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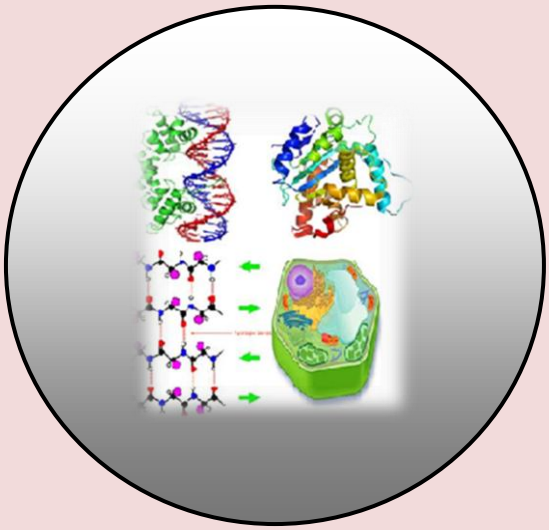

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Structure Elucidation of a Novel Hexasaccharide 'Tososе' from Jersy Cow Colostrum by 2D NMR and Mass Spectrometry

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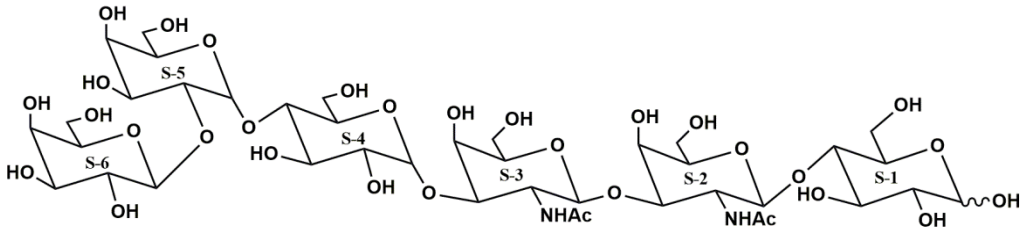
ABSTRACT



TOSOSE

The importance of cow milk as immunostimulant, brain developer, bone strengthening and eye-sight enhancer is defined in ancient Indian literature. Cow milk contains protein, fat and carbohydrate. The carbohydrate content is made up of lactose and oligosaccharides. Recent researches have shown that the oligosaccharide present in any milk is responsible for its biological activity. In the present study we have worked on Jersy cow colostrum for its oiligosaccharide content. The Jersy cow colostrum was collected in bulk and processed by modified method of Kobata and Ginsburg, resulting into the isolation of crude oligosaccharide mixture which was further acetylated for its easy purification of column chromatography and removing the spectral degeneracy while interpreting the NMR spectrum. In this process, a novel hexasaccharide, Tososе was isolated from Jersy cow colostrum and its structure was elucidated by chemical degradation, chemical transformation 1D and 2D NMR and Mass spectrometry.

Keywords: Milk, Oligosaccharides, Tososе, ^{1}H and ^{13}C NMR, HSQC, TOCSY, COSY, Mass spectrometry.

INTRODUCTION

The medicinal values and glory of cow milk are well explained in ancient Indian literature of Charak Samhita and Ayurveda. It is also beneficial for the growth of infants and adults. Cow milk is also considered as a substitute for mother's milk. In ancient Indian medicinal literature, it has been defined as AMRITA (Ghopal et al, 2015; Mana et al, 2021). It is constituted of protein, fat, or carbohydrate; however, it is not clearly defined that which part or constituent of milk is responsible for its biological activity. Recent researches by glycochemists and glycobiologists has revealed that oligosaccharides of any milk, play a decisive role in its biological activity. These oligosaccharides have shown to promote the development of the immune system, brain, nervous system, and bones (Ghopal et al, 2015). Additionally, cow milk also exhibit biological activities such as anti-inflammatory, antioxidant, and immunological effects (Gangwar et al, 2018; Mohapatra et al, 2019, Singh et al, 2016; Singh et al, 2019). Notably, it also enhances the production of milk in lactating women (Angella et al, 2011). Oligosaccharides isolated from cow milk have unique medicinal benefits, such as promoting the growth of beneficial bifidobacteria in the intestine, which helps in preventing the infections by inhibiting the adhesion of pathogenic bacteria to the intestinal mucosa.

Furthermore, these oligosaccharides have applications as tumor markers, brain enhancers, and anti-infective agents (Khan et al, 2019; Angela et al, 2011). The oligosaccharides found in cow milk and colostrum consists of glucose, galactose, GlcNHAc, and GalNHAc (Gunjan et al, 2019; Sinha et al, 2017). These monosaccharides are linked by O-glycosidic linkage at various positions, with different configurations (α or β). In our previous studies, we isolated oligosaccharides containing four to twelve monosaccharide units, with both straight and branched-chain structures. Some identified oligosaccharides, such as aurose, asose, rusose, usose, and taurose, were isolated from different cow species (Gunjan et al, 2019; Sharma et al, 2022). In the present study, we have collected 10 liter of Jersy cow colostrum from Lucknow, Uttar Pradesh, India, and was processed by modified method of Kobata and Ginsburg, (Gunjan et al, 2019; Sharma et al, 2022) incorporating deprotination, centrifugation, microfiltration, gel filtration, and lyophilization. The isolation of crude oligosaccharide mixtures was further acetylated by acetic anhydride and pyridine for converting it into their non-polar acetyl derivatives. The oligosaccharides were purified by silica column chromatography and the homogeneity of the oligosaccharide mixture was confirmed by high-performance liquid chromatography. This process led to the isolation of a novel hexasaccharide named Tosose (Compound F). The stereoscopic structure of the new oligosaccharide was elucidated with the help of chemical degradation, chemical transformation, ^{1}H, ^{13}C, 2D NMR (COSY, TOCSY, HSQC and HMBC) experiments along with ES-Mass spectrometry.

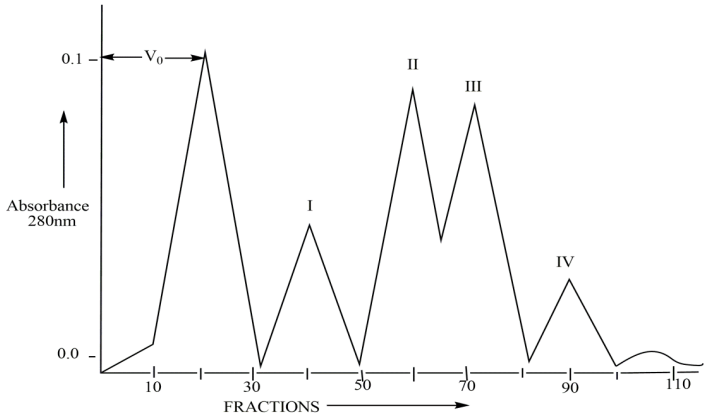
EXPERIMENTAL

The sugars were visualized on TLC with 50% aqueous H_2SO_4 reagent and on paper chromatography, sugars were visualized with acetyl acetone and p-dimethyl amino benzaldehyde reagents. The absorbent for TLC was silica gel G (SRL) and CC silica gel (SRL, 60-120 mesh).

Freeze drying of the compound was done with the help of CT 60e (HETO) Lyophylizer and centrifuged by a cooling centrifuge Remi instruments C-25 at 5000 rpm. The ^{1}H and ^{13}C NMR spectra of oligosaccharides were recorded in $CDCl_3$ at 25^{o}C on a Bruker AM 300 MHz NMR spectrometer. The electron spray mass spectra were recorded on a MICROMASS QUATTRO II triple quadruple mass spectrometer. For mass spectroscopy the sample was dissolved in suitable solvent such as methanol/acetonitrile/water and was introduced into the ESI source through a syringe pump at the rate 5µl per min. The ESI capillary was set at 3.5 KV and the cone voltage was 40 V.

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

10-liter cow colostrum (1-5 day) was collected from a domestic cow of district Lucknow, Uttar Pradesh. The milk was fixed by addition of equal amount of ethanol. The preserved milk was taken to laboratory and then 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. More ethanol was added to clear filtrate to a final concentration of 68% and the resulting solution was left over night 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 and lyophilized affording crude oligosaccharides mixture (205 gm).



Graph 1. Spehadex G-25 chromatography of Cow colostrum Oligosaccharides Detected by Phenol Sulphuric Acid Method. Elution was made with TDW.

Sephadex G-25 Gel Filtration of Cow Oligosaccharides mixture

The repeated gel filtration was performed by Sephadex G-25 chromatography of crude colostrum oligosaccharide mixture. Colostrum oligosaccharide mixture was packed in a column (1.6 x 40 cm) (void volume = 25 ml) equilibrated with glass triple distilled water (TDW) and it was left for 10-12 hrs to settle down. The material was applied on a Sephadex G-25 column and was eluted for separation of protein and glycoprotein from oligosaccharide (low molecular weight component). Presence of neutral sugars was monitored in all eluted fractions by phenol-sulphuric acid test.

In this U.V. monitored Sephadex G-25 chromatography of cow colostrum oligosaccharide mixture showed four peaks i.e. I, II, III and IV. Fractions under peaks II and III gave a positive phenol-sulphuric acid test for sugars which showed the presence of oligosaccharide mixture in cow colostrum. These fractions (peak II and III) were pooled and lyophilized (Graph 1 and Table 1).

Table 1. Cow Colostrum Oligosaccharides Mixture Chromatographed Over Sephadex G-25 Column Chromatography.

Fraction No.	Peak	Compound (in gm)	Phenol-H_2SO_4 test for sugar	Further investigation
1-31	-	3.53	-ve	
32-46	-	1.47	+ve	
47-64	Peak II	4.50	+++ve	Peak II and III were taken for further investigation (11gm)
65-78	Peak III	6.50	+++ve	
79-100	Peak IV	0.52	-ve	

Acetylation of Oligosaccharide mixture [Singh et al., 2019]

10 gm Oligosaccharides mixture was 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 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 (10.8gm). 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 four compounds were finally separated by column chromatography over silica gel using varying proportions of hexane, chloroform and methanol as eluents. Detection of the spots were done by spraying with 50% H_2SO_4.

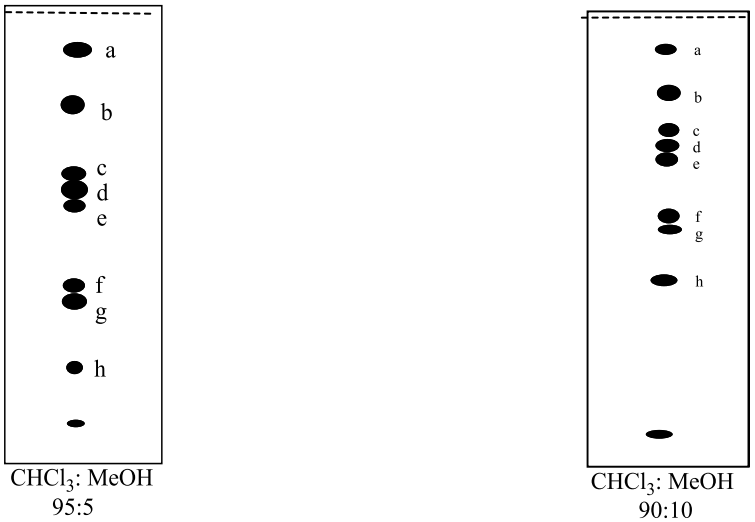


Figure 1. TLC of Acetylated Oligosaccharides Mixture at Different Polarity Proportions.

Purification of acetylated oligosaccharide mixture on Silica gel chromatography

Purification of the acetylated oligosaccharide mixture (10 gm) was carried out over silica gel (500 gm) using varying proportion of $CHCl_3$, $CHCl_3$: MeOH as eluents, collecting fraction of 500 ml each. All these fractions were checked on TLC and those showing similar spots were taken together for further investigations. Repeated column chromatography was done and combined fractions of 135-154 (750mg) from chromatography 1 containing d, e and f were chromatographed over 50 gm silica gel. The elution was carried out using Hexane: $CHCl_3$, $CHCl_3$ and $CHCl_3$: MeOH as eluent in different proportion and collecting fraction of 50 ml each.

Fractions No. 135-154 (750mg) Mixture Chromatographed over 50 gm silica gel.

Fraction no.	**Solvent**	**Eluted Residue Amorphous (mg)**	**Spots on TLC**	**Further Investigation**
1-15	Hexane:$CHCl_3$ (10:90)	_	_	_
16-32	Pure $CHCl_3$	_	_	_
33-40	Pure $CHCl_3$	20mg	streaking	_
41-60	MeOH:$CHCl_3$ (0.1:99.9)	_	_	_
61-78	MeOH:$CHCl_3$ (0.1:99.9)	70mg	streaking	_
79-101	MeOH:$CHCl_3$ (0.2:99.8)	65mg	d with streaking	CC-5
102-125	MeOH:$CHCl_3$ (0.2:99.8)	223mg	d, e	_
126-143	MeOH:$CHCl_3$ (0.2:99.8)	138mg	d, e, f	_
144-165	MeOH:$CHCl_3$ (0.5:99.5)	105mg	e, f	CC-6
166-180	MeOH:$CHCl_3$ (0.5:99.5)	92mg	f	CC-7
181-189	MeOH:$CHCl_3$ (1:99)	14mg	washing	_

Deacetylation of compound f, Tososе Acetate: [Kumar et al., 2019]

Compound **'f'** (92 mg) was obtained from column chromatography of acetylated oligosaccharide mixture. 35 mg of compound f was dissolved in acetone (3 ml) and 3.5 ml of NH_4OH was added in it and was left overnight in a stoppered hydrolysis flask. After 24 hours ammonia was removed under reduced pressure and the compound was washed thrice with $CHCl_3$ (5 ml) (to remove acetamide) and water layer was finally freeze dried giving the deacetylated oligosaccharide **F** (29 mg).

Methyl glycosidation/Acid hydrolysis of compound F (Agnihotri et al., 2019)

Compound **F** (8 mg) was ref1uxed with absolute MeOH (2 ml) at 70ºC for 18 h in the presence of cation exchange IR-120 (H) resin. The reaction mixture was filtered, while hot and filtrate was concentrated. To this reaction mixture of methylglycoside F, 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. The hydrolysis was complete after 24 hr. The hydrolysate was neutralized with freshly prepared $BaCO_3$ filtered and concentrated under reduced pressure to afford α-and β-methylglucosides along with the Glc, Gal and GalNAc. Their identification of compound F were confirmed by comparison with authentic samples (TLC, PC) of α-and β-methylglucosides along with the Glc, Gal and GalNAc.

Killiani hydrolysis of compound F [Ranajan et al., 2015; Gunjan et al., 2019]

Compound **F** (5 mg) was dissolved in 2 ml Killiani mixture (AcOH-H_2O-HCl, 7:11:2) and heated at 100°C for 1 hrs 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 evaporated under reduced pressure to afford glucose and Gal on comparison with authentic samples of glucose, Gal and GalNAc.

Compound F, TOSOSE

On deacetylation of 35 mg of compound **f** with NH_4OH/acetone, it afforded substance B (29 mg) $[\alpha]_D^{25}$ = -8º (*c* 1% H_2O). For experimental analysis, this compound was dried over P_2O_5 at 100°C and 0.1 mm pressure for 8 hr.

$C_{40}H_{68}O_{31}N_2$	%C	%H	%N
Calculated	**44.77**	**6.34**	**2.61**
Found	**44.78**	**6.34**	**2.61**

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

δ6.15[d, 1H, J=3.6Hz, *α*-Glc(S-1) H-1], δ5.67[d, 1H, J=8.1Hz, *β*-Glc(S-1) H-1], δ5.36[d, 1H, J=3.3Hz, *α*-Glc(S-4) H-1], δ5.35[d, 1H, J=3.6Hz, *α*-Gal(S-5) H-1], δ4.58[d, 1H, J=8.1Hz, *β*-Gal(S-6) H-1], δ4.50[d, 2H, J=8.1Hz, *β*-GalNAc(S-2) & (S-3) H-1], δ3.90[m, 1H, *α*-Gal(S-5) H-2], δ3.80[m, *β*-GalNAc(S-2 & S-3) H-3 & *α*-Glc(S-4) & *β*-Glc(S-1) H-4].

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

δ89.88[1C, *α*-Glc(S-1) C-1], δ89.12[2C, *α*-Glc(S-4) & *α*-Gal(S-5) C-1], δ90.35[1C, *β*-Glc(S-1) C-1], δ100.83[2C, *β*-GalNAc(S-2) & *β*-GalNAc(S-3) C-1], δ101.23[1C, *β*-Gal(S-6) C-1].

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

δ5.72[d, 1H, J=3.9Hz, *α*-Glc(S-1) H-1], δ5.21[d, 2H, J=3.6Hz, *α*-Glc(S-4) & *α*-Gal(S-5) H-1], δ4.65[d, 1H, J=8.1Hz, *β*-Glc(S-1) H-1], δ4.51[d, 1H, J=7.8Hz, *β*-Gal(S-6) H-1], δ4.45[d, 1H, J=7.5Hz, *β*-GalNAc(S-3) H-1], δ4.44[d, 1H, J=7.8Hz, *β*-GalNAc(S-2) H-1], δ3.27[t, *β*-Glc(S-1) H-2], δ2.00[s, 3H, ($NHCOCH_3$), *β*-GalNAc(S-3)], δ1.98[s, 3H, ($NHCOCH_3$), *β*-GalNAc(S-2)].

ES MASS

1095 $[M+Na]^+$, 1072$[M]^+$,1054, 1020, 960, 910, 899, 875, 846, 841, 778, 748, 743, 713, 690, 677, 654, 633, 623, 586, 577, 527, 461, 460, 459, 427, 415, 410, 383, 334, 304, 180, 162.

RESULT AND DISCUSSION

Compound Tosose, $C_{40}H_{68}O_{31}N_2$ $[\alpha]_D^{25}$= -8° gave positive Phenol-sulphuric acid test, Fiegl test (Kumar et al, 2010, Kumar et al, 2019) and Morgan-Elson test (Waren, 1960) showing the presence of normal and amino sugars moietie(s) in the Compound F. The HSQC spectrum of acetylated Tosose showed the presence of six cross peaks of seven anomeric protons and carbons in their respective region at δ6.15x89.88, δ5.67x90.35, δ5.36x89.12, δ5.35x89.12, δ4.58x101.23 and δ4.50x100.83 suggested the presence of seven anomeric protons and carbon in Compound F in its reducing form (Fig-2).

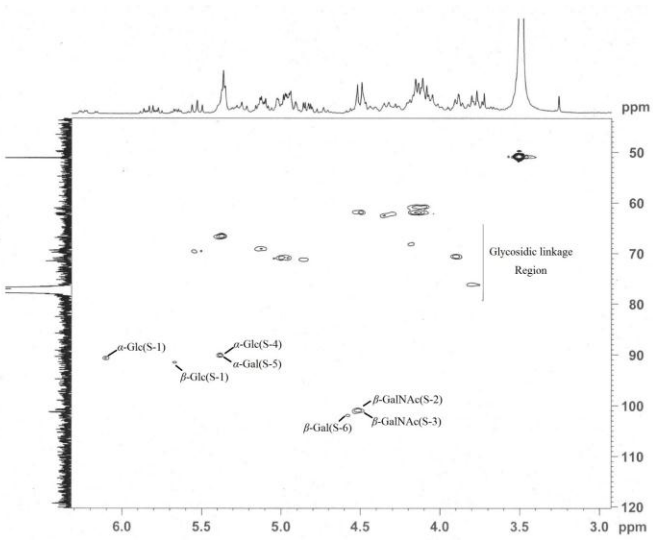
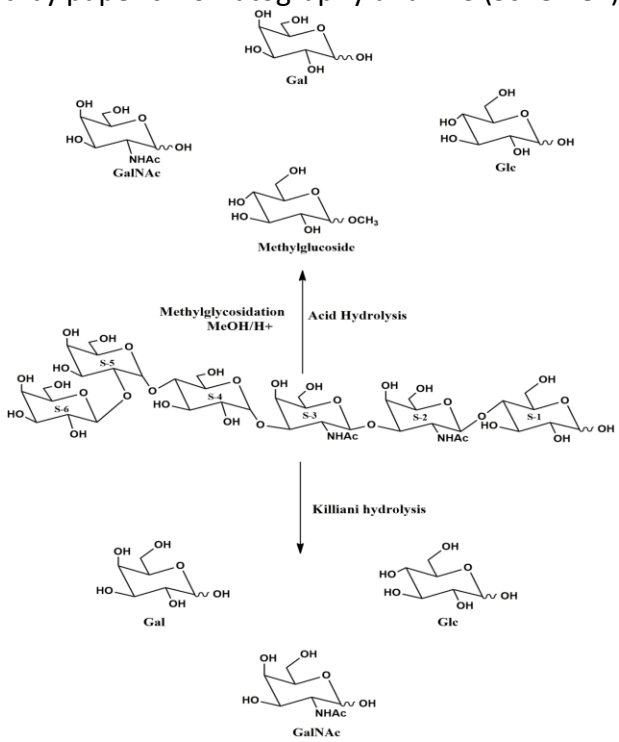


Figure 2. HSQC Spectrum of Tososе acetate in $CDCl_3$ at 300 MHz.

The reducing nature of Tososе was further confirmed by its methylglycosylation MeOH/H^+ followed by its acid hydrolysis, which led to the isolation of *α* and *β*-methylglucosides along with Glc, Gal and GalNAc suggested the presence of glucose at the reducing end, for convenience all six monosaccharides were denoted as S-1, S-2, S-3, S-4, S-5 and S-6. The monosaccharide constituents in Tososе were confirmed by its Killiani hydrolysis[148] under strong acidic condition, followed by paper chromatography and TLC (Scheme I).



Scheme I: Methylglycosidation/Acid Hydrolysis and Killiani Hydrolysis of Tososе

In this hydrolysis three spots were found identical with the authentic samples of Glc, Gal and GalNAc by co-chromatography. Thus, the Hexasaccharide contained three types of monosaccharide units i.e. Glc, Gal and GalNAc. Presence of six doublets for seven anomeric protons at δ6.15(1H), δ5.67(1H), δ5.36(1H), δ5.35(1H), δ4.58(1H) and δ4.50(2H) in the ^{1}H NMR of Tosose Acetate in $CDCl_3$ at 300 MHz confirmed the presence of a Hexasaccharide in its reducing form (Fig-3).

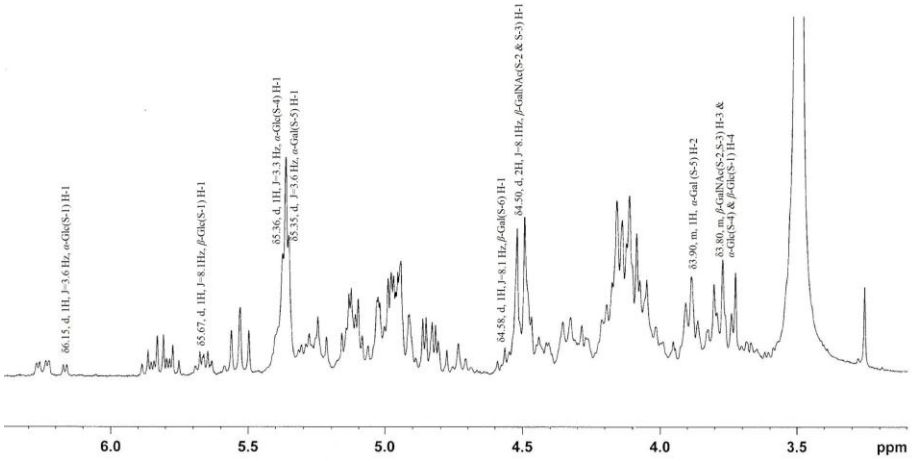


Figure 3. ^{1}H NMR Spectrum of Tosose acetate in $CDCl_3$ at 300 MHz.

Further the presence of five anomeric peaks for seven anomeric carbon at δ89.12(2C), δ89.88(1C), δ90.35(1C), δ100.83(1C) and δ101.23(2C) in the ^{13}C NMR spectrum of acetylated Tosose in $CDCl_3$ at 300 MHz confirmed Tosose as a Hexasaccharide in its reducing form (Fig-4).

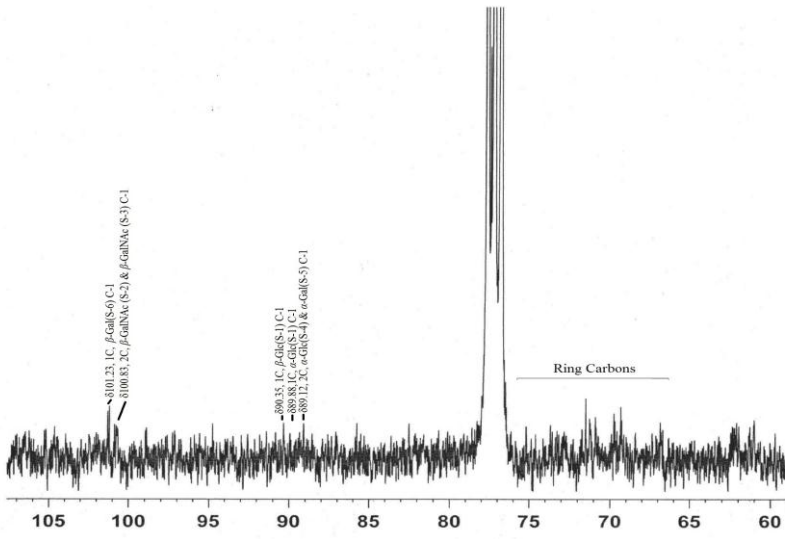


Figure 4. ^{13}C NMR Spectrum of Tosose acetate in $CDCl_3$ at 300 MHz.

The Hexasaccharide nature of Tososе was further supported by the presence of six anomeric proton doublets for seven anomeric protons at δ5.72 (1H), δ5.21(2H), δ4.65(1H), δ4.51(1H), δ4.45(1H) and δ4.44(1H) in ^{1}H NMR spectrum of Tososе in D_2O at 300 MHz. ^{1}H and ^{13}C NMR spectra justifying the seven anomeric signals for Hexasaccharide with total integral intensity of six anomeric proton/carbon. The molecular formula $C_{40}H_{68}O_{31}N_2$ was in agreement with mass ion peak obtained from ES-MS spectrum of Tososе which showed the highest mass ion peak at m/z 1095 and m/z 1072 due to $[M+Na]^+$ and $[M]^+$ respectively for a Hexasaccharide. The ^{1}H NMR spectrum of Tososе in D_2O at 300 MHz contain two anomeric doublets at δ5.72 (J=3.9Hz) and δ4.65 (J=8.1Hz) for *α* and *β* anomers of reducing monosaccharides (S-1) i.e. Glc (Fig-5).

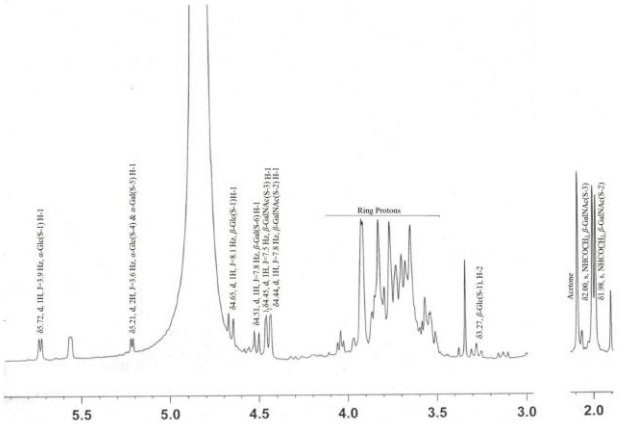


Figure 5. ^{1}H NMR Spectrum of Tososе in D_2O at 300 MHz.

Simultaneously ^{1}H NMR and ^{13}C NMR spectrum of Tososе acetate also showed downfield shifted *α* and *β* anomeric proton and carbon of reducing monosaccharides (S-1) i.e. Glc (S-1) at δ6.15, δ5.67 and δ89.88, δ90.35 respectively. The anomeric protons signal present at δ5.67 assigned for *β*-Glc (S-1) in TOCSY Spectrum of Tososе acetate contains three cross peaks at δ5.67x3.80, δ5.67x5.02 and δ5.67x5.24 (Fig-6).

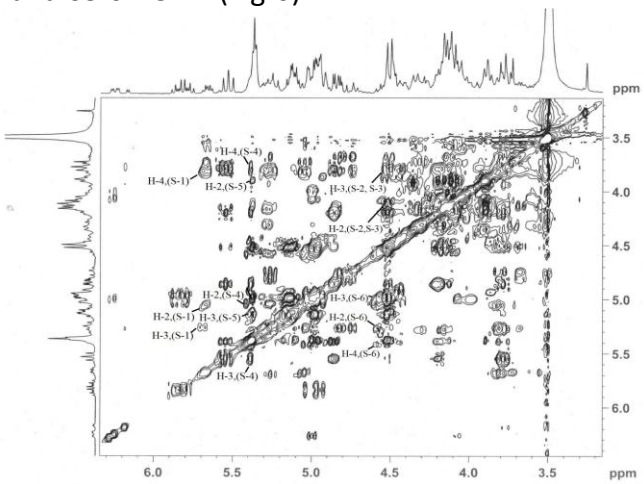


Figure 6. TOCSY Spectrum of Tososе acetate in $CDCl_3$ at 300 MHz.

The chemical shift of the cross peak at δ5.67x3.80 suggested that in sugar S-1 only one position was available for glycosidic linkage by the next monosaccharide unit, which was later identified as H-4 of S-1 by COSY spectrum of Tosose acetate (Fig-7).

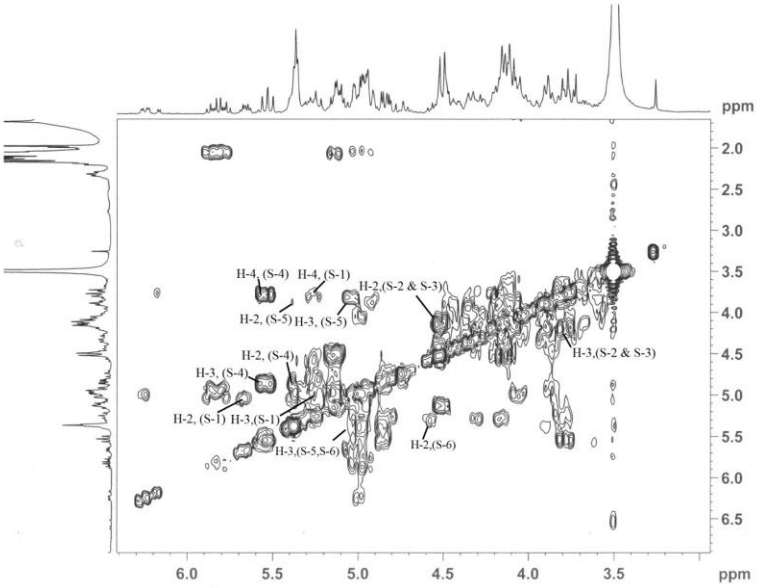


Figure 7. COSY Spectrum of Tosose acetate in $CDCl_3$ at 300 MHz.

Further the presence of another anomeric proton doublet at δ4.44 (J=7.8Hz) along with singlet of three proton of amide methyl at δ1.98 in ^{1}H NMR spectrum of Tosose in D_2O and anomeric proton doublet at δ4.50 (J=8.1Hz) in ^{1}H NMR spectrum of Tosose acetate showed the presence of *β*-GalNAc (S-2) in Tosose. The presence of anomeric signals of Glc and GalNAc along with a triplet at δ3.27 for H-2 of *β*-Glc suggested the presence of Lactose type of structure in which Gal was replaced with GalNAc i.e. GalNAc-(1→4)-Glc at the reducing end of Tosose hence confirming the (1→4) linkage between S_2 and S_1. The appearance of H-4 signal of *β*-Glc at δ3.80 in the ^{1}H NMR of Tosose acetate confirmed the (1→4) linkage between S_2 and S_1 which was assigned by COSY and TOCSY spectrum of Tosose acetate. This linkage was also supported by the presence of cross peak at δ3.80x76.95 in glycosidic region of HSQC spectrum of Tosose acetate in $CDCl_3$. Larger coupling constant (J), 7.8Hz of anomeric proton at δ4.44 confirmed *β* glycosidic linkage between S-2 & S-1. The anomeric proton doublet at δ4.50 assigned for *β*-GalNAc (S-2) in ^{1}H NMR spectrum of Tosose acetate contain three cross peaks at δ4.50x3.80, δ4.50x4.12 and δ4.50x4.98 in its TOCSY spectrum. The chemical shift value of the cross peaks at δ4.50x3.80 and δ4.50x4.12 suggested that in sugar (S-2) two positions were available for substitution which was later identified as H-2 and H-3 of S-2 by COSY spectrum of Tosose acetate. Further it was confirmed by COSY spectrum of Tosose Acetate that H-2 position of *β*-GalNAc (S-2) at δ4.12 was substituted by –NHAc group and the multiplet present at δ3.80 in ^{1}H NMR spectrum of Tosose acetate suggested that the H-3 of *β*-GalNAc (S-2) was available for glycosidation by the next monosaccharide moiety (S-3). Further another anomeric proton doublet appeared at δ4.50 (d, J=8.1Hz) in the ^{1}H NMR spectrum of Tosose acetate gave its

complimentary signal at δ100.83 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*-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 Tosose was supported by appearance of anomeric proton signal at δ4.45 (J= 7.5Hz) along with singlet of amide methyl at δ2.00 ^{1}H NMR spectrum of Tosose in D_2O at 300 MHz (Table-2).

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

Moieties	In D_2O		In $CDCl_3$	
	^{1}H NMR(δ)	Coupling constant(J)	^{1}H NMR(δ)	Coupling constant(J)
α-Glc(S-1)	5.72	3.9Hz	6.15	3.6Hz
β-Glc(S-1)	4.65	8.1Hz	5.67	8.1Hz
β-GalNAc(S-2)	4.44	7.8Hz	4.50	8.1Hz
β-GalNAc(S-3)	4.45	7.5Hz	4.50	8.1Hz
α-Glc(S-4)	5.21	3.6Hz	5.36	3.3Hz
α-Gal(S-5)	5.21	3.6Hz	5.35	3.6Hz
β-Gal(S-6)	4.51	7.8Hz	4.58	8.1Hz

Since it was ascertained by the COSY and TOCSY spectrum of Tosose acetate that the *β*-GalNAc (S-2) has two vacant position i.e. H-2 and H-3, and it was already confirmed that H-2 of S-2 was linked with -NHAc hence the left over H-3 position of *β*-GalNAc (S-2) at δ3.80 must be linked to *β*-GalNAc (S-3).

The (1→3) linkage between S-3 and S-2 was supported by the presence of H-3 signal of S-1 at δ3.80 in upfield region of ^{1}H NMR spectrum of Tosose acetate. This linkage was further supported by the presence of cross peak at δ3.80x76.95 in glycosidic region of HSQC spectrum of Tosose acetate in $CDCl_3$. The anomeric proton signal present at δ4.45 for GalNAc (S-3) had a J value of 7.5Hz confirmed the *β*-(1→3) glycosidic linkage between S-2 and S-3. The anomeric proton doublet at δ4.50 assigned for *β*-GalNAc (S-3) in ^{1}H NMR spectrum of Tosose acetate in $CDCl_3$ showed three cross peaks at δ4.50x3.80, δ4.50x4.12, and δ4.50x4.98 in its TOCSY spectrum, 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 δ3.80 represented the linkage region of *β*-GalNAc (S-3) which was later identified as H-3 of *β*-GalNAc (S-3) by COSY spectrum of Tosose acetate which was available for (1→3) glycosidic linkages by the next monosaccharide unit (S-4).

Next anomeric proton doublet appeared at δ5.36 in ^{1}H NMR spectrum of Tosose acetate showed its complimentary signal at δ89.12 in its HSQC spectrum. The chemical shift value of anomeric carbon and anomeric proton were having resemblance with literature value of anomeric chemical shift value of *α*-Glc (S-4) hence S-4 monosaccharide was confirmed as *α*-Glc (S-4). Presence of *α*-Glc (S-4) in Tosose was confirmed by the appearance of anomeric proton doublet at δ5.21 in its ^{1}H NMR spectrum in D_2O. Since, H-3 position of S-3 was available for glycosidic linkage by the next monosaccharide unit (S-4), hence *α*-Glc (S-4) must be attached to H-3 of S-3.

The (1→3) linkage between *α*-Glc (S-4) and *β*-GalNAc (S-3) was further supported by the ^{1}H NMR spectrum of Tososе acetate in which the signal for H-3 of *β*-GalNAc (S-3) appeared at δ3.80 which was later confirmed by COSY and TOCSY spectrum of Tososе acetate. This linkage was also supported by the presence of cross peak at δ3.80x76.95 in glycosidic region of HSQC spectrum of Tososе acetate in $CDCl_3$. The small coupling constant, J=3.6Hz of *α*-Glc (S-4) confirmed the *α*-(1→3) glycosidic linkage between *α*-Glc (S-4) and *β*-GalNAc (S-3). The anomeric proton signal at δ5.36 assigned for *α*-Glc (S-4) in ^{1}H NMR spectrum of Tososе acetate gave four cross peaks at δ5.36x3.80, δ5.36x4.22, δ5.36x4.86 and δ5.36x5.56 in its TOCSY spectrum, out of which one cross peak arised at δ5.36x3.80 suggested that in sugar S-4, only one position was available for glycosidation by the next monosaccharide moiety (S-5), which was identified as H-4 of *α*-Glc (S-4) by COSY spectrum of Tososе acetate. The next anomeric proton signal which appeared at δ5.35 (J=3.6Hz) in the ^{1}H NMR spectrum of Tososе acetate had its complimentary signal at δ89.12 in HSQC spectrum of Tososе acetate. The chemical shift value of anomeric carbon and anomeric proton were having resemblance with literature value of anomeric chemical shift value of *α*-Gal hence S-5 monosaccharide was confirmed as *α*-Gal (S-5). Further the presence of *α*-Gal (S-5) (J=3.6Hz) as the next monosaccharide in Tososе was supported by the appearance of anomeric proton doublet at δ5.21 in the ^{1}H NMR spectrum of Tososе in D_2O at 300 MHz. Since it was ascertained by the TOCSY and COSY spectrum of Tososе acetate that the H-4 position of S-4 was available for glycosidic linkage with next monosaccharide unit (S-5), thus H-4 position of *α*-Glc (S-4) must be linked with *α*-Gal (S-5). This linkage was further supported by ^{1}H NMR spectrum of acetylated Tososе in which the signal for H-4 of S-4 appeared at δ3.80 which was later confirmed by COSY, TOCSY and HSQC spectrum of Tososе acetate. The (1→4) linkage between S-5 and S-4 was further confirmed by the presence of cross peak at δ3.80x76.95 in glycosidic region of HSQC spectrum of Tososе acetate in $CDCl_3$. The small coupling constant (J), 3.6Hz of Gal (S-5) confirmed the *α*-glycosidic linkage between S-5 and S-4. The anomeric proton signal assigned for *α*-Gal (S-5) at δ5.35 gave three cross peaks at δ5.35x3.90, δ5.35x4.98 and δ5.35x5.13 in its TOCSY spectrum. Out of which one cross peak arised at δ5.35x3.90 suggested the position of linkage, which was later identified as H-2 of *α*-Gal (S-5) by COSY spectrum of Tososе acetate, suggesting that the H-2 of *α*-Gal (S-5) was available for glycosidation by the next monosaccharide (S-6). Further next anomeric proton doublet appeared at δ4.58 in the ^{1}H NMR spectrum of Tososе acetate gave its complementary signal at δ101.23 in its HSQC spectrum in $CDCl_3$. The chemical shift values of anomeric carbon and anomeric proton were having resemblance with literature value of anomeric chemical shift value of *β*-Gal hence S-6 monosaccharide was confirmed as *β*-Gal (S-6). Further the presence of *β*-Gal (S-6) (J=7.8Hz) as the next monosaccharide in Tososе was supported by the appearance of anomeric proton doublet at δ4.51 in the ^{1}H NMR spectrum of Tososе in D_2O at 300 MHz (Fig-5).
The appearance of H-2 signal of S-5 at δ3.90 in the ^{1}H NMR spectrum of Tososе acetate suggested that *β*-Gal (S-6) may be linked to *α*-Gal (S-5). This linkage was also supported by the presence of cross peak at δ3.90x71.21 in glycosidic region of HSQC spectrum of Tososе acetate in $CDCl_3$. The coupling constant of anomeric signal (S-6) at δ4.51 with J value of 7.8 Hz confirmed the *β*-(1→2) configuration of the glycosidic linkage between S-6 and S-5.

The anomeric proton signal assigned for *β*-Gal (S-6) at δ4.58 in ^{1}H NMR spectrum of Tososе acetate gave three cross peaks at δ4.58x4.98, δ4.58x5.05 and δ4.58x5.26 in its TOCSY spectrum since this anomeric proton does not showed any cross peak in the linkage region i.e. δ3.5-4.2ppm, hence confirmed that *β*-Gal (S-6) 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 Tososе acetate in $CDCl_3$ at 300 MHz. All the ^{1}H NMR assignments for ring protons of monosaccharide units of Tososе 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 ^{1}H and ^{13}C NMR of acetylated and deacetylated oligosaccharide. The glycosidic linkages in Tososе were assigned by the cross peaks for glycosidically linked carbons with their protons in the HSQC spectrum of acetylated Tososе. All signals obtained in ^{1}H and ^{13}C NMR of compound Tososе were in conformity with the assigned structure and their position were confirmed by 2D NMR viz. COSY, TOCSY, HSQC experiments. Thus based on the pattern of chemical shifts of ^{1}H NMR, ^{13}C NMR, COSY, TOCSY and HSQC experiments it was interpreted that the compound was a Hexasaccharide having following structure as:

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

TOSOSE

The Electronspray Mass Spectrometry data of Tososе not only confirmed the derived structure but also supported the sequence of monosaccharide in Tososе (Fig-8).

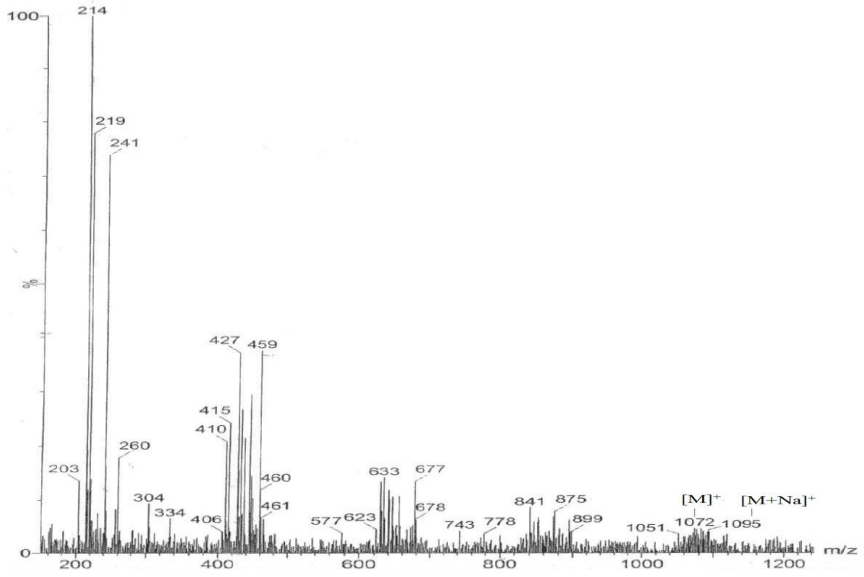
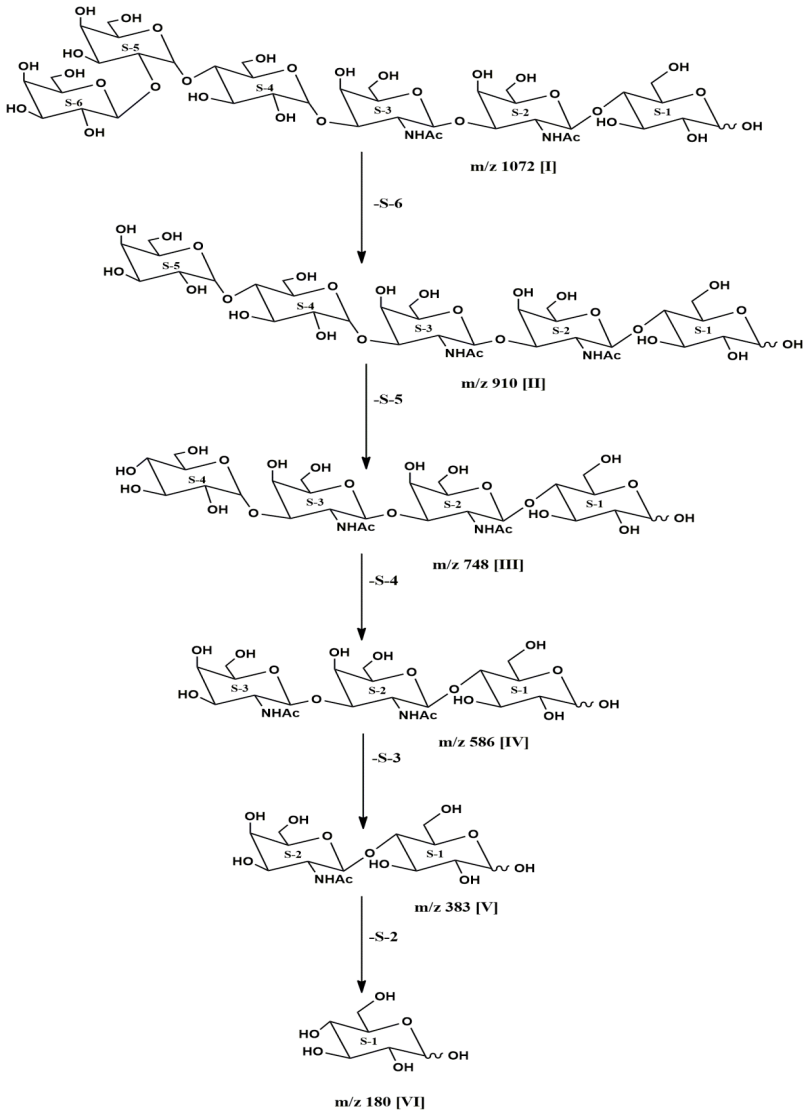


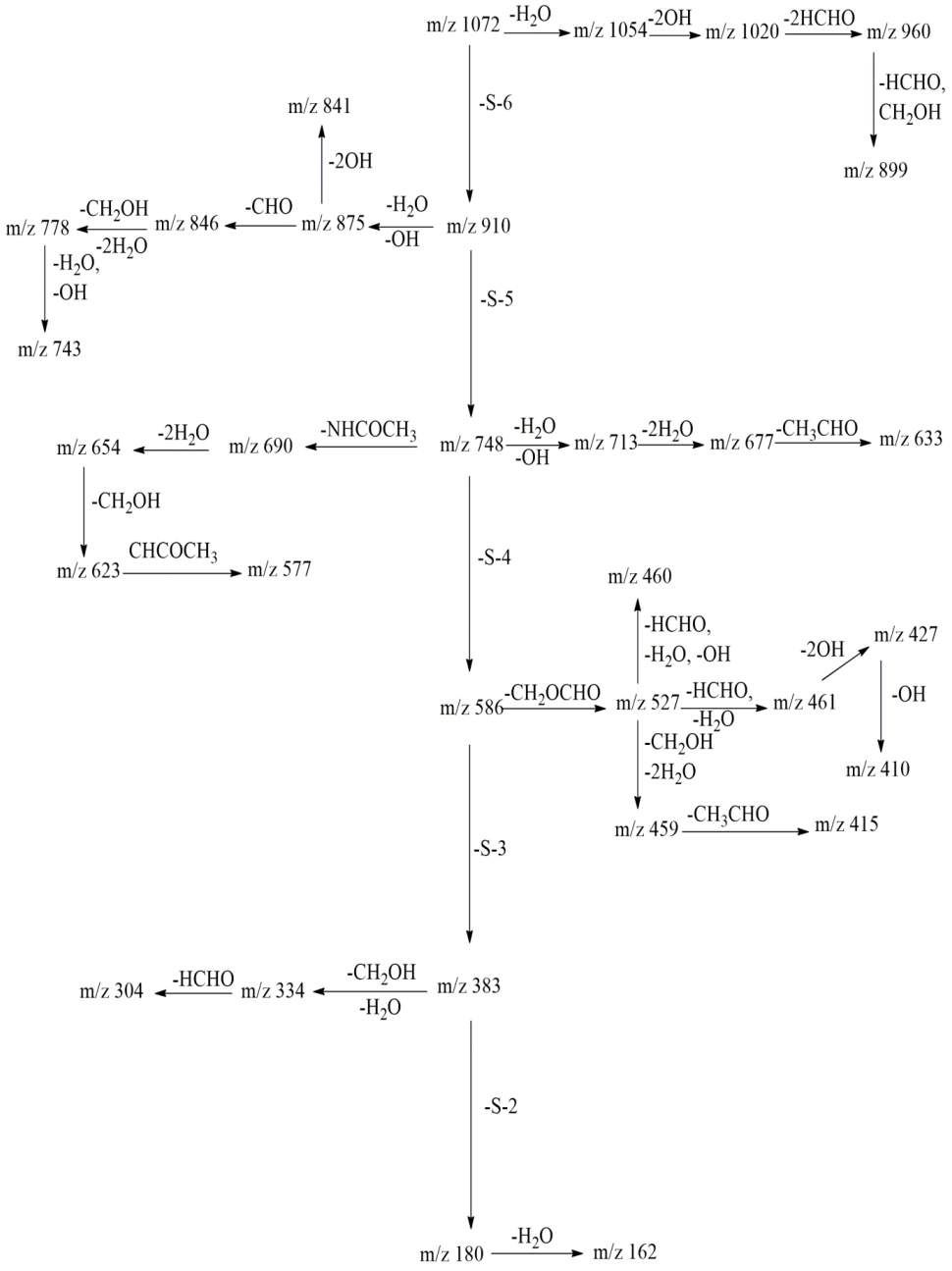
Figure 8. ES-MS Spectrum of compound F, Tososе.

The highest mass ion peaks were recorded at m/z 1095 and 1072 which were due to $[M+Na]^{+}$ and $[M]^{+}$ respectively, confirming the molecular weight of Tososе as 1072 and was in agreement with its molecular formula.



Scheme II: Mass fragmentation of compound F, Tososе

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 Hexasaccharide m/z 1072 (I) fragmented to give mass ion at m/z 910 (II) [1072-S_6], this fragment was arise due to the loss of terminal *β*-Gal (S_6) moiety from hexasaccharide indicating the presence of *β*-Gal (S_6) at the non-reducing end. It further fragmented to give mass ion peak at m/z 748 (III) [910-S_5] which was due to loss of *α*-Gal (S_5) moiety from pentasaccharide. This fragment of 748 further fragmented to give mass ion peak at m/z 586 (IV) [748-S_4] which was due to loss of *α*-Glc (S_4) moiety from the tetrasaccharide. This fragment of 586 further fragmented to give mass ion peak at m/z 383 (V) [586-S_3] which was due to loss of *β*-GalNAc (S_3) moiety from the trisaccharide. This disaccharide unit again fragmented to give mass ion peak at m/z 180 (VI) [383-S_2], which was due to loss of *β*-GalNAc (S_2) moiety from disaccharide.

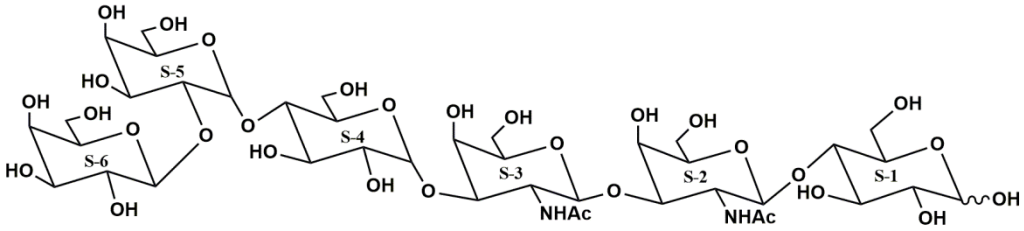


Scheme III: ES-MS fragments of compound F, Tososе.

The other fragmentation pathway in ES Mass spectrum of compound F m/z 1072 shows the mass ion peak at 1054[1072-H_2O], 1020[1054-2OH], 960[1020-2HCHO], 910[1072-S-6], 899[960-HCHO, CH_2OH], 875[910-H_2O,-OH], 846[875-CHO], 841[875-2OH], 778[846-CH_2OH, $2H_2O$], 748[910-S-5], 743[778-H_2O, -OH], 713[748-H_2O,-OH], 690[748-$NHCOCH_3$], 677[713-$2H_2O$], 654[690-$2H_2O$], 633[677-CH_3CHO], 623[654-CH_2OH], 586[748-S-4], 577[623-$CHCOCH_3$], 527[586-CH_2OCHO 461[527-HCHO, -H_2O], 460[527-HCHO,-H_2O,-OH],

459[527-CH_2OH,-$2H_2O$], 427[461-2OH], 415[459-CH_3CHO], 410[427-OH], 383[586-S-3], 334[383-CH_2OH,-H_2O], 304[334-HCHO], 180[383-S-2] and 162[180-H_2O]. 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 and HSQC spectra of Tososе acetate and Tososе, the structure and sequence of isolated Novel oligosaccharide Tososе structure was deduced as:

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



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