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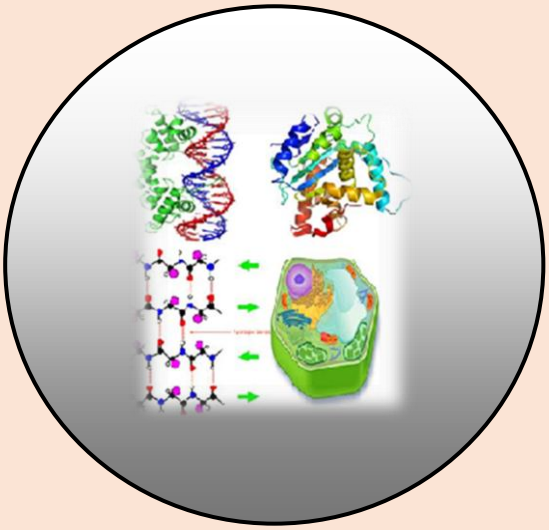

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RESEARCH PAPER

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Isolation and Structure Elucidation of a Novel Octasaccharide 'Athisose' from Milk of Rathi Cow by 2D NMR and Mass Spectrometry

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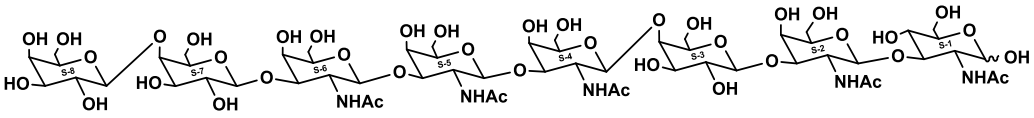
ABSTRACT

Human milk and cow milk share significant similarities, making cow milk an important nutritional source for neonates. Historically, the value of cow milk has been recognized in ancient medicinal systems such as Charak Samhita and Ayurveda for its benefits, including heart muscle strengthening, improved eyesight, enhanced immune system function, and increased milk production in lactating women. The medicinal properties of milk are due to its oligosaccharide content. Oligosaccharides are composed of monosaccharide units like Glucose (Glc), Galactose (Gal), N-Acetylglucosamine (GlcNHAc), and N-Acetylgalactosamine (GalNHAc), are linked together by O-glycosidic linkages, varying in configuration and conformation. Given the importance of cow milk oligosaccharides, our study, investigated the oligosaccharide content in different cow species, including Jersey, Sahiwal, Gir, Frasier, and Tharparkar. Specifically, we focused on Rathi cow milk from Panchmukhi district in Rajasthan, India. We processed Rathi cow milk using modified method of Kobata and Ginsburg to obtain its oligosaccharide mixture, this led to the isolation of several milk oligosaccharides, namely Rathose, Thisose, and Hisose. In this paper, we report the isolation and purification of another novel octasaccharide, Athisose, from Rathi cow milk. We elucidated its structure by using advanced physicochemical techniques, including ^{1}H, ^{13}C NMR, COSY, TOCSY, HSQC, HMBC, and mass spectrometry, along with chemical transformation and degradation methods.



Athisose

Gal-α-(1→4)-Gal-α-(1→3)-GalNHAc-β-(1→3)-GalNHAc-β-(1→3)-GalNHAc-β-(1→4)-Gal-β-(1→3)-GalNHAc-β-(1→3)-GlcNHAc

Keywords: Milk, Oligosaccharide, Athisose, NMR and Mass spectrometry.

INTRODUCTION

The resemblance of human milk with cow milk has made it more important for the nutrition of neonates, moreover the importance of the cow milk is very well defined in ancient medicinal system of Charak Samhita and Ayurveda. The cow milk helps to strengthen the heart muscles and is also relevant for better eye sight. It provides the strengthening of the immune system (Li et al, 2020)and elevates the milk output inlactating women. It was postulated by glycobiologists that all the medicinal properties residing in a particular milk is due to its oligosaccharide content. Oligosaccharides are straight or branched chain oligomers of monosaccharides consisted of Glucose, Galactose, GlcNHAc and GalNHAc building blocks which are linked together by O-glycosidic linkages at different positions of monosaccharides. Their constitution is also varied due to configuration of glycosidic linkages i.e. α andβ and conformation of sugars in their $^4C_1$ and $^1C_4$ conformations. Keeping it in mind the importance of cow milk oligosaccharides, different species of cows were investigated for the oligosaccharide content of their milk. In this series, different cow species like Jersey, Sahiwal, Gir, Frasier, and Tharparkar were probed for their oligosaccharide contents. In our present investigation, we have collected the Rathi Cow milk from Panchmukhi district of Rajasthan state of India (Chouhan et al, 2023) and processed it by modified method of Kobata and Ginsburg (Kumar, et al, 2018) for obtaining its oligosaccharide mixture, which was further proceeded by following the established methods (Kumar, et al 2018) which were defined in our earlier communications. This investigation resulted into the isolation of number of milk oligosaccharides and the structures of Rathose(Chauhan et al, 2024), Thisose (Chauhan et al, 2024) and Hisose(Chauhan et al, 2024) has already been published. In the present article we have discussed the isolation and purification of a novel octasaccharide namely Athisose from Rathi Cow milk. We have reported the structure elucidation of this novel octasaccharide by recent physico chemical techniques like ^1H, ^{13}C NMR, COSY, TOCSY, HSQC (Kay et al, 1992), HMBC (Davis, 1985) and Mass spectrometry (Barr et al, 1991) along with chemical transformation and chemical degradation.

EXPERIMENTAL

General Procedure

General Procedure were same as described in our earlier communication (Chauhan et al, 2024)

ISOLATION OF RATHI COW MILK OLIGOSACCHARIDES BY MODIFIED METHOD OF KOBATA AND GINSBURG (Chauhan et al, 2024)

10 litre Rathi cow milk was collected from a domestic cow of Panchmukhi district of Rajasthan state of India. The milk was fixed by addition of equal amount of ethanol and was processed by modified method of Kobata and Ginsburg (Chauhan et al, 2024) affording 283gm of oligosaccharide mixture.

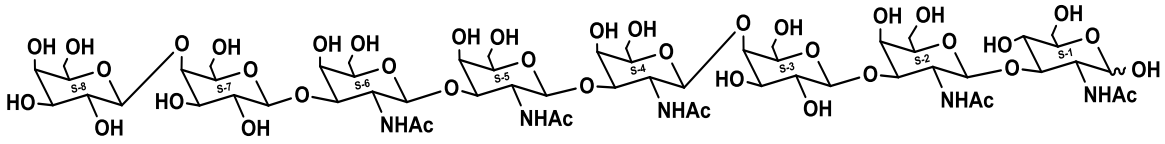
Acetylation of Oligosaccharide Mixture

10.2 gm oligosaccharide mixture was acetylated with pyridine (10.2 ml) and acetic anhydride (10. 2ml) at 60°C and the solution was stirred overnight.

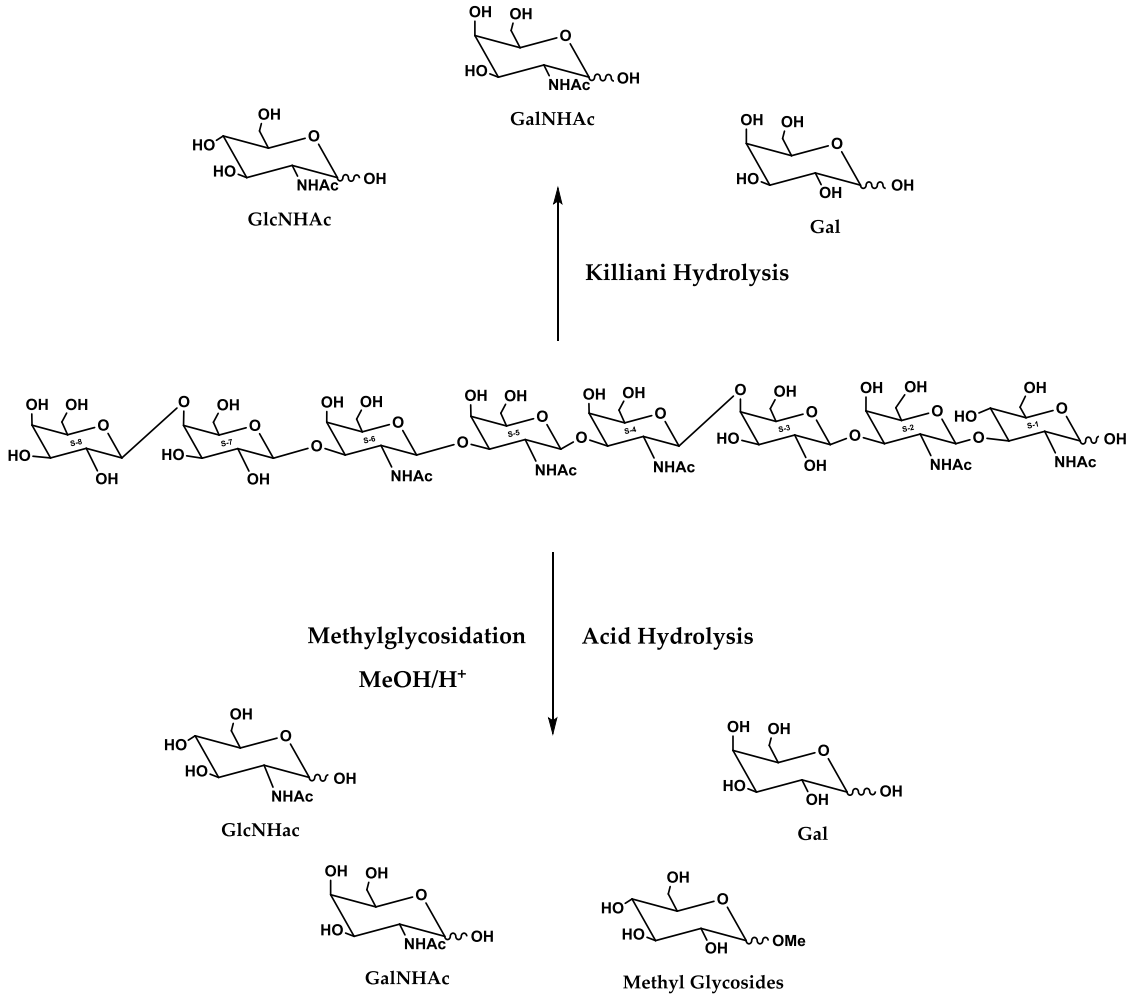
The mixture was evaporated under reduced pressure and the viscous residue was taken in $CHCl_3$ (2 ×500 ml) and it was washed in sequence with 2N-HCl (1 × 250 ml), ice cold 2N-$NaHCO_3$ (2 × 250 ml) and finally with H_2O (2 × 250 ml). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding the acetylated mixture (10.6 gm). The TLC showed five spots i.e. a, b, c, d and e in the acetylated oligosaccharide mixture of Rathi cow milk.

Deacetylation of Compound 'd', Athisose Acetate (**Chauhan et al, 2024**)

Compound 'd' (69 mg) was obtained from column chromatography of acetylated oligosaccharide mixture. 40 mg of compound 'd' was dissolved in acetone (4 ml) and 4 ml of NH_4OH was added to it and left overnight in a stoppered hydrolysis flask. After 24 hrs ammonia was removed under reduced pressure and the compound was washed thrice with $CHCl_3$ (10 ml) (to remove acetamide) and the water layer was finally freeze dried giving the deacetylated oligosaccharide '**D'** (32 mg).



Compound D, ATHISOSE



Scheme 1: Killiani Hydrolysis and Methylglycosidation/Acid hydrolysis of Athisose

Methyl Glycosidation/Acid Hydrolysis of Compound D, Athisose (Chauhan et al, 2024)
Compound '**D'** (10 mg) was refluxed with absolute MeOH (2 ml) at 70ºC for 18 hrs in 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 D, 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 GlcNHAc, Gal and GalNHAc. Identification of monosaccharides in compound D was confirmed by comparison with authentic samples (TLC, PC) of *α*-and *β*-methylglucosides along with the Glc, Gal and GalNHAc.

Killiani Hydrolysis of Compound 'D', Athisose (Khan et al; 2019)
Compound D (5 mg) was dissolved in 2 ml Killiani mixture (AcOH-H_2O-HCl, 7:11:2) and heated at 100°C for 1 hr followed by evaporation under reduced pressure. It was dissolved in 2 ml of H_2O and extracted twice with 3 ml $CHCl_3$. The aqueous residual solution was made neutral by addition of 1-2 drops of 2N NaOH and was evaporated under reduced pressure to afford GlcNHAc, Gal and GalNHAc on comparison with authentic samples of GlcNHAc, Gal and GalNHAc.

DESCRIPTION OF COMPOUND D, ATHISOSE
Compound d (69 mg) was obtained from column chromatography. On deacetylation of 50 mg of substance 'd' with NH_4OH/acetone, it afforded substance 'D' (36 mg) $[\alpha]_D^{25}$ = -20º (*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 (Partridge et al 1949), Feigl test (Feigl, 1975) and Morgan-Elson test (Warren, 1960).

$C_{58}H_{97}O_{41}N_5$		%C	%H	%N
	Calculated	45.82	6.38	4.61
	Found	45.83	6.37	4.60

^{1}H NMR of Compound 'd', Athisose Acetate in $CDCl_3$ at 300 MHz
δ6.14 [d, 1H, J=3.6 Hz, α-GlcNHAc(S-1) H-1], δ5.60 [d, 1H, J=8.1 Hz, β-GlcNHAc(S-1) H-1], δ5.38 [d, 1H, J=3.3 Hz, α-Gal(S-7) H-1], δ5.36 [d, 1H, J=2.7 Hz, α-Gal(S-8) H-1], δ4.70 [d, 1H, J=7.5 Hz, β-Gal(S-3) H-1], δ4.53 [d, 1H, J=7.8 Hz, β-GalNHAc(S-6), H-1], δ4.52 [d, 2H, J=8.1 Hz, β-GalNHAc(S-5 & S-2), H-1], δ4.52 [d, 1H, J=8.1 Hz, β-GalNHAc(S-4), H-1], δ3.82 [m, 4H, β-GalNHAc(S-2, S-4, S-5, S-6) H-3], δ3.82 [m, α-Gal(S-7) H-4], δ3.79 [m, 1H, β-GlcNHAc(S-1) H-3]

^{13}C NMR of Compound- 'd' Athisose Acetate in $CDCl_3$ at 300 MHz
δ90.25[2C, *α*-Gal(S-7) &*α*-Gal(S-8) C-1], δ91.38[1C, *α*-GlcNHAc(S-1) C-1], δ93.92[1C, *β*-GlcNHAc(S-1) C-1], δ95.44[1C, *β*-Gal(S-3) C-1], δ100.98[1C, *β*-GalNHAc(S-2) C-1], δ101.14[1C, *β*-GalNHAc(S-4) C-1], δ101.26[1C, *β*-GalNHAc(S-5) C-1], δ102.06[1C, *β*-GalNHAc(S-6) C-1].

^{1}H NMR of Compound- 'D' Athisose in D_2O at 300 MHz
δ5.72[d, 1H, J=4.2Hz *α*-GlcNHAc(S-1) H-1], δ5.22[d, 2H, J=3.6Hz, *α*-Gal(S-7) &*α*-Gal(S-8) H-1],

δ4.66[d, 1H, J=8.1Hz, *β*- GlcNHAc (S-1) H-1], δ4.54[d, 1H, J=8.7Hz, *β*-Gal (S-3) H-1],δ4.52[d, 2H, J=7.8Hz, *β*-GalNHAc(S-2 & S-4) H-1], δ4.46[d, 2H, J=7.8Hz, *β*-GalNHAc(S-5 & S-6) H-1], δ2.08[s, 3H, ($NHCOCH_3$), *β*-GlcNHAc(S-1)], δ1.99[s, 6H, ($NHCOCH_3$), *β*-GalNHAc(S-2 & S-4)], δ1.90[s, 6H, ($NHCOCH_3$), *β*-GalNHAc (S-5 & S-6)].

ES MS of compound 'D', Athisose

1519[M^+],1477[1519-CH_2CO], 1419[1477-$NHCOCH_3$], 1385[1419-2OH], 1367[1385-H_2O], 1357[1519-(S-8)], 1333[1367-2OH], 1315[1357-CH_2CO], 1285[1315-HCHO], 1195[1357-(S-7], 1194[1195-H^+], 1152[1195-CH_2CO], 1094[1152-$NHCOCH_3$], 1076[1094-H_2O], 1040[1076-$2H_2O$], 992[1195-(S-6)], 961[992-CH_2OH], 893[961-CH_3OH,$2H_2O$], 875[893-H_2O], 841[875-2OH], 789[992-(S-5)], 741[789-HCHO,H_2O], 711[741-HCHO], 683[741-$NHCOCH_3$], 677[711-OH], 646[677-CH_2OH], 633[677-2OH], 586[789-(S-4)], 536[586-2HCHO], 492[536-2OH], 467[586-$CH_2OHCOCHOHCHOH$], 466[586-HOCHCHOH,-CH_2OHCHO], 406[586-(S-1)], 424[586-(S-3)], 406[424-H_2O], 365[424-CH_2OCHO], 333[CH_3OH], 291[365-$CH_2OHCHCHOH$], 260[291-CH_2OH,], 241[260-H_3O^+], 221[424-(S-2)] 204[221-OH].

RESULT AND DISCUSSION OF COMPOUND 'D', ATHISOSE

Compound D, ATHISOSE$C_{58}H_{97}N_5O_{41}$,$[\alpha]_D^{25}$= -20º gave positive Phenol-sulphuric acid test(Partridge et al 1949), Feigl test (Feigl, et al 1975)and Morgan-Elson test test(Warren, 1960) showing the presence of normal and amino sugars moietie(s) in the compound D. The name of the compound ATHISOSE was originated from name of the animal RATHI and was designated as 'D' while its acetylated form was explained as 'd'. The HSQC spectrum of acetylated Athisose showed the presence of nine cross peaks of nine anomeric protons and carbons in their respective region at δ6.14 × 91.38, δ5.60×93.92, δ5.38×90.25, δ5.36×90.28, δ4.70×95.44, δ4.53×102.06 δ4.52×101.26, δ4.52×100.98 and δ4.52×101.14 suggested the presence of nine anomeric protons and carbon in compound D in its reducing form. Its reducing nature was confirmed by its methylglycosylation MeOH/H^+ followed by its acid hydrolysis, which led to the isolation of *α* and *β*-N-acetylmethylglucosides along with Gal and GalNHAc suggesting the presence of N-acetylglucosamine at the reducing end and it is the first report of any milk oligosaccharides where the GlcNHAc was present at the reducing end of the oligosaccharide. All eight monosaccharides present in the oligosaccharide were denoted as S-1, S-2, S-3, S-4, S-5, S-6, S-7 and S-8 for convenience. The monosaccharide constituents of Athisose were confirmed by its acid hydrolysis under strong acidic condition i.e. Killiani hydrolysis (Khan et al; 2019), which was monitored on paper chromatography and TLC. In the hydrolysis three spots were found identical with the authentic samples of GlcNHAc, Gal, and GalNHAc by co-chromatography on paper and TLC. Thus the octasaccharide Athisose contained three types of monosaccharide units i.e. GlcNHAc, Gal, and GalNHAc. Further the Presence of eight doublets for nine anomeric protons at δ6.14(1H), δ5.60(1H), δ5.38(1H), δ5.36(1H), δ4.70(1H), δ4.53(1H), δ4.52(2H) and δ4.52(1H) in the ^{1}H NMR of Athisose Acetate in $CDCl_3$ at 300 MHz. confirmed the presence of a Octasaccharide present in its reducing form.

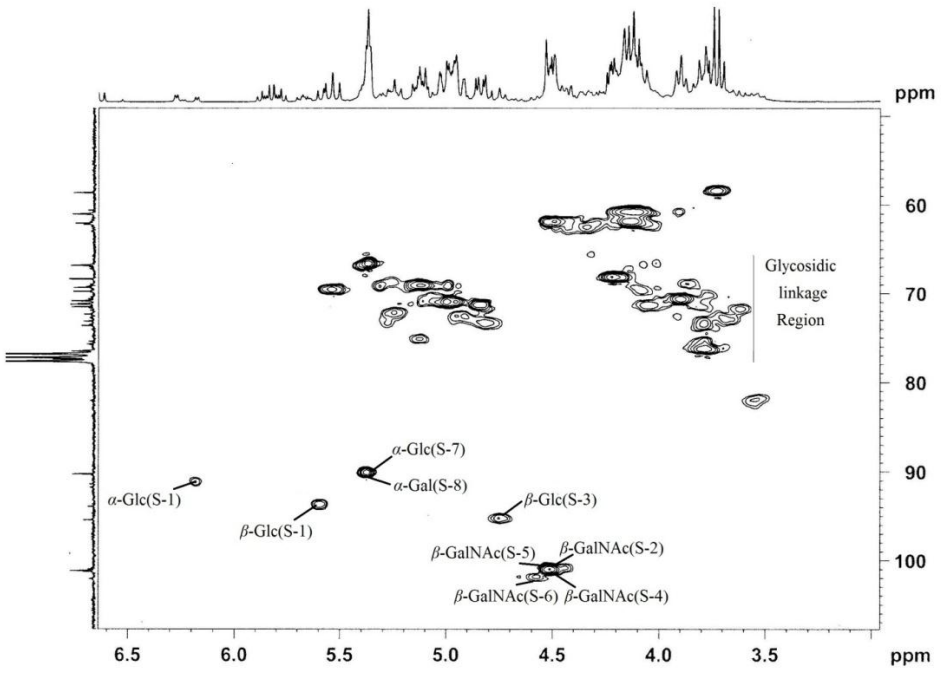


Figure 1. HSQC Spectrum of Athisose acetate in $CDCl_3$ at 300 MHz.

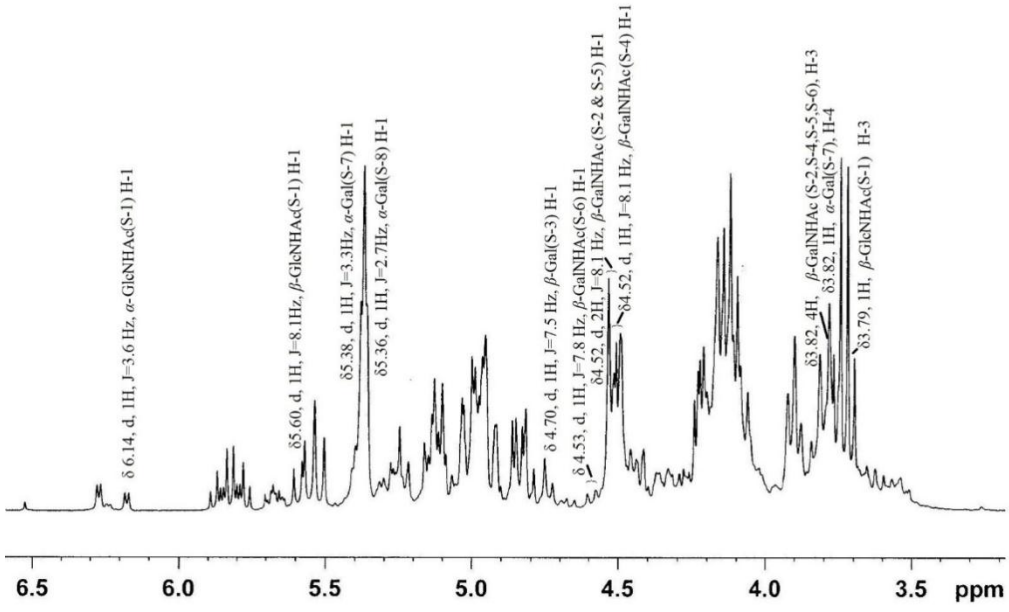


Figure 2. ^{1}H NMR Spectrum of Athisose acetate in $CDCl_3$ at 300 MHz.

Further the presence of eight anomeric peaks for nine anomeric carbon at δ90.28(2C), δ91.38(1C), δ93.92(1C), δ95.44(1C), δ100.98(1C), δ101.14(1C), δ101.26(1C) and 102.06(1C) in the ^{13}C NMR spectrum of acetylated Athisose in $CDCl_3$ at 300 MHz. also confirmed that Athisose was a Octasaccharide in its reducing form.

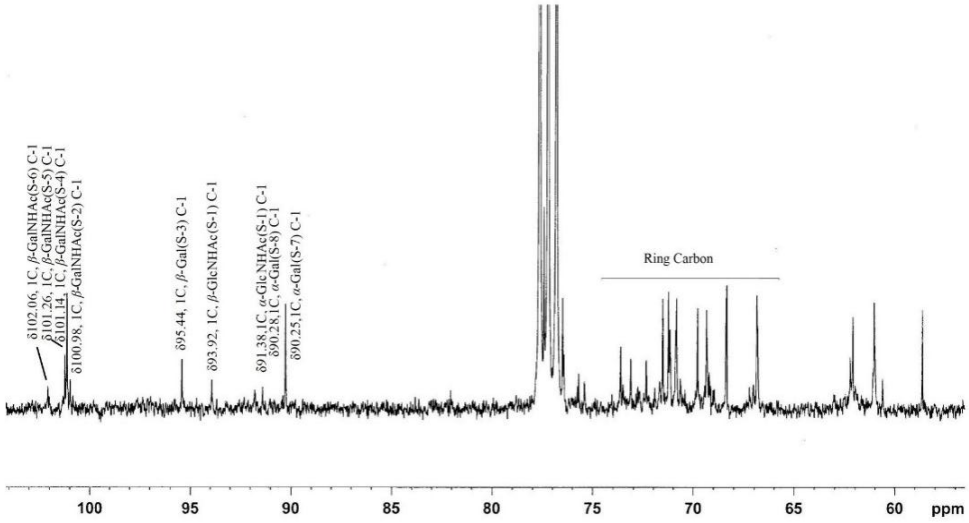


Figure 3. ^{13}C NMR Spectrum of Athisose acetate in $CDCl_3$ at 300 MHz.

The Octasaccharide nature of Athisose was further supported by the presence of six anomeric proton doublets for nine anomeric protons at δ5.72(1H), 5.22(2H), 4.66(1H), 4.54(1H), 4.52(2H) and 4.46(2H) in ^{1}H NMR spectrum of Athisose in D_2O at 300 MHz.

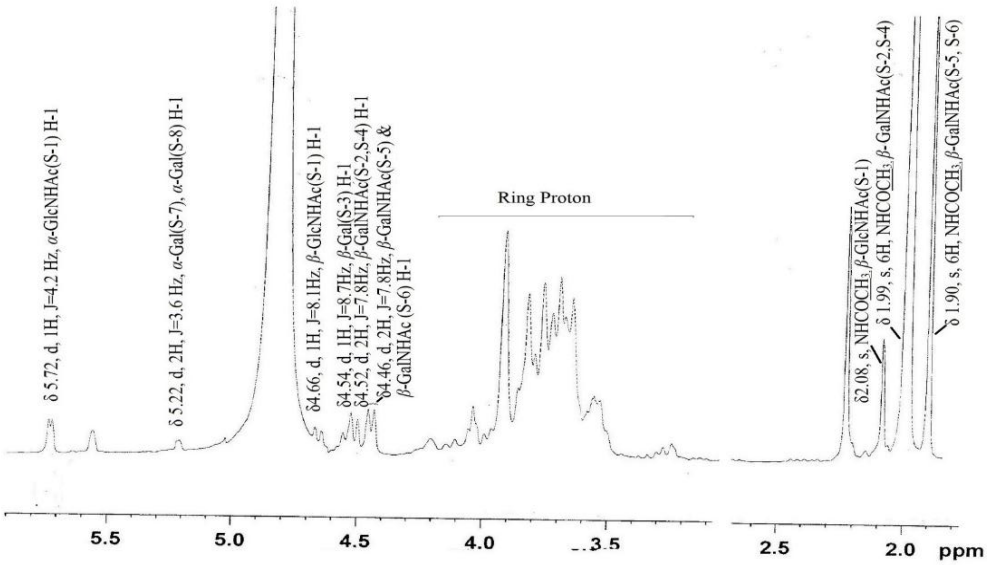


Figure 4. ^{1}H NMR Spectrum of Athisose in D_2O at 300 MHz.

^{1}H and ^{13}C NMR spectra justifies the nine anomeric signals of Octasaccharide with total integral intensity of eight anomeric proton/carbon. The molecular formula $C_{58}H_{97}O_{41}N_5$ was in agreement with mass ion peak obtained from ES-MS spectrum of Athisose which showed the highest mass ion peak at m/z 1519 $[M]^+$ for a Octasaccharide.

Table 1. Anomericproton/carbon assignments in AthisoseAcetate by HSQC Spectrum.

Anomeric Proton × Anomeric Carbon	Cross-peaks of Anomeric Proton × Anomeric Carbon
H-1(S-1) × C-1(S-1α)	δ 6.14 × 91.38
H-1(S-1) × C-1(S-1β)	δ 5.60 × 93.92
H-1(S-2) × C-1(S-2)	δ 4.52 × 100.98
H-1(S-3) × C-1(S-3)	δ 4.70 ×95.44
H-1(S-4) × C-1(S-4)	δ 4.52 ×101.14
H-1(S-5) × C-1(S-5)	δ 4.52 ×101.26
H-1(S-6) × C-1(S-6)	δ 4.53 × 102.06
H-1(S-7) × C-1(S-7)	δ 5.38 × 90.25
H-1(S-8) × C-1(S-8)	δ 5.36 × 90.28

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

Moieties	In D_2O		In $CDCl_3$	
	^{1}HNMR (δ)	Coupling constant (J)	^{1}HNMR (δ)	Coupling constant (J)
α-GlcNHAc(S-1)	5.72	4.2Hz	6.14	3.6Hz
β-GlcNHAc(S-1)	4.66	8.1Hz	5.60	8.1Hz
β-GalNHAc(S-2)	4.52	7.8Hz	4.52	8.1Hz
β-Gal(S-3)	4.54	8.7Hz	4.70	7.5Hz
β-GalNHAc(S-4)	4.52	7.8Hz	4.52	8.1Hz
β-GalNHAc(S-5)	4.46	7.8Hz	4.52	8.1Hz
β-GalNHAc(S-6)	4.46	7.8Hz	4.53	7.8Hz
α-Gal(S-7)	5.22	3.6Hz	5.38	3.3Hz
α-Gal(S-8)	5.22	3.6Hz	5.36	2.7Hz

The ^{1}H NMR spectrum of Athisose in D_2O at 300 MHz contain two doublets at δ5.72 (J= 4.2Hz) and δ4.66 (J= 8.1Hz) along with singlet of three proton of amide methyl at δ2.08 confirmed the presence of N-acetyl glucosamine at the reducing end in the octasaccharide. Further ^{1}H NMR and ^{13}C NMR spectrum of Athisose acetate showed downfield shifted *α* and *β* anomeric proton and carbon of reducing N-acetyl glucosamine (S-1) at δ6.14(J= 3.6Hz), δ5.60(J= 8.1Hz) and δ91.38, δ93.92 respectively confirmed that Athisose was a octasaccharide in its reducing form and N-acetyl glucosamine was present at reducing end. The anomeric proton signal present at δ5.60 in ^{1}H NMR Spectrum of Athisose Acetate assigned to *β*-GlcNHAc (S-1) gave three cross peaks at δ5.60×3.70, δ5.60×3.80 and δ5.60×5.16 in its TOCSY spectrum in $CDCl_3$ at 300MHz.

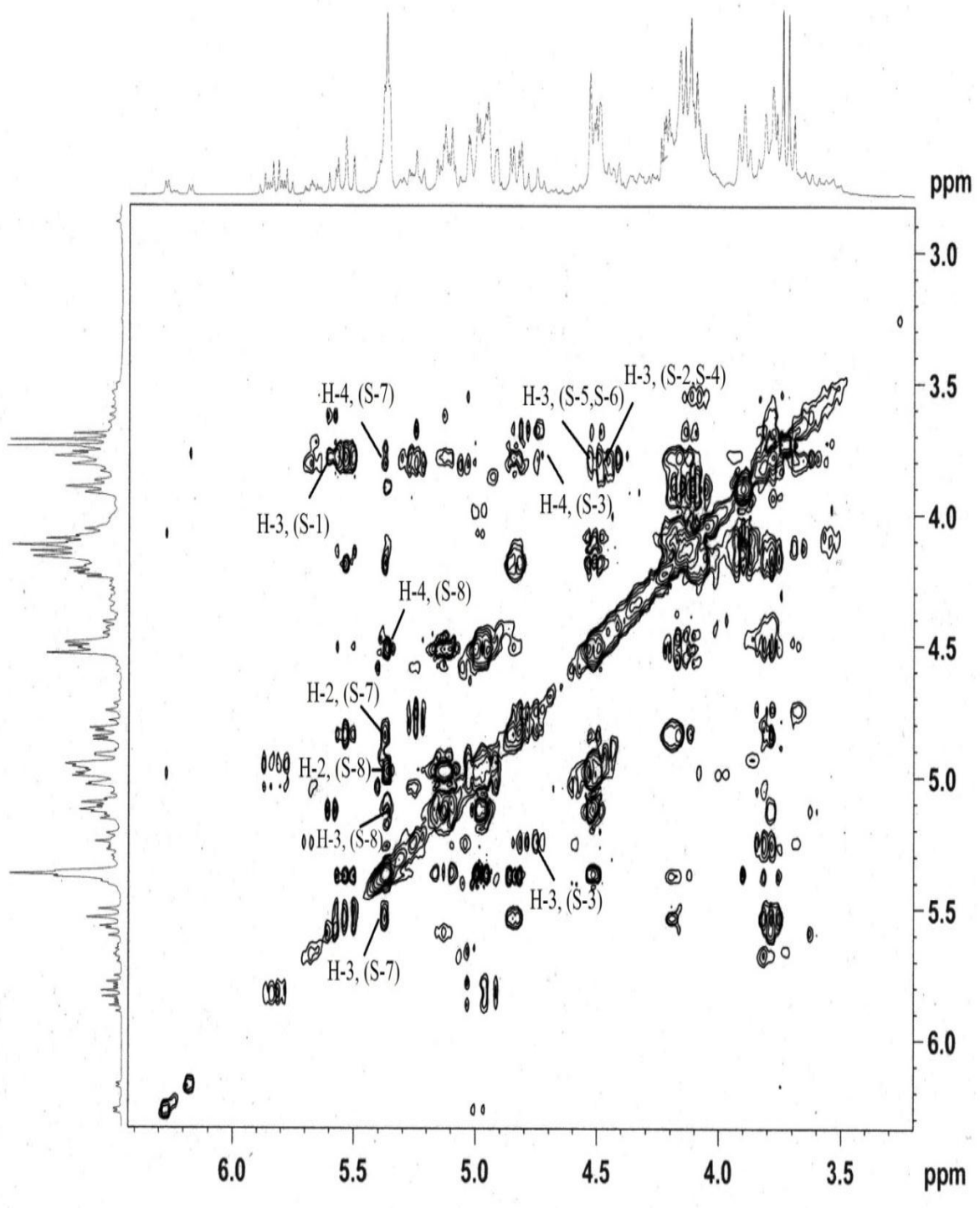


Figure 5. TOCSY Spectrum of Athisose acetate in $CDCl_3$ at 300 MHz.

The chemical shift of the cross peak at δ5.60×3.68 and δ5.60×3.79 suggested that in sugar S-1 two positions were available for substitution, which was later identified as H-2 and H-3 of *β*-GlcNHAc (S-1) by COSY spectrum of Athisose acetate in $CDCl_3$.

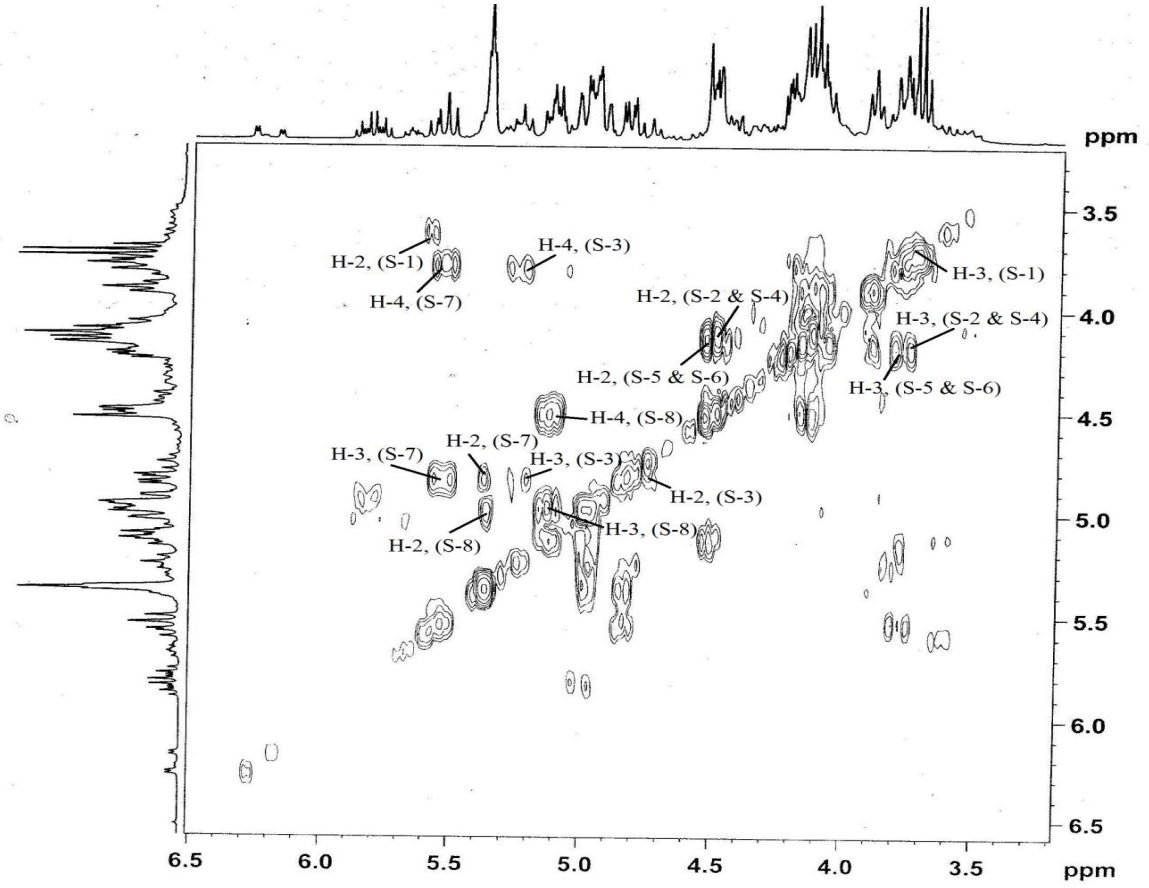


Figure 6. COSY Spectrum of Athisose acetate in $CDCl_3$ at 300 MHz.

Further it was confirmed by COSY spectrum of Athisose Acetate that H-2 position of *β*-GlcNHAc (S-1) was substituted by –NHAc group while the multiplet present at δ3.79 assigned for H-3 of S-1 showed long range coupling with anomeric carbon of next monosaccharide (S-2) at δ3.79×100.98 in its HMBC spectrum, confirmed the (1→3) linkage between S-2 and S-1. The anomeric carbon signal present at δ100.98 had its complimentary signal at δ4.52 in HSQC spectrum of Athisose acetate in $CDCl_3$ at 300 MHz.

The chemical shift values of anomeric carbon and anomeric proton of S-2 at δ100.98 and δ4.52 were having resemblance with literature value of anomeric chemical shift value of GalNHAc hence S-2 monosaccharide was confirmed as GalNHAc(Bush, 1988). Further the presence of *β*-GalNHAc (S-2) as next monosaccharide in Athisose was supported by appearance of anomeric proton signal at δ4.52 (J=7.8Hz) along with singlet of three proton at δ1.99 in ^{1}H NMR spectrum of Athisose in D_2O at 300 MHz. The coupling constant of anomeric signal *β*-GalNHAc (S-2) with larger value of 7.8 Hz showed a*β*-configuration of the *β*-GalNHAc (S-2). The (1→3) linkage between *β*-GlcNHAc (S-1) and *β*-GalNHAc (S-2) was supported by the presence of H-3 signal of S-1 at δ3.82 in upfield region of ^{1}H NMR spectrum of Athisose acetate which was confirmed by the TOCSY and COSY spectrum of Athisose acetate in $CDCl_3$ at 300 MHz. At the stage of structure assignment two novelty were determined; the diversity was presence of GlcNHAc at the reducing end and second monosaccharide was a 1→3 linkage between S-1 and S-2 in GlcNHAc and GalNHAc.

The anomeric proton signal at δ4.52 in TOCSY Spectrum of Athisose acetate assigned to *β*-GalNHAc (S-2) gave three cross peaks at δ4.52×3.82, δ4.52×4.12 and δ4.52×5.15 suggested that in sugar (S-2) two positions were available for substitution which was later identified as 4.12 (H-2) and 3.82 (H-3)and 5.15 (H-4) of S-2 by COSY spectrum of Athisose acetate. Further it was confirmed by COSY spectrum of Athisose Acetate that H-2 position of *β*-GalNHAc (S-2) δ4.12 was substituted by –NHAc group and the multiplet present at δ3.82 in ^{1}H NMR spectrum of Athisose acetate suggested that the H-3 of *β*-GalNHAc (S-2)was available for glycosylation by the next monosaccharide moiety (S-3). Further anomeric proton doublet present at δ4.70 in the ^{1}H NMR spectrum of Athisose acetate in $CDCl_3$ at 300 MHz gave its complimentary signal at δ95.44 in HSQC spectrum of Athisose acetate in $CDCl_3$. The chemical shift value of anomeric proton and anomeric carbon were having resemblance with literature value of anomeric chemical shift value of *β*-Gal (S-3) hence S-3 monosaccharide was confirmed as *β*-Gal(Bush, 1988). Further the presence of*β*-Gal (S-3) in Compound D was supported by the presence of anomeric proton doublet at δ4.54 in ^{1}H NMR spectrum of Athisose in D_2O. Since it was ascertained by the COSY and TOCSY spectrum of Athisose acetate that the *β*-GalNHAc (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*-GalNHAc (S-2) at δ3.82 must be linked to *β*-Gal (S-3). The (1→3) linkage between S-3 and S-2 was further supported by the presence of downfield H-3 proton signal at δ3.82 in the ^{1}H NMR spectrum of Athisose acetate in $CDCl_3$ at 300 MHz. The coupling constant of anomeric signal (S-3) with J value 8.7Hz confirmed the *β*-configuration of the *β*-Gal (S-3). The anomeric proton signal at δ4.70 in TOCSY Spectrum of Athisose acetate assigned to *β*-Gal (S-3) gave three cross peaks at δ4.70×3.82, δ4.70×4.83 and δ4.70×5.21. The chemical shift of the cross peak at δ4.70×3.82 suggested that in sugar S-3 only one position was available for glycosidic linkage by the next monosaccharide unit(S-4) which was later identified as H-4 of *β*-Gal (S-3) by COSY spectrum of Athisose acetate. Further HMBC spectrum of Athisose acetate showed long range coupling between H-4 of *β*-Gal (S-3) and anomeric carbon of next monosaccharide (S-4) at δ3.82×101.14 confirmed the (1→4) linkage between S-3 and S-4. The anomeric carbon signal present at δ101.14 gave its complimentary signal at δ4.52 in HSQC spectrum of Athisose acetate in $CDCl_3$ at 300 MHz. The chemical shift values of anomeric carbon and anomeric proton were having resemblance with literature value of anomeric chemical shift value of *β*-GalNHAc hence S-4 monosaccharide was confirmed as *β*-GalNHAc (Bush, 1988). Further the presence of *β*-GalNHAc (S-4) as next monosaccharide in Athisose was supported by appearance of anomeric proton signal at δ4.52 along with a singlet of three protons of amide methyl at δ1.99 in ^{1}H NMR spectrum of Athisose in D_2O. The anomeric proton signal at δ4.52 in ^{1}H NMR spectrum of Athisose acetate assigned for*β*-GalNHAc (S-4) gave three cross peaks at δ4.52×3.82, δ4.52×4.12 and δ4.52×5.15 in its TOCSY spectrum in $CDCl_3$ at 300 MHz,out of which proton signal arised at δ4.12 corresponded to H-2 position of *β*-GalNHAc (S-4) and another proton signal arised at δ3.82 represented the linkage region of *β*-GalNHAc (S-4) which was later identified as H-3 of *β*-GalNHAc (S-4) by COSY spectrum of Athisose acetate which was available for (1→3) glycosidic linkages by the next monosaccharide unit (S-5).

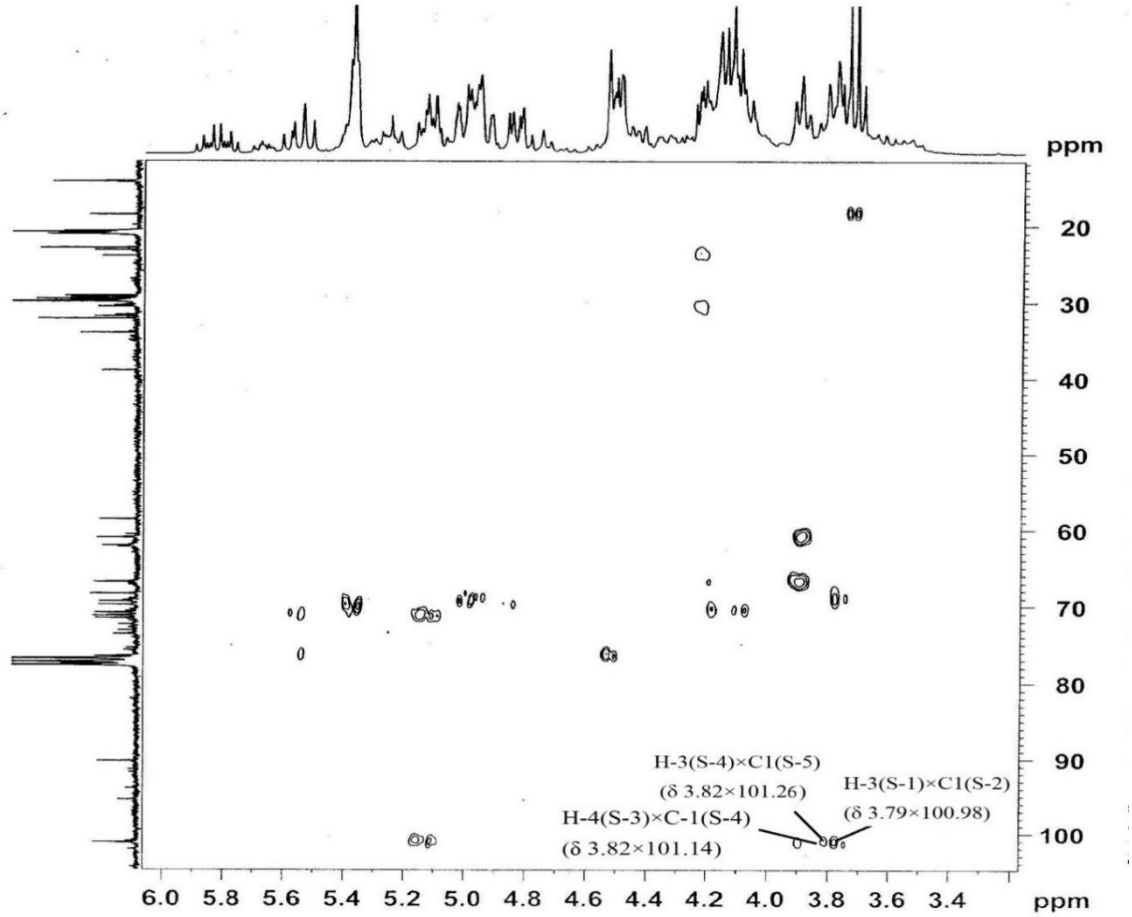
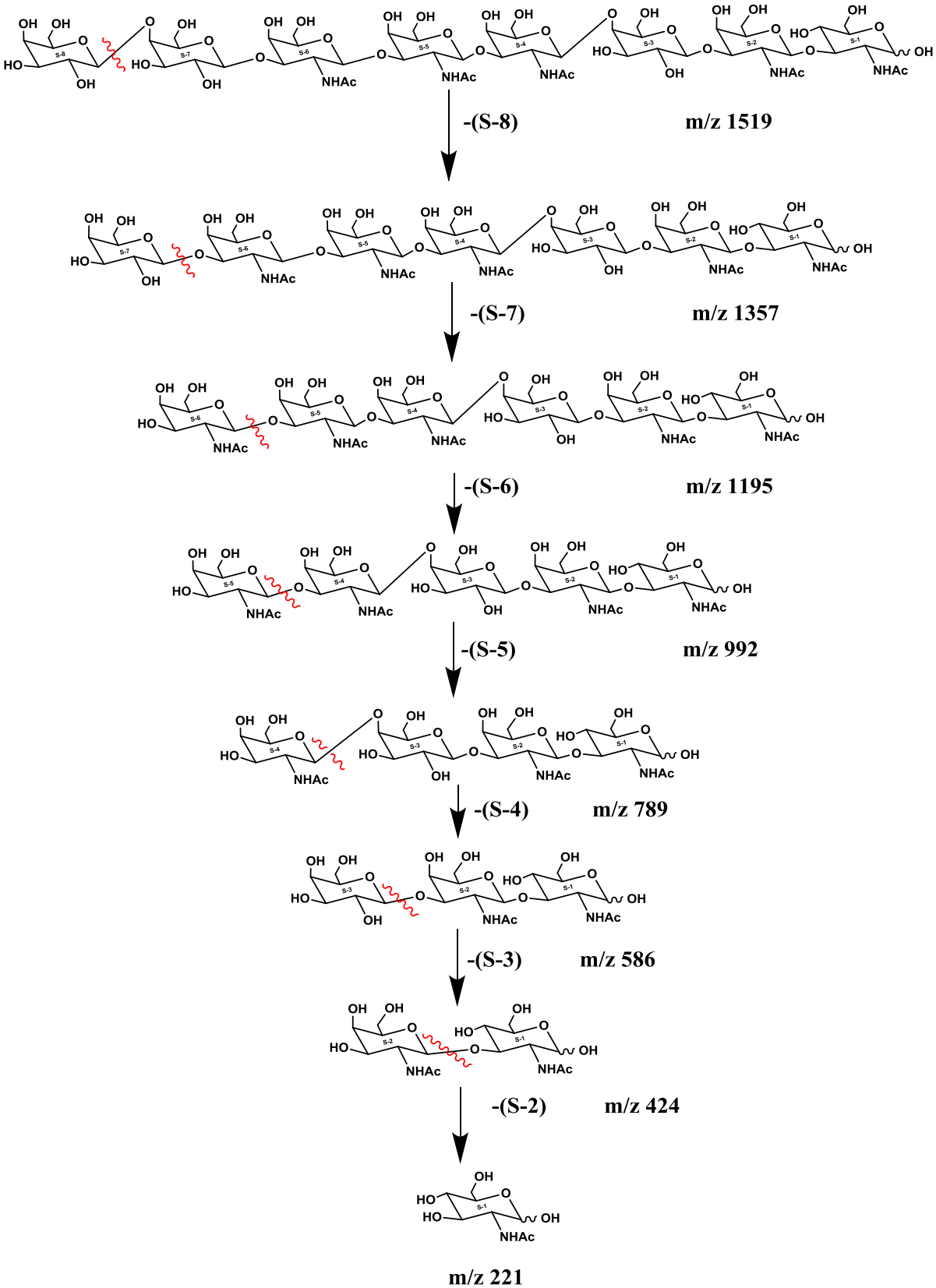


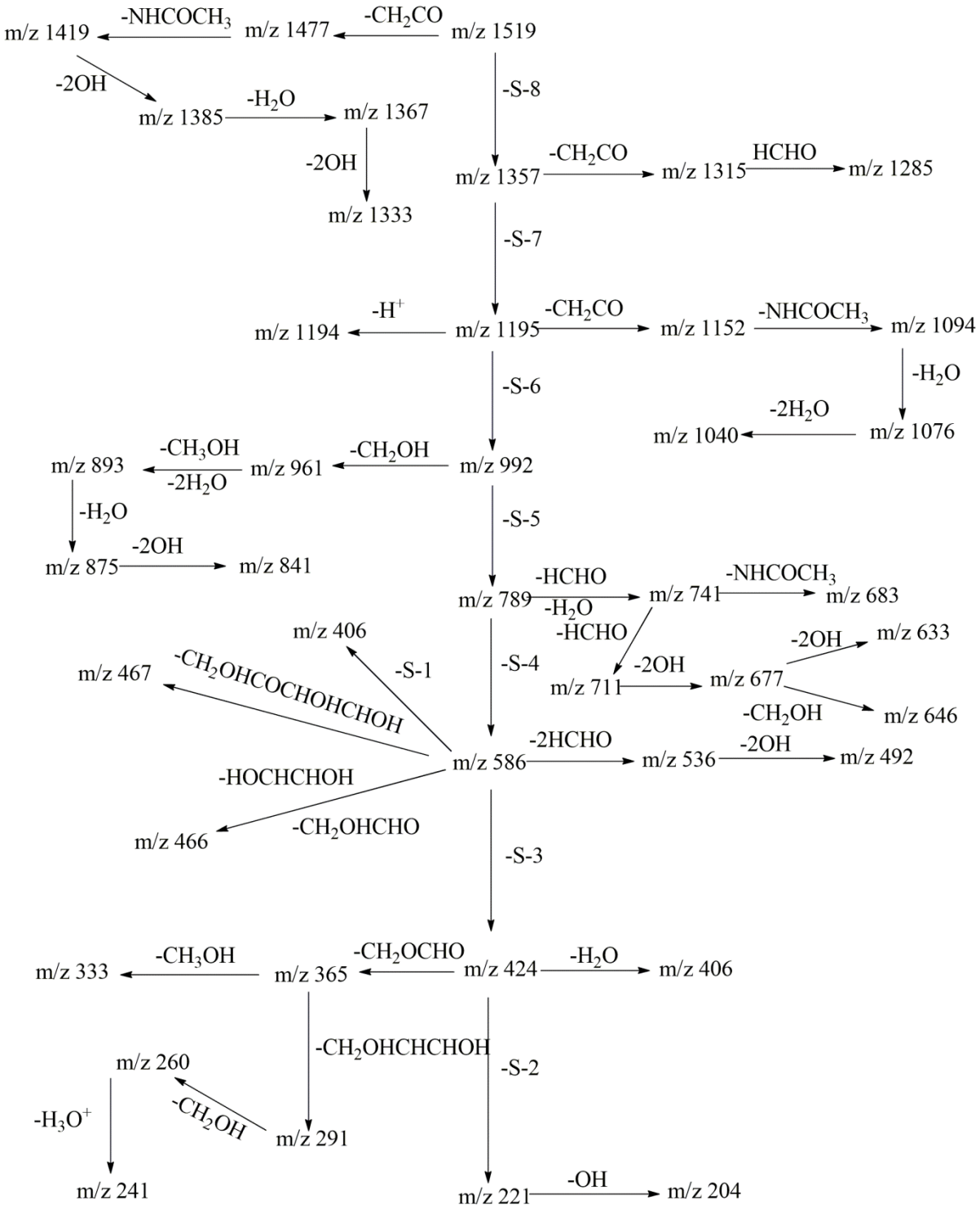
Figure 7. HMBC Spectrum of Athisose acetate in $CDCl_3$ at 300 MHz.

Further HMBC spectrum of Athisose acetate showed long range coupling between H-3 of *β*-GalNHAc(S-4) and anomeric carbon of next monosaccharide (S-5) at δ3.82×101.26 confirmed the (1→3) linkage between S-5 and S-4. The anomeric carbon signal present at δ101.26 had its complimentary signal at δ4.52 in HSQC spectrum of Athisose acetate in $CDCl_3$ at 300 MHz. The chemical shift values of anomeric carbon and anomeric proton were having resemblance with literature value of anomeric chemical shift value of GalNHAc hence S-5 monosaccharide was confirmed as GalNHAc(Bush, 1988). Further the presence of *β*-GalNHAc (S-5) as next monosaccharide in Athisose was supported by appearance of anomeric proton signal at δ4.46 along with a singlet of three protons of amide methyl at δ1.90 in ^{1}H NMR spectrum of Athisose in D_2O. The coupling constant of anomeric signal *β*-GalNHAc (S-5) with larger value of 7.8 Hz showing the *β* configuration of the *β*-GalNHAc (S-5).The anomeric proton signal at δ4.52 in TOCSY Spectrum of Athisose acetate assigned to *β*-GalNHAc (S-5) gave three cross peaks at δ4.52×3.82, δ4.52×4.12 and δ4.52×5.15, out of which proton signal arised at δ4.12 corresponded to H-2 position of *β*-GalNHAc (S-5) and another proton signal arised as multiplet at δ3.82 was later identified as H-3 of *β*-GalNHAc (S-5) by COSY spectrum of Athisose acetate which was available for (1→3) glycosidic linkage by the next monosaccharide unit (S-6). Further anomeric proton doublet appeared at δ4.53 in ^{1}H NMR spectrum of Athisose acetate gave its complimentary signal atδ102.06 in its HSQC spectrum of Athisose acetate in $CDCl_3$ at 300 MHz.

The chemical shift values of anomeric carbon and anomeric proton were having resemblance with literature value of anomeric chemical shift value of *β*-GalNHAc hence S-6 monosaccharide was confirmed as *β*-GalNHAc (Bush, 1988). Further the presence of *β*-GalNHAc (S-6) as next monosaccharide in Athisose was supported by appearance of anomeric proton signal at δ4.46 along with a singlet of three protons of amide methyl at δ1.90 in ^{1}H NMR spectrum of Athisose in D_2O. As ascertained by COSY and TOCSY spectrum of Athisose acetate H-3 position of S-5 was available for glycosylation by the next monosaccharide (S-6), hence S-6 must be linked to H-3 of S-5. i.e. (1→3) linkage between S-6 & S-5. The (1→3) linkage between *β*-GalNHAc (S-6) and *β*-GalNHAc (S-5) was further supported by the ^{1}H NMR spectrum of acetylated Athisose in which the signal for H-3 of *β*-GalNHAc (S-5) appeared at δ3.82 which was later confirmed by COSY, TOCSY and HSQC spectrum of acetylated Athisose at 300 MHz in $CDCl_3$. The coupling constant of anomeric signal *β*-GalNHAc (S-6) with larger value of 7.8 Hz showing the *β* configuration of the *β*-GalNHAc (S-6). Further the presence of *β*-GalNHAc (S-6) as next monosaccharide in Athisose was supported by appearance of anomeric proton signal at δ4.46 along with a singlet of three protons of amide methyl at δ1.90 in ^{1}H NMR spectrum of Athisose in D_2O.Further (1→3) linkage between *β*-GalNHAc (S-6) and *β*-GalNHAc (S-5) was also supported by the downfield shifted position of H-3 of S-5 at δ3.82 in acetylated spectrum of Athisose at 300 MHz in $CDCl_3$. The anomeric proton signal at δ4.53 ^{1}H NMR spectrum of Athisose acetate assigned for *β*-GalNHAc (S-6) contain three cross peaks at δ4.53×3.82, δ4.53×4.12 and δ4.53×5.15 in TOCSY spectrum of Athisose acetate in $CDCl_3$ at 300 MHz, out of which proton signal arised at δ4.12 corresponded to H-2 position of *β*-GalNHAc (S-6) and another proton signal arised at δ3.82 represented the linkage region of *β*-GalNHAc (S-6) which was later identified as H-3 of *β*-GalNHAc (S-6) by COSY spectrum of Athisose acetate which was available for (1→3) glycosidic linkage by the next monosaccharide unit (S-7). Next anomeric proton doublet appeared at δ5.38 in ^{1}H NMR spectrum of Athisose acetate in $CDCl_3$ gave its complimentary signal at δ90.25 in HSQC spectrum of Athisose 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*-Gal hence S-7 monosaccharide was confirmed as *α*-Gal(Bush, 1988). Further the presence of *α*-Gal (S-7) was supported by presence of anomeric proton doublet at δ5.22 in ^{1}H NMR spectrum of Athisose in D_2O at 300MHz.As ascertained by COSY and TOCSY spectrum of Athisose acetate H-3 position of S-6 was available for glycosylation by the next monosaccharide (S-7), hence S-7 must linked to H-3.of S-6. i.e. (1→3) linkage between S-7 & S-6. The (1→3) linkage between *α*-Gal (S-7) and *β*-GalNHAc (S-6) was further supported by the ^{1}H NMR spectrum Athisose acetate in which the signal for H-3 of β-GalNHAc (S-6) appeared at δ3.82 which was later confirmed by COSY, TOCSY and HSQC spectrum of Athisose acetate at 300 MHz in $CDCl_3$. The coupling constant of anomeric signal *α*-Gal (S-7) with larger value of 3.6 Hz showing the *α*configuration of the *α*-Gal (S-7). The anomeric proton signal of *α*-Gal (S-7) at δ5.38 in the ^{1}H NMR spectrum of Athisose acetate in $CDCl_3$ at 300 MHz. gave four cross peak at δ5.38×3.82, 5.38×4.87, and 5.38×5.58 in its TOCSY spectrum in $CDCl_3$ at 300 MHz., suggested that in sugar S-7 only one position was available for glycosidic linkage by the next monosaccharide unit (S-8), later it was confirmed by COSY spectrum of Athisose acetate that the signal arised at δ3.82 was due to the H-4 of S-7. Further anomeric proton doublet appeared at δ5.36 in ^{1}H NMR spectrum of Athisose acetate showed its complimentary signal at δ90.28 in its HSQC spectrum in



Scheme 2: MASS fragmentation of compound D, Athisose



Scheme 3. ES-MS MASS fragment of compoundD, Athisose.

$CDCl_3$ 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*-Gal hence S-8 monosaccharide was confirmed as *α*-Gal(Bush, 1988). Further the presence of *α*-Gal (S-8) as next monosaccharide in Athisose was supported by presence of anomeric proton doublet at δ5.22 in ^{1}H NMR spectrum of Athisose in D_2O.

The coupling constant of anomeric signal *α*-Gal (S-8) with smaller value of 3.6 Hz showing the *α* configuration between *α*-Gal (S-8). The anomeric proton doublet at δ5.36 in ^{1}H NMR spectrum of Athisose acetate contain four cross peak at δ5.36×4.55, δ5.36×4.19, δ5.36×5.04 and δ5.36×5.17 in its TOCSY spectrum in $CDCl_3$ at 300 MHz. Since TOCSY spectrum of Athisose acetate does not contain any methine protons in glycosidic linkage region i.e., δ3.5-4.2 ppm showed that none of -OH group of *α*-Gal (S-8) were involved in glycosidic linkage hence confirmed that *α*-Gal (S-8) was present at non-reducing end and none of their -OH group were available for glycosidic linkages which was confirmed by the TOCSY and COSY. All the ^{1}H NMR assignments for ring protons of monosaccharide units of Athisose were confirmed by COSY and TOCSY experiments. The positions of glycosi0dation in the oligosaccharide were confirmed by position of anomeric signals, Structure reporter groups (SRG) (Vliegenthart et al 1982) and comparing the signals in ^{1}H and ^{13}C NMR of acetylated and deacetylated oligosaccharide. All signals obtained in ^{1}H and ^{13}C NMR of compound Athisose were in conformity 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 an Octasaccharide having structure as:

Gal-α-(1→4)-Gal-α-(1→3)-GalNHAc-β-(1→3)-GalNHAc-β-(1→3)-GalNHAc-β-(1→4)-Gal-β-(1→3)-GalNHAc-β-(1→3)-GlcNHAc

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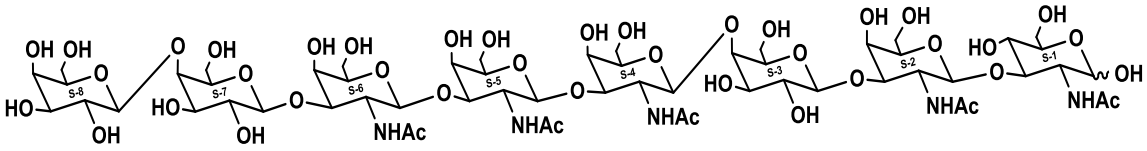
The Electronspray Mass Spectrometry data of Athisose not only confirmed the derived structure but also supported the sequence of monosaccharide in Athisose. The highest mass ion peak was recorded at m/z 1519 which was due to $[M^{+}]$ confirming the molecular weight of Athisose as 1519 and was in agreement with its molecular formula $[C_{58}H_{97}O_{41}N_5]$.

Further the mass fragments obtained formed by repeated H^{+} transfer in the oligosaccharide and was accompanied by the elimination of terminal sugar less water. The Octasaccharide m/z 1519 (I) fragmented to give mass ion at m/z 1357 (II) [1519-(S-8)], this fragment was arised due to the loss of terminal *α*-Gal (S-8) moiety from Octasaccharide indicating the presence of *α*-Gal (S-8) at the non-reducing end. It further fragmented to give mass ion peak at m/z 1195 (III) [1357-S_7] which was due to loss of *α*-Gal (S-7) moiety from heptasaccharide. This fragment of 1195 further fragmented to give mass ion peak at m/z 992(IV) [1195-(S-6)] which was due to loss of *β*-GalNHAc (S-6) moiety from the hexasaccharide. This pentasaccharide unit again fragmented to give mass ion peak at m/z 789(V) [992-(S-5)], which was due to loss of *β*-GalNHAc (S-5) moiety from pentasaccharide. The tetrasaccharide m/z 789 (VI) fragmented to give mass ion at m/z 586 (VII) [789-(S-4)], this fragment was arised due to the loss of *β*-GalNHAc (S-4) moiety from tetrasaccharide indicating the presence of *β*-GalNHAc (S-4). It further fragmented to give mass ion peak at m/z 383 (III) [586-(S-3)] which was due to loss of *β*-Gal (S-3) moiety from tetrasaccharide. This fragment of 383 further fragmented to give mass ion peak at m/z 180 (IV) [383-(S-2)] which was due to loss of *β*-GalNHAc (S-2) moiety from the disaccharide. The other fragmentation pathway in ES Mass spectrum of compound 'D' m/z 1519 shows the mass ion peak at 1477[1519-CH_2CO], 1419[1477-$NHCOCH_3$], 1385[1419-2OH], 1367[1385-H_2O], 1357[1519-(S-8)], 1333[1367-2OH], 1315[1357-CH_2CO], 1285[1315-HCHO], 1195[1357-(S-7)], 1194[1195-H^{+}], 1152[1195-CH_2CO], 1094[1152-$NHCOCH_3$], 1076[1094-H_2O], 1040[1076-$2H_2O$], 992[1195-(S-

6)], 961[992-CH_2OH], 893[961-CH_3OH,2H_2O], 875[893-H_2O], 841[875-2OH], 789[992-(S-5)], 741[789-HCHO,H_2O], 711[741-HCHO], 683[741-$NHCOCH_3$], 677[711-2OH], 646[677-CH_2OH], 633[677-2OH], 586[789-(S-4)], 536[586-2HCHO], 492[536-2OH], 467[586-$CH_2OHCOCHOHCHOH$], 466[586-HOCHCHOH,-CH_2OHCHO], 406[586-(S-1)], 424[586-(S-3)], 406[424-H_2O], 365[424-CH_2OCHO], 333[CH_3OH], 291[365-$CH_2OHCHCHOH$], 260[291-CH_2OH,], 241[260-H_3O^+], 221[424-(S-2)] 204[221-OH].

Based on result obtained from chemical degradation/acid hydrolysis, Chemical transformation, Electro spray mass spectrometry and 1D NMR viz. ^{1}H NMR, ^{13}C NMR, and 2D NMR viz. COSY, TOCSY, HMBC and HSQC spectra of Athisose acetate and Athisose, the structure and sequence of isolated Novel oligosaccharide Athisose structure was deduced as-:

Gal-α-(1→4)-Gal-α-(1→3)-GalNHAc-β-(1→3)-GalNHAc-β-(1→3)-GalNHAc-β-(1→4)-Gal-β-(1→3)-GalNHAc-β-(1→3)-GlcNHAc



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CONCLUSION

Rathi cow milk was investigated for its oligosaccharide contents which resulted in the isolation of a novel Octasaccharide "ATHISOSE" comprised of GlcNHAc, GalNHAc and galactose linked together with α and β glycosidic linkages. This is the first report in the structure of any milk oligosaccharide where GlcNHAc was present at the reducing end of the oligosaccharide.

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