3D Structure Assignment of a Novel Hexasaccharide 'Aliose' from Gaddi Sheep Milk, by 2D-NMR, Mass Spectrometry and its DFT Studies

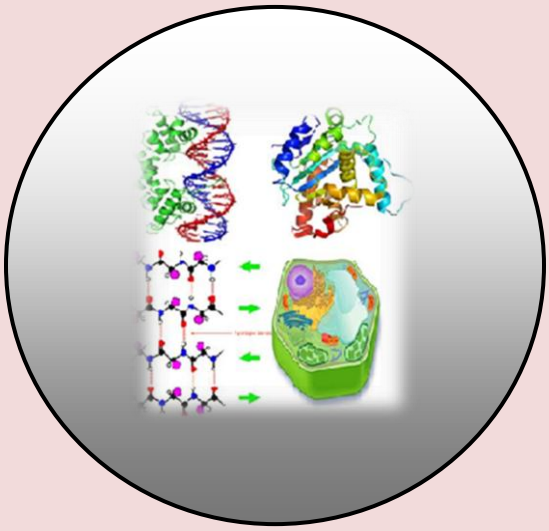
By

Lubna Jamal, Manisha Shukla, Anil Mishra and Desh Deepak

ISSN 2319-3077 Online/Electronic
ISSN 0970-4973 Print

Index Copernicus International Value
IC Value of Journal 82.43 Poland, Europe (2016)
Journal Impact Factor: 4.275
Global Impact factor of Journal: 0.876
Scientific Journals Impact Factor: 3.285
InfoBase Impact Factor: 3.66

J. Biol. Chem. Research
Volume 40 (1), 2023 Pages No. 85-107



Journal of Biological and Chemical Research

An International Peer Reviewed / Referred Journal of Life Sciences and Chemistry

Indexed, Abstracted and Cited: Index Copernicus International (Europe), World Science Index, Polish Ministry of Science and Higher Education (Poland, Europe) Research Bible (Japan), Scientific Journals Impact Factor Master List, Directory of Research Journals Indexing (DRJI), Indian Science. In, Database Electronic Journals Library (Germany), Open J-Gate, J Gate e-Journal Portal, Info Base Index, International Impact Factor Services (IIFS) (Singapore), Scientific Indexing Services (USA), International Institute of Organized Research (I2OR), Cosmos Science Foundation (Germany), Science Library Index (UAE), Eye Source, Swedish Scientific Publication, World Cat, International Innovative Journal Impact Factor, Einstein Institute for Scientific Information {EISI} and Impact Factor.pl - Kompendiumwiedzy o czasopismachnaukowych, Philadelphia citefactor.org journals indexing Directory Indexing of International Research Journals

Published by Society for Advancement of Sciences®

J. Biol. Chem. Research. Vol. 40, No. 1, 85-107, 2023
(An International Peer Reviewed / Refereed Journal of Life Sciences and Chemistry)
Ms 40/01/40/2023

ISSN 2319-3077 (Online/Electronic)
ISSN 0970-4973 (Print)

Lubna Jamal
http:// www.sasjournals.com
http:// www.jbcr.co.in
jbiolchemres@gmail.com

RESEARCH PAPER

Received: 04/04/2023 Revised: 29/06/2023 Accepted: 30/06/2023

3D Structure Assignment of a Novel Hexasaccharide 'Aliose' from Gaddi Sheep Milk, by 2D-NMR, Mass Spectrometry and its DFT Studies

Lubna Jamal, Manisha Shukla, Anil Mishra and Desh Deepak
Department of Chemistry, University of Lucknow, Lucknow-226007, India

ABSTRACT

Milk is an excellent source of biologically active oligosaccharides. They have good biocompatibility which enhances the drug delivery in the human body. While examining the oligosaccharide contents of various milk it was found that the sheep milk exhibited varied biological activities which were due to the presence of Fucose and Neuraminic acid at the non-reducing end of the oligosaccharides. The sheep milk contains vitamins, minerals and amino acids. It also contains the calcium and phosphate in high percentage. The natural ability of sheep milk to moisturize and nourish the skin is well known. It aggravates hiccups and dyspnoea. It is found to enhance *pitta*, *kapha* and reduces excess body fat. In recent times it was used for enhancing the blood platelet count that was reduced drastically during dengue fever. In view of the above facts related to the biological importance of sheep milk, we examined the milk of a rare sheep species i.e. 'Gaddi sheep'. The milk was collected and processed by modified method of Kobata and Ginsburg, followed by the regular process of chromatographic techniques that resulted in the isolation of a novel hexasaccharide 'Aliose'. Its structure was determined by ^{1}H, ^{13}C and 2D- NMR experiments involving COSY, TOCSY, HSQC and HMBC along with Mass spectrometry. The rarity of the compound Aliose was that it contained β-Gal (1→2) Glc instead of β-Gal (1→4) Glc (Lactose) present at the reducing end which is found in most of the milk oligosaccharides. Its DFT studies were also discussed. The structure of Aliose was determined as under-

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

Aliose

Keywords: Milk, Gaddi sheep, Oligosaccharide, Aliose, NMR, Mass spectrometry.

INTRODUCTION

Breast milk is the perfect food source for the development of any infant. It is responsible for enhancement of the immune system, brain growth, gut protection and bone formation of a new born. In Indian medicinal system the importance of different milk is well described. The biological ability of milk depends on oligosaccharide contents of particular milk. These oligiosaccharides contain two to fifteen monosaccharide units containing Glc, Gal, GlcNAc, GalNAc, Fucose and Sialic acid. They are arranged either in straight or branched chain structures and are linked together with o- glycosidic linkages by α and β glycosidic configurations. They are used as a medicine or an additive for enhancing the lyophilicity of the drug. Oligosaccharides have excellent biocompatibility that enhances the drug absorption in the body. It is due to its high polarity, multi-functionality and diversity of functional group orientation. It is well reported that the sheep milk contains Fucose and Sialic acid at their non-reducing end. Sheep milk oligosaccharides show varied biological properties like immunomodulatory (Shahi et al, 2020), anti-bacterial (Craft et al, 2019) and anti-oxidant (Caroprese et al, 2019). It also contains high levels of vitamins, minerals and amino acids, which are essential for the healing process and show homeostasis (Farag et al, 2020).It has highest amount of calcium and phosphate and is used in cosmetic industry as skin bars (Coni et al, 1996). It has natural ability to moisturize and nourish the skin. The sheep milk aggravates hiccups and dyspnoea (Sushrut Sanhita). It elevates *pitta* and *kapha.* It also decreases the fat. In recent time goat and sheep milk has been used for enhancing the blood platelet count decreased during Dengue fever (Mahendru et al, 2011).

Keeping in mind the biological activity of the sheep milk and the importance of the oligosaccharides, the milk of a rare species of sheep found at high altitudes of the Himalayan region (Gaddi sheep) was collected and processed by modified method of Kobata and Ginsburg (Kumar et al, 2016) incorporating centrifugation, micro filtration and lyophilization. Further it was purified by gel filtration and its homogeneity was confirmed by HPLC to obtain the oligosaccharide mixture, which was acetylated and purified by column chromatography to obtain a novel hexasaccharide 'Aliose'. Its stereoscopic structure was determined by ^{1}H, ^{13}C, 2D NMR experiments (COSY, TOCSY, HSQC, HMBC) and Mass spectrometry along with chemical degradation and chemical transformation. Its DFT studies have also been discussed in this paper.

MATERIALS AND METHODS

GENERAL PROCEDURE

The general procedures were same as described in earlier communications (Gangwar et al, 2018).

Isolation of Gaddi sheep milk oligosaccharide by modified method of Kobata and Ginsburg (Kumar et al, 2016)

9 Liters of Gaddi sheep milk was collected from a domestic sheep of Kangara district of Himanchal Pradesh. Preservation of milk was done by addition of 9 Liters ethanol. It was then taken to the laboratory and centrifuged for 30 min at 5000 rpm at -4°C, followed by its filtration through glass wool column in cold. Ethanol was further added to a final concentration of 68%. It was further centrifuged and washed twice with 68% ethanol at 0°C.

The supernatant and washings were combined and filtered through a micro filter, and was further lyophilized, affording crude oligosaccharide mixture (256 gm).

Sephadex G-25 gel Chromatography of Gaddi Sheep Milk Oligosaccharides (Gangwar et al, 2018)

13.50 gm of oligosaccharide mixture was chromatographed over Sephadex G-25 column for separation of glycoproteins, proteins and oligosaccharides. It was eluted by glass distilled water at a flow rate of 3 ml/min. Camel milk oligosaccharide mixture was packed in a column (1.6×40 cm and void volume 25 ml). The experiment was performed under conditions described in our earlier communication. Presence of neutral sugars was monitored in all eluted fractions by phenol-sulphuric acid test. Repeated Sephadex Chromatography yielded 26 gm of oligosaccharide mixture.

Acetylation of Gaddi sheep milk oligosaccharide mixture (Gangwar et al, 2018)

Oligosaccharide mixture obtained after Sephadex Chromatography (13 gm) was acetylated with pyridine (13 ml) and acetic anhydride (13 ml) at 60°C and the solution was stirred overnight. The reaction mixture was worked up by standard procedures and dried over reduced pressure, yielding the acetylated oligosaccharide mixture (13.7gm). TLC, gave eight spots i.e. a, b, c, d, e, f, g and h. Purification of the acetylated oligosaccharide mixture (13.7 gm) was carried out over silica gel (800 gm) using varied proportion of hex:$CHCl_3$, $CHCl_3$, $CHCl_3$: MeOH and as eluents, collecting fraction of 400 ml each. All these fractions were checked on TLC and the fractions showing similar spots (R_f values) were pooled together for further investigations. Repeated column chromatography of fractions III (6-30), 256 mg led to the isolation of one chromatographically pure compound Aliose acetate 'c' (63 mg).

Deacetylation of Aliose acetate (Gangwar et al, 2018)

Compound 'c' (40 mg) was dissolved in acetone (4 ml) and 4 ml of NH_4OH were added to it and was left overnight in a stoppered hydrolysis flask. After 24hr, reaction mixture was concentrated under reduced pressure to dryness and the compound was washed with $CHCl_3$ (5 ml ×3), further the water layer was freeze dried giving the deacetylated natural oligosaccharide C (35mg).

Methyl glycosidation / Acid hydrolysis of Aliose (Shukla et al, 2023)

Compound C (10 mg), was refluxed with absolute MeOH (3 ml) at 70°C for 18 hr in the presence of a cation exchange IR-120 (H) resin. The reaction mixture was filtered while hot and filtrate was concentrated which resulted in the formation of methyl glycoside of C (PC). In the solution of methylglycoside of C, 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°C. After completion of reaction (PC, TLC), it was filtered and concentrated under reduced pressure to afford α-and β-methylglucosides along with the Glc and Gal. Their identification was confirmed by comparison with authentic samples (TLC, PC).

Kiliani hydrolysis of Aliose (Ranjan et al, 2023)

Compound C (5 mg), was dissolved in 2 ml Killiani 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 glucose and Gal on comparison with authentic samples of glucose and Gal.

Description of isolated compound Aliose

Substance 'C' (63 mg), obtained from fractions 23-31 of column chromatography 3. On deacetylation of this acetylated hexasaccharide (40 mg) with NH_3/Acetone, it afforded compound Aliose (35 mg) as a viscous mass. For experimental analysis, this compound was dried over P_2O_5 at 10^0C and 0.1 mm pressure for 10 hr. It gave positive Phenol-sulphuric acid test.

$C_{36}H_{62}O_{31}$:	%C	%H
Calculated:	43.63	6.26
Found:	43.64	6.26

^{1}H NMR of Aliose Acetate at 800 MHz (in $CDCl_3$)

δ 6.22 [d, 1H, α-Glc (S-1), H-1)], δ 5.65 [d, 1H, β-Glc (S-1), H-1)], δ 4.46 [d, 1H,J=8.0 Hz, β-Gal (S-2) H-1], δ 5.65 [d, 1H, J=2.3 Hz, α-Glc (S-3), H-1], δ 4.44 [d,1H, J=8.0, β-Gal (S-4), H-1)], δ 4.44 [d, 1H, J=8.0, β-Gal (S-5), H-1], δ 4.05 [d,1H, J=7.52, β-Gal (S-6) H-1], δ 3.80 [m, 1H, β-Glc (S-1) H-2], δ 3.79 [m, 1H, β-Gal(S-2) H-3], δ 3.83 [m, 1H, β-Glc (S-3) H-2], δ 3.85 [m, 1H, β-Gal (S-4) H-3], δ 3.86[m, 1H, β-Gal (S-5) H-4].

^{13}C NMR of Aliose Acetate at 800 MHz (in $CDCl_3$)

δ 89.06 [1C, α-Glc (S-1), C-1], δ91.62 [1C,Glc (S-1), C-1], δ 101.06 [1C,Gal (S-2), C-1], δ 91.62 [1C, α-Glc(S-3), C-1], δ 101.32 [1C,Gal (S-4), C-1], δ 101.06 [1C,Gal (S-5), C-1], δ101.06 [1C,Gal (S-6), C-1].

ES-Mass Fragments of Aliose

m/z1052 [M+Na+K]+, 1029 [M+K]+, 990 [M]+, 951 [M-2H_3O+-H], 908 [951-CH_3CO], 857 [908-CH_3OH-H_3O+], 833 [908-CH_3-CH_2OHCHO], 828 [857-CHO],803 [833-HCHO], 763 [828-2CH_3OH-H+], 761[763-2H+] OR [828-OH-H_2OCH_3OH],760 [761-H+], 711[761-H_2O-CH_3OH], 710[760-H_2O-CH_3OH], 666 [MS6-S5], 633 [666-HCHO], 617 [633-CH_3-H+], 575 [617-CH_2CO], 533 [575-CH_2CO]OR [617-2CH_2CO], 505 [575-3H_2O-CH_3-H+], 445 [505-HOCHCHOH], 430 [445-CH_3], 371 [505-CH_2OHCHO-HCHO-CH_3CHO] OR [430-CH_2OCHO], 315 [331-CH_3-H+], 289 [331-CH_2CO], 267 [331-CH_3CHO-H_3O+-H], 247 [331-2CH_2CO] OR[289-CH_2CO], 231[247-CH_3-H+], 214 [231-OH], 181 [342+H+-162], 170 [214-CH_3CHO].

RESULTS AND DISCUSSION

Compound C, $C_{36}H_{62}O_{31}$'Aliose' was isolated from Gaddi sheep milk in its acetylated form and designated as 'c'. It gave positive phenol-sulphuric acid test (Dubois et al, 1956) and Feigel test (Feigel et al, 1924) showing the presence of normal sugars in oligosaccharide. The HSQC spectrum of acetylated Aliose observed in $CDCl_3$ at 800 MHz showed the presence of seven cross peaks of anomeric protons and carbons in the respective region at δ6.22× 89.05, δ5.64×91.61, δ4.46×101.06, δ5.65×91.61, δ4.44×101.32, δ4.43×101.06 and δ4.05×101.06 suggesting the presence of seven anomeric protons and carbons in it. The presence of seven anomeric protons was further confirmed by the presence of seven anomeric proton doublets at δ6.23 (1H), δ5.64 (1H), δ4.46 (1H), δ5.65 (1H), δ4.44 (1H), δ4.44 (1H) and δ4.05 (1H) in ^{1}H NMR spectrum of acetylated Aliose in $CDCl_3$ at 800 MHz. The ^{13}C NMR spectrum of Aliose acetate in $CDCl_3$ at 800 MHz also confirmed the presence of seven anomeric carbons at δ89.06 (1C), δ 91.61(2C), δ101.06 (3C) and δ101.32 (1C).

The chemical shifts of α and β anomeric proton/carbon signals respectively at δ6.23, δ5.65 and δ89.05, δ91.61 suggested that the reducing monosaccharide was glucose. The reducing nature of compound Aliose was further confirmed by its methylglycosidation by MeOH / H^+ followed by its acid hydrolysis which led to the isolation of α and β-methyl glucoside along with glucose and galactose leading to the presence of glucose at the reducing end in the oligosaccharide.

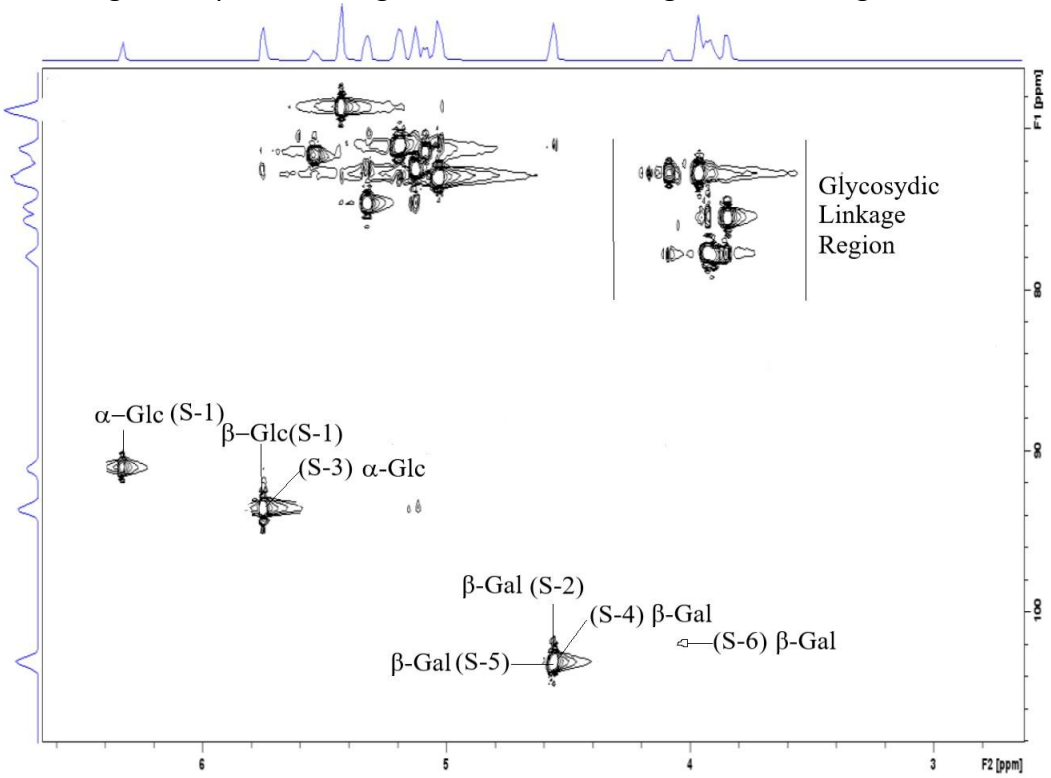


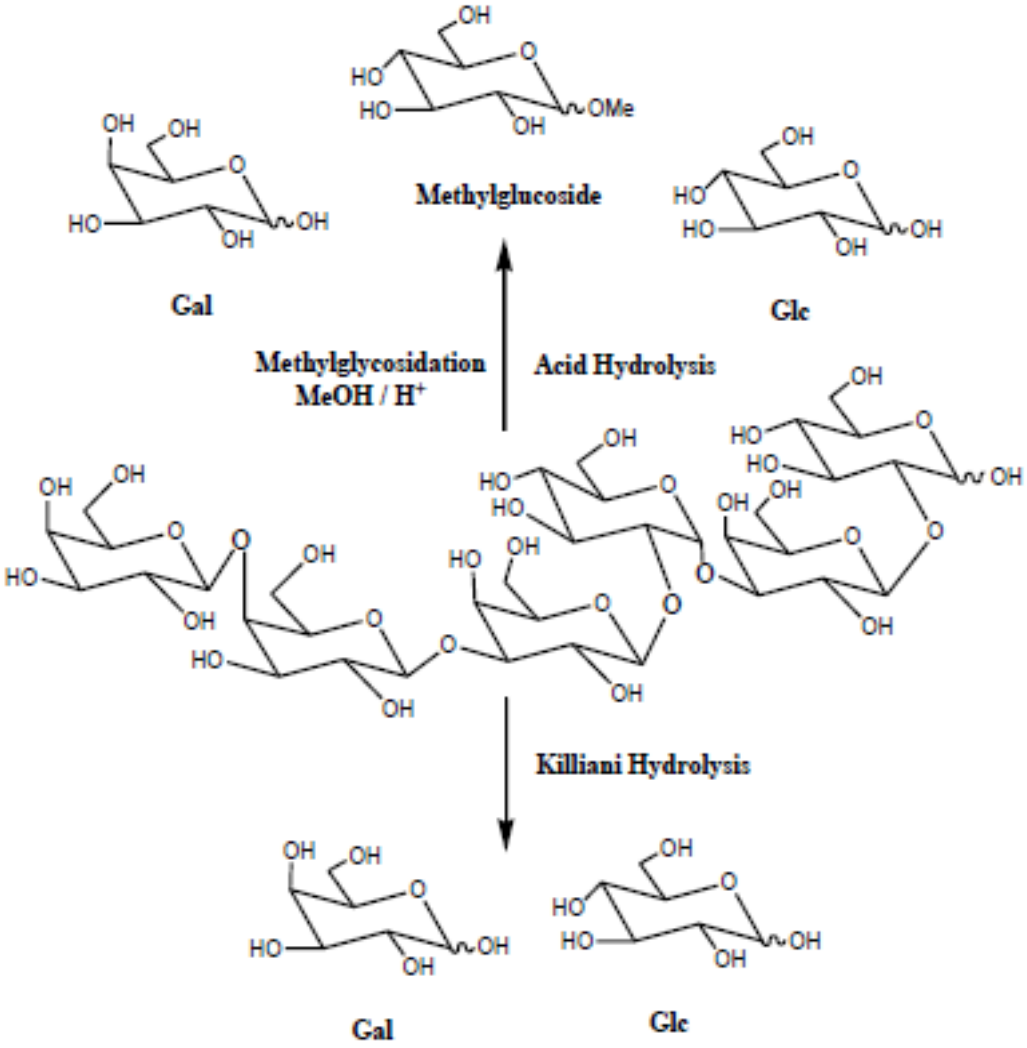
Fig : HSQC Spectrum of Aliose Acetate at 800 MHz in $CDCl_3$

Table 1. Assignment of anomeric protons and carbons of Aliose acetate by HSQC spectrum.

Sugar	^{13}C	^{1}H
S1	91.617	5.646
S2	101.063	4.460
S3	91.617	5.646
S4	101.318	4.44
S5	101.063	4.439
S6	101.063	4.046

The six monosaccharide constituents of Aliose have been designated as S1, S2, S3, S4, S5 and S6 for convenience starting from the reducing end. To confirm the monosaccharides present in Aliose, it was hydrolyzed under strong acidic conditions (Kiliani Hydrolysis). In Kiliani hydrolysis the reducing hexasaccharide gave spots on TLC and paper chromatography which were identified as Glc and Gal with authentic samples (paper chromatography) confirming that the

reducing hexasaccharide was made up of these two types of monosaccharide units i.e. Glc and Gal. The ^{1}H and ^{13}C NMR spectra of Aliose justify the seven anomeric signals for hexasaccharide with total integral intensity of six anomeric protons and carbons. Further the mass ion peak at 990 $[M]^{+}$ present in ES-MS of Aliose was in agreement with molecular formula **$C_{36}H_{62}O_{31}$**.



Methylglycosidation / Acid Hydrolysis and Killiani Hydrolysis of Aliose

The anomeric proton signal present at δ5.65 in ^{1}H NMR spectrum of Aliose acetate assigned to β-Glc(S-1) gave three cross peaks at δ5.65×3.80, δ5.65×5.00 and δ5.65×5.20 in TOCSY spectrum of Aliose acetate which was later identified as H-2, H-3 and H-4 of reducing Glc respectively by COSY spectrum of Aliose acetate. The chemical shift of the cross peak at δ5.65×3.80 suggested that in glucose S-1, H-2 position was available for glycosidic linkage by next monosaccharide unit. Further the ^{1}H signal present at δ3.80 assigned to H-2 of reducing Glc (S-1) gave a cross peak at δ3.80x101.06 in HMBC spectrum of Aliose acetate which was between H-2 of reducing Glc and C-1 of S-2, confirmed the (1→2) linkage between Glc (S-1) and S-2.

The anomeric carbon of S-2 at δ101.06 gave its complimentary anomeric proton signal at δ4.46 (8.0 Hz) in the HSQC spectrum of Aliose acetate. The chemical shift values of anomeric carbon at δ101.06 and anomeric proton at δ4.46 were having resemblance with literature value of anomeric chemical shift value of Gal hence S-2 was confirmed as Gal (Yoon et al, 2003).The large coupling constant J=8.0 Hz of Gal (S-2) confirmed the β-configuration of the linkage between Glc (S-1) and Gal (S-2).

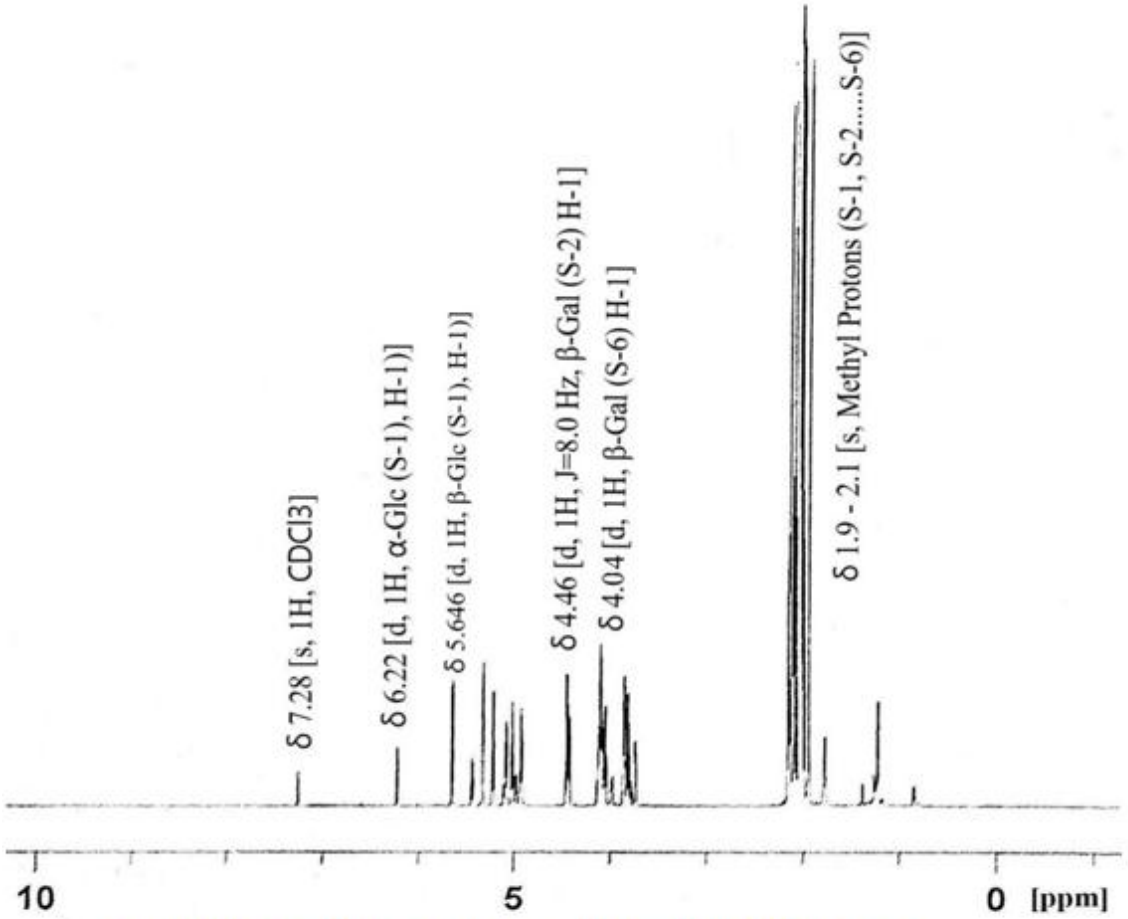


Fig: ^{1}H NMR spectrum of Aliose acetate at 800 MHz in $CDCl_3$

The next anomeric proton signal present at δ4.46 in ^{1}H NMR spectrum of Aliose acetate assigned to β-Gal(S-2) gave three cross peaks at δ4.46×3.79, δ4.46×4.30 and δ4.46×4.80 in TOCSY spectrum of Aliose acetate, which was later identified as H-3, H-4 and H-2 respectively with COSY spectrum of Aliose acetate. Out of these signals one proton signal at δ3.79 corresponded to H-3 position of β-Gal (S-2) suggested that H-3 was available for glycosidic linkages by the next monosaccharide unit. Further the ^{1}H signal present at δ3.79 assigned to H-3 of Gal.

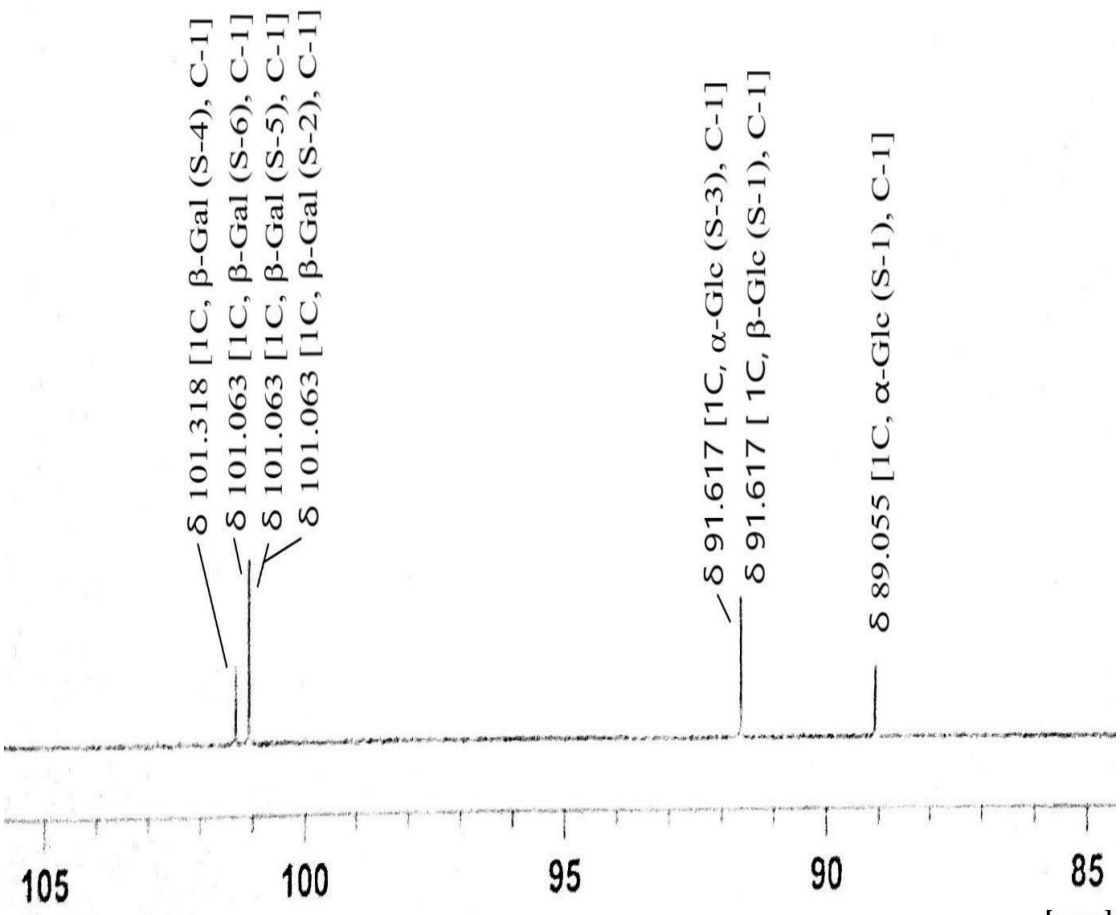


Fig : ^{13}C NMR Spectrum of Aliose Acetate at 800 MHz in $CDCl_3$

(S-2) gave a cross peak at δ3.79 ×91.61 in HMBC spectrum of Aliose acetate which was between H-3 of Gal (S-2) and C-1 of S-3, confirmed the (1→3) linkage between Gal (S-2) and S-3. The anomeric carbon of S-3 atδ91.61 gave its complimentary anomeric proton signal at δ5.64 (2.3 Hz) in the HSQC spectrum of Aliose acetate.The chemical shift values of anomeric carbon at δ91.61 and anomeric proton at δ5.64 were having resemblance with literature value of anomeric chemical shift value of Glc hence S-3 was confirmed as Glc (Yoon et al, 2003).The small coupling constant J=2.3 Hz of Glc (S-3) confirmed the α-configuration of the linkage between Gal(S-2)and Glc (S-3).The next anomeric proton signal present at δ5.64 in ^{1}HNMR spectrum of Aliose acetate assigned to Glc (S-3)gave thr cross peaks at δ5.64×3.83, δ5.64×4.41 and δ5.64×4.65 in TOCSY spectrum of Aliose acetate, which was later identified as H-2, H-3 and H-4 respectively with COSY spectrum of Aliose acetate. Out of these signals one proton signal at δ3.83 corresponded to H-2 position of Glc (S-3) suggested that H-2 was available for glycosidic linkage by the next monosaccharide unit. Further the ^{1}H signal present at δ3.83 assigned to H-2 of Glc (S-3) gave a cross peak at δ3.83×101.31 in HMBC spectrum of Aliose acetate which was between H-2 ofGlc (S-3) and C-1 of S-4, confirmed the (1→2) linkage between Glc (S-3) and S-4.

The anomeric carbon of S-4 at δ101.31 gave its complimentary anomeric proton signal at δ4.44 (8.0 Hz) in the HSQC spectrum of Aliose acetate. The chemical shift values of anomeric carbon at δ101.31 and anomeric proton at δ4.44 were having resemblance with literature value of anomeric chemical shift value of Gal (Yoon et al, 2003) hence S-4 was confirmed as Gal. The large coupling constant J=8.0 Hz of β-Gal (S-4) confirmed the β-configuration of the linkage between Glc (S-3) and Gal (S-4).The next anomeric proton signal present at δ4.44 in ^{1}H NMR spectrum of Aliose acetate assigned to Gal (S-4) gave three cross peaks at δ4.44×3.85, δ4.44×5.00 and δ4.44×5.10 in TOCSY spectrum of Aliose acetate, which were later identified as H-3, H-2 and H-4 respectively with COSY spectrum of Aliose acetate. Out of these signals one proton signal at δ3.85 corresponded to H-3 position of Gal (S-4) suggested that H-3 was available for glycosidic linkages by the next monosaccharide unit. Further the ^{1}H signal present at δ3.85 assigned to H-3 of Gal (S-4) gave a cross peak at δ3.85×101.06 in HMBC spectrum of Aliose acetate which was between H-3 of Gal (S-4) and C-1 of S-5, confirmed the (1→3) linkage between Gal (S-4) and S-5. The anomeric carbon of S-5 at δ101.06 gave its complimentary anomeric proton signal at δ4.44 (8.0 Hz) in the HSQC spectrum of Aliose acetate. The chemical

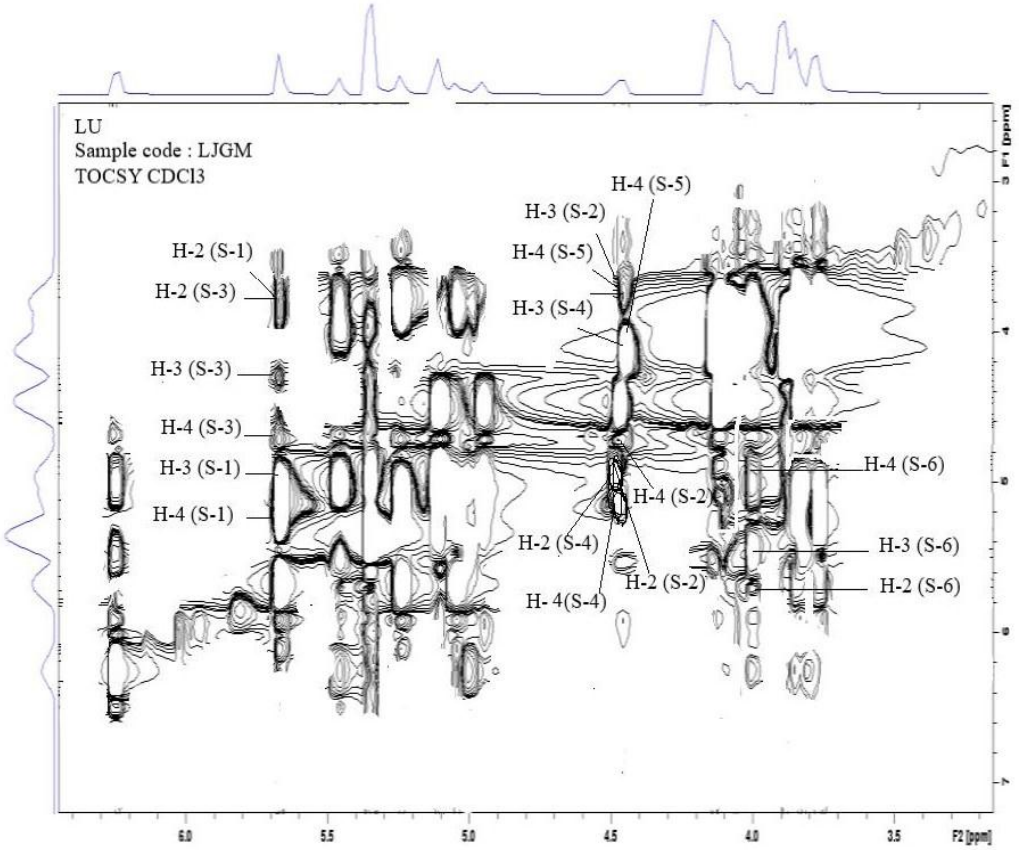


Fig : TOCSY Spectrum of Aliose Acetate at 800 MHz in $CDCl_3$

shift values of anomeric carbon at δ101.06 and anomeric proton at δ4.44 were having resemblance with literature value of anomeric chemical shift value of Gal (Yoon et al, 2003) hence S-5 was confirmed as Gal. The large coupling constant J=8.0 Hz of Gal (S-5) confirmed the β-configuration of the linkage between Gal (S-4) and Gal (S-5).

The next anomeric proton signal present at δ4.44 in ^{1}H NMR Spectrum of Aliose acetate assigned to Gal (S-5) gave three cross peaks at δ4.44×3.86, δ4.44×4.00 and δ4.44×4.20 in TOCSY spectrum of Aliose acetate, which were later identified as H-4, H-3 and H-2 respectively with COSY spectrum of Aliose acetate.

Out of these signals one proton signal at δ3.86 corresponded to H-4 position of Gal (S-5) suggested that H-4 was available for glycosidic linkages by the next monosaccharide unit. Further the ^{1}H signal present at δ3.86 assigned to H-4 of Gal (S-5) gave a cross peak at δ3.86×101.06 in HMBC spectrum of Aliose acetate which was between H-4 of Gal (S-5) and C-1 of S-6, confirmed the (1→4) linkage between Gal (S-5) and S-6. The anomeric carbon of S-6 at δ101.06 gave its complimentary anomeric proton signal at δ4.05 (7.52 Hz) in the HSQC spectrum of Aliose acetate. The chemical shift values of anomeric carbon at δ101.06 and anomeric proton at δ4.05 were having resemblance with literature value of anomeric chemical shift value of Gal hence S-6 was confirmed as Gal (Yoon et al, 2003).

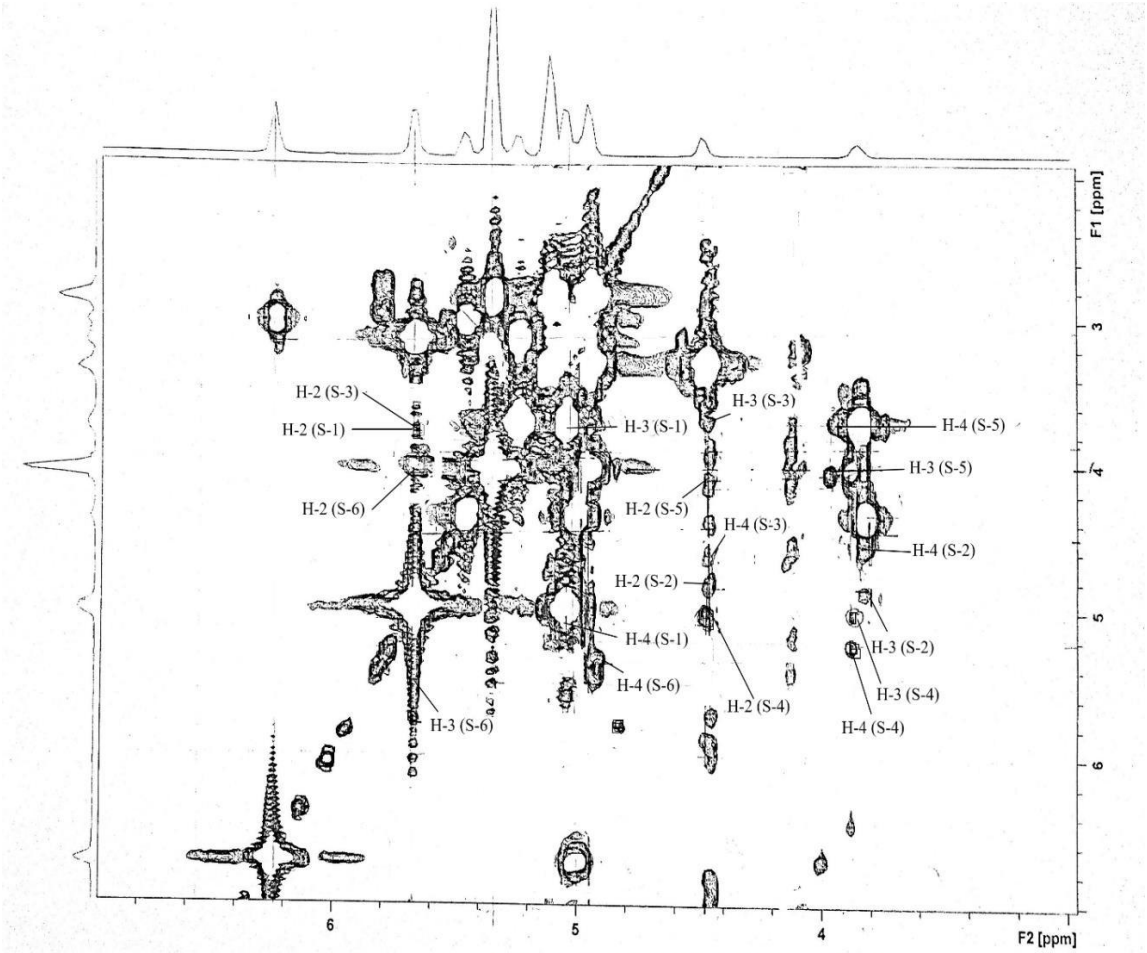


Fig : COSY Spectrum of Aliose Acetate at 800 MHz in $CDCl_3$

The large coupling constant J=7.52 Hz of Gal (S-6) confirmed the β-configuration of the linkage between Gal (S-5) and Gal (S-6). Further next anomeric proton signal present at δ4.05 in ^{1}H NMR Spectrum of Aliose acetate assigned to Gal (S-6) gave three cross peaks at δ4.05×4.80, δ4.05×5.70 and δ4.05×5.88 in TOCSY spectrum of Aliose acetate, which was later identified as H-4, H-2 and H-3 respectively with COSY spectrum of Aliose acetate. Since in the TOCSY spectrum of Aliose acetate did not show any complementary methine signals of Gal (S-6) in glycosidic linkage region i.e., δ3-4 showed that none of their OH groups were involved in glycosidic linkages suggested that Gal (S-6) was present at the non-reducing end. All the ^{1}H NMR assignments for ring proton of monosaccharide units of Aliose were confirmed by HOMO COSY and TOCSY experiments.

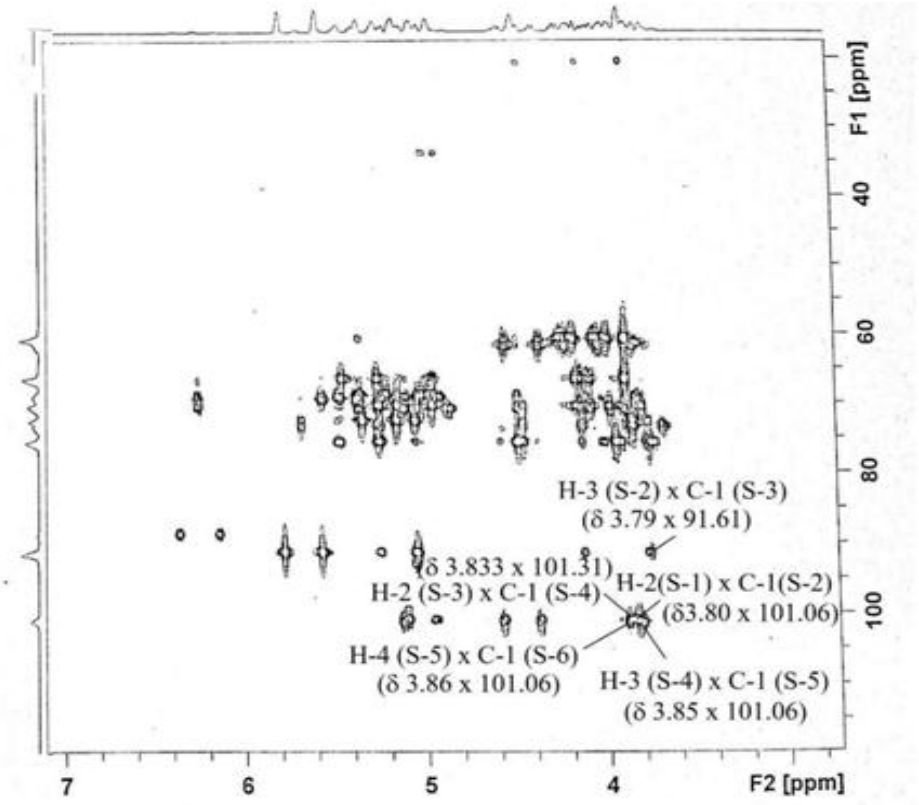


Fig : HMBC Spectrum of Aliose Acetate at 800 MHz in $CDCl_3$

Table 2. Order of ring protons in monosaccharides assigned by TOCSY spectrum.

Sugars / Ring Protons	S1	S2	S3	S4	S5	S6
H1	5.65	4.46	5.65	4.44	4.44	4.05
H2	5.20	4.80	4.65	5.10	4.20	5.88
H3	5.00	4.30	4.41	5.00	4.00	5.70
H4	3.80	3.79	3.83	3.85	3.86	4.80

The Heteronuclear Single Quantum Coherence (HSQC) spectrum of acetylated compound Aliose confirmed linkages in ^{1}H and ^{13}C NMR spectra by showing cross peaks of Glc (S-1) H-2 and C-2 at (δ3.80x75.06) showed (1→2) linkage of S-2 and S-1 i.e. its 2-position of Glc (S-1) were involved in linkage, Gal (S-2) H-3 and C-3 (δ3.79x72.61) showed (1→3) linkage of S-3 → S-2, Glc (S-3) H-2 and C-2 at (δ3.83x76.28) showed (1→2) linkage of S4→S3, Gal (S-4) H-3 and C-3 (δ 3.85 x 75.61) showed (1→3) linkage of S-5→ S-4, Gal (S-5) H-4 and C-4 at (δ3.86 x 73.28) showed (1→4) linkage of S-5 and S-6 showing in the same chemical region in acetylated and deacetylated spectra. It was further confirmed by the presence of same peaks in COSY and TOCSY spectrum. Thus based on the pattern of chemical shifts of ^{1}H, ^{13}C, HOMO COSY, and TOCSY, HSQC NMR experiments, it was interpreted that the compound Aliose was a hexasaccharide comprised of two Glc and four Gal moieties in it. The rarity of the compound Aliose was that it contained β-Gal (1→2) Glc instead of β-Gal (1→4) Glc (Lactose) present at the reducing end which is found in most of the milk oligosaccharides. The stereoscopic structure of Aliose was confirmed as under-

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

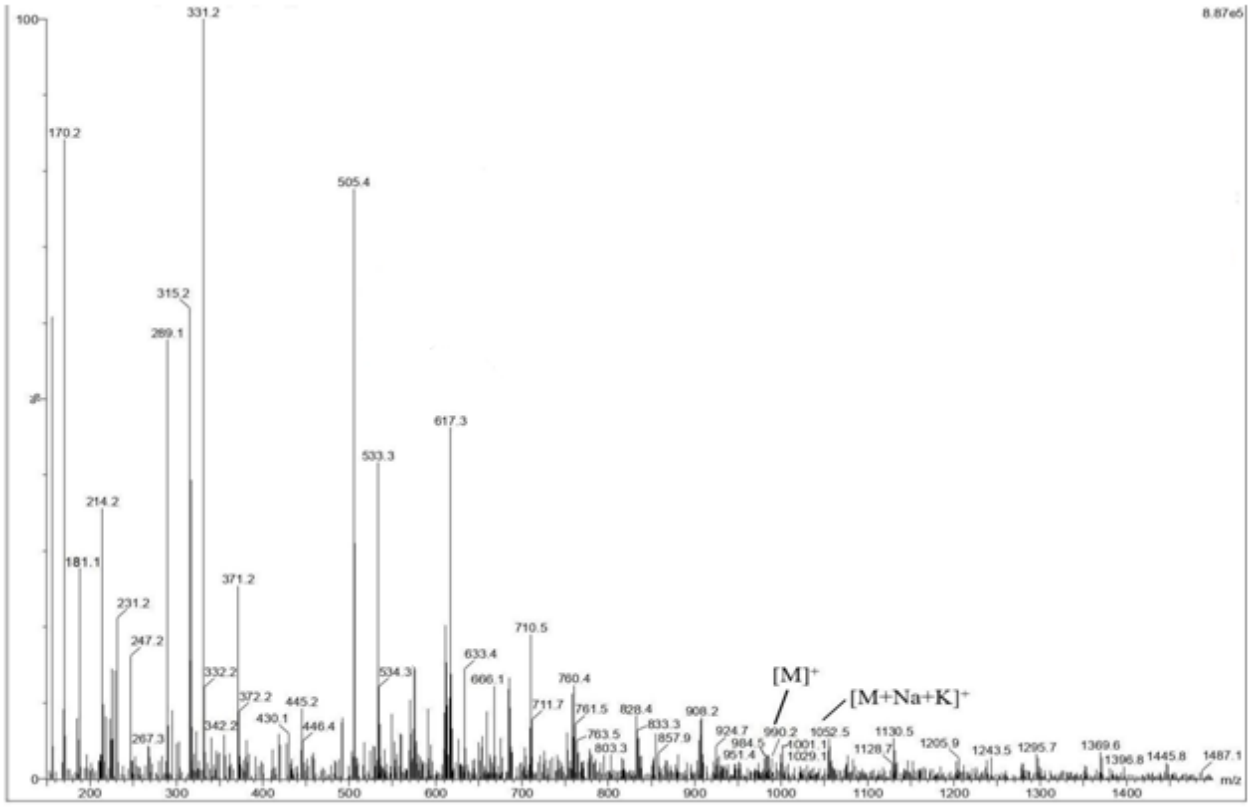


Fig: Mass spectrum of Aliose

Table 3. Assignment of ring proton correlation by COSY spectrum of Aliose acetate.

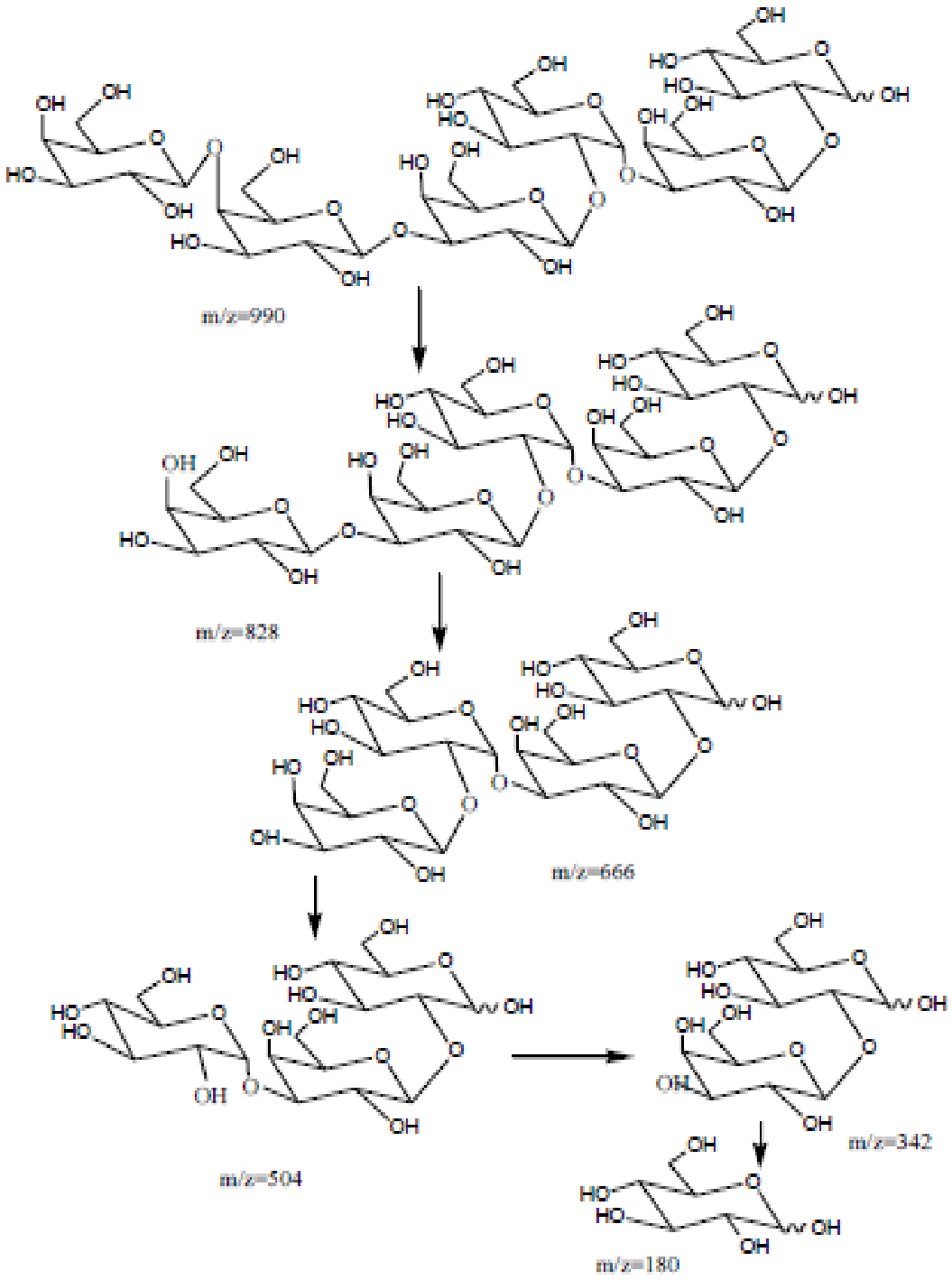
Monosaccharide / Ring Protons	S1	S2	S3	S4	S5	S6
H1	5.65	4.46	5.65	4.44	4.45	4.05
H2	3.80	4.80	3.83	5.00	4.20	5.70
H3	5.00	3.79	4.41	3.85	4.00	5.88
H4	5.20	4.30	4.65	5.10	3.86	4.80

Table 4. Assignment of glycosidic linkages by HMBC spectrum of Aliose acetate.

Monosaccharide Linkages	HMBC cross peaks	Types of Linkages
S1-S2	δ5.65×3.80	1→2 (β-GalS2→β-GlcS1)
S2-S3	δ3.79 ×91.61	1→3 (α-GlcS3→β-GalS2)
S3-S4	δ3.83×101.31	1→2 (β-GalS4→α-GlcS3)
S4-S5	δ3.85x101.06	1→3 (β-GalS5→β-GalS4)
S5-S6	δ3.86×101.06	1→4 (β-GalS6→β-GalS5)

Mass Fragmentation

The Electro spray Mass Spectroscopy data of compound not only confirmed the derived structure but also supported the sequence of monosaccharide in Aliose. The highest mass ion peak was recorded at m/Z 1052 and 1029 which was due to $[M+Na+K]^+$ and $[M+K]^+$ respectively. The other mass ion peak recorded at m/z 990 was due to $[M]^+$ confirming the molecular weight of Aliose as 990 and was in agreement with its molecular formula $C_{36}H_{62}O_{31}$. The mass fragments were formed by repeated H transfer in the hexasaccharide and was accompanied by the elimination of terminal sugar less H_2O.The hexasaccharide m/z 990 (I) fragmented to give mass ion at m/z 828 (II) [990-162(S6)], this fragment arose due to the loss of Gal (S-6) moiety from hexasaccharide. The fragment (II) at m/Z 828 arose due to the loss of Gal (S-6) was further fragmented to give mass ion peak at m/z 666 (III) [828 -162(S5)] which was a tetrasaccharide (III) arose due to loss of Gal (S-5) moiety from pentasaccharide (II).The tetrasaccharide (III) again fragmented to give mass ion peak at m/z 504 (IV) [666-162 (S4)], which was due to the loss of Gal (S-4) moiety from tetrasaccharide (III). The trisaccharide (IV) was again fragmented to give mass ion peak at m/z 342 (V) [504-162 (S-3)], which was due to the loss of Glc (S-3) moiety. The disaccharide (V) was again fragmented to give mass ion peak at 180 (VI) [342-162(S2)], which was due to the loss of Gal (S-2) moiety from disaccharide (V). These five mass ion peaks II, III, IV, V and VI appeared due to the consequent loss of S-6, S-5, S-4, S-3 and S-2 from original molecule. The other fragmentation pathway for the compound Aliose, m/z 990 are 951 [M-$2H_3O$+-H], 908[951-CH_3CO], 857[908-CH_3OH-H3O+], 833[908-CH_3-CH_2OHCHO], 828[857-CHO], 803[833-HCHO], 763[828-$2CH_3OH$-H+], 761[763-2H+] OR [828-OH-H_2O-CH_3OH], 760[761-H+], 711[761-H_2O-CH3OH], 710[760-H_2O-CH_3OH], 666[M-S6-S5], 633[666-HCHO], 617[633-CH_3-H+], 575[617-CH_2CO], 533[575-CH_2CO] OR [617-$2CH_2CO$], 505[575-$3H_2O$-CH_3-H+], 445[505-HOCHCHOH], 430[445-CH_3], 371[505-CH_2OHCHO-HCHO-CH_3CHO] OR [430-CH_2OCHO], 315 [331-CH_3-H+], 289[331-CH_2CO], 267[331-CH_3CHO-H_3O^+-H], 247[331-$2CH_2CO$] OR [289-CH_2CO], 231[247-CH_3-H+], 214 [231-OH], 181 [342+H^+-162], 170[214-CH_3CHO].

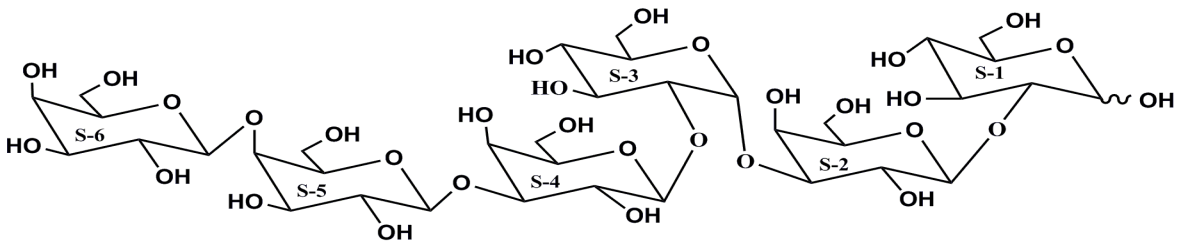


Mass-Fragmentation of Compound Aliose

Based on results obtained from chemical degradation/acid hydrolysis, chemical transformation, Electro spray mass spectrometry and $^{1}H,^{13}C$ NMR and HOMO COSY,TOCSY, HSQC and HMBC 2-D NMR techniques the structure and sequence of isolated sheep milk oligosaccharide Aliose was deduced as under-

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

Aliose



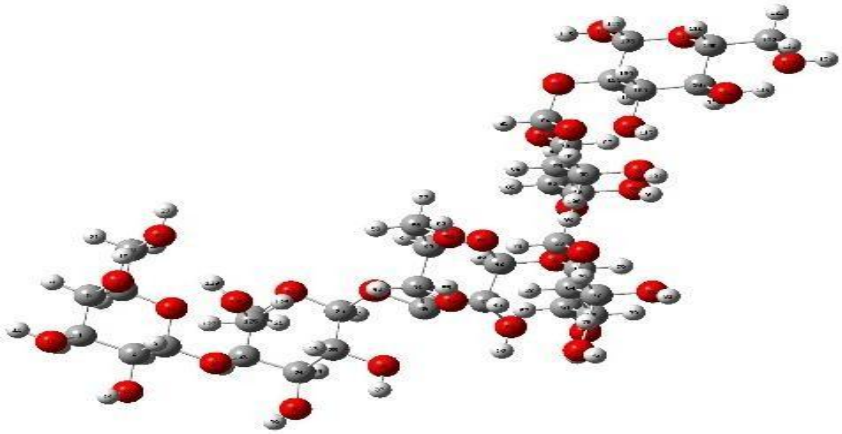
Aliose

COMPUTAIONAL ANALYSIS OF ISOLATED OLIGOSACCHARIDE ALIOSE

The computational studies were performed on Aliose using Density Functional Theory (DFT) of Gaussian 09 W programme. For this, structure of Aliose was drawn on Gauss View 5.0 and was further optimized. The optimized structure was utilized for the calculations of molecular orbitals, i.e., HOMO and LUMO. Other evaluations like bond lengths, bond angles, and molecular electrostatic potential and mulliken charges were also performed from the optimized geometry.

Optimised Structure of Aliose (Tiwari et al, 2023)

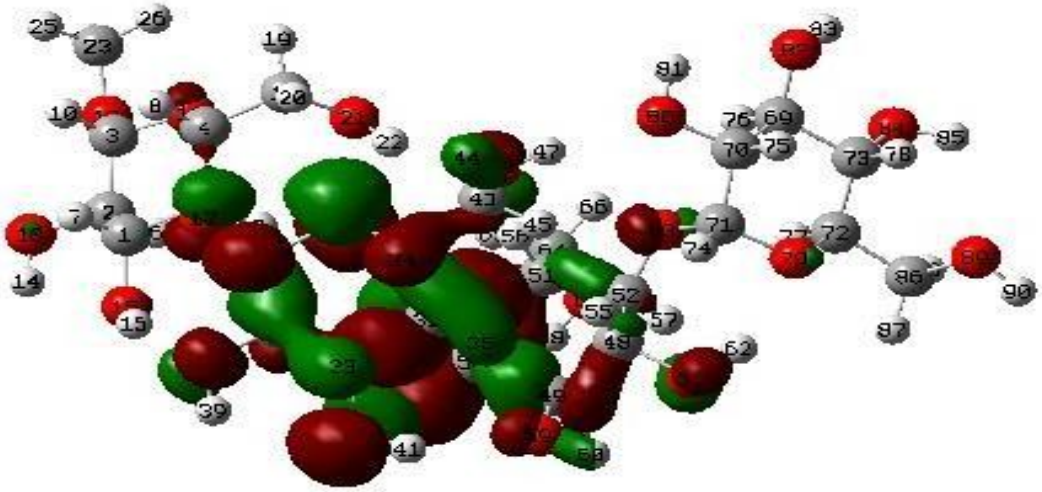
The structural geometry was optimized by minimizing its energies compared to all geometrical variables without forcing any molecular symmetry restrictions. The molecular structure of the optimized compounds was drawn by Gauss View 5.0.



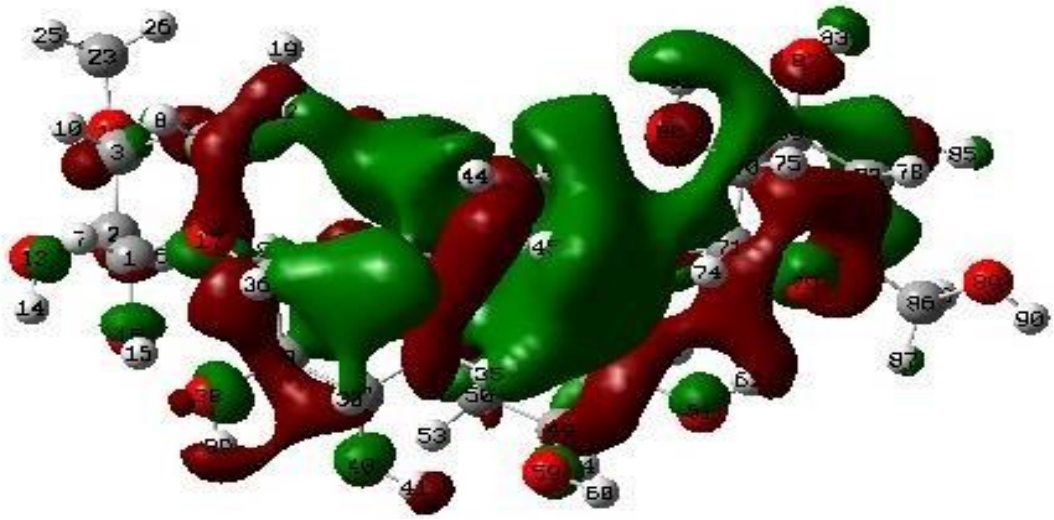
Frontier Molecular Orbitals

Frontier molecular orbitals (FMOs) are the highest occupied molecular orbital (HOMO) with electrons, so it is an electron donor and the lowest unoccupied molecular orbital (LUMO) that has a space to accept electrons, so it is an electron acceptor. These orbitals control the mode of the interaction of the compounds with the receptors. Moreover, HOMO and LUMO are very important quantum chemical parameters to determine the reactivity of the molecules and are used to calculate many important parameters such as the chemical reactivity descriptors.

Structure of HOMO of Aliose



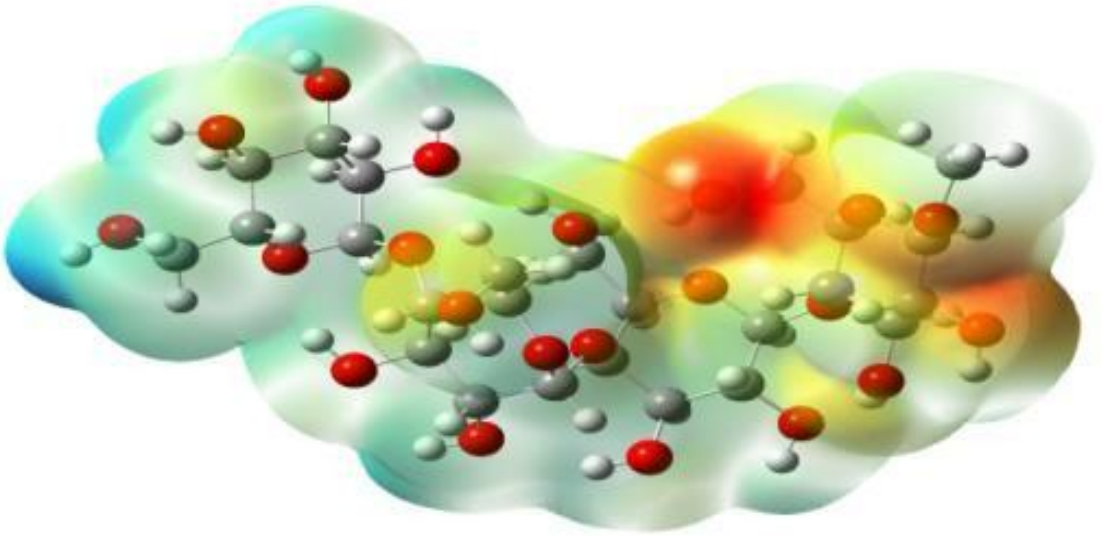
Structure of LUMO of Aliose



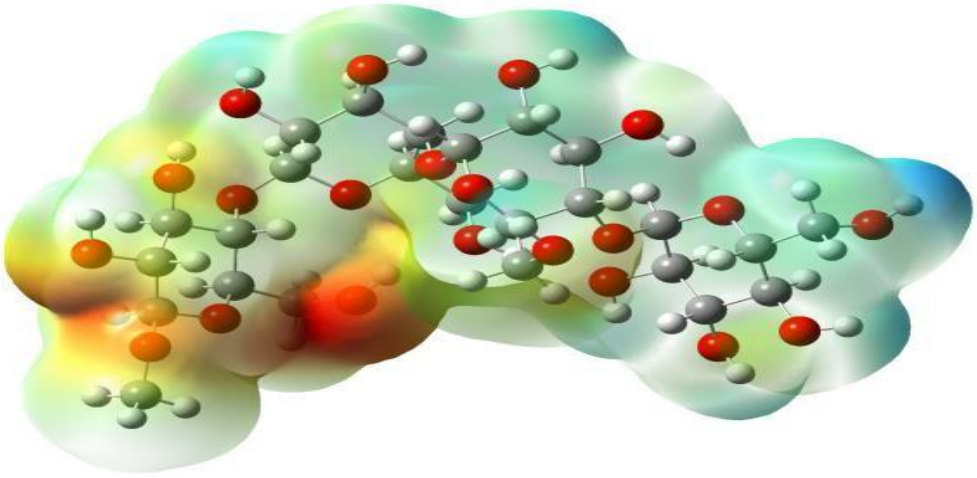
Molecular Electrostatic Potential (Ghous et al, 2023)

Regions of high and low electron density were obtained from MESP and ESP plots. Red colour indicates the nucleophilic region while blue colour indicates the electrophilic region. Light blue colour, green and yellow colour shows electron deficient, neutral and electron rich centers.

Total electron density of Aliose



Total Electron Density of Aliose



Global Reactivity Descriptors

A good approach to predict global reactivity trends is to compute reactivity descriptors such as electro negativity (χ) = –1/2(εLUMO + εHOMO), chemical potential (μ) = 1/2(εLUMO + εHOMO), global hardness(η) =1/2(εLUMO–εHOMO), global softness(S)= 1/2η and electrophilicity index (ω) = μ2 /2η electro negativity, chemical potential (μ), global hardness (η), global softness (S), ΔNmax, and electro philicity index (ω) had been calculated and listed in following table.

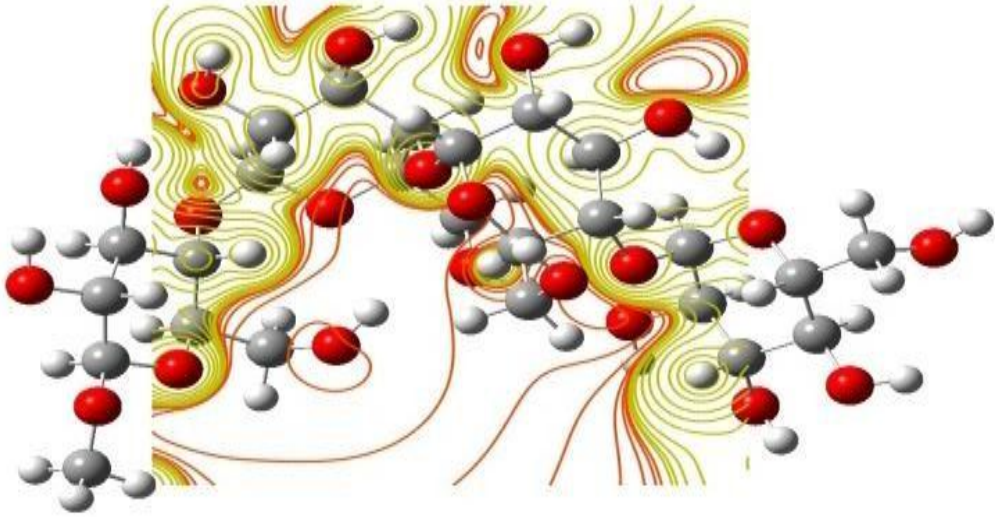
According to Koopman's theorem, the first ionization theory of a closed shell molecular system (Hartree-Fock theory (HF)) is equal to the negative of the orbital energy of the highest occupied molecular orbital (HOMO),this ascertains chemical reactivity and site selectivity. Global hardness (η) measures the resistance to change in the electron density around the molecule.

Sugar	HOMO	LUMO	E	χ	η	S	ω	μ
Aliose	-1.91	-8.80	6.89	5.35	3.4	0.09	1.57	5.35

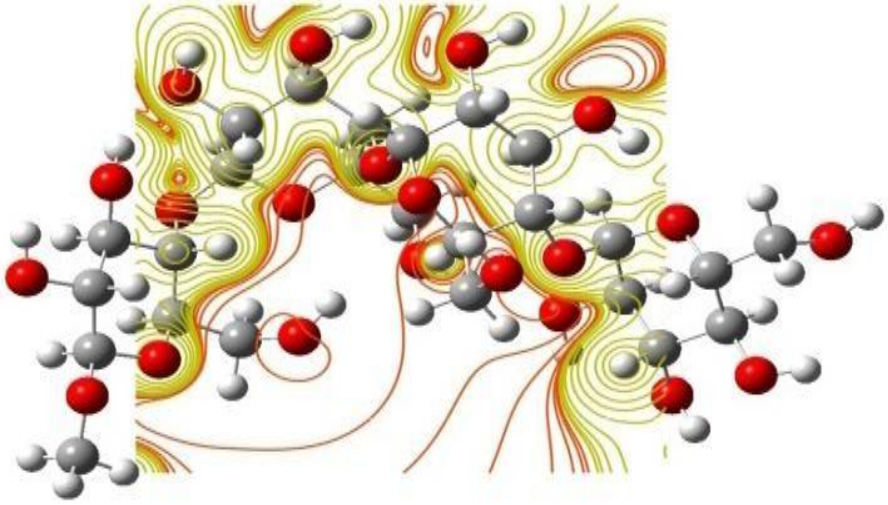
Contour Surfaces

Various contour surfaces such as electrostatic potential contour surfaces, HOMO contour surfaces and LUMO contour surfaces of the isolated hexasaccharide Aliose were obtained by DFT calculations using Gauss09W programme and B3LYP basis set and mentioned below-

1. HOMO Contour Surface of Aliose



2. LUMO Contour Surface of Aliose



Mulliken Charges

The Mulliken atomic charges of the estimated compounds were calculated by the DFT using B3LYP basis set, the data for compound is arranged in the following Table. The negative charge was delocalized on O11, O12, O13, O15, O17, O37, O38, O40, O42, O46, O58, O59, O61, O79, O80, O82, O84 and O86. C-5 has negative mulliken charge because it involve in glycosidic linkage with the S2 ring. The carbons attached to O have high value of charges. The positively charged centers are the most susceptible sites for nucleophilic attacks. However, the most negatively charged centers are the most susceptible sites for electrophilic one. Sum of mulliken charges is-0.0000.

S.No.	Atom	Charge	S.No.	Atom	Charges
1	C1	0.076690	25	O46	-0.093813
2	C2	0.119201	26	C48	0.212980
3	C3	0.363926	27	C49	0.491889
4	C4	0.264944	28	C50	0.347319
5	C5	-0.081258	29	C51	0.256386
6	O11	-0.327697	30	C52	-0.172313
7	O12	-0.282947	31	O58	-0.204848
8	O13	-0.121949	32	O59	-0.155341
9	O15	-0.114877	33	O61	-0.133859
10	O17	-0.226101	34	O63	-0.152151
11	C18	0.037860	35	C64	-0.326051
12	O21	-0.056921	36	O67	-0.126788
13	C23	0.308498	37	C69	0.130410
14	C27	-0.194011	38	C70	-0.065358
15	C28	0.514576	39	C71	0.094168
16	C29	0.039237	40	C72	0.548971
17	C30	0.400948	41	C73	0.168311
18	C31	0.264796	42	O79	-0.2804110.
19	O37	-0.389193	43	O80	-0.147759
20	O38	-0.164735	44	O82	-0.166363
21	O40	-0.112907	45	O84	-0.171337
23	O42	-0.130613	46	C86	0.072211
24	C43	-0.116877	47	O89	-0.196742

Bond Length (Calais et al, 1993)

The theoretical calculation done by DFT using Gauss09W programme, the C-C, C-H and C-O bond lengths were obtained and arranged in the following table. These calculations showed that the bond length for C-C single bond was approximately 1.5299A$^{\circ}$ while bond length between C-H was approximately 1.1021A$^{\circ}$.The bond length for O-H was 0.9688A$^{\circ}$ while C-O bond length was 1.427 A$^{\circ}$.

S. No.	Parameters	Bond Length	S. No.	Parameters	Bond Length
1	R(C1,C2)	1.5299	31	R(C49,H54)	1.0978
2	R(C1,C5)	1.5313	32	R(C49,O59)	1.4332
3	R(C1,H7)	1.1021	34	R(C50,H53)	1.0932
4	R(C1,O15)	1.4249	35	R(C50,O58)	1.405
5	R(C2,C3)	1.5265	36	R(C51,C52)	1.5464
6	R(C2,H6)	1.0996	37	R(C51,H56)	1.0929
7	R(C2,O13)	1.4207	38	R(C51,O58)	1.4418
8	R(C3,H10)	1.108	39	R(C51,C64)	1.5243
9	R(C3,O11)	1.427	40	R(C52,H57)	1.0973
10	R(C3,12)	1.3859	41	R(C52,O63)	1.4511
11	R(C4,C5)	1.541	42	R(O59,H60)	0.9688
12	R(C4,H8)	1.1052	43	R(O61,H62)	0.9721
13	R(C4,O11)	1.4275	44	R(C48,O61)	1.4325
14	R(C4,O18)	1.5305	45	R(O63,C71)	1.3933
15	R(C5,H9)	1.0933	46	R(C64,H65)	1.0972
16	R(C5,O17)	1.4508	47	R(C64,H66)	1.0925
17	R(O12,C23)	1.4245	48	R(C64,O67)	1.4264
18	R(O13,H14)	0.9686	49	R(O67,H68)	0.9672
19	R(O15,H16)	0.9745	50	R(C69,C70)	1.5248
20	R(C17,O29)	1.3898	51	R(C69,H76)	1.0983
21	R(C18,H19)	1.0977	52	R(C69,O82)	1.4253
22	R(C18,H20)	1.0952	53	R(C70,C71)	1.5324
23	R(C18,O21)	1.4173	54	R(C70,H75)	1.0991
24	R(O21,H22)	0.9713	55	R(C70,H80)	1.4257
25	R(C23,H24)	1.0916	56	R(C71,H74)	1.0948
26	R(C23,H25)	1.0999	57	R(C71,O79)	1.4293
27	R(C23,H26)	1.0952	58	R(C72,C73)	1.5408
28	R(C27,C28)	1.5273	59	R(C72,H77)	1.0974
29	R(C27,C31)	1.536	60	R(C72,O79)	1.4487
30	R(C27,H33)	1.1026	61	R(C72,C86)	1.5208

Dihedral Angle

A dihedral angle is defined as the angle between two planes both of which pass through the same bond. There is much weaker preference for particular values of the dihedral angle around single bonds. Usually, the value of 0^{o} (eclipsed) is avoided, and values of around 60^{o} (staggered) to 90^{o} are somewhat preferred, depending on the number of lone pairs on the termini. Maximum dihedral angle is 177.3679° between C5, C4, C18 and H19atomsin the compound Aliose.

PARAMETER	DIHEDRAL ANGLE	PARAMETER	DIHEDRAL ANGLE
D(C5,C1,C2,C3)	-52.0355	D(C28,C27,O40,H41)	166.1652
D(C5,C1,C2,H6)	65.6911	D(C31,C27,O40,H41)	45.2192
D(C5,C1,C2,O13)	-172.3125	D(H33,C27,O40,H41)	-75.9602
D(H7,C1,C2,C3)	66.1709	D(27,28,29,17)	175.7344
D(H7,C1,C2,H6)	176.1025	D(O40,C27,C28,O38)	60.2934
D(H7,C1,C2,O13)	54.1061	D(C28,27,31,30)	58.8135
D(O15,C1,C2,H6)	-58.799	D(C28,C27,C31,H35)	176.1541
D(O15,C1,C2,O13)	63.1975	D(C28,C27,C31,O42)	-59.8669
D(C2,C1,C5,C4)	50.8354	D(H33,O27,C31,H35)	57.6796
D(C2,C1,C5,H9)	-69.7792	D(O11,C4,C5,O17)	-169.532
D(C2,C1,C5,O17)	168.6839	D(C18,C4,C5,C1)	-173.5013
D(H7,CCC1,5,4)	-67.7703	D(C18,CC4,C5,H9)	-51.3941
D(H7,C1,5C,H9)	171.6151	D(C18,C4,C5,O17)	69.8949
D(H7,C1,C5,O17)	50.0782	D(H33,C27,CO31,42)	-178.3413
D(O15,C1,C5,C4)	171.2674	D(O40,C27,C31,C30)	178.5095
D(O15,C1,C5,H9)	50.6528	D(C18,C4,O11,C3)	-173.3118
D(O15,C1,C5,O17)	-70.8841	D(C5,C4,C18,H19)	177.3679
D(C2,C1,O15,H16)	-177.4025	D(H33,O27,C31,C30)	-59.661
D(H7,C1,O15,H16)	-61.1348	D(H8,C4,O11,C3)	-56.7819
D(C1,C2,C3,O11)	55.3843	D(C5,C4,O11,C3)	61.6567
D(C1,C2,C3,O12)	173.7472	D(O11,C4,C5,H9)	69.1791
D(H6,C2,C3,O10)	176.4269	D(O11,C4,C5,1C)	-52.9282
D(H6,C2,C3,O11)	-63.5766	D(H8,C4,C5,O17)	-49.9955
D(H6,C2,C3,H12)	54.7863	D(H8,C4,C5,H9)	-171.2844
D(O13,C2,C3,O10)	56.0934	D(H8,C4,C5,C1)	66.6083
D(O13,C2,C3,O11)	176.0899	D(O11C,3,O12,C23)	-69.5215
D(O13,C2,C3O,12)	-65.5472	D(H10,C3,O12,C23)	50.109
D(C1,C2,O13,H14)	-48.6282	D(C2,C3,O12,C23)	171.5727
D(H6,C2,O13,H14)	72.6436	D(O12,C3,O11,H4)	178.0728
D(C2,C3,O11,H4)	-63.1376	D(HC10,3,O11,C4)	57.553
D(C5,C4,C18,H20)	-65.9763	D(C5,C4,C18,O21)	58.1775

D(H8,C4,C18,H19)	-62.415	D(H8,C4,C18,H20)	54.2408
D(H8,C4,C18,O21)	178.3945	D(O11,C4,C18,H19)	55.6595
D(O11,H4,C18,H20)	172.3153	D(O11,C4,C18,O21)	-63.531
D(C1,C5,O17,C29)	120.4412	D(C4,C5,O17,C29)	-120.9546
D(H9,C5,O17,C29)	-1.1504	D(C3,O12,C23,H24)	-175.126
D(C3,O12,C23,H25)	-56.644	D(C3,O12,C23,H26)	65.4486
D(C5,O17,C29,C28)	-59.7685	D(C5,O17,C29,H36)	-178.6434

Dipole Moment

Dipole moment is arises in any system in which there is a separation of charge. In compound Aliose carbon, oxygen and hydrogen atoms are present and among them carbon and hydrogens are positively charged whereas oxygen is negatively charged. Due to this charge separation, dipole moment was generated in the molecule. Dipole moment (field-independent basis, Debye) for the isolated hexasaccharide Aliose was also calculated or obtained by DFT calculations and mentioned below-

X=-11.8901 Y=-4.1202 Z=-3.6607 Tot=13

Conclusion of DFT studies

After complete study, an oligosaccharide 'Aliose' from Gaddi sheep (*Ovis aries orientalis*) milk oligosaccharide was isolated and its structure was elucidated with the help of 1-D and 2-D NMR spectroscopic techniques. Further DFT calculations were performed using Gauss09W software and B3LYP/6-31+G (d.p.) basis set which indicates that compound was found to be stable and different electrophilic and nucleophilic centres were present in the compound which indicates the reactive regions of the compound. The data of bond lengths, bond angle, dihedral angles and mulliken charges of the compound were also reported.

CONCLUSION

A Novel Hexasaccharide Aliose was isolated and its stereoscopic structure was elucidated with the help of 2D NMR and Mass spectrometry. It was found that Aliose contained β-Gal (1→2) Glc instead of β-Gal (1→4) Glc (Lactose) present at the reducing end which was found in most of the milk oligosaccharides. It also showed its rarity by having 1→2 and 1→3 glycosidic linkages in its structure. It is the first repot of this rare oligosaccharide from any natural or synthetic source.

ACKNOWLEDGEMENTS

Authors are thankful to the university grant commission New Delhi for Financial assistance, Director CBMR-SGPGI Lucknow for running the NMR spectrum and Head Department of Chemistry, University of Lucknow for providing Laboratory and Library facilities.

REFERENCES

Ashatnghridaye – (**Ashatnghridaye Dravdravya Vighania Adhyay 5, Dudhadhivarg, Shloka no. 27)** *Sushrut Sanhita* **(Sushrut Sanhita- Part I, Vol. 156, Shloka no. 54.**

Calais, J. L. (1993). Density-functional theory of atoms and molecules. *International Journal of Quantum Chemistry*, 47, 101.

Caroprese, M., Ciliberti, M. G., Albenzio, M., Marino, R., Santillo, A. and Sevi, A. (2019). Role of antioxidant molecules in milk of sheep. *Small Ruminant Research,* 180.79-85.

Coni, E.A., Bocca, P., Coppolelli, S., Caroli, C., Cavallucci and Trabalza, M. (1996). Minor and trace element contents in sheep and goat milk and dairy products. *Food Chemistry*, 57(2), 253-260.

Dubois, M., Gilles, K., Hamilton, J.K., Rebers, P.T. and Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry,* 28(3), 350-356.

Farag, M.A., Jomaa, S.A., Abd El-Wahed, A. and R El-Seedi, H. (2020). The Many Faces of Kefir Fermented Dairy Products: Quality Characteristics, Flavor Chemistry, Nutritional Value, Health Benefits, and Safety. *Nutrients*, 12, 346.

Fiegel, F. (1956). Spot test in organic analysis. *Elsevier publishing comp*., Amsterdam. Houston-London-New York.

Gangawar, L., Singh, R., Deepak, D. (2018). Structure Elucidation of a novel oligosaccharide (Medalose) from camel milk. *Journal of Molecular Structure*, 1153, 157-161.

Ghous, F., Shukla, S., Singh, R., Parveen, S., Banerjee, M., Bishnoi, A. (2023). Synthesis, crystal structure, computational investigation, molecular docking analysis and anti-lung cancer activity of novel (Z)-3-amino-2-(cyclohexylidenehydrazono) thiazolidin-4-one. *Journal of Molecular Structure*, 1285, 1354-62.

Kumar, K., **Srivastav, A. K., Deepak, D. (2016).** Isolation of a novel oligosaccharide from goat milk. *J. Biol. Chem. Research,* 33(1), 381-387.

Ranjan,A. K., Sharma, M., Shukla, M. and Deepak, D. (2023). Structure elucidation of a novel pentasaccharide 'Ariesose' from oviesaries milk by 2-dimentional NMR and mass spectrometry. *Trends in Carbohydrate Research*. 15(2), 9-21.

Shahi, S., Sigh, H.K., Shukla, C.S., Deepak, D. and Singh, S. K. (2020). The biological utilization of Gaddi sheep's milk oligosaccharides. *Journal of critical reviews,* 7(15), 2061-2068.

Shukla, M., Sharma, M., Singh, R. and Deepak, **D.(2023).**Structural characterization of novel milk hexasaccharide Omedose from Camel milk**.** *J. Biol. Chem. Research,* 40(1), 39-52.

Tiwari, N., Jamal, N. and Mishra, A. (2023). Virtual Screening of Phytochemicals in Search of a Potential Drug Candidate for COVID-19: DFT Study and Molecular Docking. *Bentham Science,* 18, 127-144.

Yoon, Y. and Sun, E. (2003). A conformational study of linkage positions in oligosaccharides investigated by 2D NMR spectroscopy and molecular modeling. *Bulletin of Korean Chemical Society*. 24(3), 339-344.

Corresponding author: Dr. Desh Deepak, Department of Chemistry, University of Lucknow, Lucknow-226007, India.
Email: deshdeepakraju@rediffmail.com