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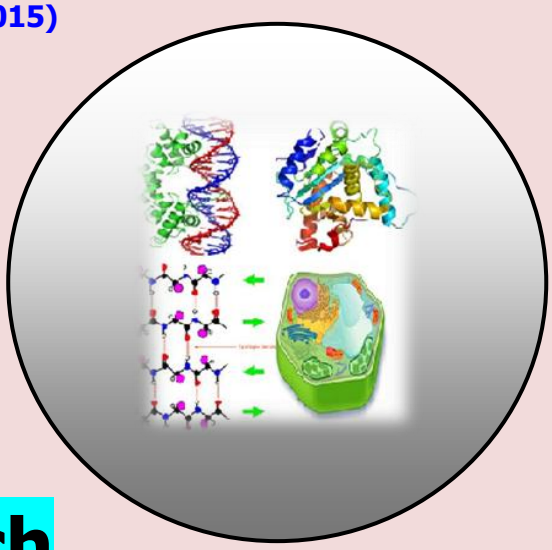

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RESEARCH PAPER

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Isolation, Purification and NMR Study of a Novel Nonasaccharide (Rieose) from 'Gaddi Sheep' Milk

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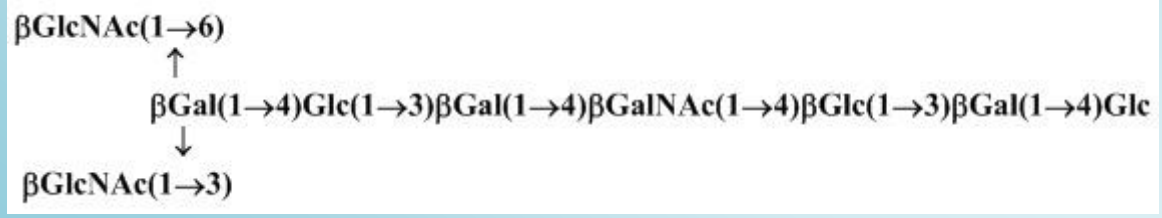
ABSTRACT

Milk is an important source of oligosaccharides which has provided number of novel biologically active oligosaccharides depending on the nature of their origin from which mammal the milk belongs. According to 'Aurveda and Unani' system of medicine, the sheep's milk has various medicinal importance, aggravates hiccup and dyspnoea, elevates pitta and kapha and decrease fat. It is used against tuberculosis in folk medicine and also helps in the enhancement of platelets count during dengue. Keeping these activities in mind Gaddi sheep's (Gaddi is a breed of sheep found at higher altitude) milk was collected and processed by Modified method of Kobata and Ginsburg and then it was purified by Sephadex G-25 Gel column. Further the acetylation of oligosaccharides mixture followed by the silica chromatography led to isolation of a novel nonasaccharide Rieose ($C_{60}H_{101}N_3O_{46}$) which gave positive chemical test for normal and amino sugars. Comparison between ^{1}H and ^{13}C signals of natural and acetylated Rieose confirmed the position of linkage in oligosaccharides and data generated from 2D NMR studies involving COSY, TOCSY, HSQC techniques along with mass spectrometry data. Structure of Rieose was confirmed as under;



RIEOSE

Key words – Milk oligosaccharides, Rieose and Kobata and Ginsburg.

INTRODUCTION

Oligosaccharides are an important constituent of milk (the third solid component after lactose and fat in human milk) and known to be effective, anti-infective agents, which can prevent adhesion of microbial pathogens to host cells [Hakkarainen et al 2005, Zivkovic et al 2011]. In the gut, milk oligosaccharide can show prebiotics activity by contribution to the growth of beneficial intestinal flora in the colon. As competitive inhibitors for binding sites on the epithelial surface, they show anti-infective and anti-adhesive properties by reducing or preventing the adhesion of pathogens [Bruggencate et al 2014, Mehra et al 2006, Kunz et al 2006, Oliveira et al 2012, Ninonuevo et al 2006, Boehm et al 2007, Mehra et al 2014]. Recent studies have demonstrated beneficial functions of milk oligosaccharides in vitro, which suggest that milk oligosaccharides have potential as a new source of microbiotic modulators with the potential to mimic the more complex oligosaccharides of human milk [Hakkarainen et al 2005, Zivkovic et al 2011]. Prebiotic oligosaccharides have been said to reduce the susceptibility to allergies in infants **[**Hoffen et al 2009, Vijay-Kumar et al 2010] and shown to influence the immune response to vaccination in mice [Vos et al 2007]. The milk oligosaccharides inhibit the adherence of pathogens to target cells, hence oligosaccharides and their derivatives are used as therapeutic agents and form the basis for the development of anti-tumor vaccines and act as effective drugs in the therapy of pathogenic diseases [Ranjan et al 2015]. Buffalo Milk oligosaccharides have ability to stimulate non-immunological resistance of the host against parasitic infections [saksena et al 1999]. Gaddi sheep's milk has highest amount of calcium and phosphate and used to produce milk skin bars (as a cleanser and moisturizer). Sheep milk protein is an important source of bioactive inhibitory and hypertensive defence and control of microbial infection. The peptides present in sheep milk have their affect in cardiovascular, nervous and immune system, besides these effects due to proteins and peptides present in sheep milk are enormous [Ranjan et al 2015]. In this present study we have worked on gaddi sheep's milk and were isolated a novel nonasaccharide, Rieose. The structures of these basic core oligosaccharides were assigned by chemical degradation and spectroscopic techniques by previous workers [Dorland et al 1977] and further, the structures of various milk oligosaccharides were determined by comparing the (1H NMR) chemical shifts of anomeric signals and other important signals of unknown milk oligosaccharides with the chemical shifts of LNT and LNnT [Dua et al 1983]. In the present study, analogies between chemical shifts of certain `structural reporter group resonances' were used to make proton resonance assignments as well as structural assignments of the oligosaccharides [Herlant-Peers et al 1981], ancillary techniques such as deacetylation, chemical degradations, 2D-NMR spectroscopy(COSY, TOCSY and HSQC) and FAB mass spectrometry were used for unambiguous determination of the structure.

MATERIAL AND METHODS

GENERAL PROCEDURE

General procedure was same as our previous paper [Gangwar et al 2017].

Isolation of gaddi sheep milk oligosaccharide by the Modified method of Kobata and Ginsberg: Isolation of Gaddi sheep milk oligosaccharides was done by the modified method of Kobata and Ginsburg [Tripathi et al 2014], which was described in our previous communication [Gangwar et al 2017] except the isolation, was done from 10 litre of buffalo milk and the yield of oligosaccharide mixture was 345.5 gm.

Acetylation of oligosaccharide mixture

For acetylation 12 g of pooled fractions obtained after Sephadex chromatography which gave positive phenol-sulphuric acid test [Dubois et al 1956] were acetylated by standard method of acetylation which yielded 13.5 gm acetylated mixture of oligosaccharides. TLC of this oligosaccharide mixture showed eight spots namely A, B, C, D, E, F, G, H, J, K, L, and M.

Deacetylation of compound

Compound L (Rieose acetate) (68.6 mg) was obtained from column chromatography 7 of acetylated oligosaccharide mixture by column chromatography. 35 mg of compound L was dissolved in acetone (2 ml) and 3 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 water layer was finally freeze dried giving the natural oligosaccharide L (Rieose) (28 mg).

Methyl glycosidation/Acid hydrolysis of compound L

Compound Rieose (12 mg) was ref1uxed with absolute MeOH (2 ml) at 70°C for 18 h in the presence of cation exchange IR-l20 (H) resin. The reaction mixture was filtered while hot and filtrate was concentrated. In the reaction mixture, 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^oC. The hydrolysis was complete after 24 h. 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 GlcNAc. Their identification was confirmed by comparison with authentic samples (TLC, PC).

Killiani hydrolysis of compound

Compound L (9 mg) was dissolved in 2 ml Killiani mixture (AcOH-H_2O-HCl, 7:11:2) and heated at 100^oC 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 glucose, Gal and GlcNAc on comparison with authentic samples of glucose, Gal and GlcNAc (Killiani et al 1930).

Description of Isolated Compound

Substance L (170.00mg) obtained from fraction 95-117 of column chromatography 13. On deacetylation of 26mg of acetylated compound with NH_3/ acetone it afforded substance L (23.6mg) as a viscous mass, $[\alpha]_D$ +114.44^0(c, 2, H_2O)

For experimental analysis, this compound was dried over P_2O_5 at 100^0 C and 0.1 mm pressure for 8 hr.

$C_{60}H_{101}N_3O_{46}$	%C	%H	%N
Calculated	45.03	5.36	2.63
Found	45.02	5.35	2.62

It gave positive Phenol-sulphuric acid test, Feigl test [Fiegl et al 1975] and Morgon-Elson test [Partridge et al 1948].

δ in D_2O: ^{1}H NMR

δ5.231 [d, 1H, J=3.0Hz, αGlc (S_1), H-1], δ4.726 [d, 1H, J=7.8Hz, βGlc (S_1'), H-1], δ4.706 [d, 1H, J=7.8Hz, βGlc (S_3), H-1], δ4.665 [d, 1H, J=8.6Hz, βGlcNAc (S_8), H-1], δ4.655 [d, 1H, J=7.6Hz,

βGlc (S_6), H-1], δ4.530 [d, 1H, J=8.1 Hz, βGlcNAc (S_9), H-1], δ4.520[d, 1H, J=8.1Hz, βGalNAc (S_4), H-1], δ4.466 [d, 1H, J=8.4Hz, βGal (S_5), H-1], δ4.412 [d,1H, J=9.1Hz, βGal (S_7), H-1] and δ4.399 [d, 1H, J=8.4Hz, βGal (S_2), H-1], δ3.290 [t, 3H, J=5.9, βGlc (S_1'), βGlc (S_3) & βGlc (S_6), H-2], δ1.921 [s, 3H, βGalNAc (S_4), $NHCOCH_3$] and δ2.004 [s, 6H, βGlcNAc (S_8) & βGlcNAc (S_9), $NHCOCH_3$].

δ in D_2O: ^{13}C NMR

δ170.50 [βGlcNAc (S_8) $NHCOCH_3$], δ169.80 [βGlcNAc (S_9), $NHCOCH_3$], δ168.20 [βGalNAc (S_4), $NHC=iOCH_3$],δ103.11 [βGal (S_7), C-1], δ102.90 [βGal (S_2) & βGal (S_5), C-1], δ102.89 [βGalNAc (S_4), C-1], δ101.10 [βGlcNAc (S_9), C-1], δ100.80 [βGlcNAc (S_8), C-1], δ95.06 [βGlc (S_6), C-1], δ95.02 [βGlc (S_3), C-1], δ91.20 [βGlc (S_1), C-1] and δ89.90 [αGlc (S_1), C-1], δ20.24 [βGlcNAc (S_8) & βGlcNAc (S_9), $NHCOCH_3$] and δ20.03 [βGalNAc (S_4), $NHCOCH_3$].

δ in $CDCl_3$: ^{1}H NMR (Acetylated)-

δ6.250 [d, 1H, J=3.0Hz, αGlc (S_1), H-1], δ5.650 [d, 1H, J=7.8Hz, βGlc (S_1'), H-1], δ4.730 [d, 1H, J=7.8Hz, βGlc (S_3), H-1], δ4.682 [d, 1H, J=8.6Hz, βGlcNAc (S_8), H-1], δ4.672 [d, 1H, J=7.6Hz, βGlc (S_6), H-1], δ4.549 [d, 1H, J=8.1 Hz, βGlcNAc (S_9), H-1], δ4.539 [d, 1H, J=8.1Hz, βGalNAc (S_4), H-1], δ4.471 [d, 1H, J=8.4Hz, βGal (S_5), H-1], δ4.436 [d, 1H, J=9.1Hz, βGal (S_7), H-1] and δ4.406 [d, 1H, J=8.4Hz, βGal (S_2), H-1], δ2.090 [s, 3H, βGalNAc (S_4), $NHCOCH_3$], δ2.083 [s, 3H, βGlcNAc (S_8), $NHCOCH_3$] and δ2.077 [s, 3H, βGlcNAc (S_9), $NHCOCH_3$].

δ In $CDCl_3$: ^{13}C NMR (Acetylated)-

δ170.80 [βGlcNAc (S_8) $NHCOCH_3$], δ170.20 [βGlcNAc (S_9), $NHCOCH_3$], δ169.71 [βGalNAc (S_4), $NHCOCH_3$], δ104.29 [βGal (S_7), C-1], δ103.97 [βGal (S_2), C-1], δ103.96 [βGal (S_5), C-1], δ103.63 [βGalNAc (S_4), C-1], δ101.62 [βGlcNAc (S_8), C-1], δ101.61 [βGlcNAc (S_9), C-1], δ96.30[βGlc (S_6), C-1], δ95.12 [βGlc (S_3), C-1], δ92.02 [βGlc(S_1'), C-1] and δ90.13[αGlc (S_1), C-1], δ20.94 [βGalNAc (S_4), $NHCOCH_3$], δ20.86 [βGlcNAc (S_8), $NHCOCH_3$] and δ20.73 [βGlcNAc (S_9), $NHCOCH_3$].

ES mass

m/z 1661$[M+Na+K]^+$, m/z 1622$[M+Na]^+$, m/z 1599$[M]^+$, m/z 1563, m/z 1541, m/z 1525, m/z 1522, m/z 1509, m/z 1503, m/z 1490, m/z 1480, m/z 1472, m/z 1431, m/z 1422, m/z 1412, m/z 1396, m/z 1372, m/z 1365, m/z 1361, m/z 1315, m/z 1283, m/z 1275, m/z 1241, m/z 1193, m/z 1159, m/z 1151, m/z 1113, m/z 1111, m/z 1109, m/z 1091, m/z 1036, m/z 1031, m/z 992, m/z 971, m/z 941, m/z 910, m/z 905, m/z 887, m/z 869, m/z 851, m/z 829, m/z 813, m/z 798, m/z 790, m/z 771, m/z 748, m/z 730, m/z 707, m/z 692, m/z 660, m/z 670, m/z 629, m/z 610, m/z 608, m/z 603, m/z 593, m/z 586, m/z 545, m/z 551, m/z 523, m/z 509, m/z 504, m/z 468, m/z 465, m/z 437, m/z 408, m/z 406, m/z 395, m/z 372, m/z 364, m/z 342, m/z 333, m/z 306, m/z 295, m/z 293, m/z 277, m/z 275, m/z 261, m/z 224, m/z 191, m/z 180, m/z 162 and m/z 144.

RESULT AND DISCUSSION

Compound Rieose, $C_{60}H_{101}N_3O_{46,}$ $[\alpha]_D$ +114.44, gave positive Phenol-sulphuric acid test [Dubois et al 1956], Feigl test [Fiegl et al 1975], Morgon-Elson test [Partridge et al 1948] showing the presence of normal and amino sugar(s) in the compound.

The HSQC spectrum of acetylated compound at 300 MHz exhibited ten cross peaks for nine anomeric proton signals at δ6.250 x 90.13, δ5.650 x 92.02, δ4.730 x 95.12, δ4.682 x 101.62, δ4.672 x 96.30, δ4.549 x 101.61, δ4.539 x 103.63, δ4.471 x 103.97, δ4.436 x 104.29, and δ4.406 x 103.97, indicating that the Rieose may be a nonasaccharide, in its reducing form giving signals for α and β anomers of glucose in its reducing end. The nonasaccharide nature of acetylated compound Reiose was further confirmed by the presence of ten-anomeric carbon and proton at δ90.13(1C), δ92.02(1C), δ95.12(1C), δ96.30(1C), δ101.61(1C), δ101.62(1C), δ103.63(1C), δ103.97(2C), and δ104.29(1C) in ^{13}C NMR and δ6.250, δ5.650, δ4.730, δ4.682, δ4.672, δ4.549, δ4.539, δ4.471, δ4.436 and δ4.406 in ^{1}H NMR, respectively. Methylglycosidation of Rieose by MeOH/H^+ followed by its acid hydrolysis led to isolation of α and β- methyl glucoside, which confirmed the presence of glucose at the reducing end of the oligosaccharide. It was also confirmed by the presence of two anomeric proton signals at δ 5.231 and δ 4.726 for α- and β-Glc in the ^{1}H NMR in D_2O. For convenience the nine monosaccharides in compound have been represented as S_1, S_2, S_3, S_4, S_5, S_6, S_7, S_8 and S_9 starting from reducing end. To confirm the monosaccharide constituents in compound L, it was hydrolysed under strong acidic conditions. In Killiani hydrolysis under strong acid condition, it gave four monosaccharides i.e. glucose, galactose, N-acetylgalactosamine and N-acetyl-glucosamine, confirming that the nonasaccharide was consisted of four types of monosaccharide units i.e. glucose, galactose, N-acetylgalactosamine and N-acetyl-glucosamine. To confirm the monosaccharide constituents and their sequence in Rieose, it was hydrolysed under mild acidic conditions (Mannich-Siewert method) followed by paper chromatography and TLC. In this hydrolysis after three days paper chromatogram showed three spots, mobility of one spot was identical in mobility with authentic sample of GlcNAc, and the other spot with the lowest mobility was identical with unreacted compound L(I), further the compound with the intermediate mobility may be the octasaccharide(II). Further after seven days one new spot was observed which was having faster mobility with octasaccharide (II), suggested it was heptasaccharide (III), no new spot of monosaccharide was observed which suggested that the next monsaccharide was also GlcNAc which was merged with already existing spot of GlcNAc. After ten days two new spots were observed of which one was identical in mobility with authentic sample of Gal and other with lower mobility may be the hexasaccharide (IV) which was having faster mobility with heptasaccharide (III) confirming the next monosaccharide as Gal. After fifteen days two new spots were observed of which one was identical in mobility with authentic sample of Glc and other with faster mobility with monosaccharide (IV) may be the pentasaccharide ((V). After eighteen days one new spot with faster mobility than pentasaccharide (V) was observed, suggested it was tetrasaccharide (VI). Further after twenty one days two new spots were observed of which one was identical in mobility with authentic sample of GalNAc and other with lower mobility may be the trisaccharide ((VII)) which was having faster mobility with tetrasaccharide (VI). After twenty five days one new spot with faster mobility than trisaccharide (VII) was observed, suggested it was disaccharide (VIII), which was having identical mobility with authentic sample of Lactose. After thirty days the hydrolysis was completed and four spots were observed on TLC and PC which was found identical with authentic sample of GlcNAc, GalNAc, Gal and Glc may be confirm in that the sequence of monosaccharide in nonasaccharide.

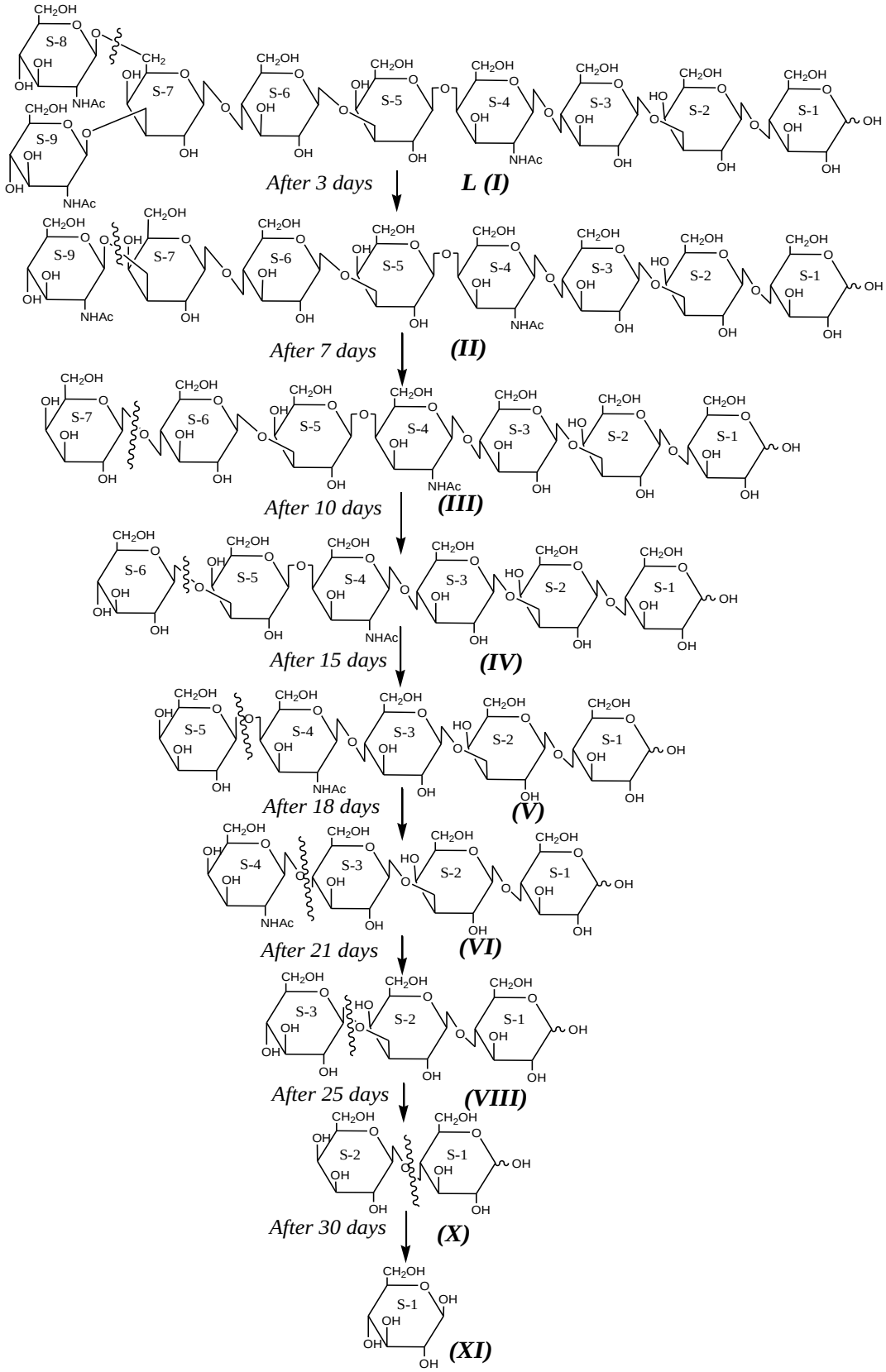
Since the glucose was present in its reducing form which was supported by ^{1}H NMR of Rieose in D_2O which contains two anomeric proton signals for α- and β-Glc at δ 5.231 (J=δ 3.0Hz) and at δ 4.726 (J=δ 7.8Hz) [Urashima et al 2002-Urashima et al 2007]. Further the presence of another anomeric proton doublet signal at 4.399 (J=δ 8.4Hz) was due to presence of β-Gal moiety in the Rieose. Since the ^{1}H NMR of Rieose showed H-2 signal of β-Glc (S_1) as a triplet at δ3.296(SRG) [Urashima et al 2007, Gronberg et al 1990], suggested the equatorial orientation of hydroxyl group at C-4 of the reducing β-Glc (S_1) were substituted and was involved in glycosidation, it suggested the presence of a lactosyl moiety i.e. β-Gal (1→4)Glc, at the reducing end of Rieose. The coupling constant of anomeric signal with J value of δ8.4Hz shows the β-configuration of anomeric linkage between S_2→S_1. The (1→4) linkage of S_1 and S_2 was also confirmed by the presence of β-Glc H-4 proton resonance at δ 3.809 in acetylated derivative of Rieose. Further another anomeric proton signal, which appeared at δ4.706(J= δ 7.8Hz), was due to presence of another Glc moiety S_3. The position of anomeric proton signal of Glc (S_3) at δ4.706 (J=δ 7.8Hz)(SRG) [Urashima et al 2000] and absence of downfield shifted H-4 proton of β-Gal(S_2) suggested that Glc was linked to β-Gal (S_2) by (1→3) linkage. The splitting pattern of anomeric signal with J value of δ7.8Hz shows the β-configuration of anomeric linkage at S_3→S_2. It was further confirmed by ^{1}H and ^{13}C NMR spectrum of acetylated Rieose, the presence of H-3 and C-3 resonance of β-Gal (S_2) at δ4.024and δ76.70 confirmed the (1→3) linkage between S_3 and S_2. The next anomeric proton signal, which appeared at δ4.520(J=δ 8.1Hz) along with signal of amide methyl group at δ 2.004 was due to presence of β-GalNAc (S_4) moiety.

Table1. ^{1}H and ^{13}C NMR values of Rieose in D_2O

Moieties	^{1}H NMR	^{13}C NMR	Coupling Constt.(J)
α - Glc (S_1)	5.231	89.90	3.0
β - Glc (S'_1)	4.726	91.20	7.8
β - Gal (S_2)	4.399	102.90	8.4
β - Glc (S_3)	4.706	95.02	7.8
β - GalNAc (S_4)	4.520	102.89	8.1
β - Gal(S_5)	4.466	102.90	8.4
β - Glc(S_6)	4.655	95.06	7.6
β - Gal (S_7)	4.412	103.11	9.1
β - GlcNAc(S_8)	4.665	100.80	8.6
β - GlcNAc(S_9)	4.530	101.10	8.1

Table 2. ^{1} H NMR values of acetylated Rieose in $CDCl_3$-

Moieties	H-1	H-2	H-3	H-4	H-5	H-6	-CH_3
α - Glc (S_1)	6.250	5.254	5.322	3.999			
β - Glc (S'_1)	5.650	4.999	5.330	3.809			
β - Gal (S_2)	4.406	4.682	4.024	4.824			
β - Glc (S_3)	4.730	4.364	4.663	4.031			
β - GalNAc (S_4)	4.539	3.967	5.100	3.920			2.090
β - Gal(S_5)	4.471	4.620	3.786	4.118			
β - Glc (S_6)	4.672	4.396	4.800	4.094			
β - Gal (S_7)	4.436	4.825	3.890	4.129	4.760	3.859	
β - GlcNAc(S_8)	4.682	3.878	4.825	4.364			2.083
β - GlcNAc(S_9)	4.549	3.890	4.806	4.342			2.077


After 3 days
L (I)
After 7 days
(II)
After 10 days
(III)
After 15 days
(IV)
After 18 days
(V)
After 21 days
(VI)
After 25 days
(VIII)
After 30 days
(X)
(XI)

The β-Glc triplet at δ3.296(SRG) [Saito et al 1984] confirmed the (1→4) linkage between β-GalNAc (S_4) and β-Glc(S_3). The coupling constant of anomeric signal with J value of δ8.1Hz shows the β-configuration of anomeric linkage at S_4→S_3. It was further confirmed by the chemical shift of β-Glc(S_3) H-4 and C-4 resonances in acetylated spectrum of Reiose which appeared at δ 4.031 and δ74.61respectively. Further next anomeric proton signal, which appeared at δ4.466(J= δ 8.4Hz) was due to presence of β-Gal (S_5) moiety. The anomeric proton value of β-Gal at δ4.466(J= δ 8.4Hz) (SRG) [Urashima et al 2004] confirmed (1→4) linkage between β-Gal (S_5) and β-GalNAc (S_4). The splitting pattern of anomeric signal with J value of δ8.4Hz shows the β-configuration of anomeric linkage between S_5→S_4. It was further confirmed by the chemical shift of β-GalNAc (S_4) H-4 and C-4 resonances in acetylated spectrum of Reiose which appeared at δ 3.920 and δ73.15respectively. Another anomeric proton signal, which appeared at δ4.655(J= δ 7.6Hz), was due to presence of another Glc (S_6) moiety. The position of anomeric proton of Glc at δ 4.655(J=δ 7.6δ) and absence of downfield shifted H-4 chemical shift of β-Gal (S_5) suggested the β-Glc (S_6) was linked to β-Gal (S_5) by (1→3) linkage (SRG) [Urashima et al 2000]. The coupling constant of anomeric signal with J value of δ7.6Hz shows the β-configuration of anomeric linkage among S_6→S_5. It was further confirmed by ^{1}H and ^{13}C NMR spectrum of acetylated Reiose, the presence of H-3 and C-3 resonance of β-Gal (S_5) at δ3.786and δ74.88(in linkage region). The next anomeric proton signal, which appeared at δ4.412(J=δ 9.1Hz) was due to presence of β-Gal (S_7) moiety. The presence of β-Glc triplet at δ3.296(SRG) [Urashima et al 2007, Gronberg et al 1990] confirmed the (1→4) linkage between β-Gal (S_7) and β-Glc (S_6). The splitting pattern of anomeric signal with J value of δ8.6Hz shows the β-configuration of anomeric linkage at S_7→S_6. It was further confirmed by the chemical shift of β-Glc (S_6) H-4 and C-4 resonances in acetylated spectrum of Reiose which appeared at δ 4.094 and δ72.78 respectively. The next two anomeric proton signal, which appeared at δ4.665(J=δ 9.1Hz) along with signal of amide methyl group at δ 2.004 and δ4.530(J=δ 8.1Hz) along with signal of amide methyl group at δ 2.004was due to presence of β-GlcNAc (S_8) and β-GlcNAc (S_9) moieties, respectively. The linkages between β-GlcNAc (S_8) and β-GlcNAc (S_9) to β-Gal(S_7) was confirmed by the position of anomeric proton values of β-GlcNAc(S_8-δ4.665 & S_9-δ4.530) with downfield shifted value of H-4 of β-Gal(S_7) (SRG) [Urashima et al 2004 , Urashima et al 2004-Urashima et al 2007].

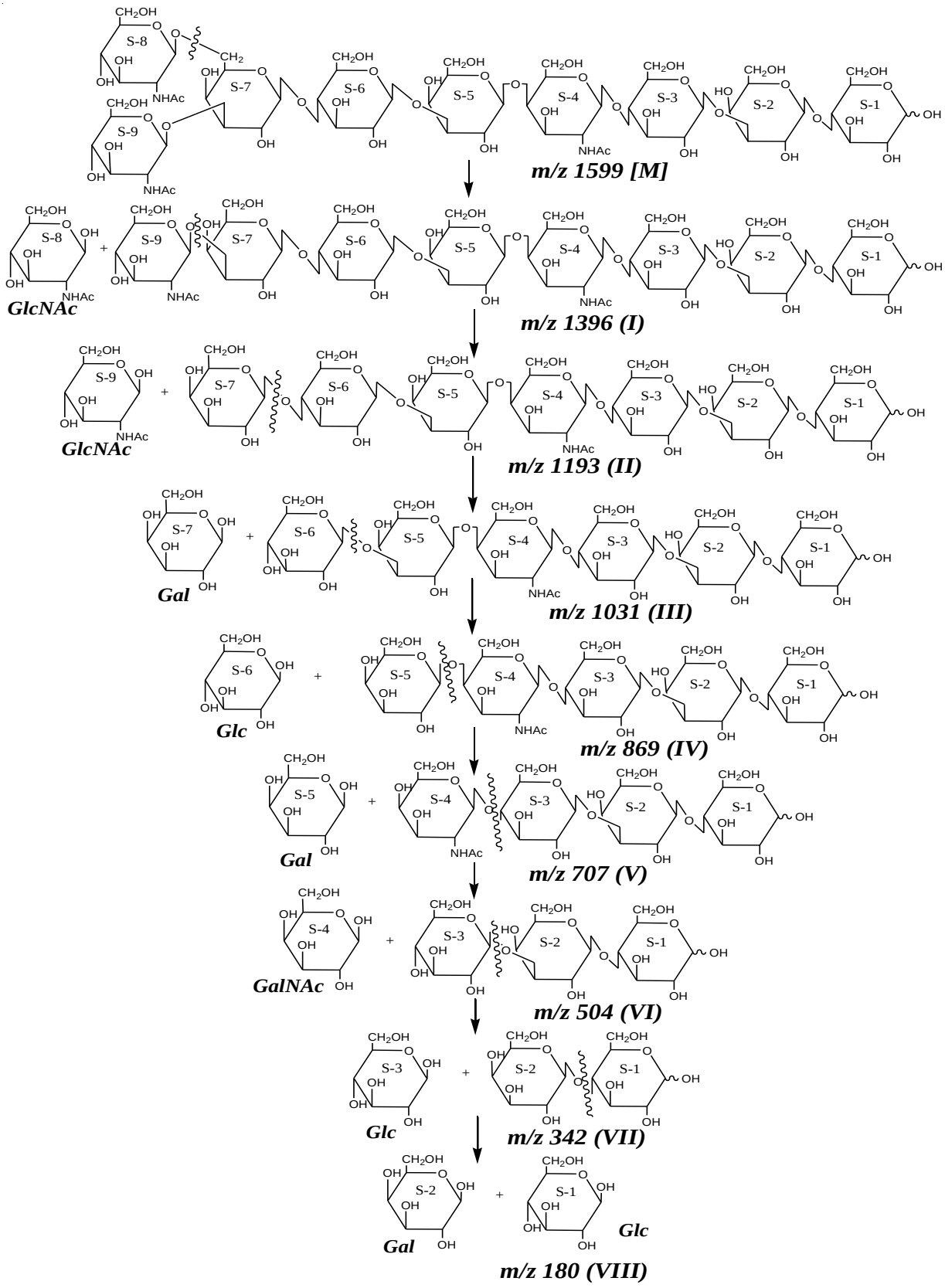
Table1. ^{13}C NMR values of acetylated Rieose in $CDCl_3$-

Moieties	C-1	C-2	C-3	C-4	C-5	C-6	-$CONH_2$	-CH_3
α - Glc (S_1)	90.13	68.61	68.43	77.02				
β - Glc (S'_1)	92.02	67.92	67.82	77.33				
β - Gal (S_2)	103.97	67.17	73.48	60.83				
β - Glc (S_3)	95.12	63.17	62.74	74.61				
β - GalNAc (S_4)	103.63	62.27	61.67	73.15			169.71	20.94
β - Gal(S_5)	103.96	61.14	74.88	67.17				
β - Glc(S_6)	96.30	68.61	68.43	72.98				
β - Gal (S_7)	104.29	66.85	72.71	67.82	68.43	72.61		
β - GlcNAc(S_8)	101.62	62.27	61.67	61.14			170.78	20.86
β - GlcNAc(S_9)	101.61	69.55	68.43	67.92			170.20	20.73

The coupling constant of anomeric signals with J value of δ8.6Hz andδ8.1Hz shows the β-configuration of anomeric linkages between $S_8 \rightarrow S_7$ and $S_9 \rightarrow S_7$, respectively. It was further confirmed by the chemical shift of β-Gal(S_7) of H-3, H-6, C-3 and C-6 resonances in acetylated spectrum of Reiose which appeared at δ3.890, δ3.859, δ72.71and δ72.61respectively, with upfield shifted H-4 value of β-Gal(S_7) at δ4.129(SRG) [Gronberg et al 1990]. The ^{13}C NMR values of anomeric carbons and ring carbons of Rieose are given in table. The various values of ring carbons are in accordance with ^{13}C value of their respective monosaccharides, which also supports the derived structure.

The nonasaccharide nature of compound was further confirmed by the spectral studies of acetylated derivative of compound. Ring hydrogens involved in linkage at δ 3.999(4-position) for $S_1 \rightarrow S_2$, δ 4.024 (3-position) for $S_2 \rightarrow S_3$, 4.031 (4-position), for $S_3 \rightarrow S_4$, δ3.920 (4-position) for $S_4 \rightarrow S_5$, δ 3.786 (3-position) for $S_5 \rightarrow S_6$, δ 4.094 (4-position) for $S_6 \rightarrow S_7$, δ 3.859 (6-position) for $S_7 \rightarrow S_8$,and δ 3.890 (3-position) for $S_7 \rightarrow S_8$, showing in region same chemical region in acetylated and deacetylated spectra. These studies were made on the basis of HOMOCOSY, TOCSY and HSQC connectivities. The heteronuclear single quantum coherence (HSQC) spectrum of acetylated compound confirmed the position of anomeric linkages in ^1H and ^{13}C NMR spectra by showing cross peaks of an α-Glc(S_1) H-4 & C-4 at (δ 3.999 x 77.02) and β-Glc(S_1) H-4 & C-4 at (δ 3.809 x 77.33). It also contains cross peak of β-Gal (S_2)H-3 & C-3 at (δ 4.024 x 73.48), β-Glc (S_3) H-4 & C-4 at (δ4.031 x 74.61), β-GalNAc (S_4) H-4& C-4 at (δ 3.920 x 73.15), β-Gal (S_5) H-3 & C-3 at (δ 3.786 x 74.88), β-Glc (S_6) H-4 & C-4 at (δ4.094 x 72.98), and β-Gal (S_7) H-6 & C-6 and H-3 & C-3 at (δ 3.859 x 72.61) and at (δ3.890 x 72.71), respectively. Based on the pattern of chemical shifts of ^1H, ^{13}C, HOMOCOSY, TOCSY and HSQC NMR experiments it was interpreted that the compound was nonasaccharid. The result obtained from the ES mass spectrum further substantiated the structure of Rieose which was derived by its ^1H and ^{13}C NMR spectra. The highest mass ion peak were recorded m/z 1661 which was due to $[M+Na+K]^+$. Other mass ion peak recorded at m/z 1622 and m/z 1599, were due to $[M+Na]^+$ and, [M] respectively, confirmed that the molecular weight of compound was 1599. Further the mass fragments were formed by repeated H transfer in the oligosaccharide and was accompanied by the elimination of terminal sugar less water. The fragmentation pathway confirmed the sequence of monosaccharide units in the nonasaccharide (scheme: 1, 2 & 3). The nonasaccharide fragment mass ion peak at m/z 1599(M) on fragmentation gave the octasaccharide mass ion peak at m/z 1396(I) which was obtained by the loss of S_8 sugar unit i.e. GlcNAc (S_8) sugar unit linked to S_7 of the oligosaccharide, which was supported by its respective fragment at m/z 239, this confirmed the presence of GlcNAc (S_8) at the non-reducing end. The octasaccharide mass ion peak fragmented to give mass ion peak at m/z 1193(II), which was due to the loss of S_9 sugar unit i.e. GlcNAc (S_9) sugar unit linked to S_5 of the oligosaccharide which was also supported by its complimentary fragment at m/z 239, this confirmed the presence of GlcNAc (S_9) at the non-reducing end. Further the heptasaccharide mass ion fragmented by the loss of other sugar i.e. Gal (S_7), gave the corresponding hexasaccharide mass ion fragment at m/z 1031(III). The mass ion peak at m/z 1031 further fragmented to give mass ion fragment for pentasaccharide moiety which was arose by loss of sugar (S_6) [Glc]. It was accounted for the mass ion fragment at m/z 869(IV). This pentasaccharide mass ion fragment on further fragmentation gave tetrasaccharide segment (V) at m/z 707, by loss of sugar (S_4) [GalNAc].

This on further fragmentation gave a trisaccharide segment (VI) at m/z 504 by loss of sugar (S_4) [GalNAc]. This trisaccharide mass ion fragment on further fragmentation gave an important disaccharide segment (VII) at m/z 342, by loss of sugar (S_3) [Gal], which on further fragmentation give a monosaccharide fragment (VIII) at 180 by loss of sugar (S_2) [Gal].(scheme-1& 2). The nonasaccharide mass ion peak at m/z 1599 in the spectrum of compound L also showed other supporting mass ion peaks which are shown in scheme-3.The other important signals obtained at m/z 1361[M-S_8,S_9], m/z 1275[M-S_1,S_2], m/z 1113[M-S_1,S_2,S_3], m/z 910[M-S_1,S_2,S_3,S_4], m/z 790[M-S_1,S_2,S_3,S_4,S_5], m/z 586[M-S_1,S_2,S_3,S_4,S_5,S_6], m/z 545[M-S_1,S_2,S_3,S_4,S_8,S_9 or M-S_1,S_2,S_6,S_7,S_8,S_9 or M-$\mathbf{S_1}$,$\mathbf{S_5}$,$\mathbf{S_6}$,$\mathbf{S_7}$,$\mathbf{S_8}$,$\mathbf{S_9}$], m/z 504[M-$\mathbf{S_1}$,$\mathbf{S_2}$,$\mathbf{S_3}$,S_4,S_8,S_9] and m/z180[M-S_3,S_4,S_5,S_6,S_7,S_8,S_9].The other supporting mass fragments obtained at m/z 1522[M-CH_2OHCHO], m/z 1480[1522-CH_2CO], m/z 1431[1480-OH,CH_2OH], m/z 1372[1431-CH_2CO,OH], m/z 1315[1372--$NHCOCH_3$], m/z 1563[M-$2H_2O$], m/z 1503[1563-CH_2OHCHO], m/z 1472[1503-CH_2OH], m/z 1422[1472-$2H_2O$,$NHCOCH_3$], m/z 1396[1472-2OH,CH_2CO], m/z 1509[M-CH_2CHO], m/z 1541[M-$NHCOCH_3$], m/z 1525[1542-OH], m/z 1490[1525-H_2O,OH], m/z 1472[1490-H_2O], m/z 1422 [1490-CH_3,HCHO] and m/z 1412[1472-CH_2OHCHO] confirmed the nonasaccharide nature of compound. The nonasaccharide m/z 1599 on fragmentation gave octasaccharide m/z 1396 (M-S_9), which was further confirmed by its other fragments ions at m/z 1365[1396-CH_2OH], m/z 1283[1396-$2H_2O$,OH], m/z 1241[1283-CH_2CO], m/z 1159[1241-CH_2OH,2OH,H_2O], m/z 1111[1159-H_2O,HCHO], m/z 1036[1111-$NHCOCH_3$,OH] and m/z 1193[1241-$NHCOCH_3$]. The octasaccharide m/z 1396 on fragmentation gave heptasaccharide m/z 1193(1396-S_8), which was further confirmed by its other fragments ions at m/z 1151[1193-CH_2CO], m/z 1109[1151-CH_2CO], m/z 1091[1109-H_2O or 1193-CH_2OHCHO,CH_2CO], m/z 1031[1091-CH_2OHCHO or 1091-2HCHO], m/z 1036[1091-OH,HCHO,H_2O], m/z 992[1036-CH_2CHOH], m/z 905[992-$NHCOCH_3$,2OH] and m/z 869[905-$2H_2O$]. The heptasaccharide m/z 1193 on fragmentation gave hexasaccharide m/z 1031 (1193-S_7), which was further confirmed by its other fragments ions at m/z 971[1031-CH_2OHCHO], m/z 941[971-HCHO], m/z 905[941-$2H_2O$], m/z 887[905-H_2O], m/z 869[887-H_2O], m/z 829[887-$NHCOCH_3$], m/z 798[830-HCHO], m/z 748 [798-CH_3,OH,H_2O], m/z 730 [748-H_2O], m/z 707[798-CH_2OHCHO, CH_2OH], m/z 813[887-CH_2OH], m/z 771[813-CH_2CO] and m/z 692[771-$NHCOCH_3$,CH_2CHO]. The hexasaccharide m/z 1031 on fragmentation gave pentasaccharide m/z 869(1031-S_6), which was further confirmed by its other fragments ions at m/z 851[869-H_2O], m/z 771[851-$2CH_2OH$,H_2O], m/z 670[771-CH_2OHCHO], m/z 610[670-$NHCOCH_3$], m/z 608[670- $2CH_2OH$], and m/z 603[670-CH_2OHCHO].The pentasaccharide m/z 869 on fragmentation gave tetrasaccharide m/z 707 (869-S_5), which was further confirmed by its other fragments ions at m/z 692[707-CH_3], m/z 660[707-HCHO,OH], m/z 629[660-CH_2OH], m/z 593[629-$2H_2O$], m/z 551[593-CH_2CO], m/z 509[551-CH_2CO], m/z 523[551-CH_2CHO], m/z 465[523-OH,CH_2OH] and m/z 504[551-HCHO,OH]. The tetrasaccharide m/z 707 on fragmentation gave trisaccharide m/z 504 (707-S_4), which was further confirmed by its other fragments ions at m/z 468[504-$2H_2O$], m/z 437[468-CH_2OH], m/z 406[437-CH_2OH], m/z 364[406-CH_2CO], m/z 395[437-CH_2CO], m/z 364[395-CH_2OH], m/z 333[364-CH_2OH], m/z 293[333-CH_3,OH,H_2O], m/z 408[468- CH_2OHCHO], m/z 372[408-$2H_2O$], m/z 295[372-CH_2OHCHO,OH], m/z 277[295-H_2O], m/z 224[277-$2H_2O$,OH], m/z 162[224-$2CH_2OH$] and m/z 342[372-HCHO].

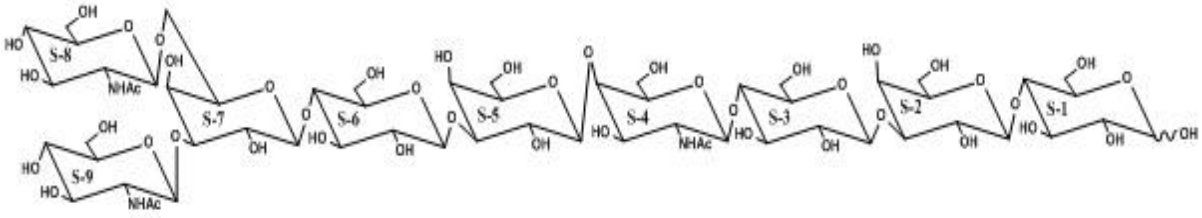


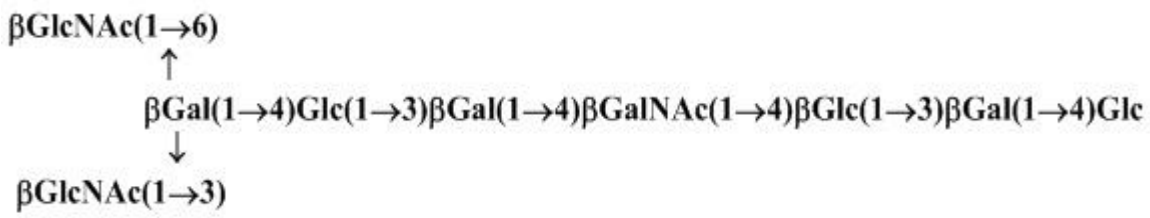
Scheme: 3-ES-Mass Fragmentation of Rieose (Compound L)

The trisaccharide m/z 504 on fragmentation gave disaccharide m/z 342 (504-S_3), which was further confirmed by its other fragments ions at m/z 306[342-2H_2O], m/z 261[306- H_2O,OH], m/z 224[261-OH,HCHO], m/z 293[342-CH_2OH], m/z 275[293-H_2O], m/z 191[275-CH_2OH,CH_2CHO] and m/z 180[275-CH_2OHCHO,H_2O,OH]. The disaccharide m/z 342 on fragmentation gave monosaccharide m/z 180 (342-S_2), which was further confirmed by its other fragments ions at m/z 162[180-H_2O] and m/z 144[162-H_2O].

CONCLUSION

Based on the results obtained from chemical degradation chemical transformation, mass spectrometry and ^{1}H , ^{13}C, HOMOCOSY, TOCSY , HSQC NMR, the structure of the isolated novel nonasaccharide, Reiose was deduced as-





REIOSE

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