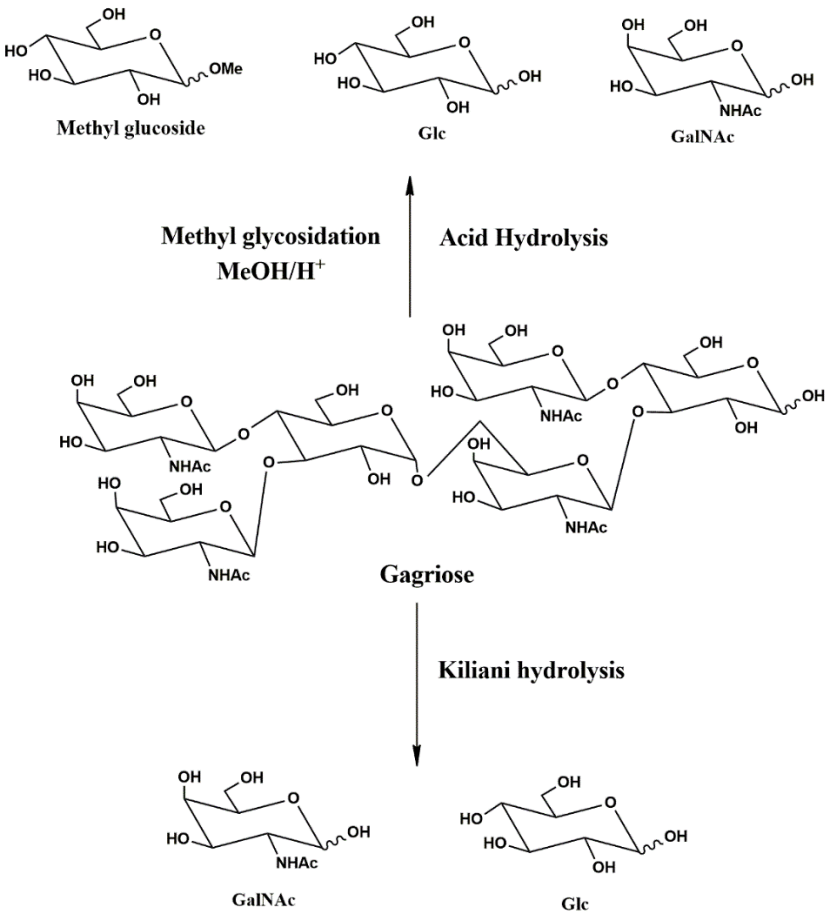
1154[M^+], 1125[1154-CHO], 1066[1125-CH_2OCHO], 1083[1125-CH_2CO], 1053[1154-CH_2OHCHO], 951[1154-S-6], 893[951- $NHCOCH_3$], 891[951- CH_2OHCHO], 875[893-H_2O], 847[891-CH_3CO], 841[875-2OH], 828[847-H_3O^+], 748[951-S-5], 706[748-HCHO], 677[706-CHO], 645[706-CH_2OH,HCHO], 646[706-2CH_2OH], 641[677-2H_2O], 640[677- H_2O,H_3O^+], 623[640-OH], 628[645-OH], 583[641-$NHCOCH_3$], 586[748-S-4], 508[586-CH2OHCHO,H_2O], 466[508-CH_2CO], 491[508-H_2O] 465[466-H^+], 410[466-CH_3COCH], 383[586-S-3], 365[383-CH_2OH], 303[365-2CH_2OH], 180[383-S-2].

RESULTS AND DISCUSSION

The structure of the novel goat milk oligosaccharide, compound **'A'** Gagriose was elucidated with the help of chemical degradation, chemical transformation, spectroscopic techniques like NMR (^1H, ^{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 by comparing the ^1H NMR data of acetylated oligosaccharides and natural oligosaccharides. The ^1H NMR assignments were made by interpretation of data of COSY, TOCSY, HSQC and HMBC experiments.

Compound A, Gagriose, $C_{44}H_{74}O_{31}N_4$ $[\alpha]_D^{25}$= -6.9º gave positive Phenol-sulphuric acid test, Fiegl test (Warren et al., 1960) and Morgan-Elson test (Fiegl et al., 1975) showing the presence of normal and amino sugars moieties in the compound A. ^1H NMR experiment of acetylated Gagriose exhibit six doublets for seven anomeric protons at δ6.27(1H), δ5.71(1H), δ5.67(1H), δ4.47(1H), δ4.44(2H) and δ4.38(1H) in $CDCl_3$ at 300 MHz indicating that Gagriose may be a hexasaccharide in its reducing form giving signals for α and β-anomers at the reducing end. Further the presence of five anomeric peaks for seven anomeric carbon at δ89.42 (1C), δ91.72 (2C), δ103.73 (1C), δ104.43 (1C) and δ104.58 (2C) in the ^{13}C NMR spectrum of acetylated Gagriose "a" in $CDCl_3$ at 300 MHz confirms hexasaccharide nature of Gagriose in its reducing form. The Hexasaccharide nature of Gagriose was further supported by the presence of six anomeric proton doublets for seven anomeric protons at δ5.26(1H), δ5.21(1H), δ4.67(1H), δ4.52(1H), δ4.47(1H) and δ4.46(2H) in ^1H NMR spectrum of Gagriose in D_2O at 300 MHz. The reducing nature of Gagriose was further confirmed by its methyl glycosidation MeOH/H^+ followed by its acid hydrolysis, which led to the isolation of α and β-methyl glucosides along with Glc and GalNAc suggesting the presence of glucose at the reducing end and presence of Glc and GalNAc in the compound Gagriose (A). The HSQC spectrum of acetylated Gagriose showed the presence of six cross peaks of seven anomeric protons and carbons in their respective region at δ6.27x89.42, δ5.67x91.72, δ5.71x91.72, δ4.47x104.43, δ4.44x104.58 and δ4.38x103.73 suggested that compound Gagriose 'a' must be hexasaccharide in it's reducing form. Thus ^1H and ^{13}C HSQC spectrum of acetylated Gagriose justify the five anomeric signals for hexasaccharide with total integral intensity of six anomeric protons/carbons. For convenience, starting from reducing end all six monosaccharides was denoted as S-1, S-2, S-3, S-4, S-5 and S-6. To confirm the monosaccharide constituents in compound Gagriose 'A', it was hydrolyzed under strong acidic conditions of Kiliani hydrolysis which gave two monosaccharides i.e.

Glc and GalNAc which were found identical with the authentic samples of Glc and GalNAc by co-chromatography (TLC and PC), confirming that the hexasaccharide compound Gagriose 'A' consist of two types of monosaccharide units i.e. Glc and GalNAc.

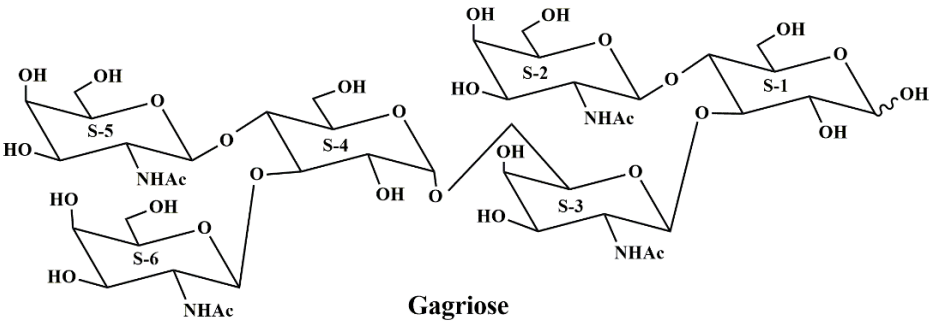


The molecular formula $C_{44}H_{74}O_{31}N_4$ was in agreement with mass ion peak obtained from ES-MS spectrum of compound Gagriose "A" which showed the highest mass ion peak at m/z 1154 $[M]^+$ for a Hexasaccharide. The ^1H NMR spectrum of Gagriose "A" in D_2O at 300 MHz contains two anomeric proton doublets at δ5.21 (d, J= 3.9 Hz) and δ4.67(d, J= 8.1 Hz) for *α* and *β* anomers of reducing monosaccharides (S-1) i.e Glc. Further the presence of another anomeric proton doublet at δ4.46 (d, J= 7.5Hz) along with a singlet of amide methyl at δ1.90 in ^1H NMR spectrum of Gagriose in D_2O was due to the presence of *β*-GalNAc (S-2) moiety as next monosaccharide in Gagriose. In addition to signals of *α*-Glc and *β*-Glc, presence of a triplet at δ3.27 which was due to H-2 of *β*-Glc (S-1) suggested the presence of Lactose type of structure in which Gal was replaced with GalNAc i.e. *β*-GalNAc-(1→4)-Glc at the reducing end of Gagriose. Simultaneously ^1H NMR and ^{13}C NMR spectrum of Gagriose acetate also showed downfield shifted *α* and *β* anomeric proton and carbon of reducing monosaccharides i.e. Glc (S-1) at δ6.27 (J= 3.9 Hz), δ5.67 (J= 8.7 Hz) and δ89.42, δ91.72 respectively. The anomeric protons signal present at δ5.67 assigned for *β*-Glc (S-1) in TOCSY Spectrum of Gagriose acetate contains three cross peaks at δ5.67 x 3.53, δ5.67 x 3.83 and δ5.67x5.05. The chemical shift of the cross peak at δ5.67 x 3.53 and δ5.67 x 3.83 suggested that in sugar S-1,

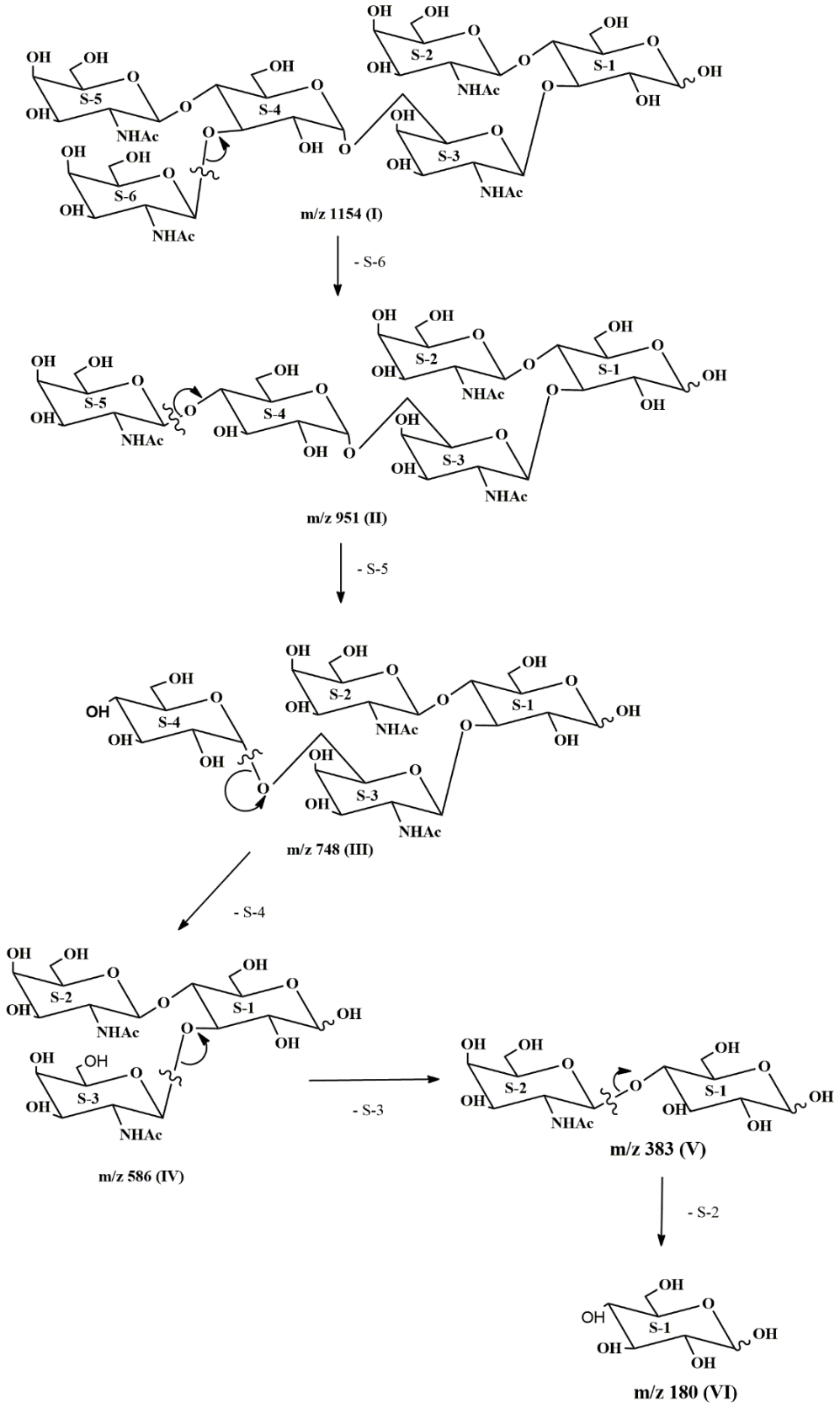
two positions were available for glycosidic linkage by the next monosaccharide unit, which was later identified as H-4 and H-3 of S-1 by COSY spectrum of Gagriose acetate. The earlier suggested (1→4) sequence of attachment of the monosaccharide unit (Glc & GalNAc) in Gagriose Acetate was finally confirmed from the HMBC spectrum of Gagriose acetate where C-H long range coupling was observed between H-4 of *β*-Glc (S-1) and C-1 of *β*-GalNAc (S-2) at δ3.53x104.58. The coupling constant of anomeric signal (S-2) at δ4.46 in D_2O with J value of 7.5 Hz confirmed the *β*-configuration of the glycosidic linkage between (S2→S1). This linkage was further supported by the presence of triplet (J= 8.7Hz) of H-4 signal of *β*-Glc (S-1) which appeared at δ3.53 in the ^{1}H NMR spectrum of Gagriose acetate. The anomeric carbon of *β*-GalNAc (S-2) at 104.58 given its complimentary proton signal at δ4.44 in the HSQC spectrum of Gagriose acetate. The chemical shift values of anomeric proton and anomeric carbon were having resemblance with literature value of anomeric chemical shift value of *β*-GalNAc hence S-2 monosaccharide was confirmed as *β*-GalNAc. Since it was ascertained by the COSY and TOCSY spectrum of Gagriose acetate that the *β*-Glc has two vacant position i.e. H-3 and H-4, and it was already confirmed that H-4 of S-1 was linked with *β*-GalNAc (S-2) whereas the left over H-3 position of *β*-Glc at δ3.83 showed a long range coupling with anomeric carbon i.e. C-1 of next monosaccharide (S-3) at δ103.73 in HMBC spectrum of Gagriose acetate in $CDCl_3$ at 300 MHz. The anomeric carbon signal at δ103.73 had its complimentary signal at δ4.38 in HSQC spectrum of Gagriose acetate in $CDCl_3$ at 300 MHz. The chemical shift value of anomeric proton and anomeric carbon were having resemblance with literature value of anomeric chemical shift value of *β*-GalNAc (S-3) hence S-3 monosaccharide was confirmed as *β*-GalNAc (S-3). Further the presence of *β*-GalNAc (S-3) as next monosaccharide in Gagriose was supported by appearance of anomeric proton signal at δ4.52 (J= 7.5 Hz) in ^{1}H NMR spectrum of Gagriose in D_2O at 300 MHz. The coupling constant of anomeric signal *β*-GalNAc (S-3) with larger value of 7.5 Hz showed that *β* configuration of the *β*-GalNAc (S-3). The (1→3) linkage between *β*-Glc (S-1) and *β*-GalNAc (S-3) was also supported by the presence of H-3 signal of S-1 at δ3.83 in the upfield region of ^{1}H NMR spectrum of Gagriose acetate which was confirmed by the TOCSY and COSY spectrum of Gagriose acetate in $CDCl_3$ at 300 MHz. The anomeric proton signal at δ4.44 in TOCSY Spectrum of Gagriose acetate assigned to *β*-GalNAc (S-2) gave three cross peaks at δ4.44 x 3.86, δ4.44 x 4.95 and δ4.44 x 5.38 suggested that in sugar (S-2) only one position was available for substitution which was later identified as H-2 position of S-2 by COSY spectrum of Gagriose acetate which was occupied with –NHAc group of GalNAc (S-2). Since the anomeric proton signal present at δ4.44 in TOCSY Spectrum of Gagriose acetate does not showed any methine proton in the linkage region except H-2 position which was already linked with –NHAc group confirmed that *β*-GalNA (S-2) was present at non-reducing end and none of its -OH group were involved in glycosidic linkage. The anomeric protons signal assigned for *β*-GalNAc (S-3) at δ4.38 in ^{1}H NMR Spectrum of Gagriose acetate gave three cross peaks at δ4.38 x 3.70, δ4.38 x 4.88 and δ4.38 x 5.37 in its TOCSY spectrum which was later assigned for H-6, H-3 and H-4 respectively by COSY spectrum of Gagriose acetate. The chemical shift of H-6 of S-3 at δ3.70 suggested that in sugar S-3 only one position was available for glycosidic linkage by next monosaccharide unit. The next anomeric proton doublet which appeared at δ5.26 (d, J= 3.6 Hz) in the ^{1}H NMR spectrum of Gagriose in D_2O was due to the presence of *α*-Glc (S-4) moiety. Further the presence of *α*-Glc (S-4) as next

monosaccharide was supported by ^{1}H NMR proton doublet at δ5.71 (J= 3.6Hz) in the ^{1}H NMR spectrum of Gagriose acetate in $CDCl_3$. This anomeric proton at δ5.71 gave its complimentary signal at δ91.72 in HSQC spectrum of Gagriose acetate. The chemical shift value of anomeric proton and carbon were having resemblance with literature value of anomeric chemical shift value of *α*-Glc. Since it was ascertained by COSY and TOCSY spectrum of Gagriose acetate that H-6 of S-3 was available for glycosidic linkage by next monosaccharide i.e. (S-4) hence S-4 (*α*-Glc) must be linked to H-6 of *β*-GalNAc (S-3). The (1→6) linkage between S-4 and S-3 was supported by the presence of cross peak at δ3.70 x 69.04 in glycosidic region of HSQC spectrum of Gagriose acetate in $CDCl_3$. Further (1→6) linkage between S-4 and S-3 was supported by presence of downfield H-6 signal at δ3.70 in ^{1}H NMR spectrum of Gagriose acetate in $CDCl_3$.The coupling constant of anomeric signal (S-4) with J value 3.6Hz confirmed the *α*-configuration of the *α*-Glc (S-4) moiety. The anomeric proton signal of *α*-Glc (S-4) at δ5.71 in the ^{1}H NMR spectrum of Gagriose acetate in $CDCl_3$ at 300 MHz. gave three cross peak at δ5.71 x 3.53, δ5.71 x 3.83, and δ5.71 x 5.05 in its TOCSY spectrum, suggested that in sugar S-4, two positions were available for glycosidic linkage by the next monosaccharide units, later it was confirmed by COSY spectrum of Gagriose acetate that the signal arised at δ3.53 and δ3.83 was due to the H-4 and H-3 of S-4 respectively. Further, HMBC spectrum of Gagriose acetate showed long range coupling between H-4 of *α*-Glc (S-4) and anomeric carbon of next monosaccharide (S-5) at δ3.53 x 104.58 confirmed the (1→4) linkage between S-5 and S-4. This (1→4) linkage between S-5 and S-4 was supported by the presence of H-4 signal of *α*-Glc (S-5) at δ3.53 in upfield region of ^{1}H NMR spectrum of Gagriose acetate which was confirmed by the TOCSY and COSY spectrum. The anomeric carbon signal present at δ104.58 had its complimentary signal at δ4.44 in HSQC spectrum of Gagriose acetate in $CDCl_3$ at 300 MHz. The chemical shift value of anomeric carbon and anomeric proton were having resemblance with literature value of anomeric chemical shift value of *β*-GalNAc (S-5) hence S-5 monosaccharide was confirmed as *β*-GalNAc (S-5). Further the presence of *β*-GalNAc (S-5) as next monosaccharide in Gagriose was supported by appearance of anomeric proton signal at δ4.46 along with a singlet of three protons of amide methyl at δ2.00 in ^{1}H NMR spectrum of Gagriose in D_2O. The coupling constant of anomeric signal *β*-GalNAc (S-5) with larger value of 7.5 Hz showing the *β* configuration of the *β*-GalNAc (S-5). The anomeric proton signal at δ4.44 in TOCSY Spectrum of Gagriose acetate assigned to *β*-GalNAc (S-5) gave three cross peaks at δ4.44 x 3.86, δ4.44 x 4.95 and δ4.44x5.38 suggested that in sugar (S-5) only one position was available for substitution. The chemical shift value of cross peak at δ3.86 was identified as H-2 of *β*-GalNAc (S-5) by COSY spectrum of Gagriose acetate which was occupied by –NHAc group. Since the anomeric proton signal present at δ4.44 assigned for *β*-GalNAc (S-5) in TOCSY Spectrum of Gagriose acetate does not showed any methine proton in the linkage region except H-2 position which was already linked with –NHAc group confirmed that *β*-GalNAc (S-5) was present at non-reducing end and none of its -OH group were available for glycosidic linkage. Since it was ascertained by the COSY and TOCSY spectrum of Gagriose acetate that the *α*-Glc (S-4) has two vacant position i.e. H-3 and H-4 and it was already confirmed that H-4 of S-4 was linked with *β*-GalNAc (S-5) whereas the left over H-3 position of *α*-Glc (S-4) at δ3.83 showed a long range coupling with anomeric carbon i.e. C-1 of next monosaccharide (S-6) at δ104.43 in HMBC spectrum of Gagriose acetate in $CDCl_3$ at 300 MHz confirmed the (1→3) linkage between S-6 and S-4.

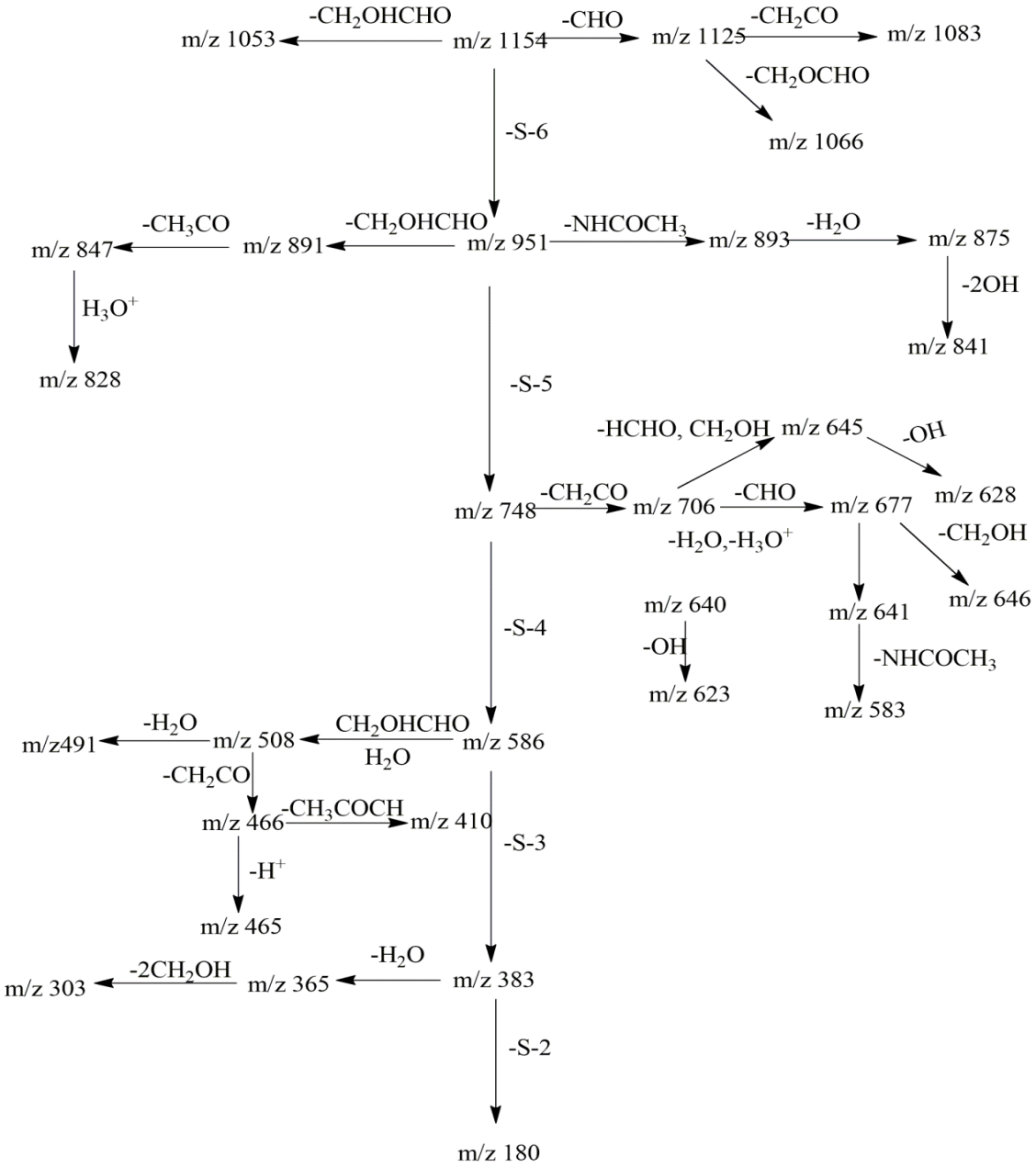
The anomeric carbon signal at δ104.43 showed its complimentary signal at δ4.47 in HSQC spectrum of Gagriose acetate in $CDCl_3$ at 300 MHz. The chemical shift value of anomeric proton and anomeric carbon were having resemblance with literature value of anomeric chemical shift value of *β*-GalNAc (S-6) hence S-6 monosaccharide was confirmed as *β*-GalNAc (S-6). Further the presence of *β*-GalNAc (S-6) as next monosaccharide in Gagriose was supported by appearance of anomeric proton doublet at δ4.47 (J= 7.5 Hz) along with singlet of three proton of amide methyl at δ2.03 (J= 7.5 Hz) in ^{1}H NMR spectrum of Gagriose in D_2O at 300 MHz. The coupling constant of anomeric signal *β*-GalNAc (S-6) with larger value of 7.5 Hz showed that *β* configuration of the *β*-GalNAc (S-6). The (1→3) linkage between *α*-Glc (S-4) and *β*-GalNAc (S-6) was supported by the presence of H-3 signal of S-4 at δ3.83 in upfield region of ^{1}H NMR spectrum of Gagriose acetate which was confirmed by the TOCSY and COSY spectrum of Gagriose acetate in $CDCl_3$ at 300 MHz. The anomeric proton signal at δ4.47 in TOCSY Spectrum of Gagriose acetate assigned to *β*-GalNAc (S-6) gave three cross peaks at δ4.47 x 3.86, δ4.47 x 4.95 and δ4.47 x 5.38 suggested that in sugar (S-6) only one position was available for substitution which was later identified as H-2 of S-6 by COSY spectrum of Gagriose acetate which was linked with –NHAc group. Since the anomeric proton signal present at δ4.47 in TOCSY Spectrum of Gagriose acetate does not showed any methine proton in the linkage region except H-2 position which was linked with –NHAc group confirmed that *β*-GalNAc (S-6) was present at non-reducing end and none of its -OH group were available for glycosidic linkage. All the ^{1}H NMR assignments for ring protons of monosaccharide units of Gagriose were confirmed by COSY and TOCSY experiments. The positions of glycosidation in the oligosaccharide were confirmed by position of anomeric signals, Structure reporter groups (S.R.G.) and comparing the signals in ^{1}H and ^{13}C NMR of acetylated and deacetylated oligosaccharide. The glycosidic linkages in Gagriose were assigned by the cross peaks for glycosidically linked carbons with their protons in the HSQC spectrum of acetylated Gagriose. The values of these cross peaks appeared as *β*-Glc (S-1) H-4 and C-4 at δ3.53 x 81.99 showed (1→4) linkage between S-2 and S-1, *β*-Glc (S-1) H-3 and C-3 at δ3.83 x 76.19 showed (1→3) linkage between S-3 and S-1, *β*-GalNAc (S-3) H-6 and C-6 at δ3.70 x 69.04 showed (1→6) linkage between S-3 and S-4, *α*-Glc (S-4) H-4 and C-4 at δ 3.53 x 81.99 showed (1→4) linkage between S-6 and S-5 and *α*-Glc (S-4) H-3 and C-3 at δ3.83 x 74.08 showed (1→3) linkage between S-6 and S-5. All signals obtained in ^{1}H and ^{13}C NMR of compound Gagriose were in conformity with the assigned structure and their position were confirmed by 2D NMR viz. COSY, TOCSY, HSQC and HMBC experiments. In the light of forgoing evidence, the structure of **Gagriose** was established as:



Gagriose



Scheme 1. 0: Mass fragmentation of compound A Gagriose.



Scheme 1.1. ES-MS fragments of compound A Gagriose.

CONCLUSION

In summary, we conclude that the structure of the isolated novel goat milk oligosaccharide, compound **'A'** Gagriose was reported for the first time from any natural source/milk and was elucidated with the help of ^{1}H, ^{13}C, 2D NMR spectroscopy and mass spectrometry.

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