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Isolation and Structure elucidation of novel Hexasaccharide Usose from Lal-Muha Cow milk by 2D NMR

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Abstract

Milk provides number of novel oligosaccharides depending on the nature of their origin and to which mammals the milk belongs and has shown varied biological activities such as anti-tumor, anticancer, anticomplementary, hypoglycemic, anti-inflammatory anticoagulant, antiviral, immunostimulant and immunological activities. Milk oligosaccharides have the potential to produce immuno-modulation effects, significant influence on intestinal mineral absorption and in the formation of the brain and central nervous system. The prebiotic properties of oligosaccharide highly depend on structural conformation, including their degree of polymerization, monosaccharide composition, and their glycosidic linkages. Determination of structures of novel oligosaccharide is challenging because of a range of molecular diversity and complexity of molecular structure. Due to compositional resemblance of cow's milk with human milk and the medicinal importance of cow's milk given in Indian medicinal system i.e. Ayurveda, a rare species of cow found in hills of Sikkim (India) commonly known as Lal-Muha at 5,410 ft was considered a potential source of biological active oligosaccharides. For this purpose Lal-Muha cow milk was collected in bulk and processed by the modified method of Kobata and Ginsburg followed by gel filtration, HPLC and column chromatography which resulted in the isolation of novel oligosaccharide namely, Usose. The structure of isolated oligosaccharides was elucidated by chemical transformations, chemical degradation, NMR of neutral and acetylated oligosaccharide (^{1}H, ^{13}C and 2D COSY, TOCSY, HSQC and HMBC) and mass spectrometry.

GalNAc-β(1→4)Gal-β(1→4)Glc-β(1→4)Gal-β(1→4)Glc
↑
GlcNAc-α(1→3)

Keywords: Milk; Isolation; Hexasaccharide; Usose and 2D NMR.

Introduction

Carbohydrates are present in the form of oligosaccharides, polysaccharides, glycoconjugates i.e. glycosaminoglycans, glycoproteins, glycopeptides, glycolipids and proteoglycans; in plants, bacteria, milk and many others likely sources are the main source of energy for living organisms and the central pathway of energy storage and supply for most cells[1-6]. A broad range of oligosaccharides and their derivatives act as an effective drug against most of acute and chronic diseases, and play an essential role in many molecular processes impacting eukaryotic biology and diseases and exhibit varied biological activities such as immunostimulant, hypoglycemic, anti-tumor, antiviral, anticancer, anticoagulant, anti-complementary, immunological and anti-inflammatory activities[7-9]. Milk is a complex mixture of lipids, proteins, carbohydrates and smaller metabolites, and thus, represents a key role in infant nourishment and development[10]. Oligosaccharides are one of the substantial bioactive components of milk which have significant influence on prebiotic activity[11], anti-adhesion activity[12-13], anti-inflammatory activity[14], glycome modifying activity, in formation of brain and central nervous system[15]. The prebiotic properties of milk oligosaccharides highly depend on structural conformation, including their degree of polymerization, monosaccharide composition, and their glycosidic linkages. Earlier the oligosaccharides has been defined as carbohydrates with a maximum number of monosaccharides up to 10[16] and as there is no physiological or chemical reason to set the limit of milk oligosaccharide, it has been seen that oligosaccharides may contain of 3 up to 19 units of monosaccharides[17]. Identification of novel oligosaccharide structures can be challenging because of a range of molecular diversity and complexity of molecular structure. Oligosaccharides generally give remarkably complex and overlapped proton NMR spectra having a large number of peaks in a proton spectrum in the chemical shifts range of δ4.0-6.0, which make the spectrum complex. Therefore, the complexity of spectrum can be resolved by using an efficient 2D NMR spectroscopy[18-20]. Milk contains fucosylated and sialylated oligosaccharides, which reduce the intact of bacteria and viruses to intestinal cells. The cow milk is important for human

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life which is written in ancient literature. Indian ancient Physician Dhanvantri confirmed that it protects the human from heart diseases and leucoderma. The Ayurveda has described the medicinal importance of black cow milk and Rigveda says that Cow milk is Amrita, protects human being from diseases, its milk have the curative and prophylactic effects. Several studies supported the constructive effects of supplementation of cow milk in diarrhea in human with immune-deficiency syndrome, NSAID-induced gastrointestinal disturbances. Cow milk oligosaccharides reduce the adhesion of enterotoxic *E. Coli* strains of the calf[21]. Keeping in mind the biological activity of Cow milk and oligosaccharide present therein, it was collected in bulk and was processed by modified method of Kobata and Ginsburg[22] followed by different chromatographic techniques like gel filtration, TLC, CC, HPLC etc. which resulted into the isolation of a new milk oligosaccharides namely Usose (C). The structures of purified milk oligosaccharides were elucidated by the using of the data generated from spectroscopic techniques like NMR (^{1}H, ^{13}C, COSY, TOCSY, HSQC and HMBC) mass spectrometry and chemical degradation, chemical transformation. The present paper deals with structure elucidates of novel oligosaccharide from Lal-Muha Cow milk namely Usose (C).

2.1 Experimental:

General procedures

Optical rotations were measured with an AA-5 series automatic polarimeter in 1dm tube. ^{1}H and ^{13}C NMR spectra of oligosaccharides were recorded in D_2O and the spectra of acetylated oligosaccharides were recorded in $CDCl_3$ at 25ºC on a Bruker AM 300 and 400 FT NMR spectrometer. The ES-MS were recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. The C, H and N analysis were recorded on CARLO-ELBA 1108 an elemental analyzer. The sugars were visualized on TLC with 50% aqueous H_2SO_4 reagent and on Paper Chromatography with acetyl acetone and p-dimethyl amino benzaldehyde reagents. The absorbent for TLC was silica gel G (SRL) and CC silica gel (SRL, 60-120 mesh). PC was performed on Whatman No.1 filter paper using solvent system ethyl acetate-pyridine (2:1) saturated with H_2O. Sephadex G–25 (PHARMACIA) was used in gel permeation chromatography. Freeze drying of the compound was done with the help of CT 60e (HETO) lyophylizer and centrifuged by a cooling centrifuged Remi instruments C-23 JJRCI 763. To check the homogeneity of the compounds reverse phase HPLC system was used equipped with Perkin Elmer 250 solvent delivering system, 235 diode array detector and G.P. 100 printer plotter. Authentic samples of N-acetylglucosamine (GlcNAc), Nacetylgalactosamine (GalNAc), galactose (Gal), glucose (Glc), fucose (Fuc) and silalic acid were purchased from Aldrich Chemicals.

2.2. Isolation of Lal Muha Cow milk oligosaccharides by Modified Kobata and Ginsberg method

10 liter Lal-muha Cow milk was collected and added equal amount of ethanol. It was centrifuged for 15 min at 5000 rpm at 4°C. The solidified lipid layer was removed by filtration through glass wool column in cold. Ethanol was added to the clear filtrate to a final concentration of 68% and the resulting solution was left overnight at 0°C. The white precipitate formed, mainly of lactose and protein was removed by centrifugation and washed twice with 68% ethanol at 0°C. The supernatant and washing were combined and filtered through a microfilter (0.24 μm) (to remove remaining lactose) and lyophilized affording crude oligosaccharide mixture (245 gm). The lyophilized material responded positively to Morgan-Elson test[23] and in the oligosaccharide mixture. This lyophilized material (mixture of oligosaccharides) was further purified by fractionating it on Sephadex G-25 column using glass double distilled water as elutant at a flow rate of 3ml/m. Each fraction was analysed for sugars by phenol-sulphuric acid reagent[24] for presence of neutral sugar.

2.3. Acetylation of oligosaccharide mixture

11.4 gm Oligosaccharides mixture was acetylated with pyridine (11.4 ml) and acetic anhydride (11.4 ml) at 60°C and solution was stirred overnight. The mixture was evaporated at 60°C with reduced pressure and viscous residue was taken in $CHCl_3$ (250 ml) and washed twice in ice cold water, evaporated to dryness yielding the acetylated mixture 11.0gm. The acetylation converted the free sugars into their non polar acetyl derivatives which were resolved nicely on TLC, giving 8 spots i.e. a, b, c, d, e, f, g, and h.

2.4. Purification of acetylated milk oligosaccharide

Isolation of the acetylated oligosaccharide (11.0 g) was purified by column chromatography. The silica was used in the ratio of 1:100 using various proportions of Hexane $CHCl_3$, $CHCl_3$and $CHCl_3$: MeOH mixture which resolved into eight fractions namely I(1.45 gm), II(1.10 gm), III(2.15 gm), IV(1.20 gm), V(2.30gm), VI(2.10gm), VII(1.40gm) and VIII(800 mg) respectively. These fractions were containing a mixture of three to four compounds. Repeated column chromatography of fractions II III, led to the isolation of chromatographically pure compound c (136 mg). The

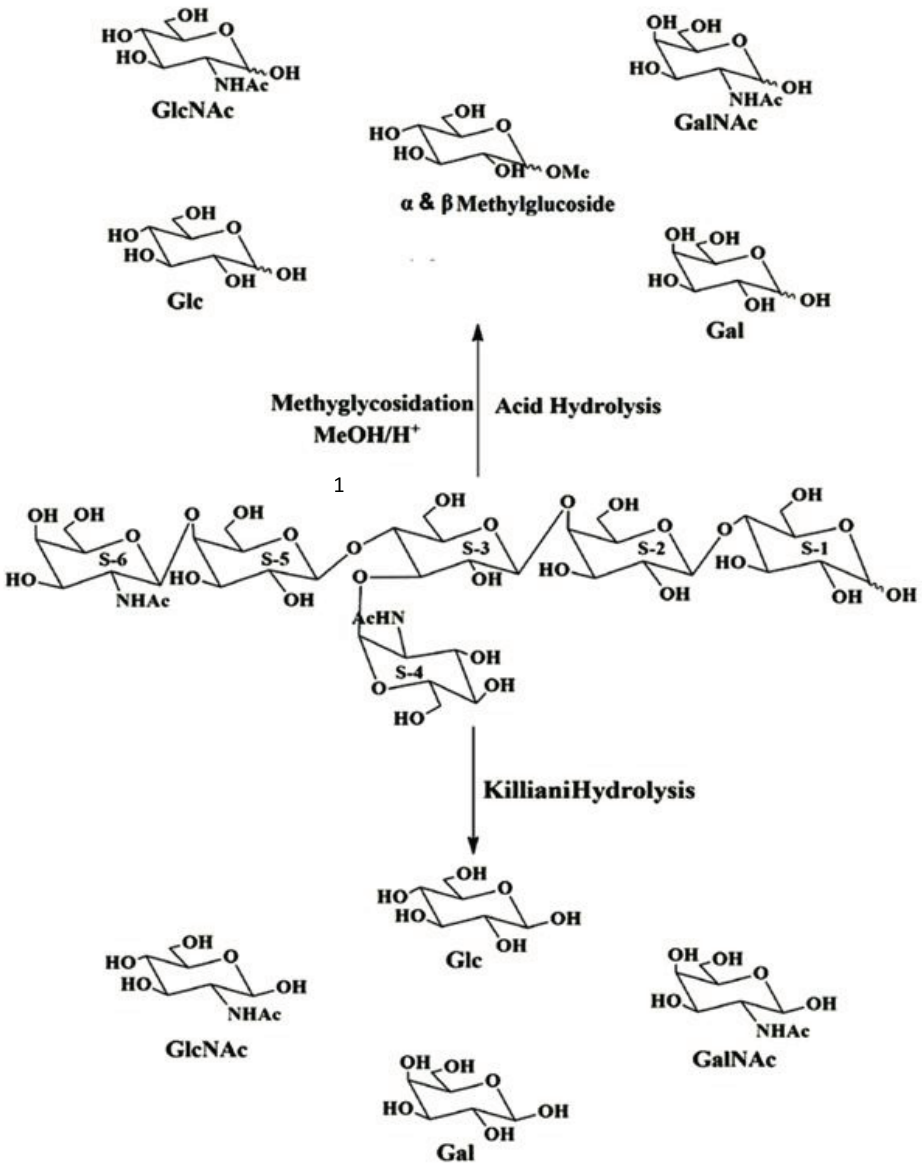
homogeneity of purified oligosaccharide was confirmed by HPLC.

2.5. Deacetylation of compounds

136 mg Compound c was obtained from column chromatography 2 of acetylated oligosaccharide mixture. 50 mg Compound c was dissolved in acetone (2ml) and NH_3 (3 ml) was added and left overnight in a stoppered hydrolysis flask. After 24h ammonia was removed under reduced pressure and the compound was washed with (3 x 5ml) $CHCl_3$ (to remove acetamide) and the water layer was finally freeze dried giving the deacetylated oligosaccharide C (44mg).

2.6. Methylglycosidation/ Acid Hydrolysis of Compound C

6 mg Compound C was refluxed with absolute MeOH (2ml) at 70°C for 18 h in the presence of cation



Scheme-1 The heptasaccharide (Usose) contained four types of sugar moieties Glc, Gal, GlcNAc and GalNAc

exchange IR-120 (H) resin. The reaction mixture was filtered while hot and filtrate was concentrated. To a solution of methylglycoside of C in 1, 4-dioxane (1ml), 0.1 N H_2SO_4 (1ml) was added and the solution was warmed for 30 minutes at 50^{0}C and solution was left over night. The hydrolysis was complete after 24h. The hydrolysate were neutralized with freshly prepared $BaCO_3$ filtered and concentrated under reduced pressure to afford α-and β-methylglucosides along with the Glc, Gal, GalNAc and GlcNAc. Their identification was confirmed by comparison with authentic samples (TLC, PC).

2.7. Killiani Hydrolysis:

5 mg Compound C was dissolved in 2 ml Kiliani mixture (AcOH-H_2O-HCI, 7:11:2) and heated at 100^{0}C for 1 h followed by evaporation under reduced pressure. It was dissolved in 2 ml of H_2O and extracted twice with 3 ml $CHCl_3$. The aqueous residual solution was made neutral by addition of 1-2 drops of 2N NaOH, to it and was evaporated under reduced pressure to afford Glc, Gal, GalNAc and GlcNAc on comparison with authentic samples of Glc, Gal, GalNAc and GlcNAc, given in **scheme-1** (Supplementary File).

2.8. Description of compounds

2.8.2 Compound C, Usose

136 mg Compound c was obtained from fraction 58-67 of chromatography–2 from acetylated oligosaccharide mixture. On deacetylation of 50 mg of substance c with NH_3/ acetone it afford substance C (44 mg) as a viscous mass, $[\alpha]_D^{25}$-46 (c, 4, H_2O). For experimental analysis, this compound was dried over P_2O_5 at 100º C and 0 .1 mm pressure for 8 hr. Compound **Usose** gave positive Phenol-sulphuric acid test[24], Feigl test[25] and Morgan-Elson test[23].

$C_{40}H_{68}O_{31}N_2$	%C	%H	%N
Calcd.	44.77	6.35	2.62
Found	44.78	6.35	2.62

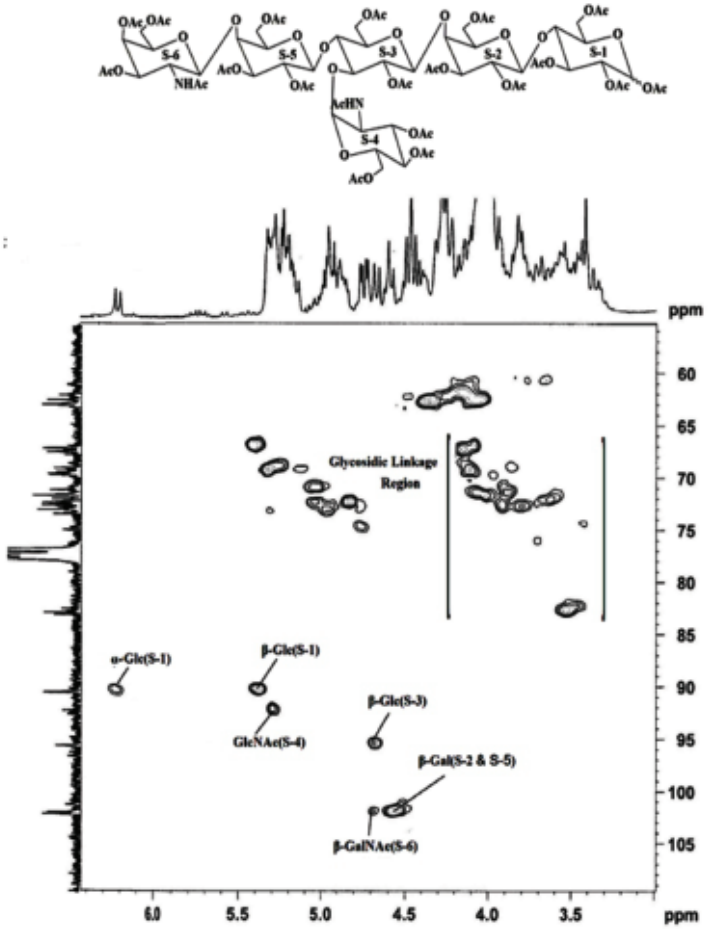


Fig-1 ^{1}H-^{13}C HSQC Spectrum of acetylated Usose in $CDCl_3$ at 300 MH_z

^{1}H NMR of Acetylated Compound c in $CDCl_3$ at 300 MHz

δ6.24 [d, 1H, J=4.4Hz, -Glc(S-1) H-1], δ5.38 [d, 1H, J=8.4Hz, β-Glc(S-1) H-1], δ5.28 [d, 1H, J=3.9Hz, -GlcNAc(S-4) H-1], δ4.68 [d, 2H, J=8.1Hz, β-Glc(S-3) & β-GalNAc(S-6) H-1], δ4.56 [d, 2H, J=9.2Hz, β-Gal(S-2 & S-5) H-1], δ3.90 [m, 2H, β-Gal(S-2 & S-5) H-4], δ3.85 [m, 1H, β-Glc(S-3) H-3], δ3.50 [m, 2H, β-Glc(S-1 & S-3) H-4].

^{13}C NMR of Acetylated Compound c in $CDCl_3$ at 300 MHz

δ90.31 [1C, -Glc(S-1) C-1], δ90.42 [1C, β-Glc(S-1) C-1], δ92.12 [1C, -GlcNAc(S-4) C-1], δ95.49 [1C, β-Glc(S-3) C-1], δ101.87 [1C, β-GalNAc(S-6) C-1], δ102.02 [2C, β-Gal(S-2 & S-5) C-1].

^{1}H NMR of Compound C in D_2O at 300 MHz

δ5.23 [d, 1H, J=4.0Hz, -Glc(S-1) H-1], δ5.18 [d, 1H, J=3.9Hz, -GlcNAc(S-4) H-1], δ4.58 [d, 1H, J=8.1Hz, β-Glc(S-1) H-1], δ4.53 [d, 1H, J=7.9Hz, β-Glc(S-3) H-1], δ4.44 [d, 2H, J=8.0Hz, β-Gal(S-2 & S-5) H-1], δ4.31 [d, 1H, J=7.6 Hz, β-GalNAc(S-6) H-1], δ3.27 [m, β-Glc(S-1), H-2] δ1.97[s, 3H, $NHCO\underline{CH}_3$, -GlcNAc(S-4)], δ1.93[s, 3H, NHCOCH, β-GalNAc(S-6)]

ES Mass

1134[M+Na+K], 1111[M+K], 1095[M+Na], 1072[M^+], 1027[1072-CH_3CHO,-H^+],879[1072-$NHCOCH_3$-$CH_2OHCOCO$,-H_2O,-HCHO], 806[869-CH_3CHO,-H_3O^+], 757[869-HOCHCHOH,-H_3O^+,-CH_3OH,H^+], 669[707-2H_3O^+], 651[669-H_2O], 489[651-2HOCHCHOH,-CH_2CO], 496[707-$NHCOCH_3$, -$CH_2OHCOCO$,-HCHO-2H_2O], 465[504-2H_3O^+,-H^+], 406[465-CH_2CO,-OH], 365[465-2CH_3OH, -2H_2O], 303[342-2H_3O^+,-H^+], 244[303-CH_2CO,-OH] and 174[244-2H_3O^+ CH_3OH].

3. Result and discussion

3.1. Structure elucidations of Usose

3.1.1. NMR spectroscopy

Compound C, Usose ($C_{40}H_{68}O_{31}N_2$) $[\alpha]_D^{25}$ = - 46° gave positive phenol-sulphuric acid test[24], Feigl test[25] and Morgan-Elson test[23] owing to the presence of normal and amino sugars in Usose. The ^{1}H NMR spectrum **Fig-4** (s. f.) of Usose showed six doublets for seven anomeric proton signals at 5.23(1H), δ5.18 (1H), 4.58(1H), 4.53(1H) 4.44(2H) and 4.31(1H) in D_2O at 300 MHz. These proton signals of Usose had two doublet at δ5.23 (J=4.0Hz) and δ4.58 (J=8.1Hz) for its α

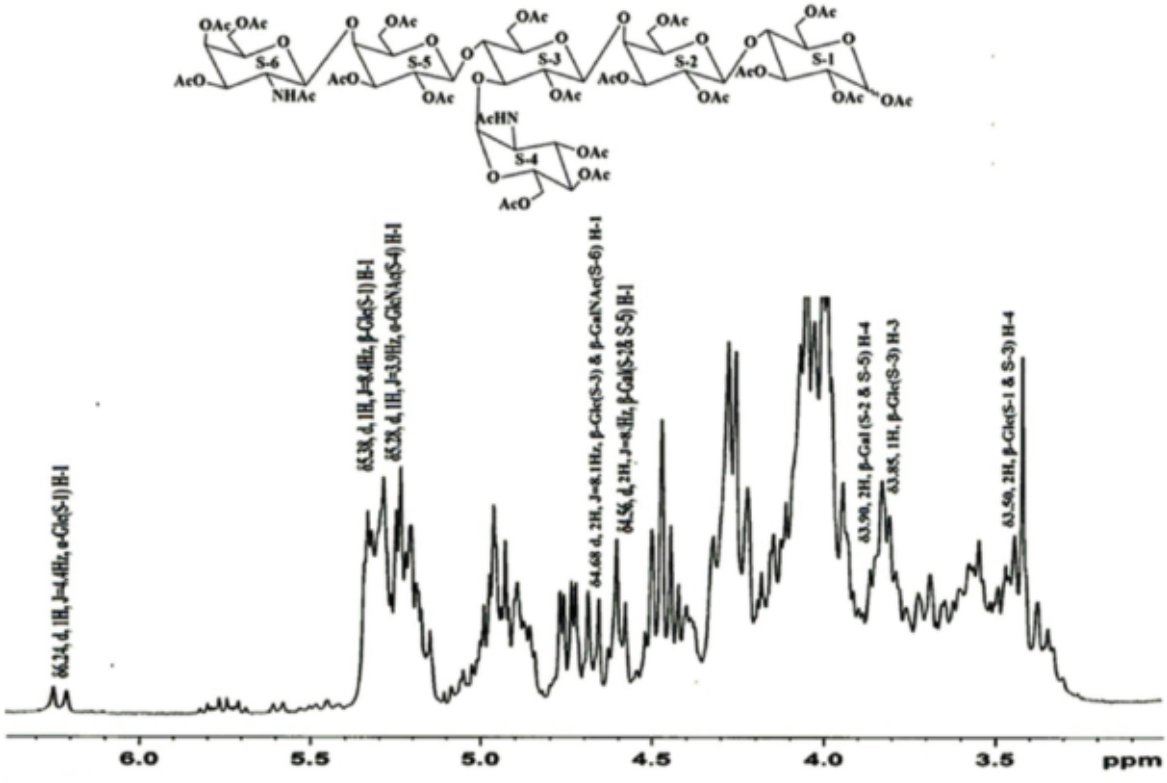


Fig-2 ^{1}H NMR Spectrum of Usose Acetate in $CDCl_3$ at 300 MH_z

and β anomers of glucose suggesting that the Usose was a hexasaccharide in its reducing form containing glucose at the reducing end. Further, the HSQC spectrum **Fig-1** of acetylated Usose indicated the presence of six cross peaks of seven anomeric protons and carbons in their respective region at δ6.24x90.31, δ5.38x90.42, δ5.28x92.12, δ4.68x101.87, δ4.68x95.49 and δ4.56x102.02. These cross peaks suggested that Usose may be a hexasaccharide in its reducing form. Cross peaks at δ6.24x90.31 and δ5.38x90.42 suggested for presence of and anomers of glucose in reducing form. Further confirmation of the reducing nature of Usose was done by its methylglycosylation MeOH/H^+ followed by its acid hydrolysis **scheme-1**. This led to the isolation of α and β-methylglucosides, along with Glc, Gal, GlcNAc and GalNAc suggesting the presence of glucose at the reducing end and presence of Glc, Gal, GlcNAc and GalNAc moieties in Usose. The monosaccharides constituents in Usose were confirmed by its Killiani hydrolysis **scheme-1 [26]** 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 (PC, TLC) which confirmed that Usose contained four types of monosaccharides units i.e. Glc, Gal, GlcNAc and GalNAc. For the sake of convenience, the six monosaccharides present in compound have been assigned as S-1, S-2, S-3, S-4, S-5 and S-6 starting from reducing end. The hexasaccharide nature of acetylated Usose was also confirmed by the presence of five doublets for seven anomeric protons at δ6.24(1H), δ5.38(1H), δ5.28(1H), δ4.68(2H) and δ4.56(2H) in the ^{1}H NMR spectrum of acetylated Usose in $CDCl_3$ at 300 MHz. Further hexasaccharide nature of Usose was confirmed by the presence of six anomeric carbon signals for seven anomeric carbon at δ90.31(1C), δ90.42(1C), δ92.12(1C), δ95.49(1C), δ101.87(1C) and δ102.02(2C) in the ^{13}C NMR spectrum **Fig-3** of acetylated Usose in $CDCl_3$ at 300 MHz. The ^{1}H and ^{13}C NMR spectra of Usose justify the seven anomeric signals for hexasaccharide with total integral intensity of seven anomeric protons and carbons. The mass ion peak at 1072 $[M]^+$ present in ES-MS of Usose was in agreement with molecular formula $C_{40}H_{68}O_{31}N_2$.

Table 1: ^{1} H NMR of anomeric proton values of compound C in $CDCl_3$ and D_2O at 300 MHz.

Moieties	In $CDCl_3$		In D_2O	
	^{1}H NMR(δ)	Coupling constant(J)	^{1}H NMR(δ)	Coupling constant(J)
α-Glc(S-1)	6.24	4.4Hz	5.23	4.0Hz
β-Glc(S-1)	5.38	8.4Hz	4.58	8.1Hz
β-Gal(S-2)	4.56	9.2Hz	4.44	8.0Hz
β-Glc(S-3)	4.68	8.1Hz	4.53	7.9H
α-GlcNAc(S-4)	5.28	3.9Hz	5.18	3.9Hz
β-Gal(S-5)	4.56	9.2Hz	4.44	8.0Hz
β-GalNAc(S-6)	4.68	8.1Hz	4.31	7.6Hz

The ^{1}H NMR spectrum **Fig-4** (s.f.) of Usose in D_2O at 300 MHz contain two doublets at δ5.23 (J= 4.0Hz) and δ4.58 (J= 8.1Hz) confirmed the presence of glucose at its reducing form[27-30] in the hexasaccharide. Further the presence of another anomeric doublet at δ4.44 (J= 8.0 Hz) in the ^{1}H NMR spectrum in D_2O suggested the presence of β-Gal[29,31] (S-2) residue as the next monosaccharide unit**.** In addition to anomeric signals of α-Glc and β-Glc, presence of a triplet at δ3.28 which was due to H-2 of β-Glc (S-1) suggested the presence of Lactose structure (structure reporter group)[32-33] i.e. β-Gal(1-4)→Glc at the reducing end of Usose. Simultaneously ^{1}H NMR spectrum **Fig-2** (s.f.) of Usose acetate showed downfield shifted α and β anomeric proton signal of the reducing glucose (S-1) i.e. Glc (S-1) at δ6.24 (J= 4.4 Hz), and δ5.38 (J= 8.4Hz)[34]. The anomeric protons signal existing at δ5.38 (J=8.4) in TOCSY Spectrum **Fig-5** (s.f.) of Usose acetate assigned to β-Glc (S-1) proton at 5.38 gave three cross peaks with 3.50, 4.56 and 5.15ppm which was later identified as H-4, H-2 and H-3 of reducing Glc respectively in the COSY spectrum **Fig-6** (s.f.) of Usose acetate. The peak at 3.50ppm assigned to H-4 of S-1 suggested that one position was available for glycosidic linkage in S-1 moiety by next monosaccharide unit. The earlier suggested (1→4) linkage between β-Glc (S-1) and β-Gal (S-2) was further confirmed by HMBC spectrum **Fig-7** (s.f.) of Usose acetate at 300 MHz which contain

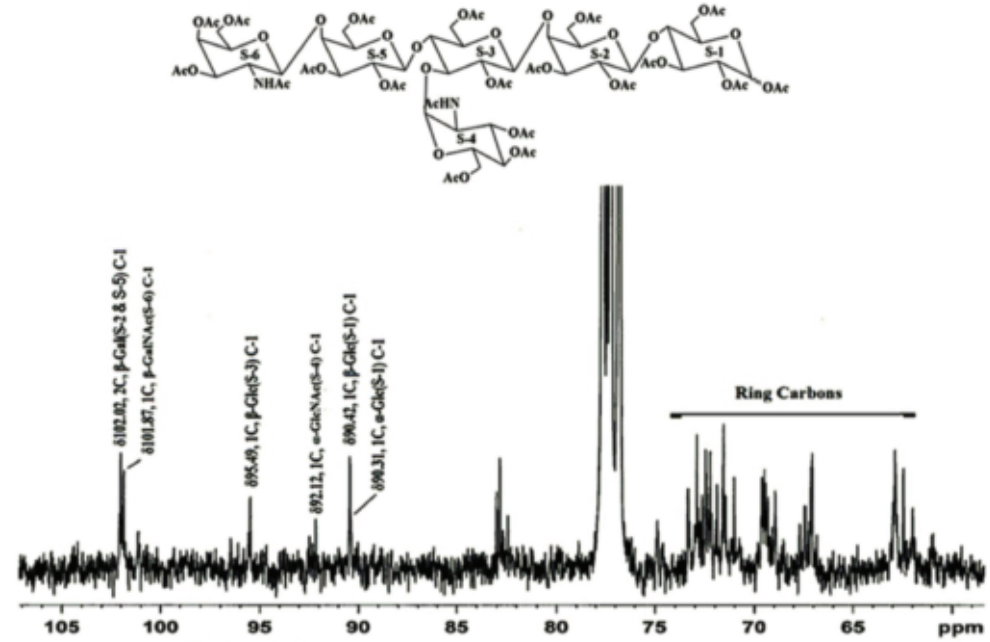


Fig-3 ^{13}C NMR Spectrum of Usose Acetate in $CDCl_3$ at 300 MH_z

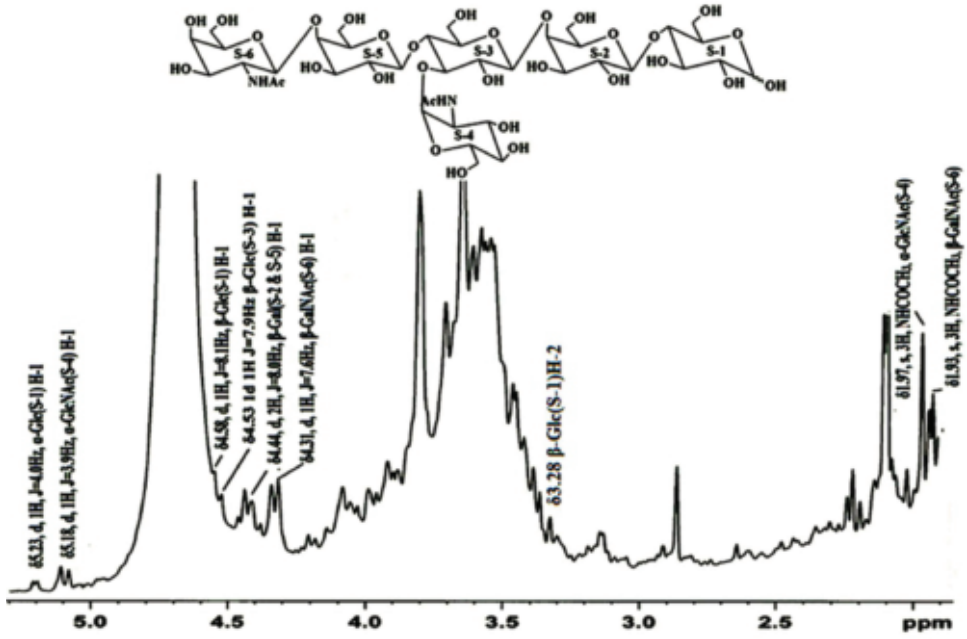


Fig-4 ^{1}H NMR Spectrum of Usose Acetate in D_2O at 300 MH_z

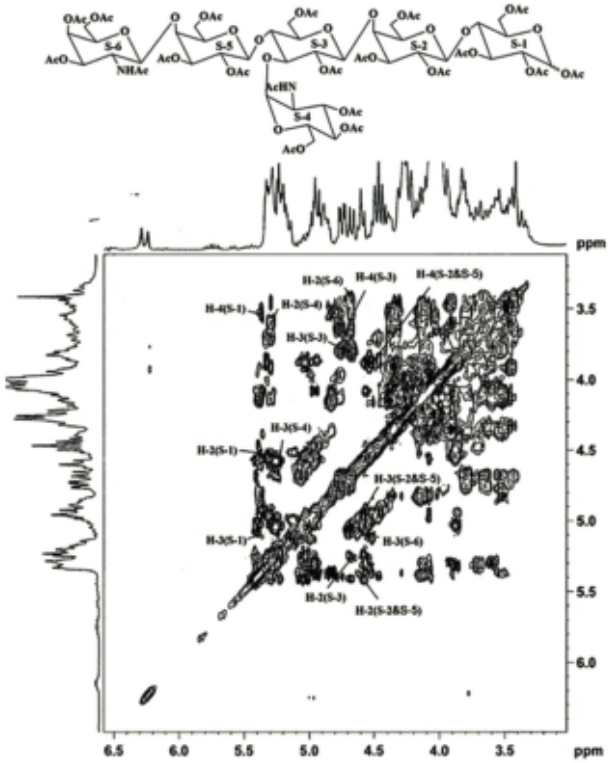


Fig-5 COSY Spectrum of acetylated Usose in $CDCl_3$ at $300MH_z$

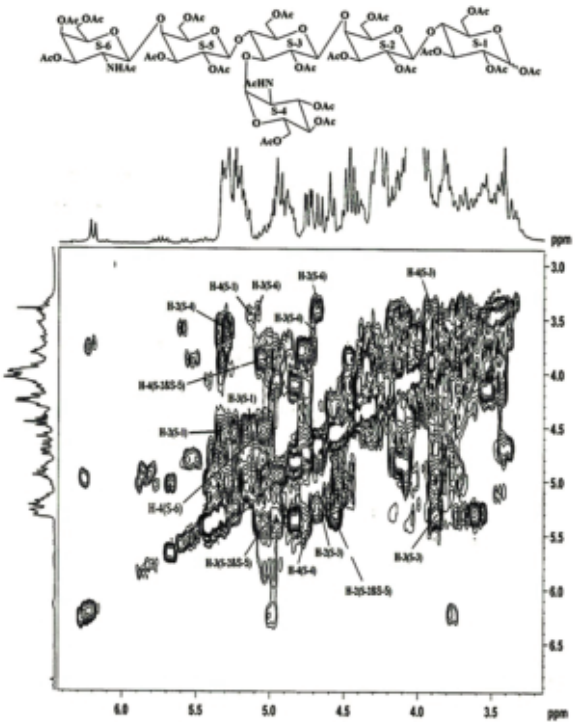


Fig-6 TOCSY Spectrum of acetylated Usose in $CDCl_3$ at $300MH_z$.

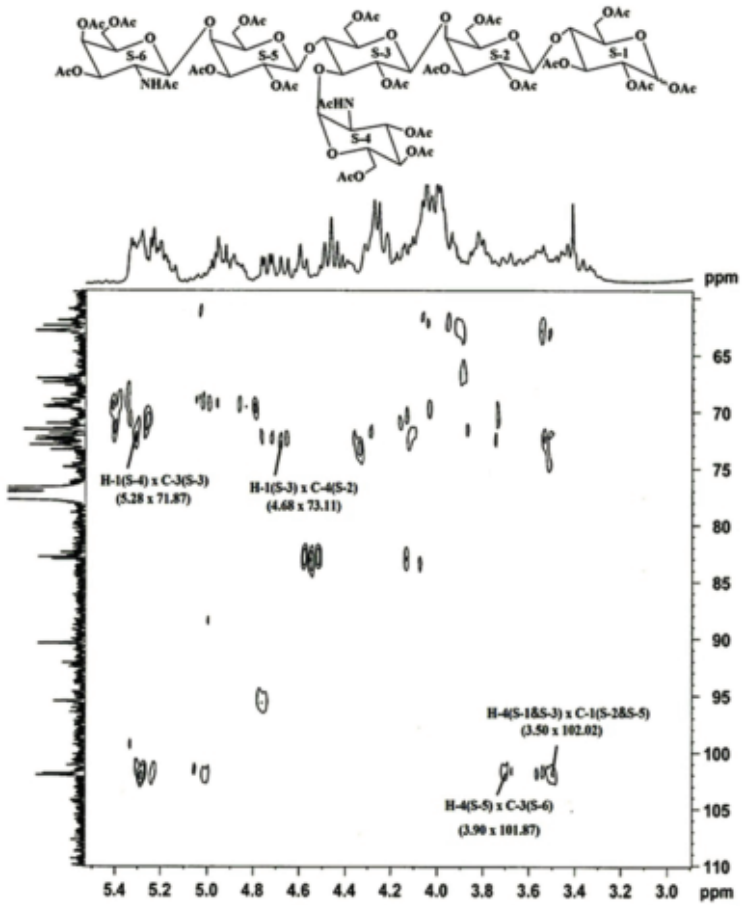


Fig-7 ^{1}H-^{13}C HMBC Spectrum of acetylated Usose in $CDCl_3$ at 300 MH_Z.

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 δ3.50x102.02. The anomeric carbon of β-Gal (S-2) at δ102.02 gave its complimentary anomeric proton signal at δ4.56 (J=9.2Hz) in the HSQC spectrum **Fig-1** (s.f.) of Usose 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 δ4.56 for β-Gal (S-2) had a J value of 9.2Hz confirming the β glycosidic linkage between S-2→S-1. The (1→4) linkage between β-Glc (S-1) and β-Gal (S-2) was also supported by the presence of H-4 signal of S-1 at δ3.50 in upfield region of ^{1}H NMR spectrum of and a cross peak at δ3.50x82.93 in glycosidic region of HSQC spectrum of Usose acetate in $CDCl_3$. The anomeric proton signal at δ4.56 in ^{1}H NMR spectrum of Usose acetate in $CDCl_3$ assigned for β-Gal (S-2) gave three cross peaks at δ4.56x3.90, δ4.56x5.05 and δ4.56x5.36 in its TOCSY spectrum which was later identified as H-4, H-3 and H-2 by COSY spectrum of Usose acetate. The cross peak at δ4.56x3.90 assigned to H-4 of S-2 suggested that one position was available for glycosidic linkage in S-2 moiety by next monosaccharide unit. The H-4 position of β-Gal (S-2) at δ3.90 showed its C-4 position at δ73.11 in HSQC spectra of Usose acetate which showed a long range coupling with anomeric proton of next monosaccharide S-3 i.e. H-1 of S-3 and C-4 of S-2 at δ4.68x73.11 in HMBC spectrum of acetylated Usose in $CDCl_3$ at 300 MHz confirming (1→4) linkage between S-3 and S-2. The anomeric proton signal at δ4.68 (J=8.1) showed its complimentary carbon signal at δ95.49 in HSQC spectrum of Usose 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-Glc (S-3), hence S-3 monosaccharide was confirmed a β-Glc (S-3). The coupling constant of anomeric proton signal present at δ4.68 for β-Glc (S-3) had a J value of 8.1Hz in $CDCl_3$ suggesting a β glycosidic linkage between S-3→S-2. Further signal of anomeric proton at δ4.53 with J value 7.9 Hz in the ^{1}H NMR of Usose in D_2O at 300 MHz also

confirmed β glycosidically linked Glc (S-3) in Usose. The (1→4) linkage between β-Gal (S-2) and β-Glc (S-3) was supported by the presence of H-4 signal of S-2 at δ3.90 in upfield region of ^{1}H NMR spectrum and a cross peak at δ3.90x73.11 in glycosidic region of HSQC spectrum of Usose acetate in $CDCl_3$. The anomeric protons signal present at δ4.68 in ^{1}H NMR Spectrum of Usose acetate in $CDCl_3$ assigned to β-Glc (S-3) gave three cross peaks at δ4.68x3.50, δ4.68x3.85, and δ4.68x5.25 in the TOCSY spectrum **Fig-5** (s.f.) of Usose acetate, which were later identified as H-4, H-3 and H-2 respectively by COSY spectrum of Usose acetate. The chemical shift of the cross peak at δ4.68x3.50 and δ4.68x3.85 suggested that in Glc S-3, two positions were available for glycosidic linkage by next monosaccharide units. The H-3 position of β-Glc (S-3) at 3.85 showed its C-3 position at δ71.87 in HSQC spectra of Usose acetate which showed a long range coupling with anomeric proton of next monosaccharide S-4 i.e. H-1 of S-4 and C-3 of S-3 at δ5.28x71.87 in HMBC spectrum of acetylated Usose in $CDCl_3$ at 300 MHz suggesting the (1→3) linkage between S-4 and S-3. The anomeric proton signal at δ5.28 showed its complimentary carbon signal at δ92.12 in HSQC spectrum of Usose 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 α-GlcNAc (S-4), hence S-4 monosaccharide was confirmed as α-GlcNAc (S-4). The coupling constant of anomeric proton signal present at δ5.28 for α-GlcNAc (S-4) had a J value of 3.9 Hz in $CDCl_3$ confirmed a α glycosidic linkage between S-4→S-3. Simultaneously the ^{1}H NMR spectrum of Usose in D_2O at 300 MHz contain anomeric proton signal at δ5.18 (J=3.6 Hz) along with a singlet of three proton of amide methyl at δ1.97 suggested that S-4 was GlcNAc. Further signal of anomeric proton at δ5.18 with J value 3.6 Hz in the ^{1}H NMR of Usose in D_2O at 300 MHz also confirmed that GlcNAc (S-4) was glycosidically linked to Glc S-3 by α glycosidic linkage. The (1→3) linkage between β-Glc (S-3) and α-GlcNAc (S-4) was supported by the presence of H-3 signal of S-3 at δ3.85 in upfield region of ^{1}H NMR spectrum and a cross peak at δ3.85x71.87 in glycosidic region of HSQC spectrum **Fig-1** (s.f.) of Usose acetate in $CDCl_3$. The anomeric proton signal at δ5.28 in ^{1}H NMR spectrum of Usose acetate in $CDCl_3$ assigned for α-GlcNAc (S-4) gave three cross peaks at δ5.28x3.95, δ5.28x5.04 and δ5.28x4.60 in its TOCSY spectrum of Usose acetate, which was later identified as H-2 (containing NHAc group), H-3 and H-4 by COSY spectrum of Usose acetate. This anomeric proton did not have any cross peak in the linkage region, hence it was confirmed that α-GlcNAc (S-4) was present at non-reducing end and no hydroxyl groups of α-GlcNAc (S-4) were involved in glycosidic linkage. Above it was already described by the COSY and TOCSY spectrum of Usose acetate that the β-Glc (S-3) had two vacant positions i.e. H-3 and H-4 and it was earlier confirmed that H-3 of S-3 was linked with α-GlcNAc (S-4) but H-4 of S-3 was still vacant further the ^{1}H NMR signal of H-4 (S-3) at δ3.50 gave a cross peak at δ3.50xδ102.02 in HMBC spectrum of Rusose acetate confirming a (1→4) linkage between (S-5) and (S-3). The anomeric carbon of β-Gal (S-5) at δ102.02 gave its complimentary anomeric proton signal at δ4.56 (J=9.2) in the HSQC spectrum of Usose 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-5), hence S-5 monosaccharide was confirmed as β-Gal (S-5). The anomeric proton signal present at δ4.56 (S-5) had a J value of 9.2Hz confirming the β glycosidic linkage between S-5→S-4. The presence of β-Gal (S-5) was further confirmed by presence of doublet at δ4.44 with J value 8.0 Hz in the ^{1}H NMR of Usose in D_2O at 300 MHz. The (1→4) linkage between β-Glc (S-3.) and β-Gal (S-5) was also supported by the presence of H-4 signal of S-1 at δ3.50 in upfield region of ^{1}H NMR spectrum and a cross peak at δ3.50x82.93 in glycosidic region of HSQC spectrum of Usose acetate in $CDCl_3$. The anomeric proton signal at δ4.56 in ^{1}H NMR spectrum of Usose acetate in $CDCl_3$ assigned for β-Gal (S-5) gave three cross peaks at δ4.56x3.90, δ4.56x5.05 and δ4.56x5.36 in its TOCSY spectrum, which was later identified as H-4, H-3 and H-2 by COSY spectrum of Usose acetate. The cross peak at δ4.56x3.90 assigned to H-4 of S-5 suggested that one position was available for glycosidic linkage in S-5 moiety by next monosaccharide units. The ^{1}H NMR signal of H-4 (S-5) at δ3.90 gave a cross peak at δ3.90xδ101.87 in HMBC spectrum of Usose acetate confirming a (1→4) linkage between (S-6) and (S-5). The anomeric carbon of (S-6) at δ101.87 gave its complimentary anomeric proton signal at δ4.68 in the HSQC spectrum of Usose acetate. 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). The anomeric proton signal present at δ4.68 (S-6) had a J value of 7.9 Hz suggesting β glycosidic linkage between S-6→S-5. Further signal of anomeric proton at δ4.31 with J value 7.6 Hz in the ^{1}H NMR of Usose in D_2O at 300 MHz also confirmed β glycosidically linked GalNAc (S-6). Simultaneously the ^{1}H NMR spectrum of Usose in D_2O at 300 MHz contain anomeric proton signal at δ4.31(J=7.6 Hz) along with a singlet of three

proton of amide methyl at δ1.93 suggested that S-6 was GalNAc **[35]**. The (1→4) linkage between β-Gal(S-5) and β-GalNAc (S-6) was also supported by the presence of H-4 signal of S-5 at δ3.90 in upfield region of ^{1}H NMR spectrum and a cross peak at δ3.90x73.11 in glycosidic region of HSQC spectrum of Usose acetate in $CDCl_3$. The anomeric proton signal at δ4.68 in ^{1}H NMR spectrum of Usose acetate in $CDCl_3$ assigned for β-GalNAc (S-6) gave three cross peaks at δ4.68x3.85, δ4.68x4.55 and δ4.68x5.14 in its TOCSY spectrum, which was later identified as H-2 (containing NHAc group), H-4 and H-3 by COSY spectrum of Usose acetate. This anomeric proton did not have any cross peak in the linkage region, hence it was confirmed that β-GalNAc (S-6) was present at non-reducing end and no hydroxyl groups of β-GalNAc (S-6) were involved in glycosidic linkage. The 2D NMR (COSY, TOCSY, HSQC and HMBC) experiments further confirming the ^{1}H NMR and ^{13}C NMR for the ring protons and carbons of the monosaccharides unit of Usose. 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. In the light of above given evidence, the structure of Usose was established as:

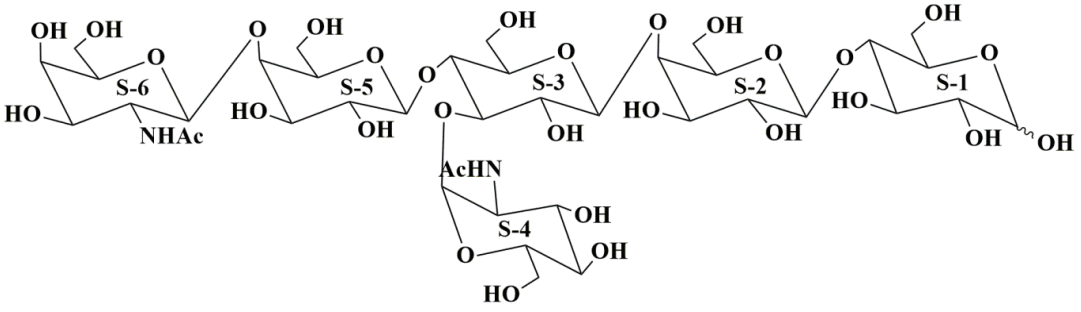
GalNAc-β(1→4)Gal-β(1→4)Glc-β(1→4)Gal-β(1→4)Glc

↑

GlcNAc-α(1→3)

Usose

The result obtained from the ES mass spectrum further substantiated the structure of Usose which was derived by its ^{1}H and ^{13}C NMR spectra. The highest mass ion peaks **Fig-8** (s.f.) were recorded at m/z 1134, 1111 and 1095 which were due to $[M+Na+K]^+$, $[M+K]^+$ and $[M+Na]^+$ respectively. It also contains the molecular ion peak at m/z 1072 confirming the molecular weight of Usose as 1072[M^+] which was in agreement with its molecular formula $C_{40}H_{68}O_{31}N_2$. 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 fragmentation pathway confirmed the sequence of monosaccharide units in the hexasaccharide. The hexasaccharide Usose m/z 1072(I) fragmented to give mass ion peak at m/z 869(II) [1072-S_6], this fragment was arised due to the loss of terminal β-GalNAc (S-6) moiety from hexasaccharide indicating the presence of β-GalNAc (S-6) at the non-reducing end. It further fragmented to give mass ion peak at m/z 707(III) [869-S_5] which was due to loss of β-Gal (S_5) moiety from the pentasaccharide. This pentasaccharide unit fragmented to give mass ion peak at m/z 504(V) [707-S_4], which was due to loss of α-GlcNAc (S_4) moiety from tetrasaccharide. The tetrasaccharide m/z 504(V) fragmented to give mass ion at m/z 342 (VI) [504-S_3], this fragment was arised due to the loss of β-Glc (S_3) moiety from trisaccharide indicating the presence of β-Glc (S_3). Further peak at fragment of 342 was 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 the disaccharide. The other fragmentation pathway in ES Mass spectrum **Fig-8** (s.f.) of compound E, Usose m/z 1072 shows the mass ion peak at 1027[1072-CH_3CHO,-H^+],879[1072-$NHCOCH_3$-$CH_2OHCOCO$,-H_2O,-HCHO], 806[869-CH_3CHO,-H_3O^+], 757[869-HOCHCHOH,-H_3O^+,-CH_3OH,H^+], 669[707-$2H_3O^+$], 651[669-H_2O], 489[651-2HOCHCHOH,-CH_2CO], 496[707-$NHCOCH_3$, -$CH_2OHCOCO$,-HCHO-$2H_2O$], 465[504 -$2H_3O^+$,-H^+], 406[465-CH_2CO,-OH], 365[465-$2CH_3OH$, -$2H_2O$], 303[342-$2H_3O^+$,-H^+], 244[303-CH_2CO,-OH] and 174[244-$2H_3O^+$ CH_3OH],. Based on result obtained from chemical degradation/acid hydrolysis, Chemical transformation, Electro spray mass spectrometry, structure reporter group and 1D-NMR viz. ^{1}H NMR, ^{13}C NMR, and 2D-NMR viz. COSY, TOCSY, HMBC and HSQC spectra of Usose acetate and Usose, the structure and sequence of isolated Novel oligosaccharide Usose was deduced as-:



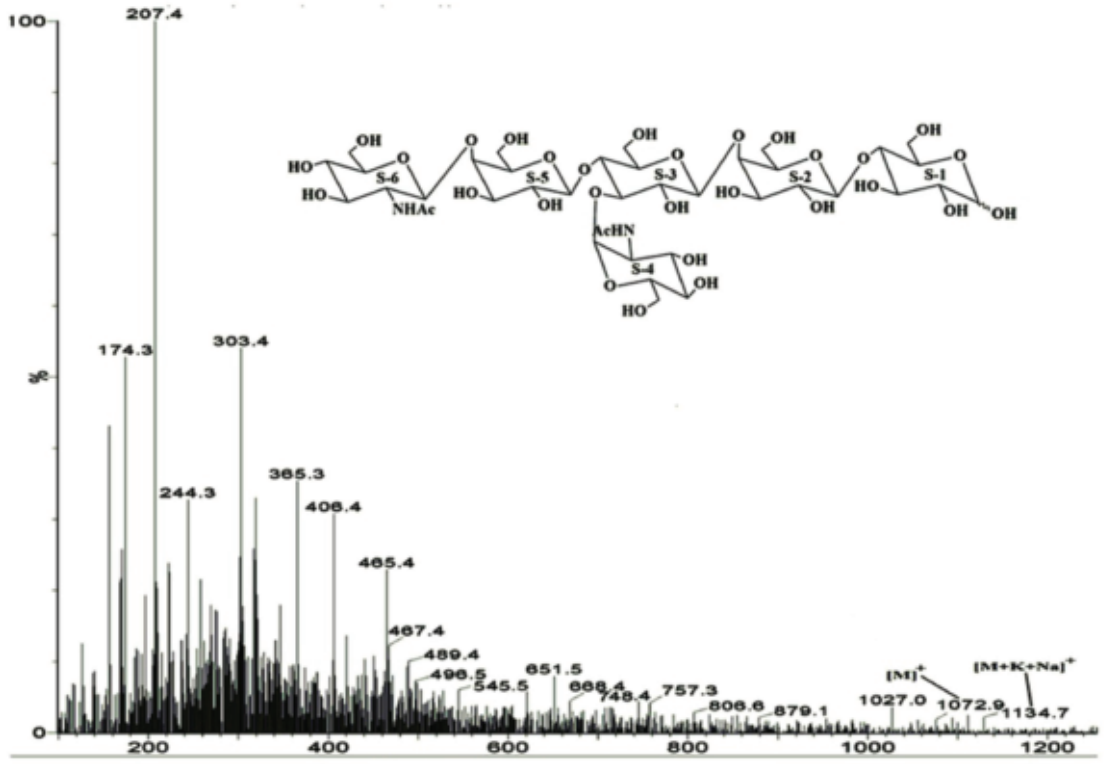


Fig-8 ES-Mass spectrum of Compound Usose.

4. Conclusion

In summary, the novel milk oligosaccharides namely as **C** (Usose) has been isolated from Lal-Muha Cow milk and its structure was elucidated with the help of ^{1}H, ^{13}C, 2D NMR spectroscopy and mass spectrometry.

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