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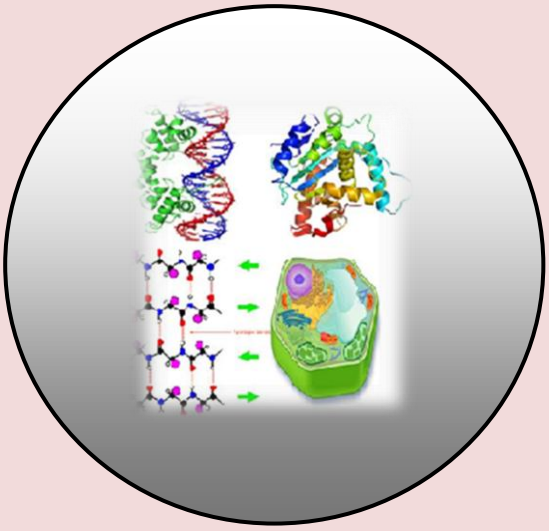

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RESEARCH PAPER

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Structure Elucidation of a Novel Nonasaccharide 'Tarose' from Jersey Cow Colostrum by 2D NMR and Mass Spectrometry

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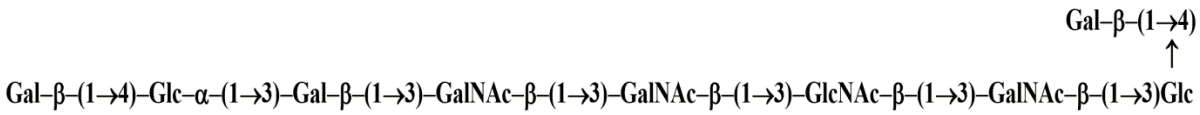
ABSTRACT

The cow milk is a replacement to mother's milk and it develops the immune system, brain and bones of a neonate. The biological activity of the milk of various cow species depends on its oligosaccharide contents. During recent time different cow species like Tharparkar, Gir, Jersey, Sahiwal, Rathi etc. were investigated for their oligosaccharide content that depends on the geographical flora fauna and food habits of the animal. It was also visualized that the colostrums carry higher percentage of oligosaccharides and hence show improved biological activity. In view of the above considerations we have collected Jersey cow colostrums and processed it by the modified method of Kobata and Ginsburg that incorporates deproteination, centrifugation, micro-filteration and gel chromatography resulting in the isolation of crude oligosaccharide mixture which was acetylated for its isolation and purification by silica column chromatography which lead to the isolation of an undescribed oligosaccharide 'Tarose'. Its structure was elucidated by chemical degradation, chemical transformation, structure reporter group theory (SRG), NMR (^{1}H, ^{13}C) and 2D NMR (COSY, TOCSY, HSQC, HMBC) along with Mass spectrometry. The structure of the novel nonasaccharide Tarose was established as under-



TAROSE

Keywords: Jersey cow, Milk, Oligosaccharide, Tarose, NMR and Mass spectroscopy.

INTRODUCTION

The traditional medicinal system of *Upnishads, Puranas*and *Vedas (Charaksutarabhan*) has elaborately defined the medicinal properties of cow milk (*Rigveda*). The medicinal properties of cow milk was also defined by Dhanvantri the physician of that time, he described that it shields the body from *pitta*, *vata* and cardiac ailments.

It was also beneficial for chronic diseases like epilepsy, jaundice, spleen enlargement and piles (Ling et al. 1961). It is good for eyes, brain and immune system (Huppertz et al 2008). Milk may be categorized as normal milk and the early milk; colostrum. The milk obtained in the first few days after the birth of a neonate is known as the colostrums, which is a rich source of all the nutrients. The most important and medicinally efficacious content of the milk is oligosaccharides present in it, which are responsible for its medicinal properties. These oligosaccharides are long chains of monosaccharides linked together by -o- glycosidic linkages incorporating variations in configuration of glycosidic linkages and conformations of the monosaccharides. Novel oligosaccharides are also formed by different glycosidic linkages (α and β) at different positions of the monosaccharides. Further, the recent researches have concluded that the colostrums have multifold medicinal importance than the regular milk. Keeping in mind the medicinal importance of cow colostrums, we have collected the colostrums of a Jersey cow, within four days of delivering the calf. The colostrums was preserved by addition of an equal volume of ethyl alcohol and further processed by modified method of Kobata and Ginsburg (Kumar et al 2018) incorporating deproteination, microfilteration and lyophilysation, to obtain the crude oligosaccharide mixture. It was further purified by Sephadex chromatography and homogeneity of oligosaccharide mixture was confirmed by HPLC. Further, for easy isolation and purification of these highly polar oligosaccharides, it was converted to its non-polar acetyl derivatives, which was subjected to silica column chromatography that resulted in the isolation of a chromatographically pure yet undescribed nonasaccharide “Tarose”. The structure of this novel nonasaccharide was established by physicochemical techniques like ^{1}H, ^{13}C, 2D NMR (HSQC, TOCSY, COSY and HMBC) and Mass spectrometry.

EXPERIMENTAL

General Procedure

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). For evaporation of alcohol from crude extract of milk oligosaccharides, Buchi Rotary evaporator was used. Freeze drying of the compounds was done with the help of CT 60e (HETO) lyophilizer and centrifuged by a cooling centrifuging machine, Remi instruments C-23 JJRCI 763. Optical rotations were measured with a Buchi automatic Polarimeter in 1.2 cm tube. The C, H and N analysis were recorded on CARLO-ELBA 1108 elemental analyzer. ^{1}H and ^{13}C NMR and 2D experiments were recorded in solvent $CDCl_3$ and D_2O at 25°C on a Bruker AM 300 MHz FT-NMR spectrometer. The Electronspray mass spectra were recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. The milk oligosaccharide sample (dissolved in water as solvent) was introduced into the ESI source through a syringe pump at the rate of 5μl per min. The ESI capillary was set at 3.5 KV and the cone voltage was 40 V. The spectra were collected in 6 s scans and the print outs are averaged spectra of 6-8 scans. Spectrum recorded in higher mass scale is computerized deconvoluted. Authentic samples of glucosamine, galactosamine, galactose and glucose were purchased from Aldrich Chemicals.

Isolation of Milk Oligosaccharides by Modified Method of Kobata and Ginsburg

Cow colostrum (10 lit.) (Kumar K et al; 2018)

Equal amount of alcohol (10lit.) was added and stored, centrifuged at 4ºC, filtered through a loosely packed glass

Lipid layer
(Residue) discarded

Supernatant
Was precipitated by addition of 68% ethanol and separated by centrifugation

Supernatant

filtered through a micro filter (0.2µ) to remove remaining lactose.
It was then lyophilized.

Protein and Lactose
(Residue) discarded

Lyophilized material (205gm)
(Mixture of oligosaccharides)

Was then fractionated on a sephadex G-25 column, eluted with triple distilled water at flow rate 3ml/min. fractions were analyzed for sugars by phenol-sulphuric acid reagent.

Carbohydrate containing fractions

Fractions were pooled, lyophilized and analyzed by HPLC

Analytical HPLC

The carbohydrate fractions were eluted with TDW (containing 0.1% TFA & CH_3CN) at a flow rate 1 ml/min, to check homogeneity of the oligosaccharide mixture. The elution was monitored by UV absorbance at 220nm.

Deacetylation

The chromatographically pure acetylated milk oligosaccharides were deacetylated by dissolving them in acetone & NH_3 and left overnight. Ammonia was removed under reduced pressure and the compound was washed with $CHCl_3$ and was finally freeze dried giving the deacetylated milk oligosaccharides.

Chemical transformation (acetylation)

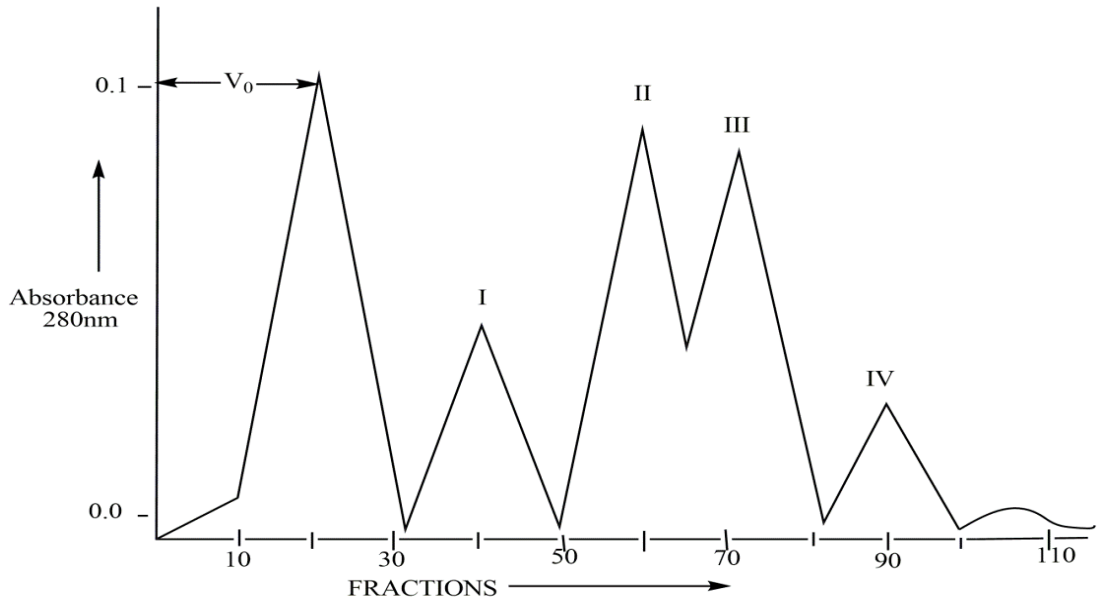
Fractions with (+) test for sugars could not be separated over silica gel, hence the oligosaccharide mixture was acetylated with Ac_2O and pyridine converting free sugar into non-polar acetyl derivatives which were resolved nicely on TLC and were separated by column chromatography over silica gel which resulted in the isolation of chromatographically pure compounds.

Isolation of Cow Colostrum Oligosaccharides by Modified Method of Kobata and Ginsburg (Kumar et al 2016)

10 litre cow colostrum (1-5 day) were 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 ºC. 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)**.**

Sephadex G-25 Gel Chromatography of Cow Oligosaccharide 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. A substantial amount of proteins, glycoproteins and serum albumin were eluted in the void volume which was confirmed by positive coloration with p-dimethylaminobenzaldehyde reagent and phenol-sulphuric acid reagent. 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 2).



Graph 1. Spehadex G-25 Chromatography of Cow Colostrum Oligosaccharides Detected by Phenol Sulphuric Acid Test. Elution was made with TDW.

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	4.50	+++ve	Peak II and III were
65-78	IIPeak III	6.50	+++ve	taken for further
79-100	Peak IV	0.52	-ve	investigation (11gm)

Confirmation of Homogeneity of Cow Colostrum Oligosaccharide by Reverse Phase HPLC

Pooled fractions obtained from Sephadex G-25 column, containing oligosaccharide mixture (11.0gm) were qualitatively analyzed by reverse phase HPLC. The HPLC system was equipped with Perkin- Elmer 250 solvent delivering system, 235-diode array detector and G.P. 100 printer plotter. The column used for this purpose was C_{18}Purosphere 25 cm × 0.4 cm × 5-µ m (from E. Merck). A binary gradient system of acetonitrile: 0.5% trifluro-acetic acid (5:95) in triple distilled water (TDW) to CH_3CN: 0.5 % TFA (60:40) within 25 min at a flow rate of 1 ml/min was used. The eluants were detected at 220 nm. Twelve peaks were noticed in the sample (pooled fraction under peaks II and III) at the varied retention times from 1.925 min to 19.892min. For convenience the peaks were numbered in their increasing order of retention time *i.e.* [1.925min (R_1), 3.250(R_2), 4.225(R_3), 4.950(R_4), 5.450(R_5), 6.725(R_6), 8.200(R_7), 10.233(R_8), 13.250(R_9), 15.433(R_{10}), 19.000(R_{11}) and 19.892(R_{12})].

Table 2. HPLC Table of Crude Cow Colostrum Oligosaccharide.

Peak	Retention time	Area	Area %	Height	Height%
1	1.925	2510836	7.210	25646	2.492
2	3.250	5392454	15.484	222236	21.594
3	4.225	3791632	10.887	83985	8.161
4	4.950	1942657	5.578	96702	9.396
5	5.450	2557166	7.343	50398	4.897
6	6.725	5147728	14.781	86464	8.402
7	8.200	3526955	10.127	39140	3.803
8	10.233	1908336	5.480	44421	4.316
9	13.250	1941528	5.575	92688	9.006
10	15.433	683106	1.961	16513	1.605
11	19.000	1899121	5.455	171444	16.659
12	19.892	197146	0.566	11938	1.160

Acetylation of Oligosaccharide Mixture

10gm oligosaccharide mixture was acetylated with pyridine (10 ml) and acetic anhydride (10ml) at 60°C and the solution was stirred overnight.

The mixture was evaporated under reduced pressure and the viscous residue was taken in $CHCl_3$ (500 ml) and it was washed in sequence with 2N-HCl (1 x 25 ml), ice cold 2N-$NaHCO_3$ (2 x 25 ml) and finally with H_2O (2 x 25 ml). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding the acetylated mixture (10.8gm).

Deacetylation of compound b, Tarose Acetate

Compound **'b'** (48 mg) was obtained from column chromatography 2 of acetylated oligosaccharide mixture. 40 mg of compound b 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 hrs ammonia was removed under reduced pressure and the compound was washed thrice with $CHCl_3$ (5 ml) (to remove acetamide) and the water layer was finally freeze dried giving the deacetylated oligosaccharide **B** (34 mg).

Methyl glycosidation/Acid hydrolysis of compound B: (Manisha et al; 2023)

Compound **B** (8 mg) was refluxed with absolute MeOH (2 ml) at 70ºC for 18 hr in the presence of cation exchange IR-l20 (H) resin. The reaction mixture was filtered while hot and filtrate was concentrated. 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 30 minutes at 50°C. The hydrolysis was complete after 24 hrs. (TLC) 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, GalNAc and GlcNAc. Their identification of compound B was confirmed by comparison with authentic samples (TLC, PC) of *α*-and *β*-methylglucosides along with the Glc, Gal, GalNAc and GlcNAc.

Killiani hydrolysis of compound B: (Muzeeb et al; 2018)

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

Description of Compound B, Tarose

Compound **b** (48 mg) was obtained from fraction 109-114 of column chromatography 2. On deacetylation of 40 mg of substance b with NH_4OH/acetone, it afforded substance **B** (34 mg) $[\alpha]_D^{25}$ =+34° (*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. 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, Tarose 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, Tarose 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, Tarose 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.

RESULTS AND DISCUSSION

Compound B, Tarose, $C_{62}H_{104}O_{46}N_4$ $[\alpha]_{\mathrm{D}}^{25}$ = +34° gave positive Phenol-sulphuric acid test(Partridge et al. 1949), Feigl test (Feigl et al. 1975) and Morgan-Elson test (Dubois, M. et al. 1956) showing the presence of normal and amino sugars moietie(s) in the compound B. The HSQC spectrum of acetylated Tarose 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 Tarose 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 Tarose 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 Tarose 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 Tarose in $CDCl_3$ at 300 MHz confirmed the presence of a nonasaccharide in its reducing form.

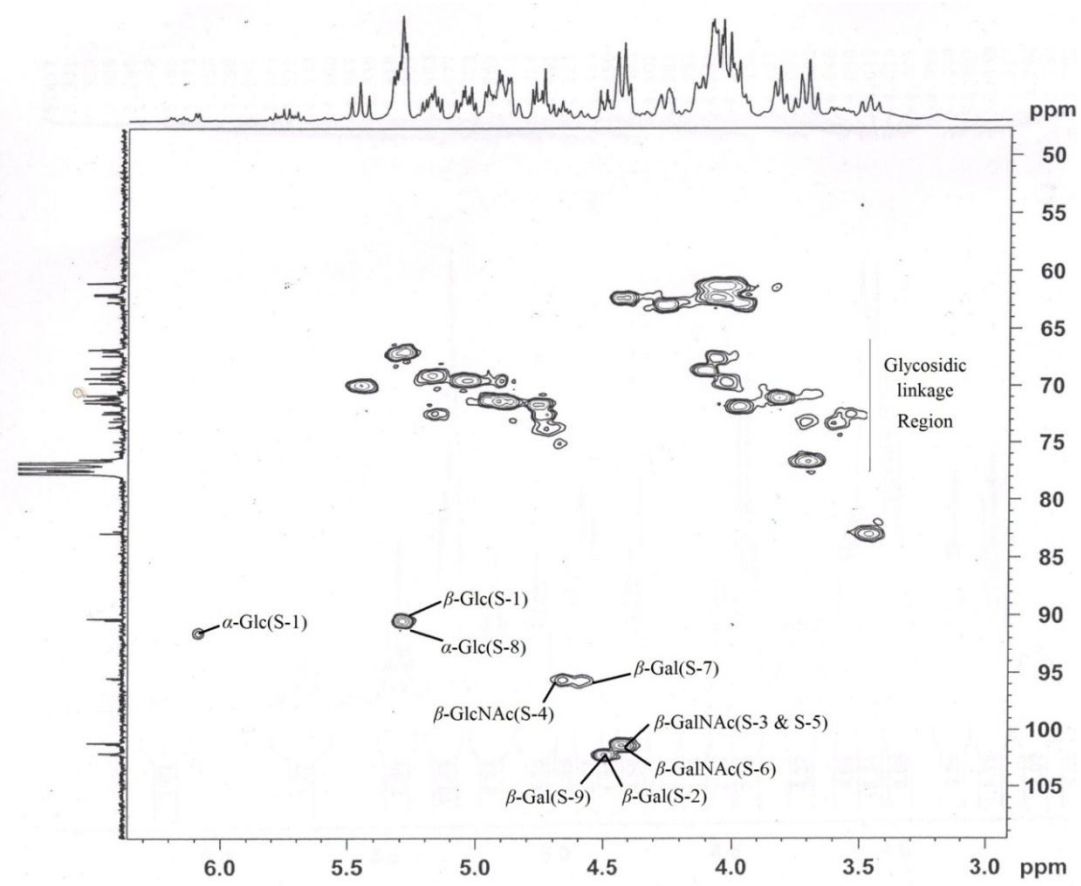


Figure 1. HSQC Spectrum of Tarose acetate in $CDCl_3$ at 300 MHz.

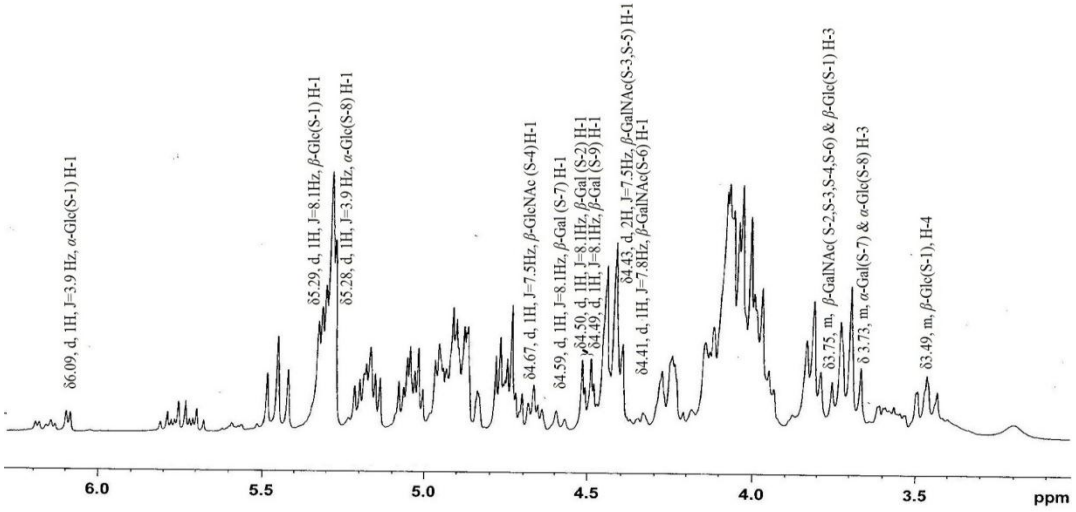


Figure 2.^{1}H NMR Spectrum of Tarose acetate in $CDCl_3$ at 300 MHz.

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 Tarose in $CDCl_3$ at 300 MHz. confirmed the presence of a nonasaccharide in its reducing form.

The nonasaccharide nature of Tarose 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 ^{1}H NMR spectrum of Tarose in D_2O at 300 MHz. The ^{1}H and ^{13}C NMR spectra of Tarose 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 Tarose was in agreement with molecular formula $C_{62}H_{104}O_{46}N_4$.

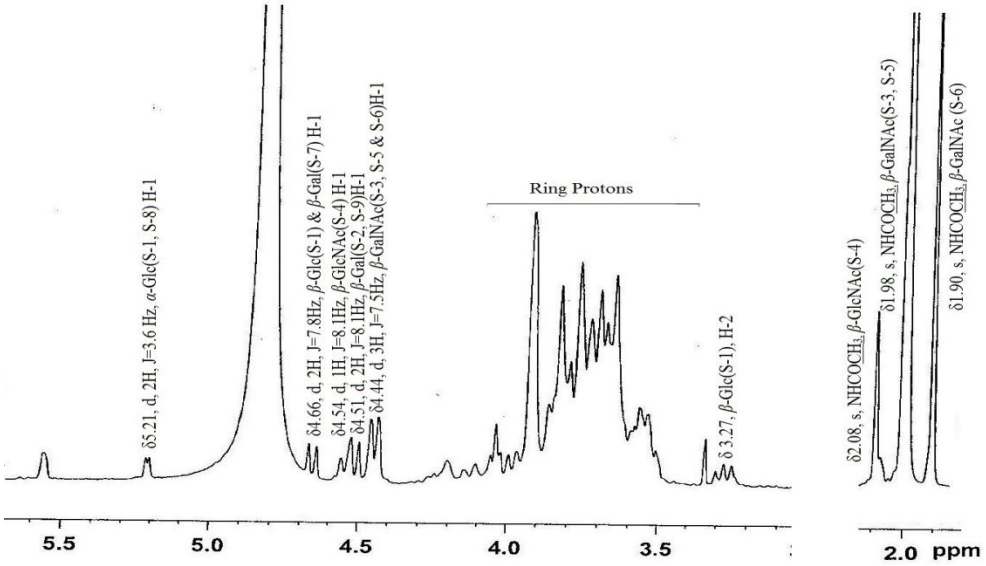


Figure3. ^{1}H NMR Spectrum of Tarose in D_2O at 300 MHz.

Table 3.^{1}H NMR values of Compound B Tarose in D_2O and $CDCl_3$ 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)	5.21	3.6Hz	6.09	3.9Hz
β-Glc(S-1)	4.66	7.8Hz	5.29	8.1Hz
β-Gal(S-2)	4.51	8.1Hz	4.50	8.1Hz
β-GalNAc(S-3)	4.44	7.5Hz	4.43	7.5Hz
β-GlcNAc(S-4)	4.54	8.1Hz	4.67	7.5Hz
β-GalNAc(S-5)	4.44	7.5Hz	4.43	7.5Hz
β-GalNAc(S-6)	4.44	7.5Hz	4.41	7.8Hz
β-Gal(S-7)	4.66	7.8Hz	4.59	8.1Hz
α-Glc(S-8)	5.21	3.6Hz	5.28	3.9Hz
β -Gal(S-9)	4.51	8.1Hz	4.49	8.1Hz

The ^{1}H NMR spectrum of Tarose 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) (Bendik et al 1999), (Vliegenthart et al, 1992) at the reducing end of Tarose. Simultaneously ^{1}H NMR and ^{13}C NMR spectrum of Tarose 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.

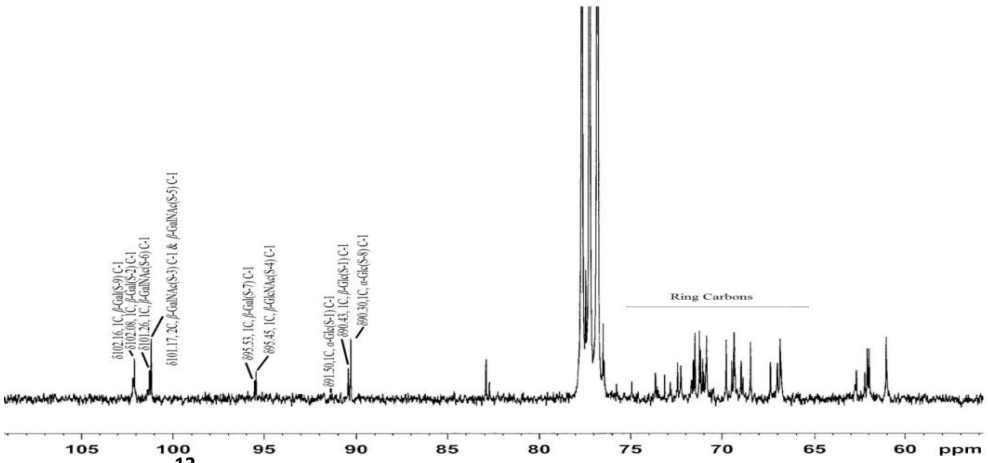
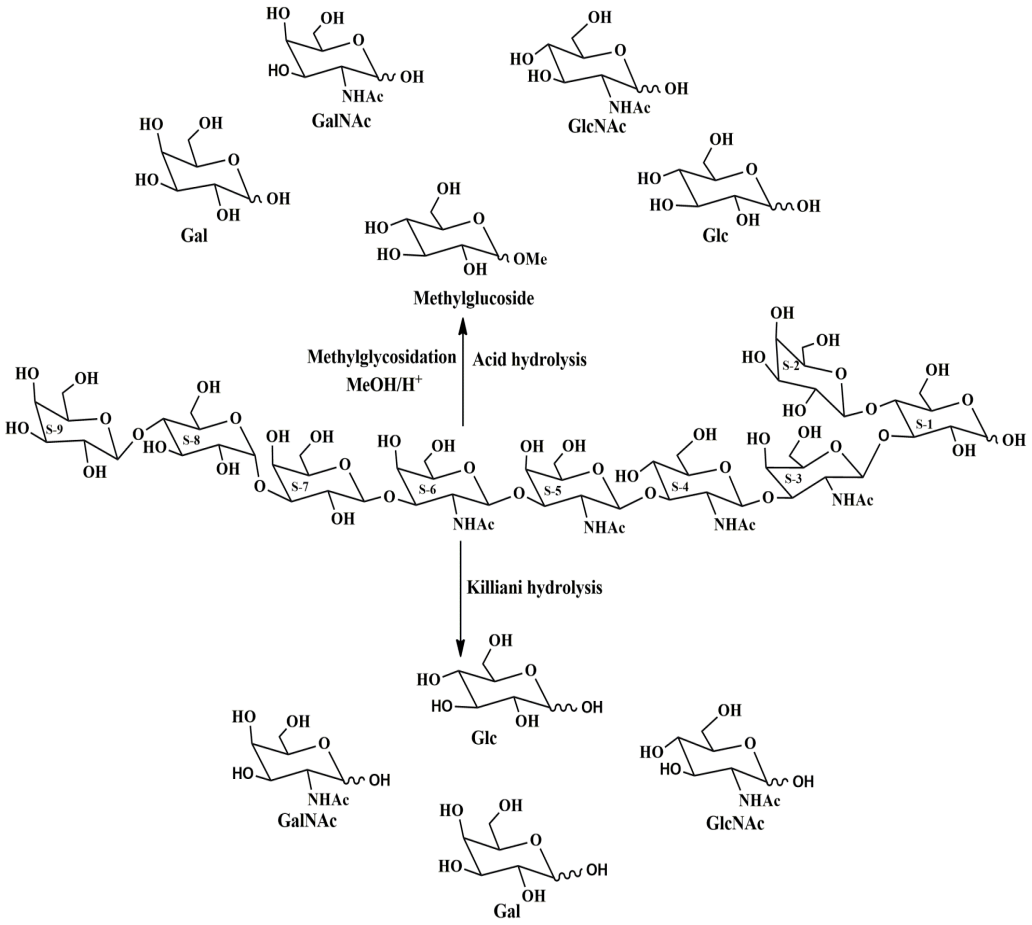


Figure 4.^{13}C NMR Spectrum of Tarose acetate in $CDCl_3$ at 300 MHz.



Scheme 1. Methylglycosidation/Acid Hydrolysis and Killiani Hydrolysis of Tarose.

The anomeric protons signal present at δ5.29 in TOCSY Spectrum of Tarose 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 Tarose 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 Tarose 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 δ3.49 x 102.08.

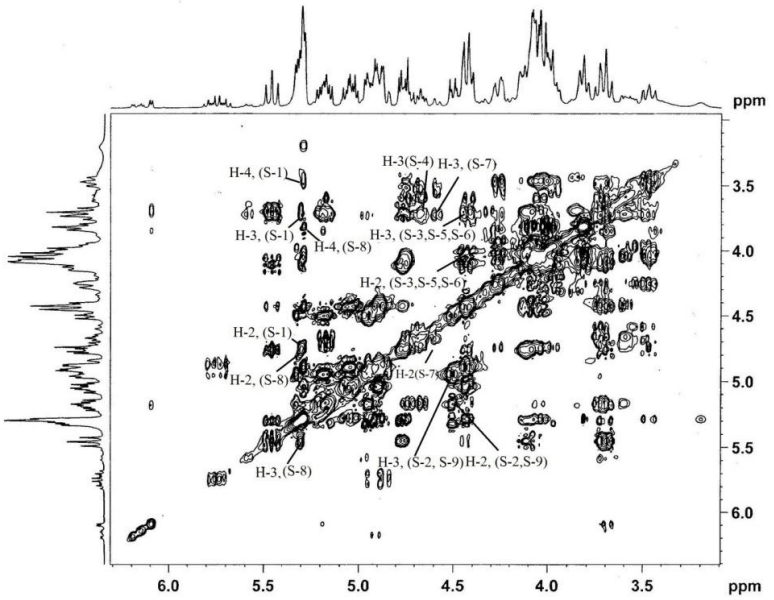


Figure 5. TOCSY Spectrum of Tarose acetate in $CDCl_3$ at 300 MHz.

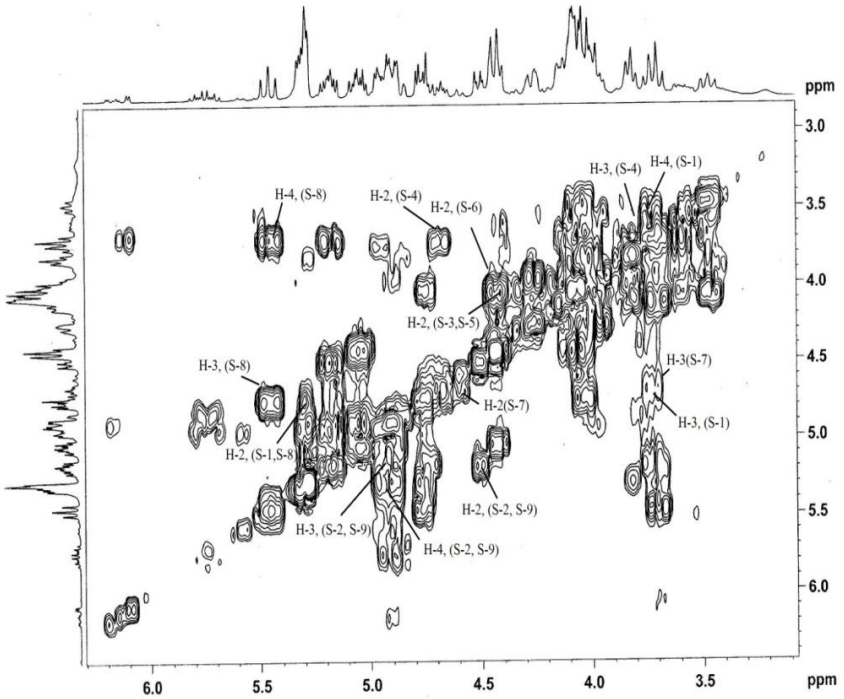


Figure 6. COSY Spectrum of Tarose acetate in $CDCl_3$ at 300 MHz.

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 Tarose 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 atδ4.51 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 Tarose 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 Tarose acetate in $CDCl_3$. The anomeric proton signal at δ4.50 in ^{1}H NMR spectrum of Tarose 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 Tarose 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 Tarose 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 Tarose 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 Tarose 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 Tarose in D_2O at 300 MHz (Uemura et al. 2005) 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 Tarose 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 Tarose acetate in $CDCl_3$.The anomeric proton signal at δ4.43 ^{1}H NMR spectrum of Tarose 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 arose at δ4.12 corresponded to H-2 position of *β*-GalNAc (S-3) and another proton signal arose 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 Tarose 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 Tarose acetate in $CDCl_3$ at 300 MHz. was due to the presence of *β*-GlcNAc (S-4) moiety. 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 Tarose 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 Tarose in D_2O[253]. Since it was ascertained by COSY and TOCSY spectrum of Tarose 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 Tarose 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 Tarose 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 Tarose 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 Tarose 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 Tarose acetate was identified as H-3 of *β*-GlcNAc (S-4) by COSY spectrum of Tarose 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 Tarose 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 Tarose 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 Tarose 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 Tarose 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 Tarose 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 Tarose acetate. The anomeric proton signal present at δ4.43 in ^{1}H NMR spectrum of Tarose 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 arose at δ4.43x4.12 corresponded to H-2 position of *β*-GalNAc (S-5) and another signal arose 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 Tarose acetate which was available for (1→3) glycosidic linkage by the next monosaccharide unit (S-6). Further, HMBC spectrum of Tarose 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.

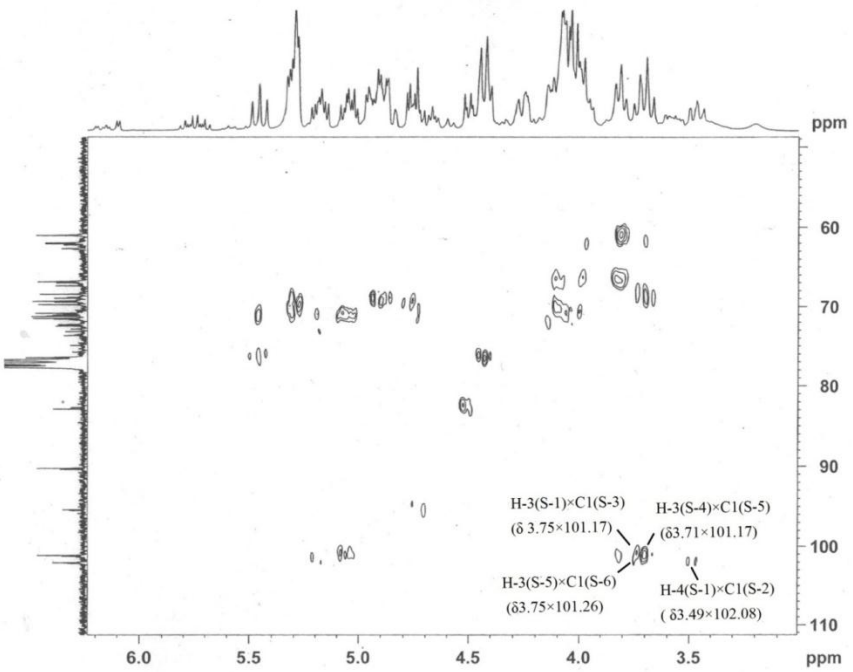
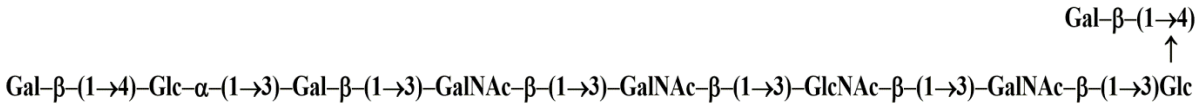


Figure 7. HMBC Spectrum of Tarose acetate in $CDCl_3$ at 300 MHz.

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 Tarose 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 Tarose 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 Tarose 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 Tarose 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 Tarose 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 Tarose 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 Tarose at 300 MHz in $CDCl_3$.The anomeric proton signal present at δ4.41 in ^{1}H NMR spectrum of Tarose 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 arose at δ4.41x4.12 corresponded to H-2 position of *β*-GalNAc (S-6) while the other signal arose 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 Tarose 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 Tarose acetate in $CDCl_3$ gave its complimentary anomeric carbon signal at δ95.53 in the HSQC spectrum of Tarose 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 Tarose was supported by appearance of anomeric proton signal at δ4.66 (J=7.8Hz) in ^{1}H NMR spectrum of Tarose in D_2O at 300 MHz. The anomeric proton signal present atδ4.66 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 Tarose 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 –NAc 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 Tarose 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 Tarose 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 arose at δ4.59x4.68 corresponded to H-2 position of *β*-Gal (S-7), however the cross peak arose 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 Tarose 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 Tarose acetate in $CDCl_3$ gave its complimentary signal δ90.30 in HSQC spectrum of Tarose 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 Tarose in D_2Oat 300 MHz. Since it was ascertained by COSY and TOCSY spectrum of Tarose 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 Tarose 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 Tarose 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 arose 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 Tarose 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 Tarose acetate in $CDCl_3$ gave its complimentary signal at δ102.16 in HSQC spectrum of Tarose 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 Tarose in D_2O at 300 MHz.

Since it was ascertained by COSY and TOCSY spectrum of Tarose 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 Tarose 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 Tarose 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 Tarose acetate in $CDCl_3$. The anomeric proton signal at δ4.49 in ^{1}H NMR spectrum of Tarose 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 Tarose acetate in $CDCl_3$ at 300 MHz. All the ^{1}H NMR assignments for ring protons of monosaccharide units of Tarose 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 Tarose were assigned by the cross peaks for glycosidically linked carbons with their protons in the HSQC and HMBC spectrum of acetylated Tarose. All signals obtained in ^{1}H and ^{13}C NMR of compound Tarose 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 ^{1}H NMR, ^{13}CNMR, COSY, TOCSY, HSQC and HMBC experiments it was interpreted that the compound was a nonasaccharide having following structure as:



TAROSE

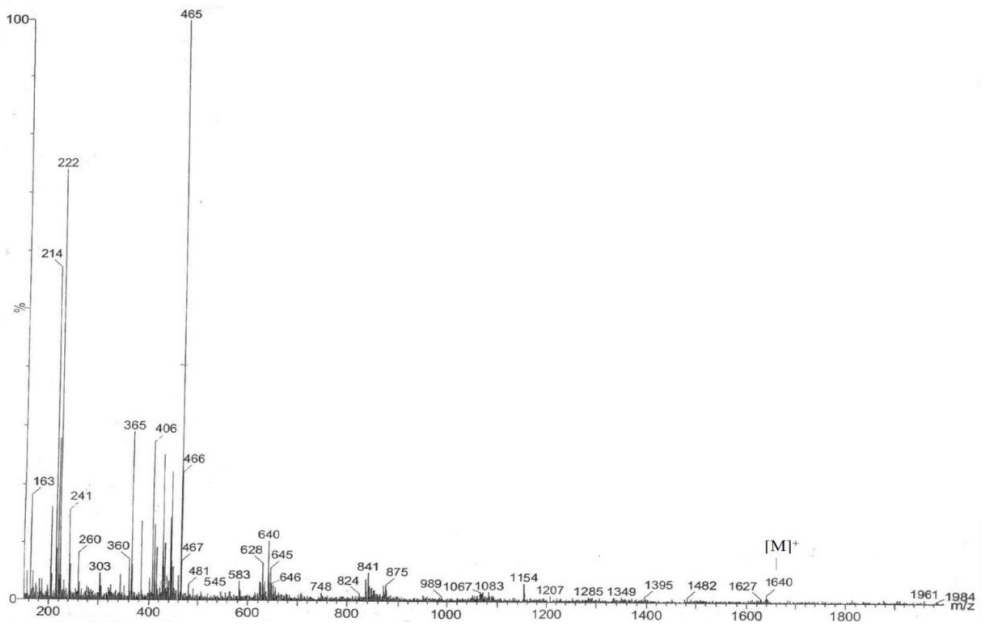
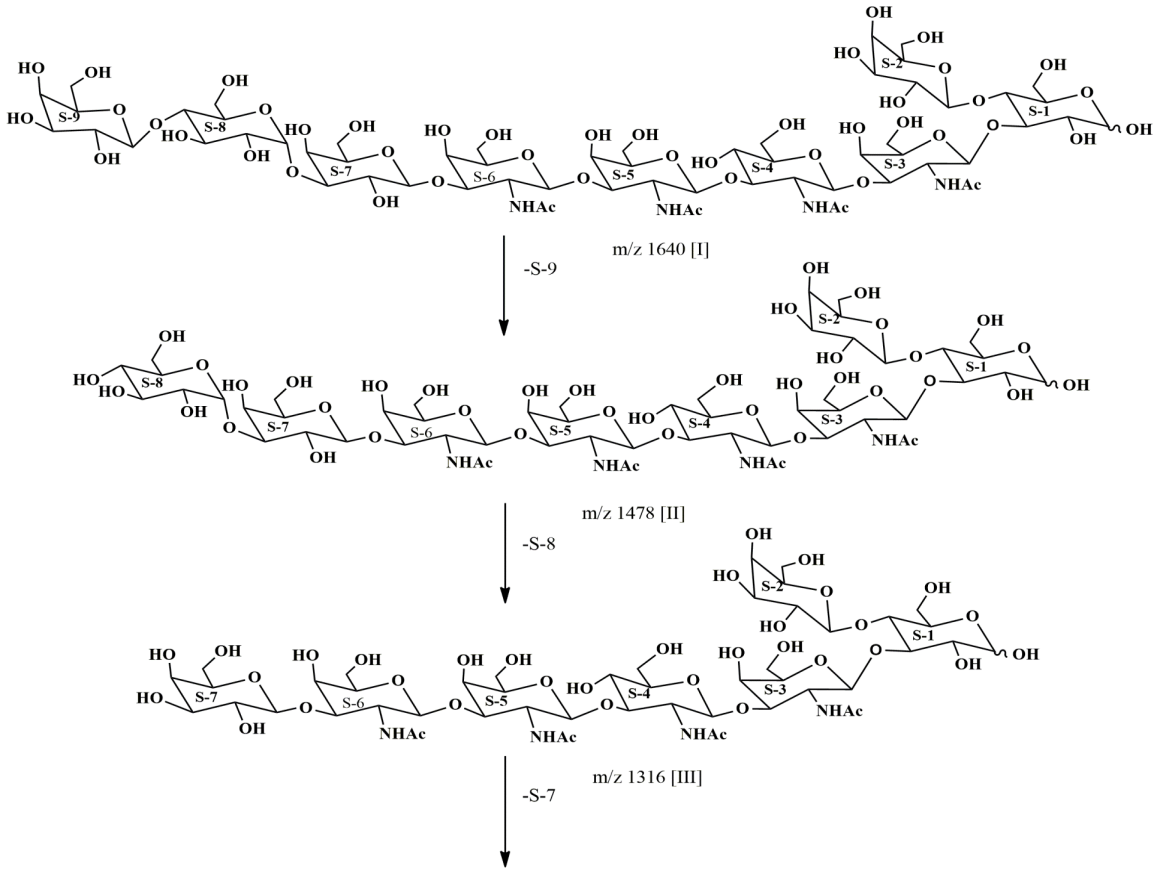


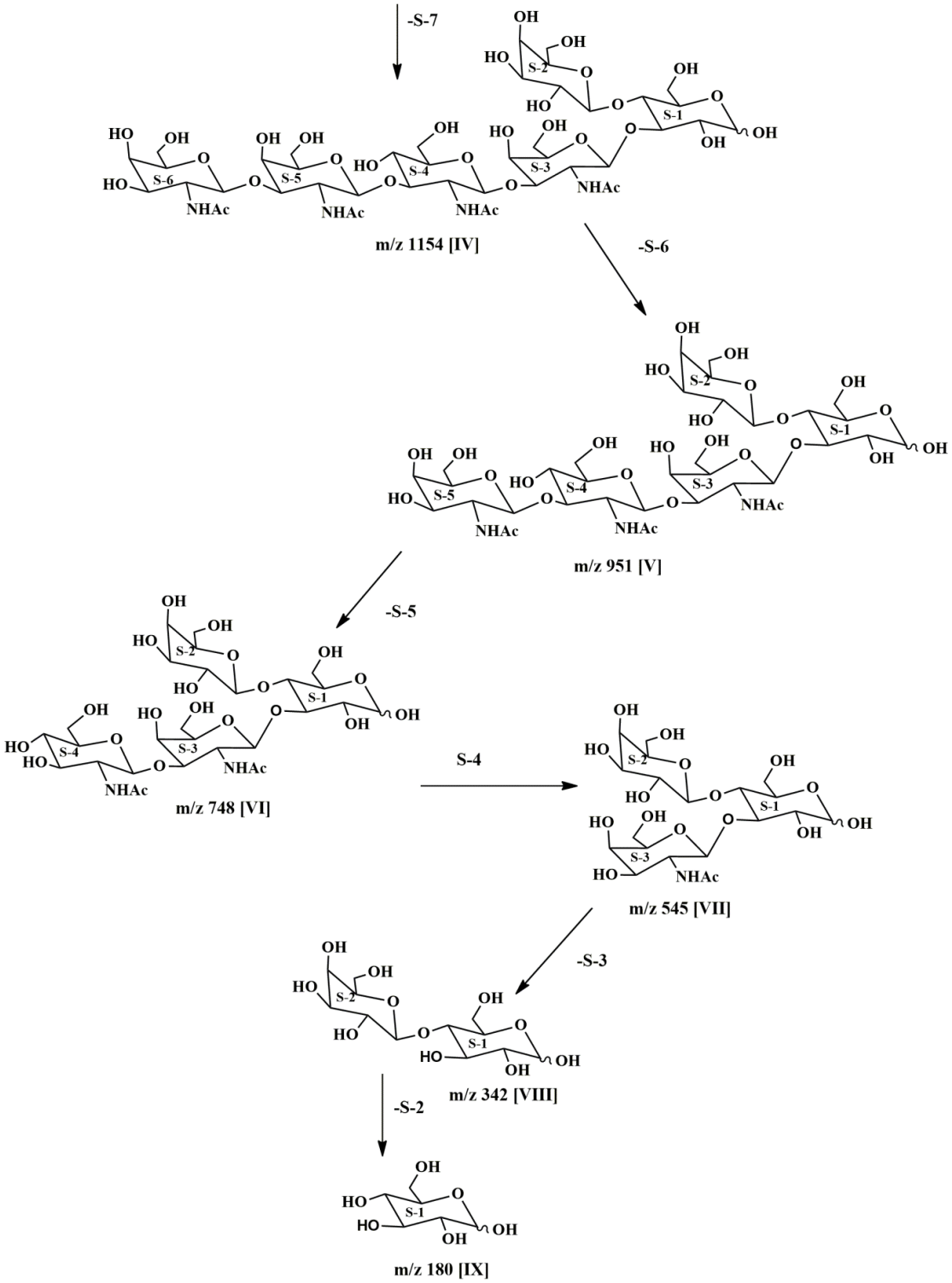
Figure 8. ES- Mass Spectrum of Tarose.

The Electronspray Mass Spectrometry data of Tarose not only confirmed the derived structure but also supported the sequence of monosaccharide in Tarose. The highest mass ion peaks were recorded at m/z 1640 which was due to [M^+] confirming the molecular weight of Tarose1640 was in agreement with its molecular formula. Further the mass fragments were formed by repeated H transfer (Barber et. al.1982) 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 arose 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.

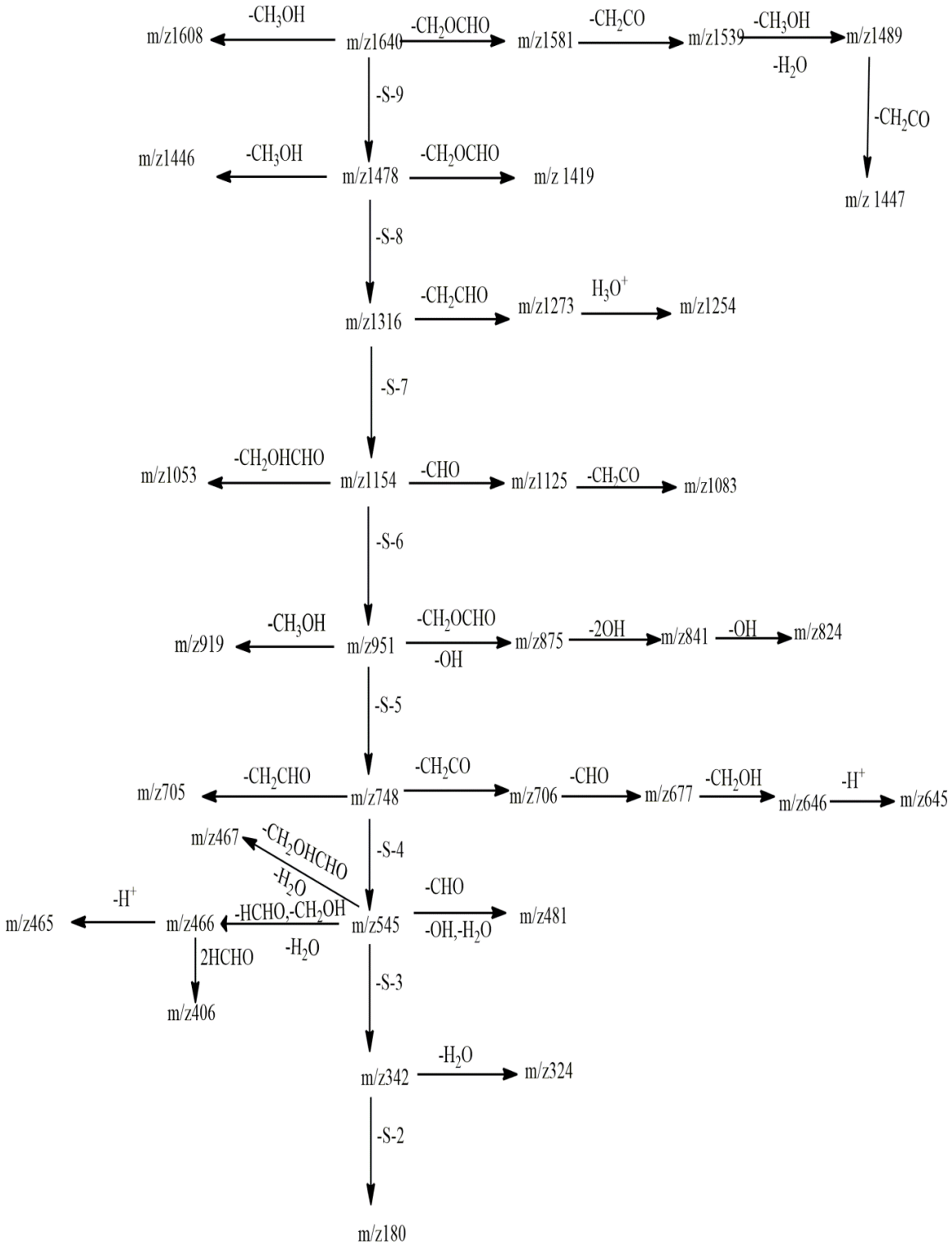


Scheme 2 Continued: Mass fragmentation of compound B, Tarose

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 arose 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.



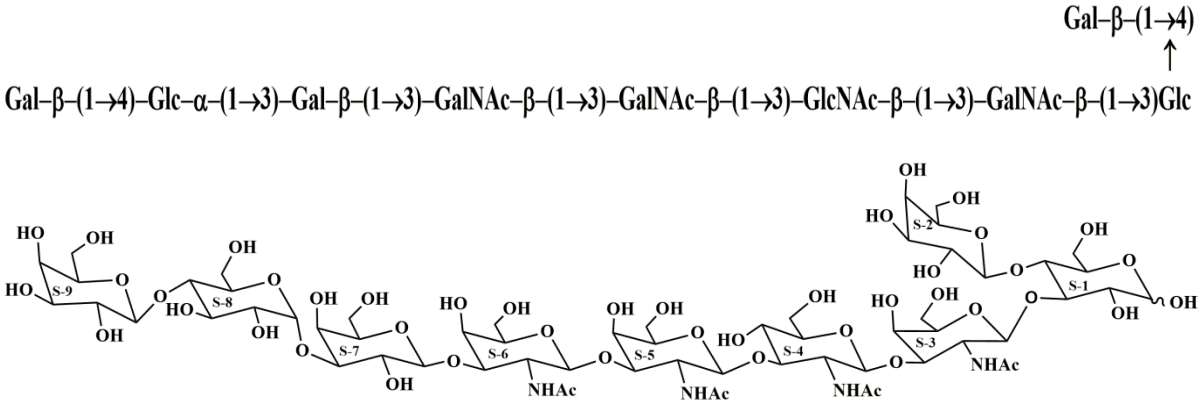
Scheme 2. Mass fragmentation of compound B, Tarose.



Scheme 3. ES-MS fragmentation of compound B, Tarose.

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], 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. ^1H NMR, ^{13}C NMR and 2D NMR (De Waard et.al.1992) viz. COSY, TOCSY, HMBC and HSQC spectra of Tarose acetate and Tarose, the structure and sequence of isolated Novel oligosaccharide Tarose structure was deduced as:



TAROSE

CONCLUSION

In conclusion of the research paper it was inferred that a novel nonasaccharide with a small branched chain was isolated from the colostrums of Jersey cow and its structure was elucidated by combining the results obtained from traditional system of structure elucidation of natural products along with recent techniques of 1D and 2D NMR along with Mass spectrometry.

ACKNOWLEDGEMENTS

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