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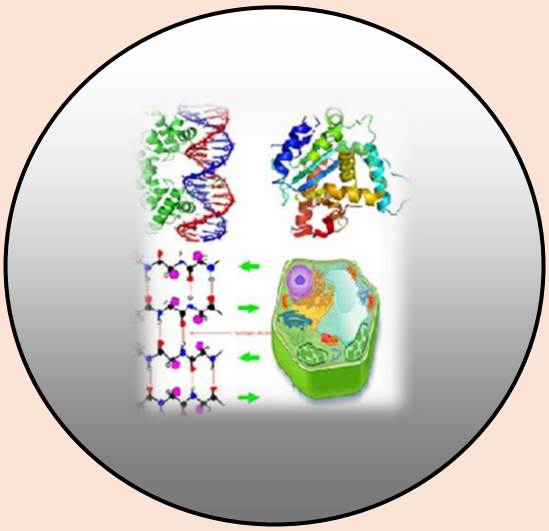

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Structural Characterization of Novel Nonasaccharide Arisose from Cow Colostrum

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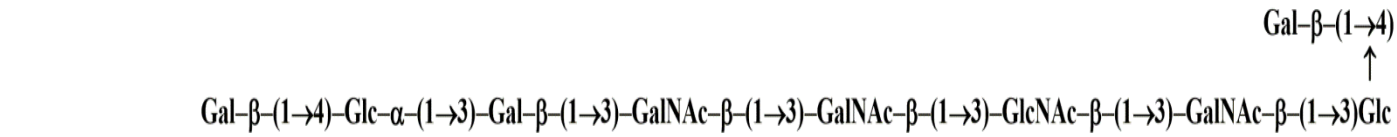
ABSTRACT

Colostrum is the highly nutritious rich fluid of the mammary glands produced by female mammals after parturition. It is recommended that mammalian neonates must ingest colostrum to develop passive immunity. It has been observed that cow colostrum is rich in protein, immunoglobulin, lactoferrin, Oligosaccharide, and growth factors. In Ancient letterature, it has been found that the importance of cow milk and cow colostrum suggested antibacterial effect and therapeutic effect for various chronic infections like bacterial, viral, parasitic or fungal. With a view of importance of isolated biologically active novel milk oligosaccharide, cow colostrum was collected in bulk (10 litre) and processed by modified method of Kobata and Ginsberg leading to isolation of oligosaccharide mixture which on gel filtration, HPLC, chemical transformation and column chromatography resulted into isolation of a novel oligosaccharide. Structure of this novel milk oligosaccharide Arisose was characterised on the basis of result obtained from ^{1}H, ^{13}C, 2D NMR (HSQC, COSY, TOCSY and HMBC) and ES-Mass spectrometry. The structure of novel nonasaccharide was deduced under,



Arisose

Keywords: Cow Colostrum, Arisose, Gel filtration, Column Chromatography and NMR.

INTRODUCTION

Milk obtained from any mammalian source is of two types. (1) is “Colostrum” i.e. early milk which is obtained after parturition and (2) is “mature milk” i.e. normal milk. The colostrum has a nutrient profile and immunological composition substantially higher than normal milk. So, colostrum is a mammary-derived source that is highly enriched in growth factors and specific proteins that promote development and improve immunity in newborns (Godhia et al., 2013 And Boudry et al., 2008). Carbohydrate is one of the other major components of milk, which contains lactose and oligosaccharides. These oligosaccharides show enormous biological activity such as immunostimulant, anti-tumour, anti-cancer, anti-inflammatory (Singh et al., 2019), anti-complementary (Saksena et al., 1999), antiviral, antimicrobial (Ying at al., And Singh at al., 2018), antioxidant, hypoglycemic activity, lipid lowering (Halas et al., 2012) and regulation of mineral absorption are reported in medicinal literature (Poeikhampha et al., 2011). From the previous investigation, it was observed that these oligosaccharides obtained from different mammals show specific properties in different areas. As Goat milk oligosaccharides have an important role in intestinal protection and repair after a change caused by DSS (dextron sodium sulphate) induced colitis and have shown their implication in human intestinal inflammation (Federico et al., 2006). Donkey milk oligosaccharides hand has the ability to stimulate non-specific and specific immunological resistance (Deepak et al., 1998). The oligosaccharide isolated from elephant milk contained a high ratio of sialyl oligosaccharides which may be significant with respect to the formation of brain components such as gangliosides of suckling claves (Osthoff et al., 2007). Mare’s milk oligosaccharides include numerous biological activities such as antioxidant and lipid lowering activities (Srivastava et al., 2012). Oligosaccharides obtained from cow milk reduce the adhesion of enterotoxin E. coli strain of the calf (Johansson et al., 2005). According to Ayurveda, cow colostrum has been described as nutritive and good for vital organs such as the eyes, brain and heart. It has promoted immunity and acts as Rasayana and Ojovardhaka and as a better immune booster. It has also improve the effect of Medha or intellect as a good memory-booster. According to Ayurveda, it is ideally suited as a brain tonic as it has the ability to pacify Vata dosha which is responsible for the proper functioning of the sensorium. However, more information about cow colostrum oligosaccharides is still developing. Cow milk or colostrum contains lactose (Gal-β-(1–4)-Glc) as the predominant carbohydrate as well as a variety of oligosaccharides, in which most of them contain lactose unit at their reducing ends (Sasaki et al., 2016 And Jenness et al., 1964). Keeping the importance of above-mentioned activities in mind Cow Colostrum was collected and processed by a modified method of Kobata and Ginsburg (Kobata and Ginsburg, 1970) and then it was purified by Sephadex G-25 Gel column. Further, the acetylation of oligosaccharides mixture was followed by the silica gel chromatography and led to the isolation of a novel oligosaccharide Arisose which gave a positive chemical test for normal and amino sugars. In the present study, we have elucidated the structure of a novel milk oligosaccharide namely Arisose with the help of chemical degradation, chemical transformation, spectroscopic techniques like (^{1}H NMR, ^{13}C NMR and 2D NMR) and Mass spectrometry.

MATERIAL AND METHODS

GENERAL PROCEDURE

General procedure was same as described in our previous communication (Maurya et al., 2017)

Isolation of Mare milk oligosaccharide by the modified method of Kobata and Ginsburg-

Isolation of cow colostrum oligosaccharides from 10 litre cow colostrum was done by the modified method of Kobata and Ginsburg method, which was described in our previous communication (Maurya et al., 2017), the isolation was done from 10 litre of cow colostrum and the yield of oligosaccharide mixture obtained 365 gm.

Acetylation of Mare milk oligosaccharide mixture

10 gm oligosaccharides mixture was obtained from cow colostrum, acetylated with pyridine (10 ml) and acetic anhydride (10 ml) at 60^{o}C and solution was stirred overnight. The mixture was evaporated at water bath at 60^{o}C under reduced pressure and viscous residue was taken in $CHCl_3$ (500 ml) and washed twice with cold water, evaporated to dryness yielding the acetylated mixture (8.1gm). The acetylation converted the free sugars into their non-polar acetyl derivatives which were resolved nicely on TLC, giving eight spots i.e. **a, b, c, d, e, f, g** and **h** of which one compound was finally separated by column chromatography over silica gel using varying proportions of hexane, chloroform and methanol as eluents. Detection of the spots was done by spraying with 50% H_2SO_4.

Purification of Acetylated Milk Oligosaccharides on Silica Gel Column:

Separation of the acetylated oligosaccharide mixture (8.1 gm) was carried out over silica gel (200 gm.) using varying proportion of $CHCl_3$, $CHCl_3$: MeOH as eluents, collecting fraction of 200 ml each. All these fractions were checked on TLC. So nine fractions, I (175mg), II (38 mg), III (1214 mg), IV (152mg), V (94mg), VI (70mg), VII (1.56g), VIII (750mg), IX (1.135mg), X (1.172mg) XI (470mg) and XII (168mg) were obtained. These fractions were the mixtures of two and three oligosaccharides which were further separated by repeated column chromatography. Fraction III was re-chromatographed on silica gel column and eluted with the varied proportion of $CHCl_3$:MeOH to yield chromatographically pure oligosaccharide 'b' (198mg).

Deacetylation of Compound (b) Arisose Acetate by NH_3/ Acetone

Compound 'b' (198 mg) was obtained from column chromatography of acetylated oligosaccharide mixture. 45mg of Compound 'b' was dissolved in acetone (3 ml) and 3.4 ml of NH_4OH 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 $CHCl_3$ and the water layer was finally freeze dried giving the deacetylated (Natural) oligosaccharide **B** (35mg).

Methylation/ acid hydrolysis of Compound B

Compound B (10 mg) was refluxed with absolute MeOH (2 ml) at 70ºC for 18 hrs in the presence of cation exchange IR-120 (H) resin. The reaction mixture was filtered while hot and filtrate was concentrated given one spot of lesser polarity.

To this reaction mixture of methylglycoside B, 1, 4-dioxane (1 ml), and 0.1N H_2S0_4 (1 ml) was added and the solution was warmed for 30minutes at 50ºC. The hydrolysis was complete after 24 hrs (TLC). The hydrolyzate showed two spots on TLC, the spot with most polar mobility was found identical mobility with the authentic sample of Gal while the spots with faster mobility on reduction with NaOMe gave methylglucoside which was confirmed by comparison with authentic samples (TLC, PC) of *α*-and *β*-methylglycosides showing that in the trisaccharide the reducing sugar was glucose

Kiliani Hydrolysis of Compound B Arisose

Compound B (5 mg) was dissolved in 2 ml Killiani mixture (AcOH-H2O-HCl, 7:11:2) and heated at 100ºC for 1 hrs. followed by evaporation under reduced pressure. It was dissolved in 2 ml of H2O and extracted twice with 3 ml CHCl3. The aqueous residual solution was made neutral by addition of 1-2 drops of 2N NaOH and was evaporated under reduced pressure to afford Glu-Gal and GalNAc which further confirmed by Paper chromatography

Description of isolated compound Arisose

Compound B, Arisose:

$[\alpha]_D^{25}$ =+34º (*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.

$C_{62}H_{104}O_{46}N_4$		%C	%H	%N
	Calculated	**45.36**	**6.34**	**3.41**
	Found	**45.37**	**6.39**	**3.41**

^{1}H NMR of Compound-b, Arisose Acetate in $CDCl_3$ at 300 MHz.

δ6.09[d, 1H, J=3.9Hz, *α*-Glc(S-1) H-1], δ5.29[d, 1H, J=8.1Hz, *β*-Glc(S-1) H-1], δ5.28[d, 1H, J=3.9Hz, α-Glc(S-8) H-1], δ4.67[d, 1H, J=7.5Hz, *β*-GlcNAc(S-4) H-1], δ4.59[d, 1H, J=8.1Hz, *β*-Gal(S-7) H-1], δ4.50[d, 1H, J=8.1Hz, *β*-Gal(S-2) H-1], δ4.49[d, 1H, J=8.1Hz, *β*-Gal(S-9) H-1], δ4.43[d, 2H, J=7.5Hz, *β*-GalNAc(S-3) & (S-5) H-1], δ4.41[d, 1H, J=7.8Hz, *β*-GalNAc(S-6) H-1], δ3.75[m, *β*-GalNAc(S-2, S-3, S-4, S-6) & *β*-Glc(S-1) H-3], δ3.73[m, 2H, *α*-Gal(S-7) & *α*-Glc(S-8) H-3], δ3.49[m, 1H, *β*-Glc (S-1) H-4].

^{13}C NMR of Compound-b, Arisose Acetate in $CDCl_3$ at 300 MHz.

δ91.5[1C, *α*-Glc(S-1) C-1], δ90.43[1C, *β*-Glc(S-1) C-1], δ90.30[1C, *α*-Glc(S-8) C-1], δ95.45[1C, *β*-GlcNAc(S-4) C-1], δ95.53[1C, *β*-Gal(S-7) C-1], δ101.17[2C, *β*-GalNAc(S-3) & (S-5) C-1], δ101.26[1C, *β*-GalNAc(S-6) C-1], δ102.08[1C, *β*-Gal(S-2) C-1], δ102.16[1C, *β*-Gal(S-9) C-1]

^{1}H NMR of Compound-B, Arisose in D_2O at 300 MHz.

δ5.21[d, 2H, J=3.6Hz, *α*-Glc(S-1) & (S-8) H-1], δ4.66[d, 2H, J=7.8Hz, *β*-Glc(S-1) & *β*-Gal(S-7) H-1], δ4.54[d, 1H, J=8.1Hz, *β*-GlcNAc(S-4) H-1], δ4.51[d, 2H, J=8.1Hz, *β*-Gal(S-2) & (S-9) H-1], δ4.44[d, 3H, J=7.5Hz, *β*-GalNAc(S-3) & (S-5) & (S-6) H-1], δ3.27[t, *β*-Glc(S-1), H-2], δ2.08[s, 3H, $NHCOCH_3$ *β*-GlcNAc(S-4)], δ1.98[s, 6H, $NHCOCH_3$ *β*-GalNAc(S-3) & (S-5)], δ1.90[s, 3H, $NHCOCH_3$ *β*-GalNAc(S-6)].

ES Mass

1640 [M^+], 1608, 1581, 1539, 1489, 1478, 1447, 1446, 1419, 1316, 1273, 1254, 1154, 1125, 1083, 1053, 951, 919, 875, 841, 824, 748, 706,705, 677, 646, 645, 545, 481, 467, 466, 465, 406, 342, 324, 180.

RESULT AND DISCUSSION

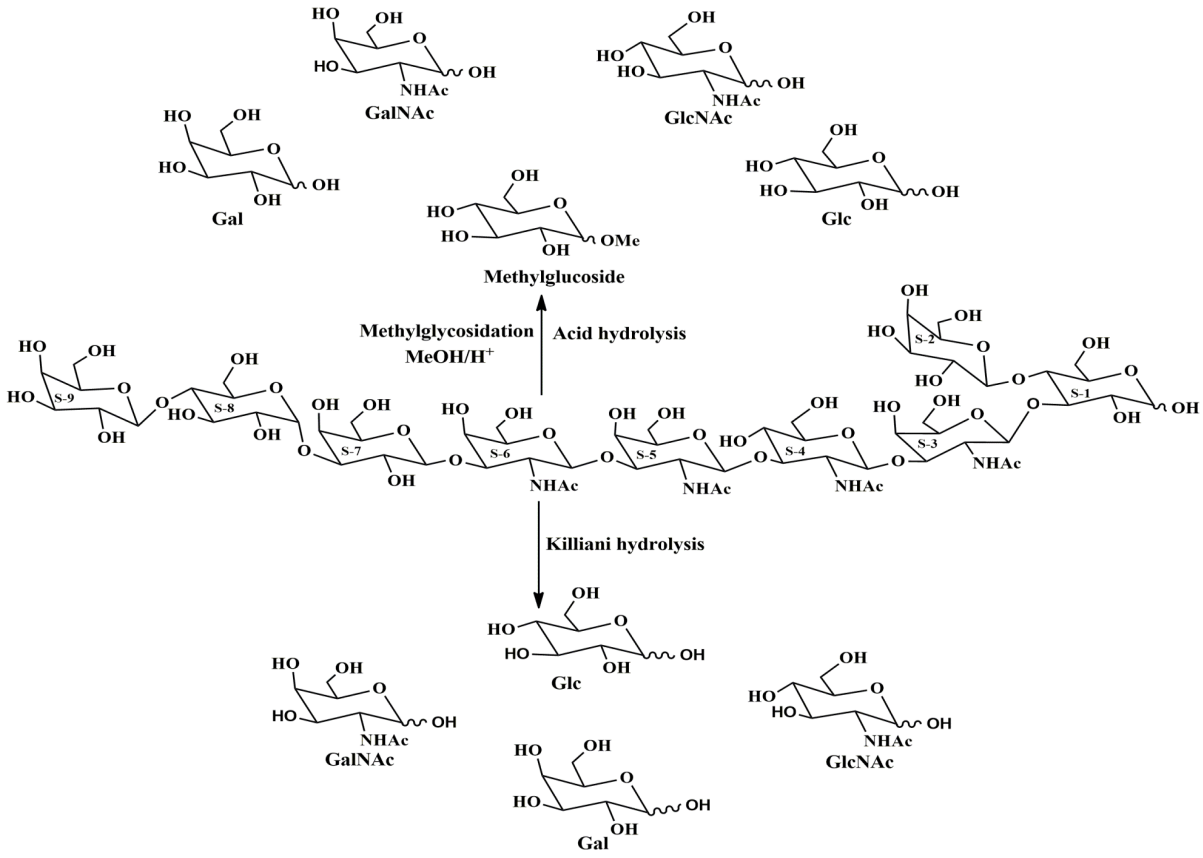
COMPOUND B (ARISOSE)

Compound B, Arisose, $C_{62}H_{104}O_{46}N_4$ $[\alpha]_D^{25}$ +34º gave positive Phenol-sulphuric acid test, Fiegl test and Morgan-Elson test showing the presence of normal and amino sugars moietie(s) in the compound B. The HSQC spectrum of acetylated Arisose showed the presence of nine cross peaks of ten anomeric protons and carbons in their respective region at δ91.5x6.09, δ90.30x5.28, δ90.43x5.29, δ95.45x4.67, δ95.53x4.59, δ101.17x4.43, δ101.26x4.41, δ102.08x4.50 and δ102.16x4.49, suggested the presence of ten anomeric protons and carbons in compound B. The reducing nature of Arisose on its methylglycosylation followed by its acid hydrolysis MeOH/H^+, led to the isolation of *α* and *β*-methylglucosides along with Glc, Gal, GlcNAc and GalNAc suggested that compound was a reducing nonasaccharide in which glucose was present at its reducing end, for convenience all nine monosaccharides were denoted as S-1, S-2, S-3, S-4, S-5, S-6, S-7, S-8 and S-9. The monosaccharides constituents in Arisose were also confirmed by its Killiani hydrolysis under strong acidic condition, followed by its paper chromatography and TLC. In this hydrolysis four spots were found identical with the authentic samples of Glc, Gal, GlcNAc and GalNAc by co-chromatography. Thus Killiani hydrolysis confirmed that Arisose contained four types of monosaccharides units i.e. Glc, Gal, GlcNAc and GalNAc. Further the presence of nine doublets for ten anomeric protons at δ6.09(1H), δ5.29(1H), δ5.28(1H), δ4.67(1H), δ4.59(1H), δ4.50(1H), δ4.49(1H), δ4.43(2H) and δ4.41(1H) in the acetylated spectrum of Arisose in $CDCl_3$ at 300 MHz confirmed the presence of a nonasaccharide in its reducing form. Further the presence of nine anomeric carbon signals for ten anomeric carbon at δ90.30 (1C), δ90.43(1C), δ91.5(1C), δ95.45(1C), δ95.53(1C), δ101.17(2C), δ101.26(1C), δ102.08(1C) and δ102.16(1C) in the ^{13}C NMR spectrum of acetylated Arisose in $CDCl_3$ at 300 MHz. confirmed the presence of a nonasaccharide in its reducing form. The nonasaccharide nature of Arisose was further supported by the presence of five anomeric proton doublets for ten anomeric protons at δ5.21(2H), δ4.66(2H), δ4.54(1H), δ4.51(2H) and δ4.44 (3H) in ^1H NMR spectrum of Arisose in D_2O at 300 MHz. The ^1H and ^{13}C NMR spectra of Arisose justify the ten anomeric signals for nonasaccharide with total integral intensity of nine anomeric proton/carbons. The mass ion peak at 1640$[M]^+$ present in ES- MS of Arisose was in agreement with molecular formula $C_{62}H_{104}O_{46}N_4$.

^1H NMR values of Compound B Arisose in D_2O and $CDCl_3$ at 300MHz

The ^1H NMR spectrum of Arisose in D_2O at 300 MHz contain two doublets at δ5.21 (J=3.6Hz) and δ4.66 (J=7.8Hz) confirmed the presence of glucose at the reducing end in the nonasaccharide. Further the presence of another anomeric doublet at δ4.51 (J=8.1 Hz) in ^{1}HNMR spectrum of D_2O suggested the presence of *β*-Gal (S-2) residue as the next monosaccharide unit.

In addition to anomeric signals of Glc and Gal, 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 i.e. *β*-Gal(1-4)→Glc (structure reporter group) at the reducing end of Arisose. Simultaneously ^{1}H NMR and ^{13}C NMR spectrum of Arisose acetate also showed downfield shifted *α* and *β* anomeric proton and carbon of reducing monosaccharide (S-1) i.e. Glc (S-1) at δ6.09 (J=3.9Hz), δ5.29 (J=8.1Hz) and δ91.5, δ90.43 respectively. The anomeric protons signal present at δ5.29 in TOCSY Spectrum of Arisose acetate assigned to *β*-Glc (S-1) gave three cross peaks at δ5.29x3.49, δ5.29x3.75 and δ5.29x4.75, which was later identified as H-4, H-3 and H-2 of reducing Glc respectively by COSY spectrum of Arisose acetate. The chemical shift of the cross peak at δ5.29x3.49 and δ5.29x3.75 suggested that in sugar S-1, two positions were available for glycosidic linkage by next monosaccharide units. The earlier suggested (1→4) linkage between *β*-Glc (S-1) and *β*-Gal (S-2) was further confirmed by HMBC spectrum of Arisose acetate at 300 MHz which contain the cross peak signal of H-4 of *β*-Glc (S-1) and anomeric carbon of next monosaccharide(S-2) i.e. *β*-Gal (S-2) at δ3.49x102.08.



Methylglycosidation/Acid Hydrolysis and Killiani Hydrolysis of Arisose

The anomeric carbon of *β*-Gal (S-2) at δ102.08 gave its complimentary anomeric proton signal at δ4.50 in the HSQC spectrum of Arisose acetate. The chemical shift value of anomeric carbon and anomeric proton were having resemblance with literature value of

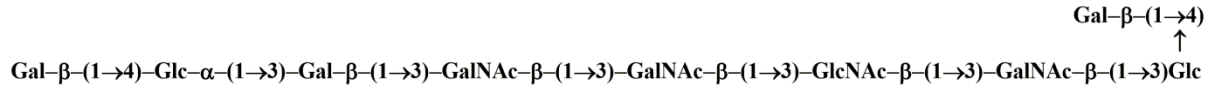
anomeric chemical shift value of *β*-Gal (S-2) hence S-2 monosaccharide was confirmed as *β*-Gal (S-2). The anomeric proton signal present at δ4.51 for *β*-Gal (S-2) had a J value of 8.1Hz confirmed the *β* glycosidic linkage between S-2→S-1. The (1→4) linkage between *β*-Glc (S-1) and *β*-Gal (S-2) was supported by the presence of H-4 signal of S-1 at δ3.49 in upfield region of ^{1}H NMR spectrum of Arisose acetate which was confirmed by the TOCSY and COSY spectrum. Further the (1→4) linkage between S-2 and S-1 was supported by the presence of cross peak at δ3.49x82.73 in glycosidic region of HSQC spectrum of Arisose acetate in $CDCl_3$. The anomeric proton signal at δ4.50 in ^{1}H NMR spectrum of Arisose acetate in $CDCl_3$ assigned for *β*-Gal (S-2) gave three cross peaks at δ4.50x4.97, δ4.50x5.15 and δ4.50x5.28 in its TOCSY spectrum since this anomeric proton does not showed any cross peak in the linkage region, hence confirmed that *β*-Gal (S-2) was present at non-reducing end and none of its -OH group were involved in glycosidic linkage. Since it was ascertained by the COSY and TOCSY spectrum of Arisose acetate that the *β*-Glc (S-1) 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*-Gal (S-2) whereas the left over H-3 position of *β*-Glc (S-1) at δ3.75 showed a long range coupling with anomeric carbon i.e. C-1 of next monosaccharide (S-3) at δ101.17 in HMBC spectrum of Arisose acetate in $CDCl_3$ at 300 MHz. The anomeric carbon signal at δ101.17 showed its complimentary signal at δ4.43 in HSQC spectrum of Arisose 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-3) hence S-3 monosaccharide was confirmed as *β*-GalNAc (S-3). Further the presence of *β*-GalNAc (S-3) as next monosaccharide in Arisose was supported by appearance of anomeric proton signal at δ4.44 (J=7.5 Hz) along with a singlet of three proton of amide methyl at δ1.98 in ^{1}H NMR spectrum of Arisose 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 supported by the presence of H-3 signal of S-1 at δ3.75 in upfield region of ^{1}H NMR spectrum of Arisose acetate which was confirmed by the TOCSY and COSY spectrum. Further the (1→3) linkage between S-3 and S-1 was supported by the presence of cross peak at δ3.75x76.48 in glycosidic region of HSQC spectrum of Arisose acetate in $CDCl_3$.The anomeric proton signal at δ4.43 ^{1}H NMR spectrum of Arisose acetate assigned for *β*-GalNAc (S-3) gave four cross peaks at δ4.43x3.75, δ4.43x4.12, δ4.43x5.05 and δ4.43x5.30 in its TOCSY spectrum in $CDCl_3$ at 300 MHz, out of which proton signal arised at δ4.12 corresponded to H-2 position of *β*-GalNAc (S-3) and another proton signal arised at δ3.75 represented the linkage methine proton of *β*-GalNAc (S-3) which was later identified as H-3 of *β*-GalNAc (S-3) by COSY spectrum of Arisose acetate which was available for (1→3) glycosidic linkages by the next monosaccharide unit (S-4). The next anomeric proton signal which appeared as doublet at δ4.67 in ^{1}H NMR spectrum of Arisose acetate in $CDCl_3$ at 300 MHz. was due to the presence of *β*-GlcNAc (S-4) moiety[254]. This anomeric proton signal present at δ4.67 gave its complimentary signal at δ95.45 in its HSQC spectrum 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*-GlcNAc (S-4) hence S-4 monosaccharide was confirmed as

β-GlcNAc (S-4). Further the presence of *β*-GlcNAc (S-4) as next monosaccharide in Arisose was supported by appearance of anomeric proton signal at δ4.54 along with a singlet of three protons of amide methyl at δ2.08 in ^{1}H NMR spectrum of Arisose in D_2O. Since it was ascertained by COSY and TOCSY spectrum of Arisose Acetate that H-3 of S-3 was available for glycosidic linkage by the next monosaccharide unit (S-4), hence *β*-GlcNAc (S-4) must be linked to H-3 of *β*-GalNAc (S-3). Further the (1→3) linkage between S-4 and S-3 was supported by the presence of cross peak at δ3.75x76.48 in glycosidic region of HSQC spectrum of Arisose acetate in $CDCl_3$. The (1→3) linkage between *β*-GlcNAc (S-4) and *β*-GalNAc (S-3) was further supported by the ^{1}H NMR spectrum of acetylated Arisose in which the signal for H-3 of *β*-GalNAc (S-3) appeared at δ3.75 which was later confirmed by COSY, TOCSY and HSQC spectrum of acetylated Arisose at 300 MHz in $CDCl_3$. The coupling constant of anomeric signal (S-4) with J value 8.1Hz confirmed the *β*-glycosidic linkage between S-4 and S-3 moiety. The anomeric protons signal present at δ4.67 assigned for *β*-GlcNAc (S-4) in the ^{1}H NMR spectrum of Arisose acetate contains three cross peaks at δ4.67x3.71, δ4.67x4.73 and δ4.67x5.18 in its TOCSY Spectrum in $CDCl_3$ at 300MHz. The chemical shift of the cross peak at δ4.67x3.71 suggested that in sugar S-4 only one position was available for glycosidic linkage by the next monosaccharide unit. Further the double doublet present at δ3.71 in ^{1}H NMR spectrum of Arisose acetate was identified as H-3 of *β*-GlcNAc (S-4) by COSY spectrum of Arisose acetate confirming that the H-3 of *β*-GlcNAc (S-4) was available for glycosidation by the next monosaccharide moiety (S-5). Further, H-3 of *β*-GlcNAc (S-4) gave a long range coupling with anomeric carbon of next monosaccharide (S-5) at δ3.71x101.17 in HMBC confirmed the (1→3) linkage between S-5 and S-4. The anomeric carbon signal present at δ101.17 had its complimentary signal at δ4.43 in HSQC spectrum of Arisose 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 Arisose was supported by appearance of anomeric proton signal at δ4.44 (J=7.5 Hz) along with a singlet of three proton of amide methyl at δ1.98 in ^{1}H NMR spectrum of Arisose in D_2O at 300 MHz. The coupling constant of anomeric signal *β*-GalNAc (S-5) with larger value of 7.5 Hz showed the *β* configuration of the *β*-GalNAc (S-5). The (1→3) linkage between S-5 and S-4 was supported by the presence of glycosidic linkage at δ3.71x71.48 in glycosidic region of HSQC spectrum of Arisose acetate in $CDCl_3$. The (1→3) linkage between *β*-GalNAc (S-5) and *β*-GlcNAc (S-4) was further supported by the ^{1}H NMR spectrum of acetylated Arisose in which the signal for H-3 of *β*-GlcNAc (S-4) appeared at δ3.71 which was later confirmed by COSY, TOCSY and HSQC spectrum of Arisose acetate. The anomeric proton signal present at δ4.43 in ^{1}H NMR spectrum of Arisose acetate in $CDCl_3$ at 300 MHz. assigned for *β*-GalNAc (S-5), contain four cross peaks at δ4.43x3.75, δ4.43x4.12, δ4.43x5.05 and δ4.43x5.35 in its TOCSY spectrum, out of which one cross peak arised at δ4.43x4.12 corresponded to H-2 position of *β*-GalNAc (S-5) and another signal arised at δ4.43x3.75 represented the linkage region of *β*-GalNAc (S-5) which was later defined as H-3 of *β*-GalNAc (S-5) by COSY spectrum of Arisose acetate which was available for (1→3) glycosidic linkage by the next monosaccharide unit

(S-6). Further, HMBC spectrum of Arisose acetate showed long range coupling between H-3 of *β*-GalNAc (S-5) and anomeric carbon of next monosaccharide (S-6) at δ3.75x101.26 confirmed the (1→3) linkage between S-6 and S-5. This (1→3) linkage between S-6 and S-5 was supported by the presence of H-3 signal of *β*-GalNAc (S-5) at δ3.75 in upfield region of ^{1}H NMR spectrum of Arisose acetate which was confirmed by the TOCSY and COSY spectrum. The anomeric carbon signal present at δ101.26 gave its complimentary signal at δ4.41 in HSQC spectrum of Arisose 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-6) hence S-6 monosaccharide was confirmed as *β*-GalNAc (S-6). Further the presence of *β*-GalNAc (S-6) as next monosaccharide in Arisose was supported by appearance of anomeric proton signal at δ4.44 along with a singlet of three protons of amide methyl at δ1.90 in ^{1}H NMR spectrum of Arisose in D_2O. The coupling constant of anomeric signal *β*-GalNAc (S-6) with J value of 7.5 Hz confirmed the *β* glycosidic linkage between S-6 and S-5. The (1→3) linkage between S-6 and S-5 was supported by the presence of cross peak at δ3.75x76.48 in glycosidic region of HSQC spectrum of Arisose acetate in $CDCl_3$. The (1→3) linkage between *β*-GalNAc (S-6) and *β*-GalNAc (S-5) was further confirmed by the ^{1}H NMR spectrum of acetylated Arisose in which the signal for H-3 of β-GalNAc (S-5) appeared at δ3.75 which was later confirmed by COSY, TOCSY and HSQC spectrum of acetylated Arisose at 300 MHz in $CDCl_3$. The anomeric proton signal present at δ4.41 in ^{1}H NMR spectrum of Arisose acetate in $CDCl_3$ at 300 MHz. assigned for *β*-GalNAc (S-6), contain four cross peaks at δ4.41x3.75, δ4.41x4.12, δ4.41x5.05 and δ4.41x5.35 in its TOCSY spectrum, out of which one cross peak arised at δ4.41x4.12 corresponded to H-2 position of *β*-GalNAc (S-6) while the other signal arised at δ4.41x3.75 corresponded to linkage region of *β*-GalNAc (S-6) which was later identified as H-3 of *β*-GalNAc (S-6) by COSY spectrum of Arisose acetate which was available for (1→3) glycosidic linkage by the next monosaccharide unit (S-7). Further anomeric proton doublet appeared at δ4.59 in^{1}H NMR spectrum of Arisose acetate in $CDCl_3$ gave its complimentary anomeric carbon signal at δ95.53 in the HSQC spectrum of Arisose acetate. The chemical shift value of anomeric carbon and anomeric proton were having resemblance with literature value of anomeric chemical shift value of *β*-Gal (S-7) hence S-7 monosaccharide was confirmed as *β*-Gal (S-7). Further the presence of *β*-Gal (S-7) as next monosaccharide in Arisose was supported by appearance of anomeric proton signal at δ4.66 (J=7.8Hz) in ^{1}H NMR spectrum of Arisose in D_2O at 300 MHz. The anomeric proton signal present at δ4.66 for *β*-Gal (S-7) had a J value of 7.8Hz confirmed the *β* glycosidic linkage between S-7→S-6.Since it was ascertained by the COSY and TOCSY spectrum of Arisose acetate that the *β*-GalNAc (S-6) has two vacant position for substitution i.e. H-2 and H-3 and it was already confirmed that H-2 of S-6 was occupied with –NHAc of S-6 hence the left over H-3 position of S-6 at δ3.75 must be linked to *β*-Gal (S-7). The (1→3) linkage between *β*-GalNAc (S-6) and *β*-Gal (S-7) was supported by the presence of H-3 signal of S-6 at δ3.75 in upfield region of ^{1}H NMR spectrum of Arisose acetate which was confirmed by the TOCSY and COSY spectrum. Further the (1→3) linkage between S-7 and S-6 was supported by the presence of cross peak at δ3.75x76.48 in glycosidic region of HSQC spectrum of Arisose acetate in $CDCl_3$.

The anomeric proton signal at δ4.59 assigned for *β*-Gal (S-7), gave two consequent complimentary anomeric signal at δ4.59x3.73 and δ4.59x4.68 in its TOCSY spectrum, out of which cross peak arised at δ4.59x4.68 corresponded to H-2 position of *β*-Gal (S-7), however the cross peak arised at δ4.59x3.73 corresponded to linkage region of *β*-Gal (S-7), which was later defined as H-3 of *β*-Gal (S-7), by COSY spectrum of Arisose acetate which was available for (1→3) glycosidic linkage by the next monosaccharide unit (S-8). The next anomeric proton doublet which appeared at δ5.28 in the ^{1}H NMR spectrum of Arisose acetate in $CDCl_3$ gave its complimentary signal δ90.30 in HSQC spectrum of Arisose acetate. The chemical shift value of anomeric proton and carbon were having resemblance with literature value of anomeric chemical shift value of *α*-Glc. Further the presence of *α*-Glc (S-8) as next monosaccharide was supported by anomeric proton doublet at δ5.21 (J=3.6Hz) in the ^{1}H NMR spectrum of Arisose in D_2O at 300 MHz. Since it was ascertained by COSY and TOCSY spectrum of Arisose acetate that H-3 of S-7 was available for glycosidic linkage by next monosaccharide i.e. (S-8) hence S-8 (α-Glc) must be linked to H-3 of *β*-Gal (S-7). The (1→3) linkage between S-8 and S-7 was confirmed by the presence of cross peak at δ3.75x76.42 in glycosidic region of HSQC spectrum of Arisose acetate in $CDCl_3$. Further (1→3) linkage between S-8 and S-7 was supported by presence of downfield H-3 signal of S-7 at δ3.75 in ^{1}H NMR spectrum of Arisose acetate in $CDCl_3$. The coupling constant of anomeric signal (S-8) with J value 3.6Hz confirmed the *α*-configuration of the S-8 moiety with *β*-Gal (S-7). The anomeric proton signal at δ5.28 assigned for *α*-Glc (S-8), gave four cross peaks at δ5.28x3.73, δ5.28x4.75, δ5.28x4.9 and δ5.28x5.46 in its TOCSY spectrum, out of which one cross peak arised at δ5.28x3.73 corresponded to linkage region of *α*-Glc (S-8), which was identified as H-4 of *α*-Glc (S-8) by COSY spectrum of Arisose acetate which was available for (1→4) glycosidic linkage by the next monosaccharide unit (S-9). The next anomeric proton signal which appeared as doublet at δ4.49 in ^{1}H NMR spectrum of Arisose acetate in $CDCl_3$ gave its complimentary signal at δ102.16 in HSQC spectrum of Arisose acetate. The chemical shift value of anomeric proton and carbon were having resemblance with literature value of anomeric chemical shift value of *β*-Gal (S-9). Hence (S-9) monosaccharide was confirmed as *β*-Gal. Further the presence of *β*-Gal (S-9) as next monosaccharide was supported by anomeric proton doublet at δ4.51 (J=8.1Hz) in the ^{1}H NMR spectrum of Arisose in D_2O at 300 MHz. Since it was ascertained by COSY and TOCSY spectrum of Arisose acetate that H-4 of S-8 was available for glycosidic linkage by next monosaccharide i.e. (S-9) hence S-9 *β*-Gal must be linked to H-4 of *α*-Glc (S-8).The (1→4) linkage between *α*-Glc (S-8) and *β*-Gal (S-9) was further supported by the ^{1}HNMR spectrum of Arisose acetate in which the signal for H-4 of *α*-Glc (S-8) appeared at δ3.73 which was already confirmed by COSY, TOCSY and HSQC spectrum of acetylated Arisose at 300 MHz in $CDCl_3$. The (1→4) linkage between S-9 and S-8 was further confirmed by the presence of cross peak at δ3.73x76.48 in glycosidic region of HSQC spectrum of Arisose acetate in $CDCl_3$. The anomeric proton signal at δ4.49 in ^{1}H NMR spectrum of Arisose acetate in $CDCl_3$ assigned for *β*-Gal (S-9) gave three cross peaks at δ4.49x4.97, δ4.49x5.15 and δ4.49x5.28 in its TOCSY spectrum since this anomeric proton does not showed any cross peak in the linkage region, hence confirmed that *β*-Gal (S-9) was present at non-reducing end and none

of its -OH group were involved in glycosidic linkage, which was confirmed by the TOCSY and COSY spectrum of Arisose acetate in $CDCl_3$ at 300 MHz. All the ^1H NMR assignments for ring protons of monosaccharide units of Arisose were confirmed by COSY and TOCSY experiments. The positions of glycosidation in the oligosaccharide were confirmed by position of anomeric signals, S.R.G. and comparing the signals in ^1H and ^{13}C NMR of acetylated and deacetylated oligosaccharide. The glycosidic linkages in Arisose were assigned by the cross peaks for glycosidically linked carbons with their protons in the HSQC and HMBC spectrum of acetylated Arisose. All signals obtained in ^1H and ^{13}C NMR of compound Arisose were in confirmity with the assigned structure and their position were confirmed by 2D NMR viz. COSY, TOCSY, HSQC and HMBC experiments. Thus based on the pattern of chemical shifts of ^1H NMR, ^{13}CNMR, COSY, TOCSY, HSQC and HMBC experiments it was interpreted that the compound was a nonasaccharide having following structure as:



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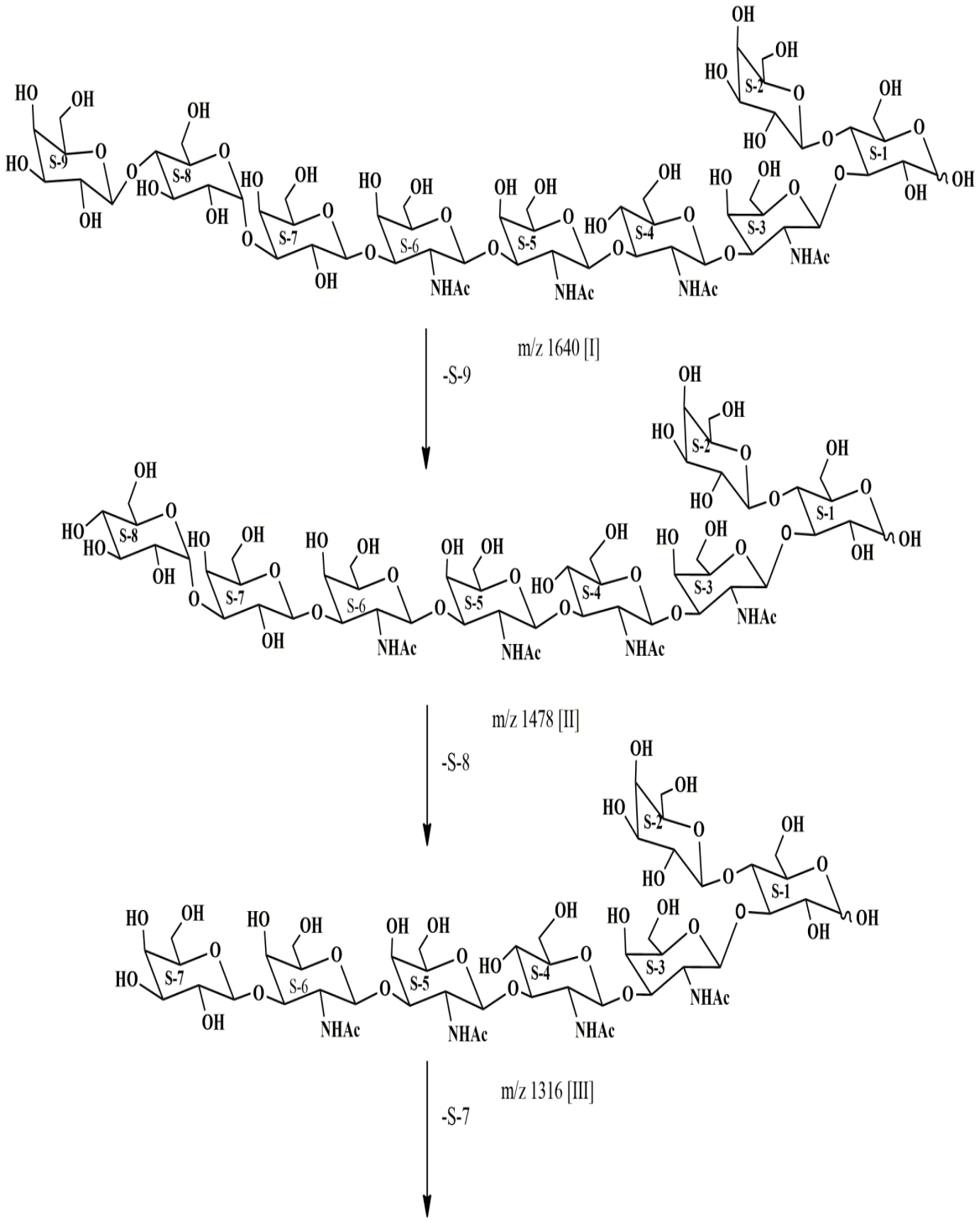
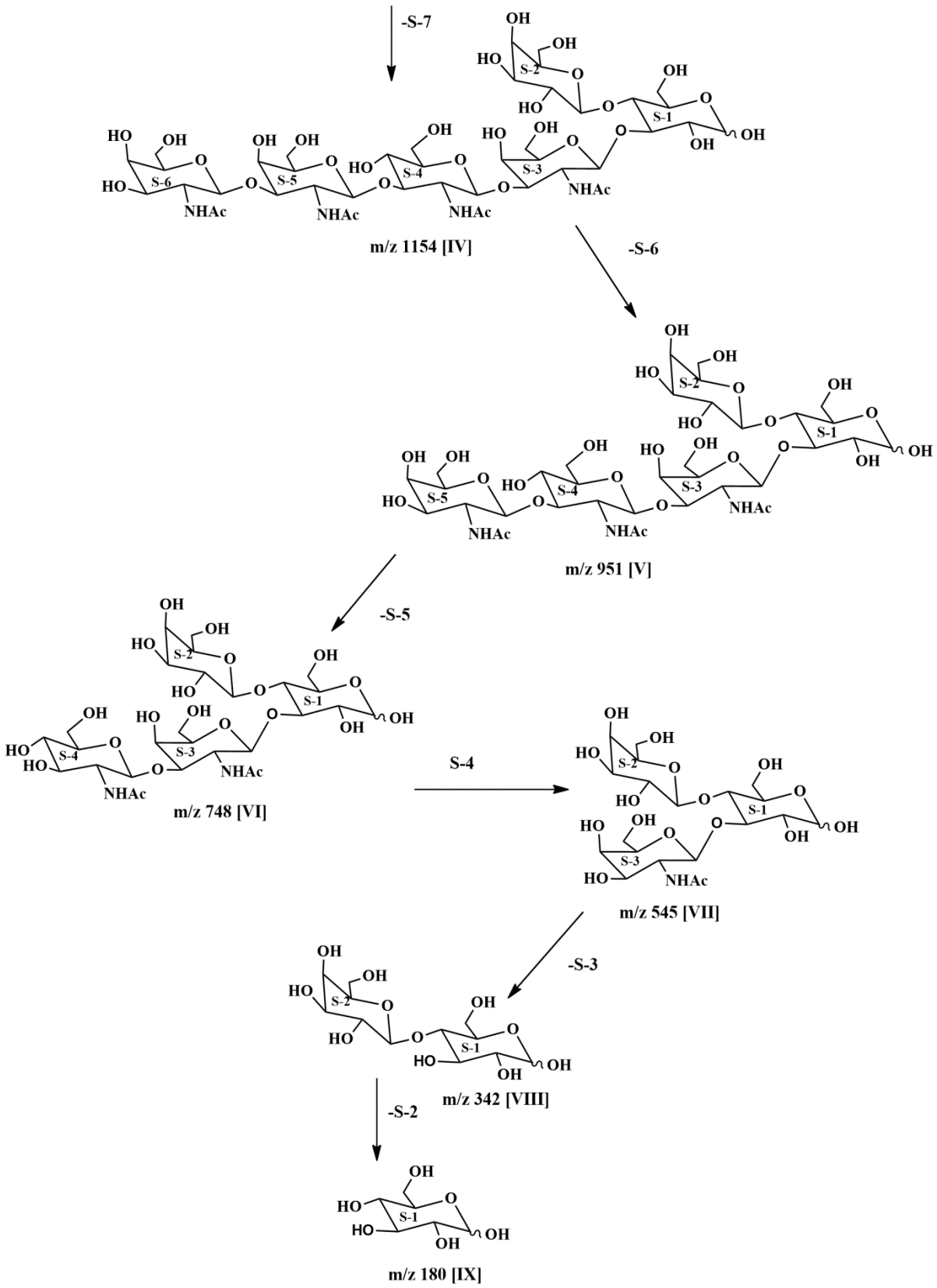
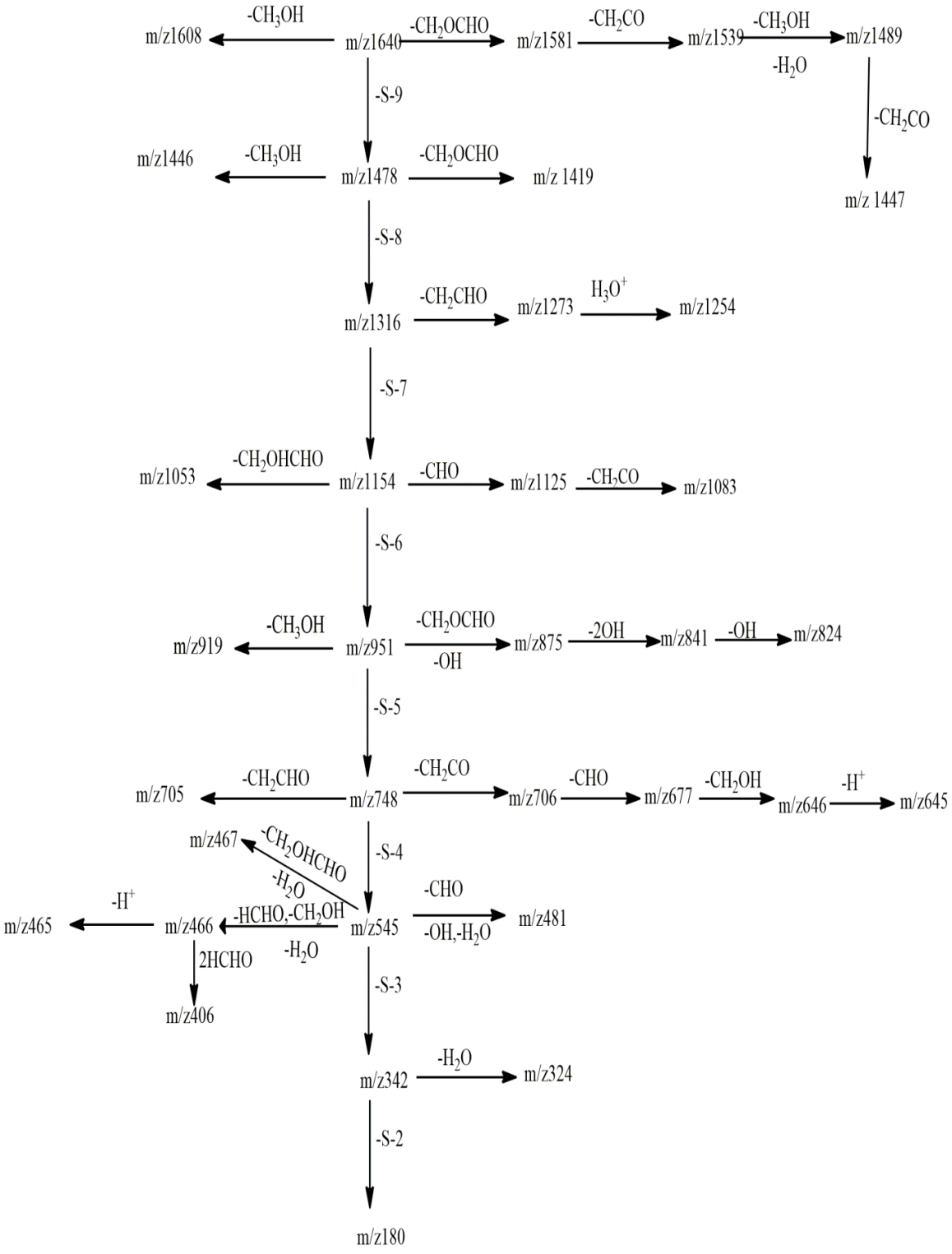
The Electronspray Mass Spectrometry data of Arisose not only confirmed the derived structure but also supported the sequence of monosaccharide in Arisose. The highest mass ion peaks were recorded at m/z 1640 which was due to $[M^+]$ confirming the molecular weight of Arisose was 1640 and in agreement with its molecular formula. 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 nonasaccharide m/z 1640 (I) fragmented to give mass ion at m/z 1478 (II) [1640-S-9], this fragment was arised due to the loss of terminal *β* -Gal (S-9) moiety from nonasaccharide indicating the presence of *β*-Gal (S-9) at the non-reducing end. It further fragmented to give mass ion peak at m/z 1316 (III) [1478-S-8] which was due to loss of *α*-Glc (S-8) moiety from octasaccharide. This fragment of 1316 further fragmented to give mass ion peak at m/z 1154(IV) [1316-S-7] which was due to loss of *β*-Gal (S-7) moiety from the heptasaccharide. This fragment of m/z 1154(IV) further fragmented to give mass ion peak at m/z 951(V) [1154-S-6], which was due to loss of *β*-GalNAc (S-6) moiety from hexasaccharide. This pentasaccharide fragment of m/z 951 further fragmented to give mass ion peak at m/z 748(VI) [951-S-5] which was due to loss of *β*-GalNAc (S-5) moiety. The tetrasaccharide unit of m/z 748(VI) again fragmented to give mass ion peak at m/z 545(VII) [748-S-4], which was due to loss of *β*-GlcNAc (S-4) moiety**.** This fragment of m/z 545 (VII) further fragmented to give mass ion at m/z 342 (VIII) [545-S-3], this fragment was arised due to the loss of *β*-GalNAc (S-3) moiety from trisaccharide indicating the presence of *β*-GalNAc (S-3) It further fragmented to give mass ion peak at m/z 180(IX) [342-S-2**]** which was due to loss of *β*-Gal (S-2) moiety from disaccharide. The other fragmentation pathway in ES Mass spectrum of compound B m/z 1640 shows the mass ion peak at 1608[1640-CH_3OH], 1581[1640-CH_2OCHO], 1539[1581-CH_2CO], 1489[1539-CH_3OH,-H_2O], 1447[1489-CH_2CO], 1478[1640-S-9], 1446[1478-CH_3OH], 1419[1478-CH_2OCHO], 1316[1478-S-8], 1273[1316-CH_2CHO], 1254[1273-H_3O^+], 1154[1316-S-7], 1125[1154-CHO], 1083[1125-CH_2CO], 1053[1154-CH_2OHCHO], 951[1154-S-6], 919[951-CH_3OH],



Mass fragmentation of compound B, Arisose



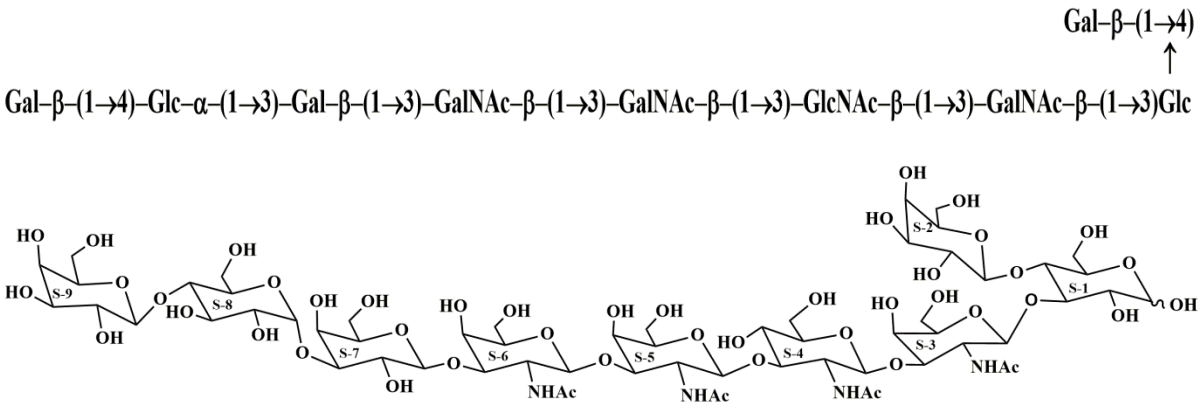
Mass fragmentation of compound B, Arisose



ES-MS fragmentation of compound B, Arisose

875[951-CH_2OCHO,OH], 841[875-2OH], 824[841-OH], 748[951-S-5], 706[748-CH_2CO], 705[748-CH_2CHO], 677[706-CHO], 646[677-CH_2OH], 645[646-H^+], 545[748-S-4], 481[545-CHO,-OH,-H_2O], 467[545-CH_2OHCHO,-H_2O], 466[545-HCHO,CH_2OH,H_2O], 465[466-H^+], 406[466-2HCHO], 342[545-S-3], 324[342-H_2O] and 180[342-S-2].

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 and 2D NMR viz. COSY, TOCSY, HMBC and HSQC spectra of Arisose acetate and Arisose, the structure and sequence of isolated Novel oligosaccharide Arisose structure was deduced as under



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CONCLUSION

In summary, the novel milk oligosaccharide isolated from cow colostrum was as a novel nonasaccharide namely Arisose and its structure was elucidated with the help of ^{1}H, ^{13}C, 2D NMR spectroscopy and mass spectrometry.

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