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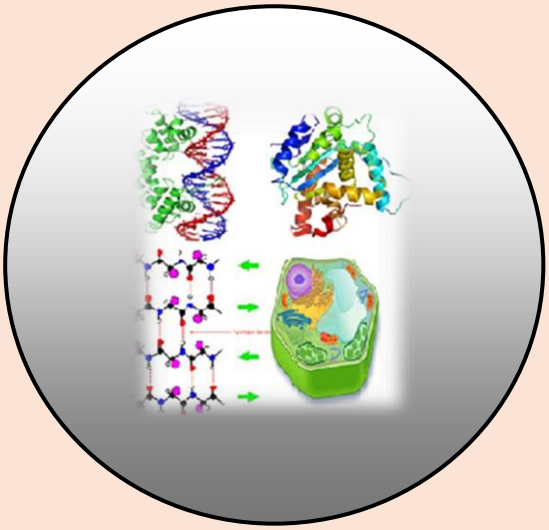

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RESEARCH PAPER

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Isolation of Biologically Active Oligosaccharide 'Rathose' from Rathi Cow Milk and Their Structure Elucidation by 2D NMR

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ABSTRACT

In the ancient literature of medicine, the biological importance of cow milk is well defined as amrata (panacea) and is prescribed for heart ailments and development of bones. It is used for chronic diseases like epilepsy, jaundice, spleen disorder and piles. With the advent of glycochemistry and glycobiology it became clear that all these medicinal properties were due to oligosaccharides present in milk. Various cow breeds carry different oligosaccharides in their milk which is due to their food habits. In view of the above facts we have collected Rathi cow milk, this cow is an inhabitant of Rajasthan state of India. Further the milk was processed by modified method of Kobata and Ginsburg for obtaining the oligosaccharide contents of Rathi cow milk. This oligosaccharide mixture was further acetylated by acetic anhydride and pyridine to get its acetylated oligosaccharide mixture and was purified by silica column chromatography resulting isolation of purified novel hexasaccharide named 'Rathose'. The Structure of this novel oligosaccharide was elucidated with the help of recent physico-chemical techniques of NMR i.e.^{1}H, ^{13}C and 2D experiments of COSY, TOCSY, HSQC and HMBC along with Mass spectrometry. The traditional methods of structure elucidation incorporating chemical degradation and chemical transformations were also performed. In the light of results obtained from the above experiments the structure of Rathose was described as under,

α-Gal-(1→3)-α-GalNHAc-(1→4)-β-Glc-(1→3)-α-GalNHAc-(1→3)-α-GalNHAc-(1→4)-Glc
RATHOSE

Keywords: Rathi Cow, Milk, Oligosaccharides, Rathose, NMR and Mass spectrometry.

INTRODUCTION

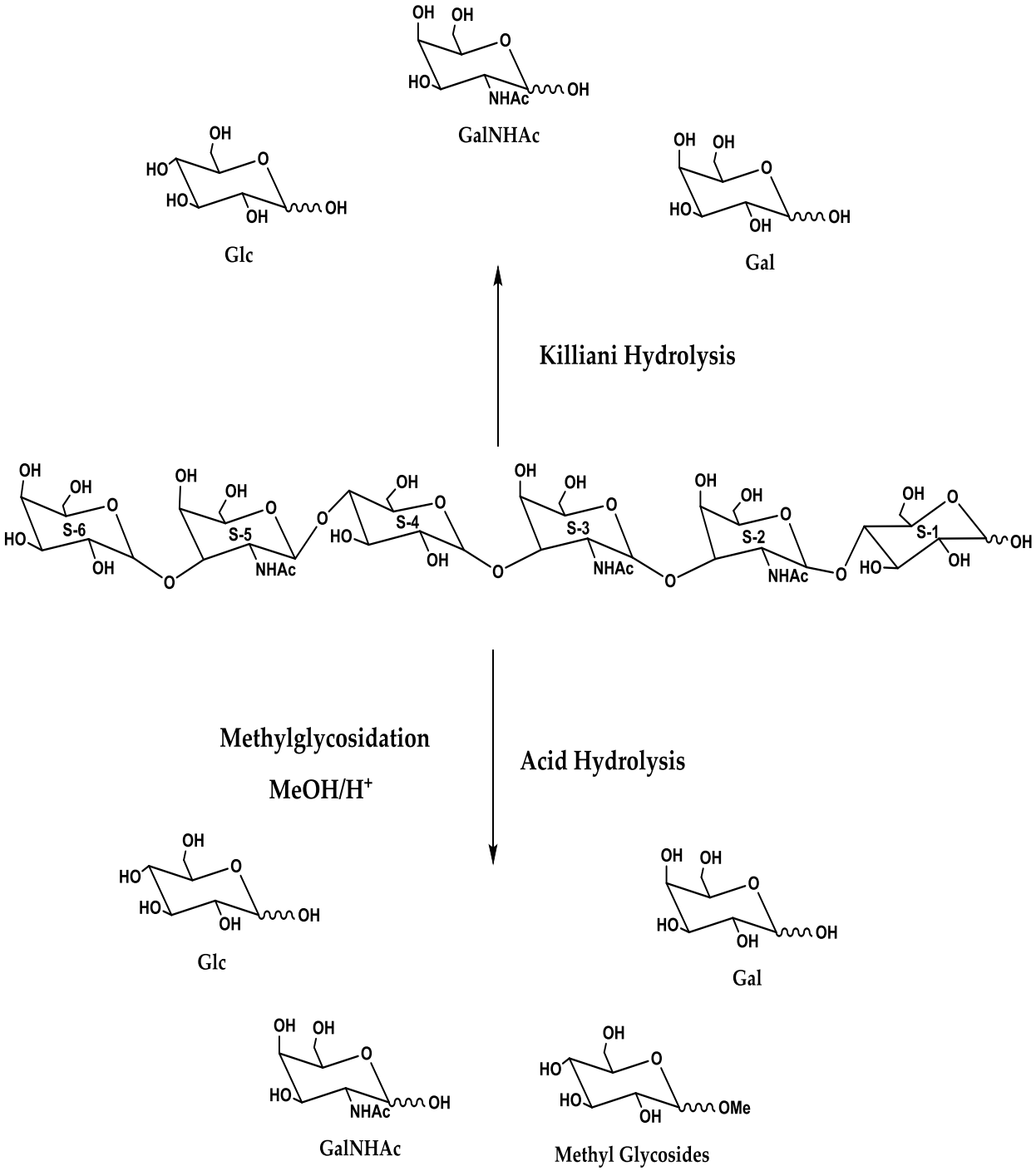
The importance of cow milk is well defined in ancient Indian literature of Ayurveda and Charak Samhita for development of immune system, brain and bones (Mana et al, 2021).

It is also reported to be helpful for the development of heart muscles. It increases the milk production by mothers and is beneficial for various chronic diseases i.e.; epilepsy, jaundice and spleen enlargement (Gholap et al, 2015). Milk is beneficial in the diseases like piles and also helpful for eyes (Ling et al 1961). Till date numbers of oligosaccharides have been isolated from the milk of different cow species found in India at various parts of the country and their structures has been elucidated. It was observed that the oligosaccharide content of milk of any cow species depends on the flora, fauna and fodder. These oligosaccharides are made up of linear or branched chains, comprised of glucose, galactose, GlcNHAc and GalNHAc, having varied combinations of α and β glycosidic linkages at different positions of monosaccharides with $^4C_1$ and $^1C_4$ conformations which directly or indirectly affects the biological activity of milk oligosaccharides. In search of more novel cow milk oligosaccharides we have collected the Rathi cow milk from Panchmukhi district of Rajasthan state, which is an arid zone of India that is why the oligosaccharide contents of Rathi cow milk are different from other cow species. The milk was collected in bulk (10 litre) and was processed by modified method of Kobata and Ginsburg (Kumar K. et al 2018) incorporating deproteination, centrifugation, microfiltration and lyophilization which resulted in isolation of crude oligosaccharide mixture which was further purified by gel filtration to obtain the protein free Oligosaccharide mixture of Rathi Cow milk. This oligosaccharide mixture was acetylated by acetic anhydride and pyridine for converting the polar oligosaccharides in their less polar acetyl derivatives for easy and smooth purification on silica gel columns which led to the isolation of a novel hexasaccharide 'Rathose'. The structure of Rathose was elucidated by combining the results obtained from traditional methods of structure elucidation i.e.; chemical transformation, chemical degradation and structure reporter group theory (Vliegenthart et al 1982, Bubb et al 2003) along with the data generated from physicochemical techniques like ^1H, ^{13}C, COSY, TOCSY, HSQC, HMBC and Mass spectrometry.

EXPERIMENTAL

General Procedure

The sugars were developed 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. ^1H 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 in 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 were averaged for 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.



Scheme 1: Killiani Hydrolysis and Methylglycosidation/Acid hydrolysis of Rathose.

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

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. The preserved milk was taken to laboratory and then 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 washings were combined and filtered through a microfilter and lyophilized affording crude oligosaccharide mixture (283 gm).

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 'a', Rathose Acetate (Kumar K et al 2018)

Compound **'a'** (45 mg) was obtained from column chromatography of acetylated oligosaccharide mixture. 40 mg of compound 'a' 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 '**A'** (31 mg).

Methyl Glycosidation/Acid Hydrolysis of Compound A: (Shukla et al; 2023)

Compound '**A'** (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 A, 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 and GalNHAc. Identification of monosaccharides in compound A 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 A: (Khan M. et al; 2019)

Compound **A** (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 Glc, Gal and GalNHAc on comparison with authentic samples of Glc, Gal and GalNHAc.

Description of Compound A Rathose

Compound '**a**' (45 mg) was obtained from fraction 109-114 of column chromatography. On deacetylation of 40 mg of substance 'a' with NH_4OH/acetone, it afforded substance '**A'** (31 mg).

For experimental analysis, this compound was dried over P_2O_5 at 100°C and 0.1 mm pressure for 8 hrs. It gave positive Phenol-sulphuric acid test, Feigl test and Morgan-Elson test:

$C_{42}H_{71}O_{31}N_3$		%C	%H	%N
	Calculated	**45.28**	**6.38**	**3.77**
	Found	**45.27**	**6.37**	**3.76**

^{1}H NMR of Acetylated Compound 'a', Rathose Acetate in $CDCl_3$ at 300 MHz

δ 6.26 [1H α-(S-1)], **δ 5.69** [1H β-(S-1)], **δ5.37** [H-4 of (S-6)], **δ5.22** [H-3 of (S-1)], **δ5.12** [H-2 of (S-4), **δ 5.12** [H-2 of (S-6)], **δ5.06** [H-2 of (S-1)], **δ4.97** [H-3 of (S-6)], **δ4.94** [H-4 of (S-2)], **δ4.92** [H-4 of (S-3)], **δ4.91** [H-4 of (S-5)], **δ 4.62** [1H (S-4)], **δ 4.50** [1H (S-2)], **δ 4.50** [1H (S-5)], **δ 4.48** [1H (S-3)], **δ4.48** [H-3 of (S-4)], **δ 4.48** [1H (S-6)], **δ4.12** [H-2 of (S-3)], **δ4.10** [H-2 of (S-2)], **δ4.10** [H-2 of (S-5)], **δ3.85** [H-3 of (S-2)], **δ3.85** [H-3 of (S-5)], **δ3.84** [H-4 of (S-4)], **δ 3.82** [H-3 of (S-3)], **δ 3.80** [H-4 of (S-1)].

^{13}C NMR of Acetylated Compound 'a', Rathose Acetate in $CDCl_3$ at 300 MHz

δ88.95 [1C, α-(S-1) C-1], **δ91.51** [1C, β-(S-1) C-1], **δ100.96** [2C, (S-3) & (S-6) C-1] **δ101.01** [2C, (S-2) & (S-5) C-1], **δ101.21** [1C, (S-4) C-1].

ES Mass ion fragments of compound A: m/z 1159, 997, 794, 631, 428, 225, 180

RESULT AND DISCUSSION

Compound A, Rathose

Compound 'A' Rathose $C_{42}H_{71}N_3O_{31}$ gave positive Phenol-sulphuric acid test (Partridge et al 1949), Feigl test (Feigl, F. et al 1975), Morgon-Elson test, confirming the presence of normal and amino sugars in compound 'A'. For the purpose of isolation, the crude oligosaccharide mixture obtained from Rathi cow milk, was acetylated with acetic anhydride and pyridine and purified by repeated column chromatography on silica gel column which resulted in the isolation of compound 'a'. Further it was deacetylated by milder-deacetylating agent i.e.; ammonia in acetone which resulted in procurement of natural oligosaccharide 'A'. Further various NMR Experiments of 1D and 2D i.e.; ^{1}H-NMR, ^{13}C-NMR, COSY, TOCSY, HSQC, and HMBC along with Mass Spectrometry were performed on acetylated and natural compound Rathose. Simultaneously, chemical transformations and chemical degradations were also performed on natural compound 'A'. The name of the compound Rathose originated from the source i.e.; Rathi cow and designated as 'A' while its acetylated derivative was defined as 'a'. Most of the NMR Experiments were performed on compound 'a' for removal of spectral degeneracy. Simultaneous experiments were also performed on compound 'A' for counting the number of amino sugars in Rathose. Further the HSQC Spectrum of compound 'a' showed presence of seven cross peaks of anomeric proton and carbon at- δ 88.95 × 6.26(S-1α), δ 91.51×5.69 (S-1β), δ 101.01×4.50 (S-2), δ 100.96 × 4.48 (S-3), δ 101.21 × 4.62(S-4), δ 101.01 × 4.50 (S-5) and δ 100.96 × 4.48 (S-6) suggesting the presence of Six anomeric protons and carbons in the oligosaccharide 'a' in its reducing form. Further the presence of seven anomeric proton doublets at δ6.26, δ 5.69, δ 4.50, δ 4.48, δ 4.62, δ 4.50 and δ 4.48in the ^{1}H NMR of 'a', which also confirmed that the compound 'a' was a hexasaccharide in its reducing form.

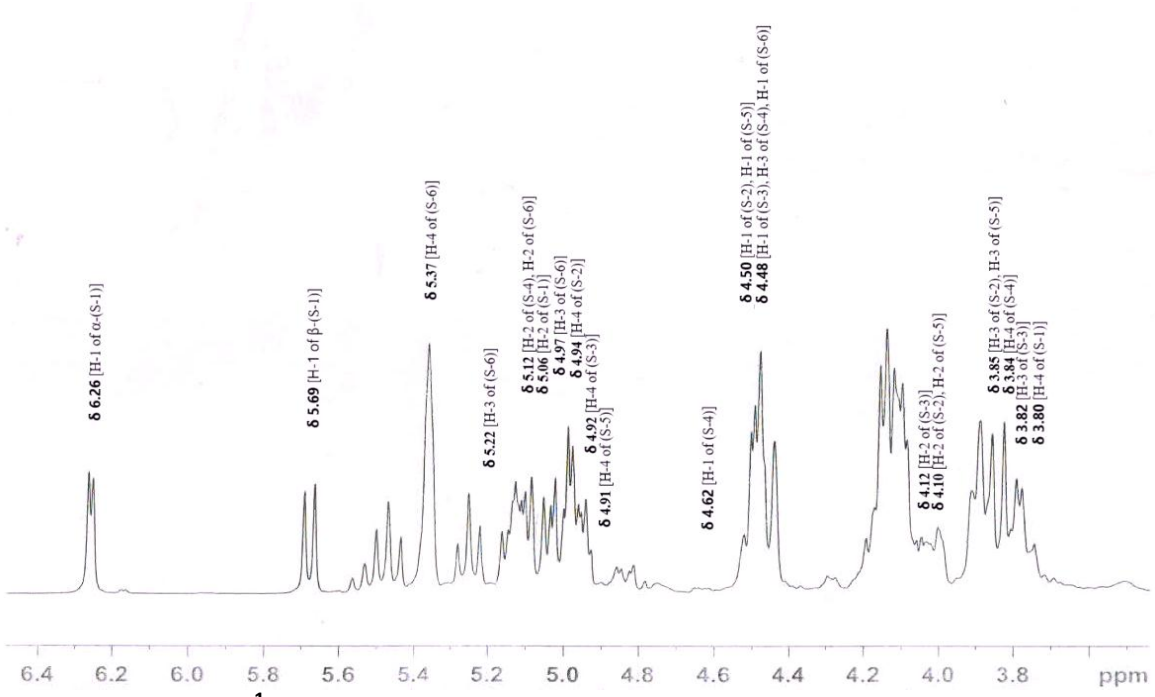


Figure 1: ^{1}H NMR spectrum of Rathose acetate in $CDCl_3$ at 300 MHz.

Table 1. Anomeric proton/carbon assignments in Rathose Acetate by HSQC Spectrum.

Anomeric Proton × Anomeric Carbon	**Cross-peaks of Anomeric Proton × Anomeric Carbon**
H-1(S-1) × C-1(S-1α)	δ 6.26 × 88.95
H-1(S-1) × C-1(S-1β)	δ 5.69 × 91.51
H-1(S-2) × C-1(S-2)	δ 4.50 × 101.01
H-1(S-3) × C-1(S-3)	δ 4.48 × 100.96
H-1(S-4) × C-1(S-4)	δ 4.62 × 101.21
H-1(S-5) × C-1(S-5)	δ 4.50 × 101.1
H-1(S-6) × C-1(S-6)	δ 4.48 × 100.96

Table 2. Chemical Shift of Anomeric Protons of Rathose Acetate in $CDCl_3$.

Moieties	**Rathose Acetate in $CDCl_3$**	
	^{1}H NMR(δ)	**Coupling Constant(J)**
α-Glc **(S-1)**	6.26	3.6 Hz
β-Glc **(S-1)**	5.69	8.4 Hz
α -GalNHAc**(S-2)**	4.50	3.3 Hz
α -GalNHAc**(S-3)**	4.48	3.9 Hz
β Glc **(S-4)**	4.62 (Not resolved)	-
α GalNHAc **(S-5)**	4.50	3.3 Hz
α Gal **(S-6)**	4.48	3.9 Hz

Table 3. Assignment of ring protons of Rathose acetate by TOCSY Spectrum in ppm.

	S-1	S-2	S-3	S-4	S-5	S-6
Anomeric Protons	**5.69**	**4.50**	**4.48**	**4.62**	**4.50**	**4.48**
Ring Protons	3.80	3.80	3.82	3.84	3.85	4.97
	5.06	4.10	4.12	4.48	4.10	5.12
	5.22	4.94	4.92	5.12	4.91	5.37

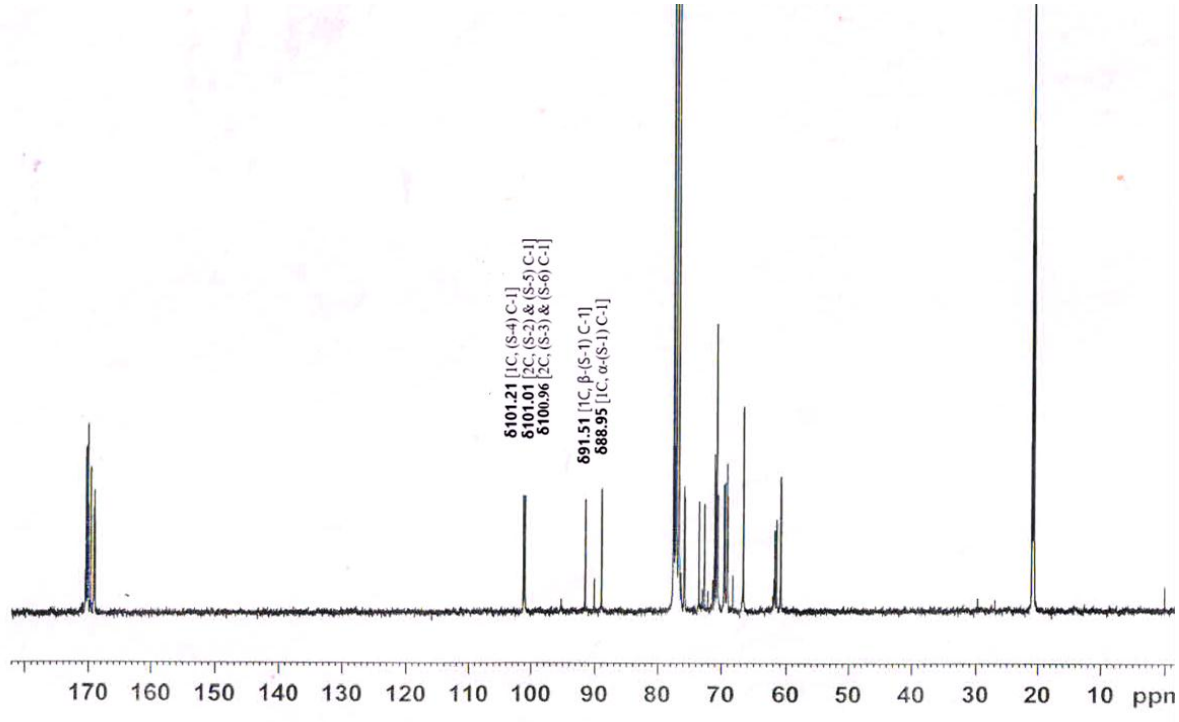


Figure 2: ^{13}C NMR spectrum of Rathose acetate in $CDCl_3$ at 300 MHz.

Table 4. Assignment of Methine protons by COSY Spectrum of Rathoseacetate in ppm.

		S-1	S-2	S-3	S-4	S-5	S-6
Anomeric Proton	**H1**	**5.69**	**4.50**	**4.48**	**4.62**	**4.50**	**4.48**
Ring Protons	**H2**	5.06	4.10	4.12	5.12	4.10	5.12
	H3	5.22	3.85	3.82	4.48	3.85	4.97
	H4	3.80	4.94	4.92	3.84	4.91	5.37

Table 5. Assignment of Glycosidic linkages by HMBC Spectrum of Rathose acetate.

Sugar	Linkage	Type of Linkage
S1-S2	δ 3.80× 101.01	β-Glc (S-1) **[1→4]** α-GalNHAc(S-2)
S2-S3	δ 3.85 × 100.96	α-GalNHAc(S-2) **[1→3]** α-GalNHAc(S-3)
S3-S4	δ 3.82 × 101.21	α-GalNHAc(S-3)**[1→3]** β Glc(S-4)
S4-S5	δ 3.84 × 101.10	β-Glc(S-4) **[1→4]** α GalNHAc(S-5)
S5-S6	δ 3.85 × 100.96	α GalNHAc(S-5)**[1→3]** α-Gal(S-6)

This result was further Substantiated by the ^{13}C anomeric carbon signals at δ 88.95 (1C), 91.51 (1C), 101.01 (2C), 100.96 (2C) & 101.21 (1C) at 300 MHz NMR spectrum of compound 'a'. Thus the ^{1}H and ^{13}C-NMR spectra along with HSQC Experiments Justified the seven anomeric signals for hexasaccharide with total integral-intensity of six anomeric protons and carbons. The mass spectrum of compound Rathose 'A' confirm edits molecular weight as **1113** which was in confirmity with the assigned structure.

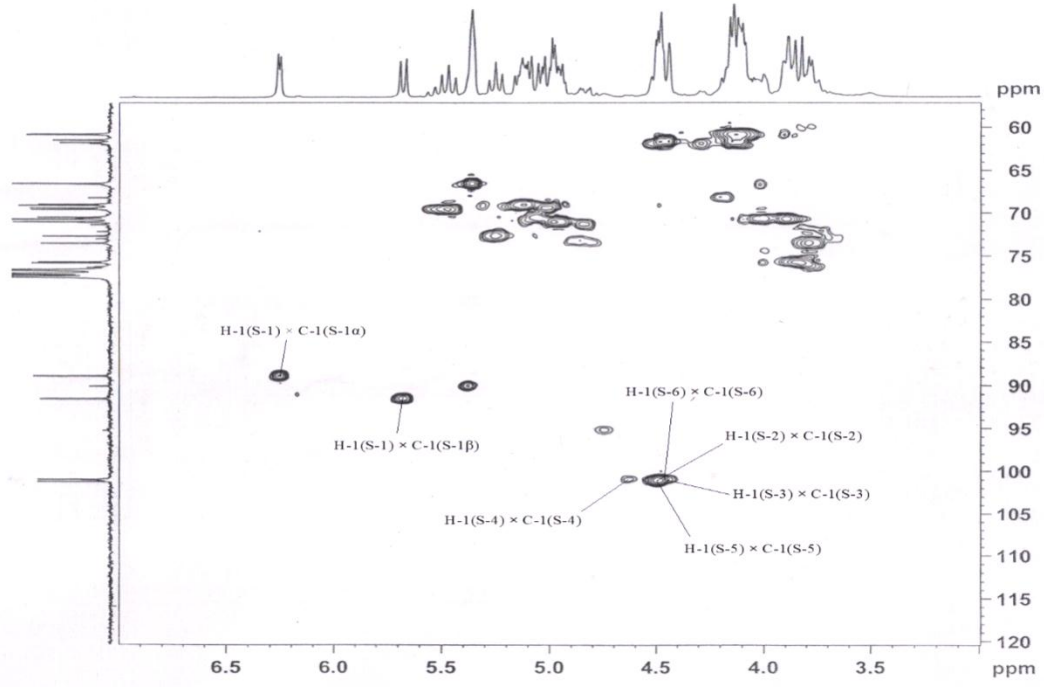


Figure 3. HSQC Spectrum of Rathose acetate in $CDCl_3$ at 300 MHz.

The reducing nature of compound 'A' was further confirmed by its methyl glycosidation followed by its acid hydrolysis, which led to the isolation of α and β methyl glucosides along with Glc, Gal and GalNHAc Confirming that the oligosaccharide 'A' contained Glc at its reducing end and also confirmed that the constituent of Oligosaccharide were Glc, Gal and GalNHAc. For convenience these monosaccharides present in oligosaccharide 'A' were designated as S-1, S-2, S-3, S-4, S-5 and S-6. The monosaccharides present in the compound 'A' were also confirmed by its Killiani hydrolysis under strong acidic conditions which was monitored on paper chromatography and TLC. On completion of Killiani hydrolysis it gave three spots of Glc, Gal and GalNHAc, which were identified by their comparison with the

authentic samples of Glc, Gal and GalNHAc, confirming that the oligosaccharide Rathose was composed of the three monosaccharide units. The ^{1}H NMR of 'A' contained three methyl signals of $NHCOCH_3$ at δ 1.896, 1.811, 2.139 showing the presence of three amino sugars in the Rathose.

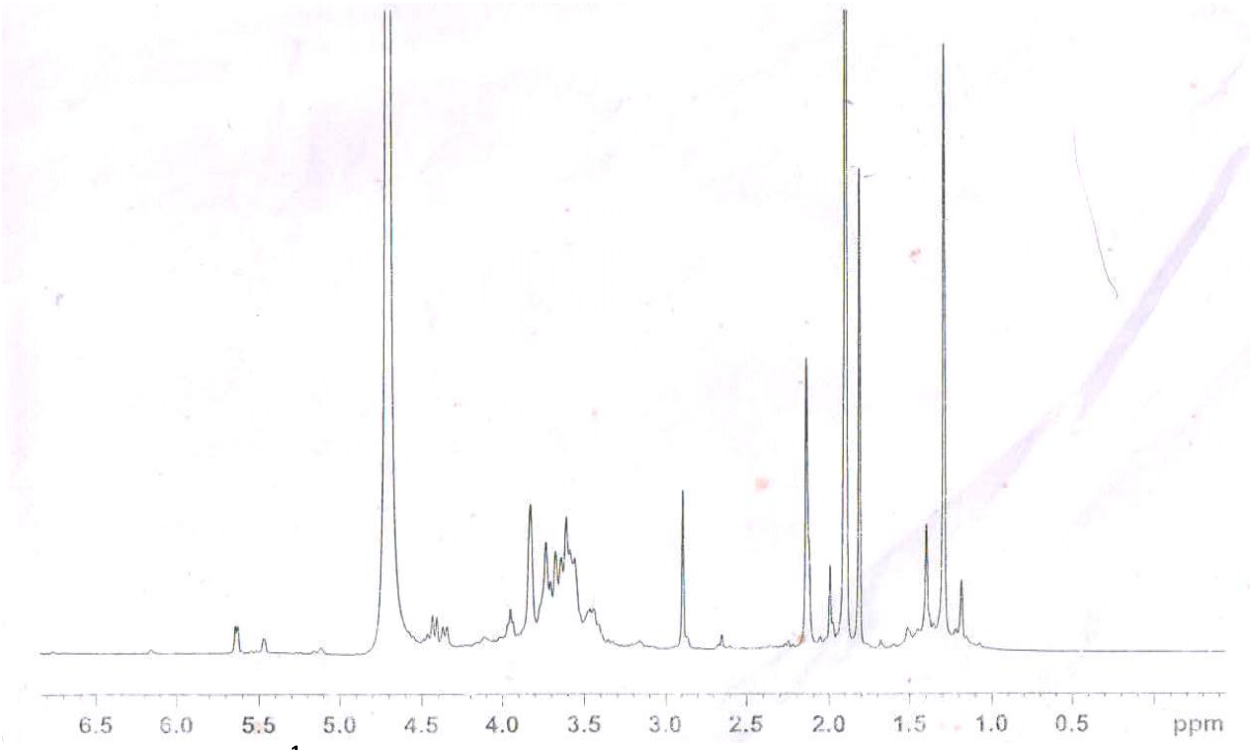


Figure 4: ^{1}H NMR spectrum of Rathose acetate in D_2Oat 300 MHz.

Further the anomeric proton signals present at δ 6.26 (S-1α) and 5.69 (S-1β) were assigned to α and β anomeric protons of Rathose acetate 'a' at 300 MHz in $CDCl_3$.The HSQC spectrum of 'a' showed a cross peak at δ 5.69 × 91.51 for monosaccharide (S-1) resembling with the literature value of Glc hence the S-1 was reconfirmed as Glucose (Bush C A et al, 1988). Further the anomeric protons present at δ 5.69(300 MHz, $CDCl_3$) showed three cross peaks at δ 5.69 × (δ 5.06, δ 5.22, δ 3.80) in the TOCSY spectrum of Rathose acetate, which were later confirmed as H-2, H-3, and H-4 of S-1 by the COSY spectrum of Rathose acetate 'a'. The chemical shift of H-4 of S-1 at δ 3.80 suggested that the position of H-4 of S-1 was available for glycosidic linkage by the next monosaccharide unit. Further, the signal for H-4 of S-1 at δ 3.80 gave a crosspeak with C1 of S-2 at δ 3.80 × 101.01, in the HMBC spectrum of Rathose acetate 'a' confirming a 1→4 glycosidic linkage between S-2 and S-1 and established that chemical shift of anomeric carbon of S-2 was at δ 101.01. The anomeric carbon/proton cross peak of S-2 generated from the HSQC spectrum of compound Rathose acetate 'a', gave a cross peak by the interaction between H1 of (S-2) and C1 of (S-2) at δ 101.01 × 4.50 in the HSQC spectrum of Rathose acetate. The anomeric carbon/proton value at δ 101.01/4.50 resembled with the anomeric proton/carbon value of GalNHAc. Hence, the monosaccharide S-2 was confirmed as GalNHAc (Bush C A et al, 1988). The anomeric proton doublet at δ 4.50 showed a J value of 3.3 Hz. and hence the GalNHAc present at S-2 was having a β configuration.

Further the anomeric proton signal present at δ 4.50 in the TOCSY spectrum of Rathose acetate 'a' exhibited three cross peaks at δ 3.85, δ 4.10, and δ 4.94. These peaks were later confirmed through the COSY spectrum as H2 (δ 4.10), H3 (δ 3.85), and H4 (δ 4.94).

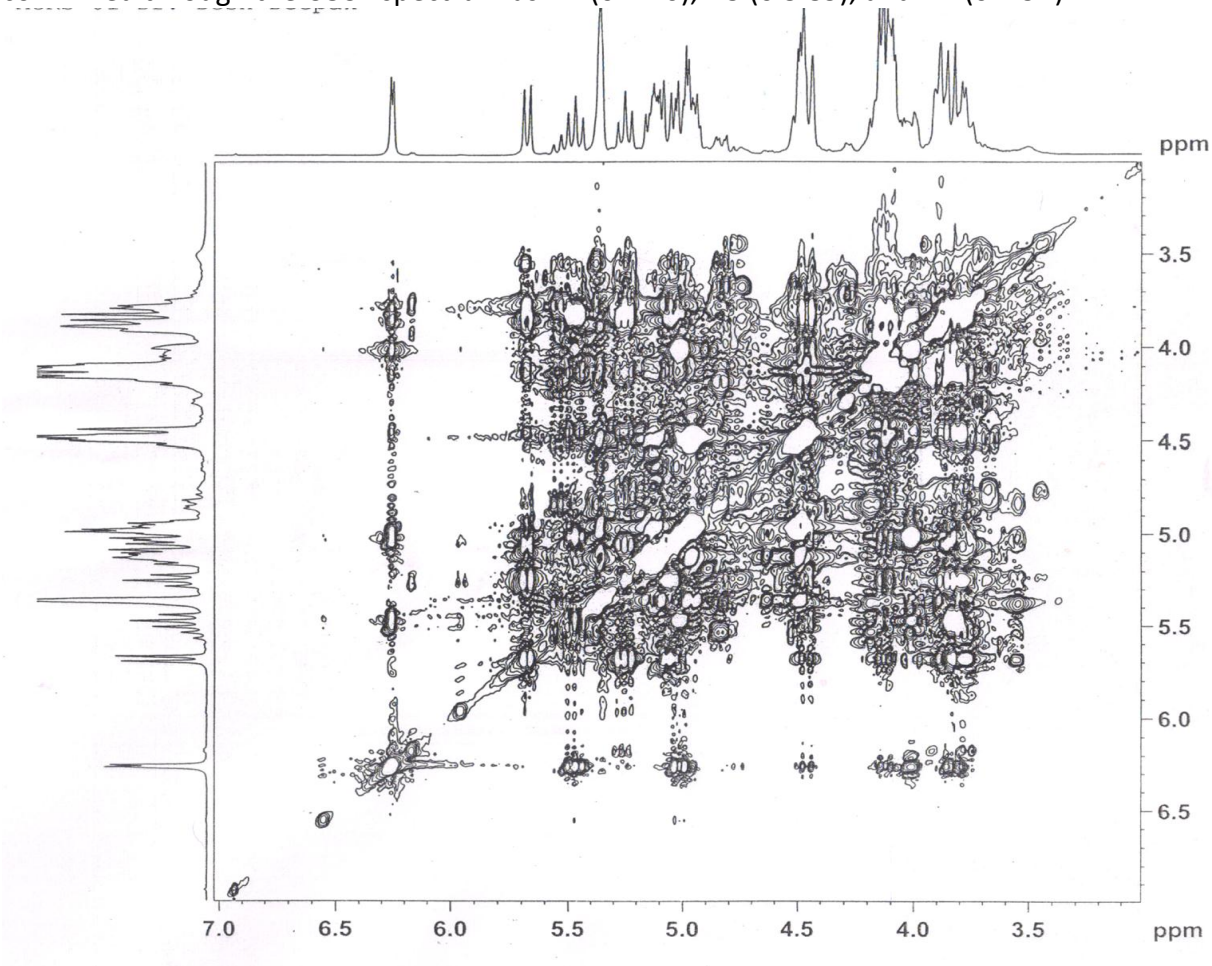


Figure 5. TOCSY Spectrum of Rathose Acetate in $CDCl_3$ at 300 MHz.

The chemical shift of H3 at δ 3.85 suggested that H3 of (S-2) was available for glycosidic linkage with the next monosaccharide unit. Further the signal present at δ 3.85 gave a cross peak at δ 3.85×100.96 in the HMBC Spectrum of Rathose acetate which was between H3 of S-2 and C1 of S-3 confirming a (1→3) linkage between S-2 and S-3. The HSQC spectrum of Rathose acetate 'a' at 300 MHz in $CDCl_3$ showed cross peaks between H1 of (S-3) and C1 of (S-3) at δ 100.96 × 4.48, confirming the anomeric proton/carbon value of S-3 unit at δ 100.96/4.48. The anomeric carbon/proton value at δ100.96 and δ4.48 confirmed that S-3 was a GalNHAc (Bush C A et al, 1988).The anomeric proton doublet for GalNHAc S-3 at δ 4.48 showed a coupling constant of 4.0 Hz and hence the configuration of GalNHAc (S-3) was α .The anomeric proton signal at δ 4.48 displayed three cross peaks at δ 3.82, δ 4.12 and δ 4.92 in the TOCSY spectrum of Rathose acetate. These were later confirmed by the COSY spectrum as H2 (δ 4.12), H3 (δ 3.82), and H4 (δ 4.92). Further the signal at δ 3.82 gave a cross peak at δ 3.82×101.21 in the HMBC spectrum of Rathose acetate 'a' confirming a (1→3) linkage between S-3 and S-4.

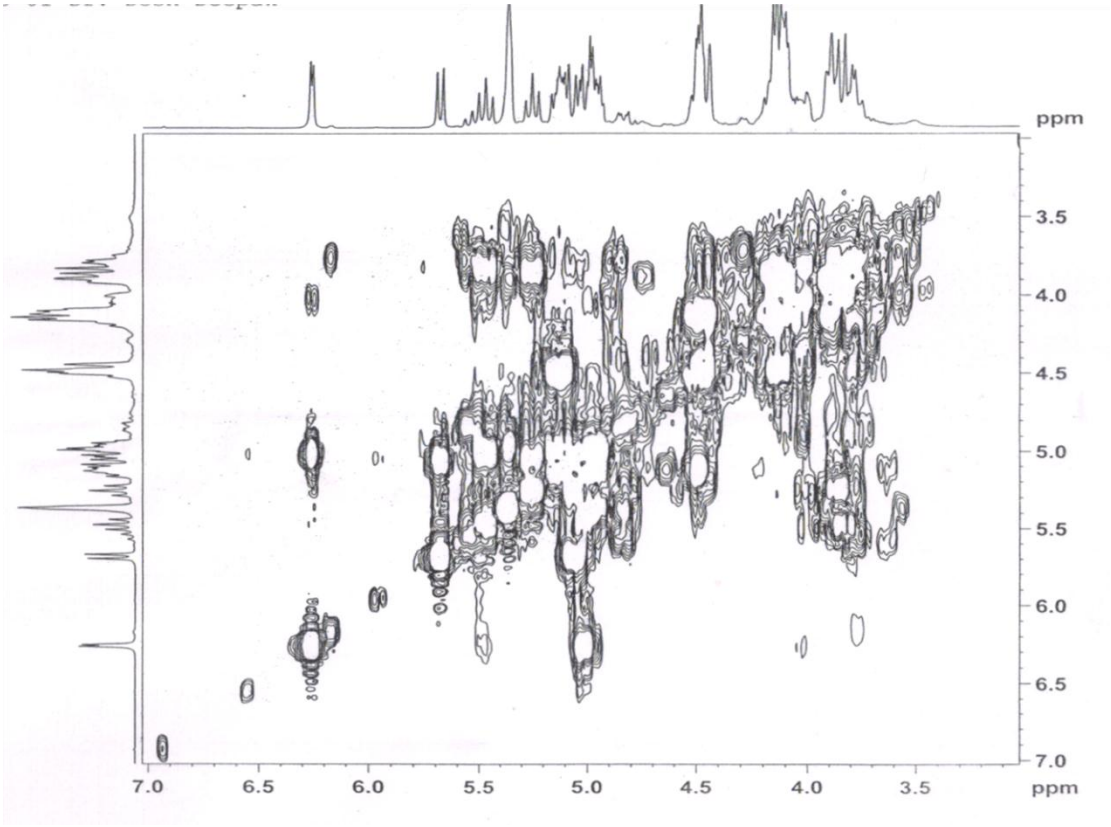


Figure 6. COSY Spectrum of Rathose Acetate in $CDCl_3$ at 300 MHz.

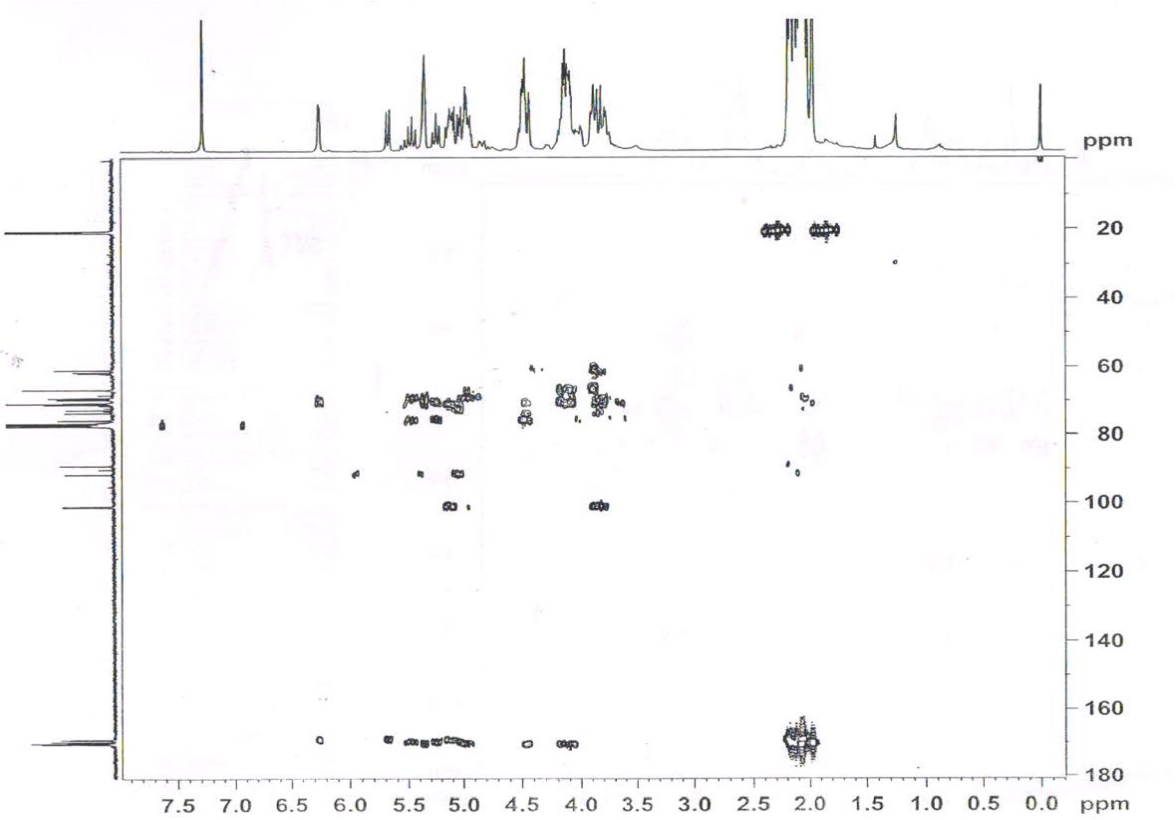


Figure 7. HMBC Spectrum of Rathose Acetate in $CDCl_3$ at 300 MHz.

The anomeric carbon value at δ 101.21 showed a cross peak at δ 101.21 ×4.62 in the HSQC spectrum of 'a' which was for monosaccharide (S-4). The chemical shift value of anomeric carbon and proton at δ 101.21 and δ 4.62 resembles with the values of Glc, hence monosaccharide S-4 was confirmed as Glucose (Bush C. A. et al, 1988). Further the anomeric proton present at δ 4.62 was not well resolved but had its anomeric carbon value at δ 101.21 confirmed it as β configuration hence S-4 was β Glucose. The anomeric proton signal at δ4.62 exhibited three cross peaks at δ4.62(δ 3.84, δ 4.48, δ 5.12) in the TOCSY spectrum, which were confirmed by COSY spectrum as H2 (δ 5.12), H3 (δ 4.48), and H4 (δ 3.84). The chemical shift of H4 at δ 3.84 indicated H4 of (S-4) was available for glycosidic linkage by the next monosaccharide unit. Further, the signal of the cross peak with C1 of the next monosaccharide unit, i.e., S-5, at δ 101.01 × 3.84, confirmed a 1→4 glycosidic linkage between S-5 and S-4, verifying the anomeric carbon of S-5 at δ 101.01. The HSQC spectrum at 300 MHz in $CDCl_3$ revealed an interaction between H1 of (S-5) and C1 of (S-5) at δ 101.01 × 4.50, with the anomeric carbon/proton value at δ 101.01/4.50.For S-5 the value δ 101.01 and δ 4.50resembles the literature value of GalNHAc hence S-5 was confirmed as GalNHAc (Bush C. A. et al, 1988). The anomeric proton value at δ 4.50 for GalNHAc (S-5) showed a J value of 3.3 Hz hence the GalNHAc S-5 had an α configuration. The anomeric proton signal at δ 4.50 showed three cross peaks at δ 3.85, δ 4.10 and 4.91 in the TOCSY spectrum of Rathose acetate, which were later confirmed by the COSY spectrum as H2 (δ 4.10), H3 (δ 3.85), and H4 (δ 4.91).

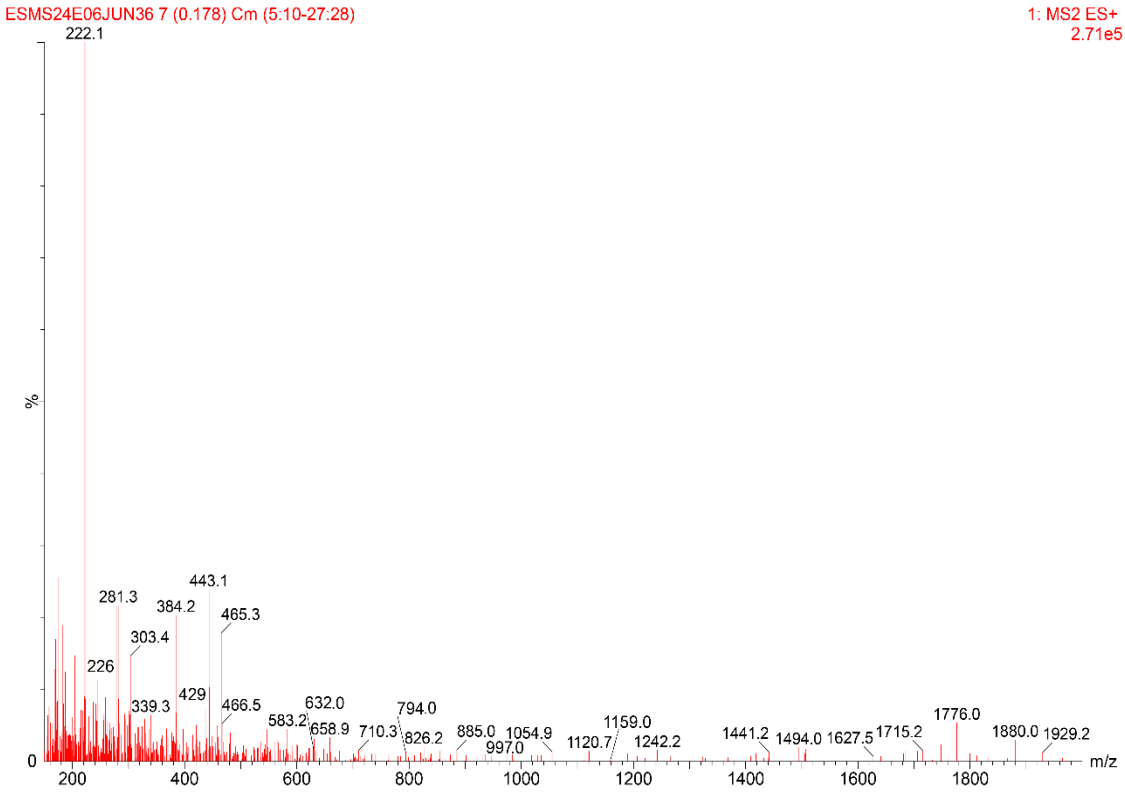
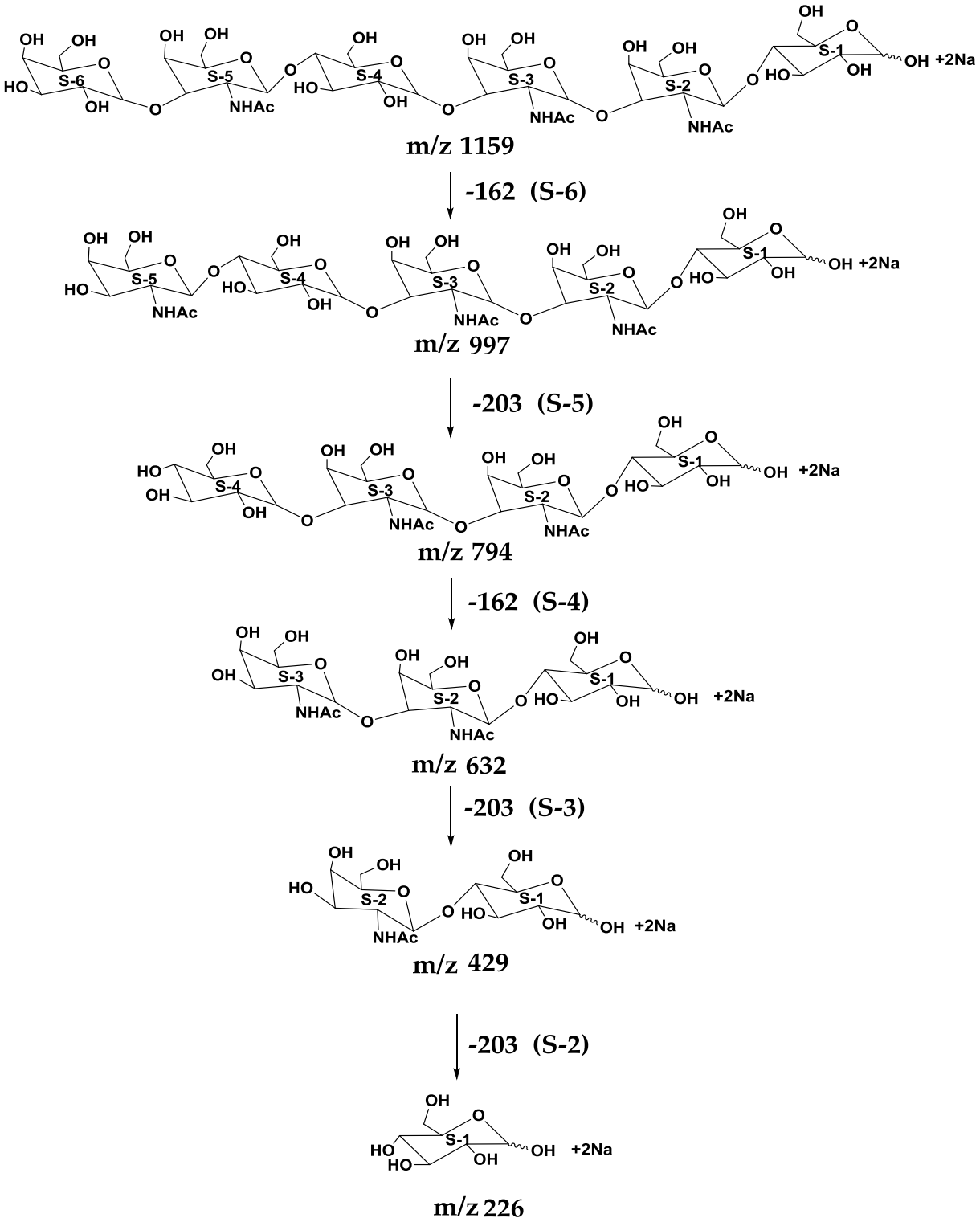


Figure 8. ES MS Spectrum of Rathose.



Scheme 2: Mass Fragmentation of Compound Rathose

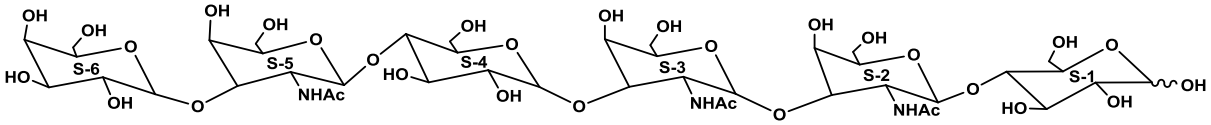
The chemical shift of H3 at δ 3.85 suggested H3 of (S-5) was available for linkage by the next monosaccharide unit. The signal at δ 3.85 gave a cross peak with the next monosaccharide unit, (S-6) in the HMBC spectrum of 'a' at δ 100.96 ×3.85, confirming a (1→3) glycosidic

linkage between (S-5) and (S-6) by HMBC Spectrum, verifying that the anomeric carbon of S-6 was present at δ 100.96. Further the HSQC spectrum at 300 MHz in $CDCl_3$ showed a cross peak between H1 of (S-6) and C1 of (S-6) at δ 100.96 × 4.48, with the anomeric carbon/proton value at δ 100.96/4.48 resembling that of galactose (Bush C. A. et al, 1988). Thus, S-6 was confirmed as galactose. The anomeric proton value of Gal (S-6) at 4.48 showed a J value of 4.2 Hz hence it was present in its α configuration. The anomeric proton signal at δ 4.48 exhibited three cross peaks at δ 4.97, δ 5.37 and δ 5.12 in the TOCSY spectrum of Rathose acetate 'a', later confirmed through the COSY spectrum as H2 (δ 5.12), H3 (δ 4.97), and H4 (δ 5.37). The Ring Proton value of monosaccharide (S-6) confirmed that none of itsring proton was in involved in glycosidic linkage by next monosaccharide unit and hence the monosaccharide (S-6) was present at the non-reducing end of the Rathose.

The structure of Rathose elucidated by 1D and 2D NMR, chemical degradation and chemical transformation, was also confirmed by Mass Spectrometry data (Barber, M. 1982). The highest ion peak obtained at m/z 1159 was due to [M+2Na] which was in confirmity with the molecular mass obtained by C-H analysis as $C_{42}H_{71}N_3O_{31,}$ this ion peak at m/z 1159 gave a fragment at m/z 997 which was generated by the loss of 162 sugar (S-6) which was Gal. This further fragmented to give another fragment ion peak at 794 generated by the loss of 203 i.e.; sugar (S-5) which was GalNHAc. This fragment of m/z 794 further fragmented by the loss of 162 i.e.; (S-4) which was Glc and gave a mass ion peak at m/z 631, This on further fragmentation gave a fragment ion peak at m/z 428 obtained by loss of (S-3) unit of the oligosaccharide, which was Galactose resulted in the fragment ion at m/z 428, which was a trisaccharide unit. This trisaccharide having mass fragment of m/z 428 further fragmented by the loss of 203, which was due to loss of GalNHAc moiety gave a peak at m/z 225, which further gave a mass ion peak at m/z 180 which was due to reducing glucose (S-1) of oligosaccharide, Rathose.

The observations obtained from the mass fragmentation patterns not only confirmed the structure of Rathose obtained by 2D NMR but also confirmed the sequence of monosaccharides in the oligosaccharide Rathose. Thus, the results obtained from the 2D NMR experiments; COSY, TOCSY, HSQC and HMBC along with mass spectrometry data confirmed the structure of Rathose as under-

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



Rathose

CONCLUSION

The results obtained from the ^{1}H, ^{13}C and 2D experiments of COSY, TOCSY, HSQC and HMBC along with Mass spectrometry for the novel hexasaccharide Rathose obtained from Rathi cow milk, concluded that this hexasaccharide comprised of six monosaccharide units made up of Glc, GalNHAc and Gal joined together with (1→4) and (1→3) glycosidic linkages having*α* and *β* glycosidic linkages.

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