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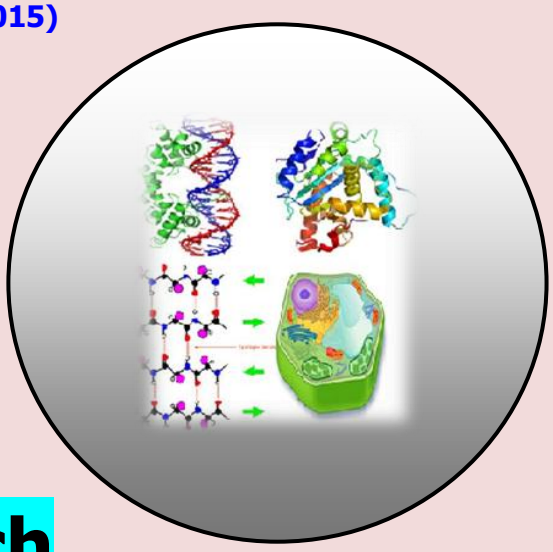

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RESEARCH PAPER

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Isolation of a Novel Sheep Milk Oligosaccharide as Biologically Active Component

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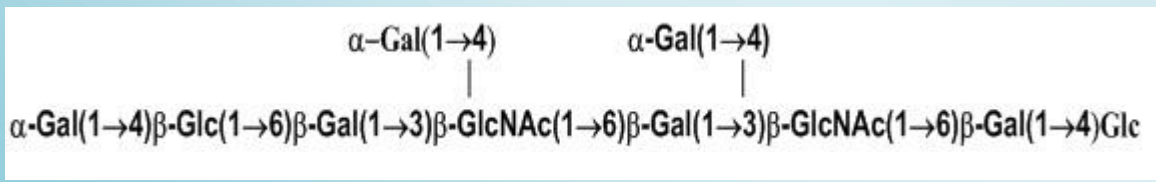
ABSTRACT

Carbohydrates are integral constituent of all living organisms and are associated with variety of vital functions, which sustain life. Oligosaccharide are amongst the most biologically diverse and important carbohydrate in biological system. 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. Sheep milk oligosaccharides contains fucose which is beneficial for skin diseases and cosmetic purposes., In the present study, to find biologically active novel milk oligoasaccharide , Sheep milk was processed by the method of Kobata and Ginsberg followed by gel filtration HPLC and column chromatography which resulted in the isolation of a novel milk oligosaccharide named Ovisose. The structure of isolated and purified milk oligosaccharide was elucidated with the help of chemical degradation, chemical transformation, spectroscopy technique like NMR (1H, 13C, and 2D NMR) and mass spectrometry. The isolated oligosaccharide Ovisose was interpretate as decasaccharide having branched structure as-



Ovisose

Key words- Milk Oligosaccharides, 2D NMR and Ovisose.

INTRODUCTION

Oligosaccharides are found as natural constituents in fruits, milk, bacteria and fungus etc and have various physiological functions such as improvement of mineral absorption and improvement of both plasma cholesterol and blood glucose level. These oligosaccharides are important model compounds for structuring the sugar specificities of various biological interaction and they have also been coupled to protein carrier for production of antibodies with defined binding specificities. Milk is rich source of biologically active oligosachharides which exhibits the various inhibitory effects on certain virulence related abilities of monocytes, lymphocytes and neutrophylls adhesion to endothelial cells (Singh et al 2016). Milk oligosachharides are known to be protected breast fed infants from a host bacterial infection. Numerous milk oligosaccharides have been isolated from milk of many mammalian including equine, bovine, and marine mammals (Kunz et al, Urashima et al,and Nakamura et al, 2004). These isolated milk oligosachharides exert various biological activities which are varied from animal to animal. Buffalo milk oligsaccharides has immune stimulant activity by increasing heamagglutination, delayed type hypersenstive reaction, and plague forming cell count in mice (Saksena et al 1999) . Donkey milk oligosaccharides have ability to non specific and specific immunological resistance (Deepak et al 1999). Goat milk oligosaccharides exhibit a specific role in human intestinal inflammation (Lara villoslada et al 2000). Human milk plays an important role in gut colonization and modulation of infant gut (A.K. Singh et al 2016). Camel milk has proven beneficial in lung ailments and treatment of tuberculosis (A.A Akhundov et al 1972). Sheep milk is very useful to aggravate hiccup and dyspnoea. It also reduces pitta, kapha and fat. It also contain fucose in its oligosaccharides which causes various biological activities (Srivastav et al 2016) chemical degradation, chemical transformation, spectroscopic techniques like (^{1}H NMR, ^{13}C NMR and 2D NMR).

MATERIAL AND METHODS

General methods were same as described in our previous articles (Ranjan et al. 2015).

Isolation of Sheep milk oligosaccharide by Kobata and Ginsberg method: 10 litres milk was collected from a Sheep and then isolated by method of Kobata and Ginsberg (Kobata A. et al., 1970). For this method, milk was stored at -20°C and centrifuged for 15 min at 5000 rpm at 4°C. The solidified lipid layer was removed by filtration through glass wool column in cold. Ethanol was added to clear filtrate to a final concentration of 68% and the resulting solution was left overnight 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. The lyophilized material responded positively to Morgan-Elson test (Partridge S.M. et al., 1948) and thiobarbituric acid assay suggesting the presence of N-acetyl sugars and sialic acid in oligosaccharide mixture. This lyophilized material (mixture of oligosaccharide) was further purified by fractionating it on sephadex G-25 chromatography using glass triple distilled water as eluant at a flow rate of 3 ml/m. each fraction was analyzed by phenol sulphuric acid reagent for the (Dubois M. et al., 1956) epresence of neutral sugar

Acetylation of Sheep milk oligosaccharide mixture

7.8gm of pooled fractions from Sephadex G-25 filtration which gave positive phenol-sulphuric acid test, were acetylated with pyridine (42 ml) and acetic anhydride (28 ml) at 60°C and the solution was stirred overnight.

The mixture was evaporated under reduced pressure and the viscous residue was taken in $CHCl_3$ (250 ml) and washed in sequence with 2N-HCl (1 x 25 ml), ice cold 2N-$NaHCO_3$ (2 x 25 ml) and finally with H_2O (2 x 25 ml). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding the acctylated mixture (10.0 g).

Deacetylation of Compound Ovisose

Ovisose accetate (53 mg) obtained from column chromatography 8 of acetylated oligosaccharide mixture was dissolved in acetone (2 ml) and 3 ml of NH_3 was added and left overnight in a stoppered hydrolysis flask. After 24 h ammonia was removed under reduced pressure and the compound was washed with (3 x 10 ml) $CHCl_3$ and the water layer was finally freeze dried giving the deacetylated oligosaccharide C (39 mg).

Methylglycosidation/ acid hydrolysis of compound Ovisose

Ovisose (8 mg) was refluxed with absolute MeOH (2 ml) at 70°C for 18 h in the presence of cation exchange IR-120 (H^+) resin. The reaction mixture was filtered while hot and filtrate was concentrated. To a solution of methylglycoside of H in 1, 4-dioxane (1 ml), 0.1 N H_2SO_4 (1 ml) was added and the solution was warmed for 30 minutes at 50°C. The hydrolysis was complete after 22 h. The hydrolysate were 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 on TLC, PC.

Kiliani Hydrolysis of Compound Ovisose

Ovisose (5 mg) was dissolved in 2 ml Kiliani mixture (AcOH-H_2O-HCl, 7: 11:2) and heated at l00^{o}C for 1 h followed by evaporation under reduced pressure. It was dissolved in 2 ml of H_2O and extracted twice with 3 ml $CHCl_3$. The aqueous residual solution was made neutral by addition of 1-2 drops of 2N NaOH to it and was evaporated under reduced pressure to afford glucose, Gal and GlcNAc. Their identification was confirmed by comparison with authentic samples on TLC, PC.

Description of Isolated Compound Ovisose

Compound Ovisose (53mg) obtained from fractions 21-48 of column chromatography-8, on deacetylation with NH_3/ Acetone, it afforded substance H (39 mg) as a viscous mass. $[\alpha]_D$ +64 ^{0}(c, 4, H_2O). For elemental analysis, this compound was dried over P_2O_5 at 100^{0} C and 0.1 mm pressure for 8 hr.

$C_{64}H_{108}O_{51}N_2$	%C	%H	%N
Calculated	44.651	6.279	1.628
Found	44.640	6.277	1.627

It gave positive Phenol-sulphuric acid test, Feigl test, Morgon-Elson test.

^{1}H NMR values of compound Ovisose in D_2O

2.006 [s,6H, $NHCOCH_3$, β-GlcNAc (S-3),(S-6)], 3.297 [t, 2H, J=8.4 Hz, β-Glc(S-1) and S-9), H-2], 3.943 [d,3H,J=3.6 Hz, β-Gal (S-2 ,S-4 and S-7), H-4], 4.462 [d,3H, J=7.5 Hz, β-Gal (S-2 S-4 and S-7), H-1], 4.569[d, 1H, (8.0 Hz , β-GlcNAc (S-3) H-1], 4.599 [d, 1H, J=7.9 Hz, β-GlcNAc (S-6) H-1], 4.679 [d, 2H, J=8.1 Hz, β-Glc (S-1) and S-9), H-1], 5.234 [d, 2H, J=3.9 Hz, α-Glc (S-1) and α-Gal (S-10),H-1], 5.270 [d,1H, J=3.4 Hz, α-Gal (S-5),H-1], 5.396[d,1H, J=3.7 Hz, α-Gal (S-8),H-1] .

^{13}C NMR values of compound Ovisose in D_2O

22.57, 57.01, 61.28, 61.41, 70.01, 71.39, 72.05, 72.25, 72.46, 72.70, 73.83, 75.11, 75.66, 76.00 ,76.08, 76.63, 79.66, 79.79, 90.50, 92.01, 93.10, 97.05, 99.36, 102.12, 104.21, 178.62

^{1}H NMR Values of acetylated compound Ovisose in $CDCl_3$

δ2.001-2.178 (NHCOC$\underline{H}_3$)], δ4.506 [β-Gal (S-2) H-1], δ4.545[β-Gal (S-4) & (S-7) H-1], δ 4.560 [β-GlcNAc (S-3), H-1], δ4.685 [β-Glc (S-9), H-1], δ4.770 [β-Glc (S1)], δ 4.748 [β-GlcNAc (S-6), H-1], δ 5.300[α-Gal (S-10),H-1], δ 5.387[α-Gal (S-5) H-1, α-Glc (S-1) H-1 and α-Gal (S-8) H-1].

^{13}C NMR Values (δ) of acetylated compound ovisose in $CDCl_3$

20.565,20.83,60.58,61.68,62.12,62.26,62.64,63.14,63.30,63.53,64.36,64.76,66.24,66.84,667.07,67.18,67.44,67.97,68.52,68.66,68.95,69.06,69.23,69.44,69.83,70.14,71.19,71.27,71.64,71.88,72.06,72.44,72.64,72.84,73.10,73.40,73.63,73.77,74.26,74.61,74.76,75.01,75.66,76.52,76.04,81.07,81.22,81.62,81.97,82.06,82.39,90.3,90.41,91.98,92.10,95.15,95.4,95.50,101.56,102.00,102.1

ES-MS PEAKS (m/z)

m/z 1759 [M+K$^+$], m/z 1720 [M$^+$] and other fragments at m/z 1702, 1689, 1686, 1685, 1672, 1660, 1645, 1642, 1629, 1624, 1558, 1541, 1523, 1516, 1505, 1500, 1485, 1483, 1481, 1480, 1464, 1440, 1396, 1378, 1365, 1361, 1360,1343, 1325, 1318,1300, 1234, 1216, 1203, 1199,
1185, 1174, 1156, 1143, 1125, 1120, 1085, 1078, 1072, 1068, 1054, 1047, 1041, 1037, 1024, 1023, 1013, 1005, 999, 997, 995,982, 965, 964, 930, 894, 869, 852, 838, 833 821, 811, 804, 794, 792, 779, 780, 775, 769, 763, 762, 746, 733, 721, 707, 702, 690, 676, 671, 661, 659, 654, 649, 642, 616, 614, 611, 545, 527,514, 504, 496, 485, 480 472, 463, 422, 342, 325, 324, 282, 267, 265, 264, 247, 180.

RESULT AND DISCUSSION

Compound Ovisose, $C_{64}H_{108}O_{51}N_2$, $[\alpha]_D$ +64^0(c,4 ,H_2O)gave positive phenol sulphuric acid test, Fiegl test Fiegl (F. et al., 1975) and Morgon-Elson test indicating the presence of normal and amino sugar in the compound-Ovisose. According to ^{1}H NMR spectrum compound Ovisose exhibited seven proton signals for eleven protons in the anomeric proton region as doublets at δ 5.396(1H), δ 5.27(1H), δ5.234 (2H), δ4.679(2H), δ 4.599(1H), δ 4.569(1H), δ4.462(3H) leading to the presence of eleven anomeric protons in it. This was further supported by the appearance of seven carbon signals for eleven anomeric carbon at δ 90.50(1C), δ 92.01(1C), δ 93.10(2C), δ 97.05 (2C), δ 99.36(1C), 102.12(1C) and δ104.21 (3H) in the ^{13}C NMR of compound Ovisose. These data led to the suggestion that compound Ovisose may be a Decasaccharide in its reducing form. The ten monosaccharides present in compound Ovisose have been designated as S-1, S-2, S-3, S-4, S-5, S-6, S-7 S-8, S-9 and S-10 for convenience. The Killiani acid hydrolysis of compound Ovisose gave three spots on the paper chromatography, which were identified as Glc, Gal and GlcNAc by co-chromatography with authentic samples. This suggested that compound Ovisose contained three types of milk sugar moieties in compound ovisose. Methylglycosidation of compound Ovisose by MeOH/H$^+$ followed by its acid hydrolysis led to the isolation of α and β methyl glucosides, suggesting that the Glucose was present in its reducing form. The reducing and free nature of Glucose was further supported by the presence of two anomeric proton signals as doublets and their coupling constant, for α and β glucose at δ5.234 (J=3.9Hz) and δ4.679(J=8.1Hz) respectively.

Next anomeric proton doublet at δ4.462 (J=7.5 Hz) in the compound Ovisose showed the presence of Gal residue as in lactosyl moiety i.e. Gal β (1→4) Glc. This was further confirmed by β-Glc (S-1) H-2 signal (a structure reporter group) (G. Gronber et al.1990) (DUA et al.1983) which appeared as a triplet at δ 3.297 (J=8.4 Hz). Further the spectrum contained third anomeric proton doublet which appeared at δ 4.569(1H) (J=8.0 Hz) was due to the presence of β-GlcNAc(S-3) moiety which was next in sequence and was linked to Lactose by glycosidic linkage. This was supported by presence of a singlet of amide methyl of N-acetyl β-Glucosamine at δ2.006 Hz. The H-4 proton resonance of β -Gal (S-2) which appeared at δ3.943 in the ^{1} H NMR spectrum of compound Ovisose, showed that β -Gal (S-2) was not substituted at C-3 position (a structure reporter group) (G.Gronber et al.1990) (DUA et al.,1983) by β -GlcNAc (S-3) moiety. This implies that the β -GlcNAc (S-3) may be 1→6 linked to β -Gal (S-2). This was confirmed by H-6 signal of β -Gal (S-2) which appeared at δ 3.876 and C-6 single of β -Gal (S-2) which appeared at δ71.88 in the ^{1}H and ^{13} C NMR spectrum of acetylated compound Ovisose. The assignment of H-6 proton was confirmed by COSY and TOSCY experiments of acetylated compound Ovisose. The chemical shift analogies of β -GlcNAc 1→6 as reported by (Dua. et al., 1983) also supported this linkage. All these views imply that the β-GlcNAc(S-3) was linked 1→6 to β –Gal (S-2). The next anomeric proton doublet present in the ^{1}H NMR spectrum of compound Ovisose at δ 4.462 (J=7.5Hz) reflected the presence of another β -Gal moiety (S-4) in the compound Ovisose. This β -Gal (S-4) was linked to β-GlcNAc(S-3) at H-3 position because the anomeric chemical shift of this β -Gal (S-4) was identical with lactosyl β -Gal (S-2) as structure reporter group(G.Gronber et al.1990) (DUA et al.1983)]. This was further confirmed by the ^{1}H NMR and ^{13}C NMR spectrum of acetylated compound Ovisose which showed that H-3 and C-3 resonance of β-GlcNAc(S-3) were appeared at δ 3.530 and δ 82.062 i.e. in the linkage region. Thus β -Gal (S-4) was linked to β -GlcNAc (S-3) by (1→3) linkage. Further another anomeric proton signal in the spectrum of compound Ovisose appeared at δ5.270 as a doublet of 3.4 Hz was due to the presence of α-Gal (S-5) as a fifth monosaccharide present in the chain. This downfield shift of the α-Gal (S-5) H-1 has resemblance with the pattern of ^{1} H NMR data reported by Prasoon et al except only with α-Gal instead of β – Gal. which suggested that this α-Gal (S-5) was glycosidically linked to 1→4 position of β-GlcNAc(S-3). This was further confirmed by ^{1}H and ^{13}C NMR spectrum of acetylated compound Ovisose which showed H-4 and C-4 resonance of β-GlcNAc(S-3) at δ 3.731 and δ 76.02 respectively. The presence of one α-Gal (S-5) and β-Gal (S-4) residue at C-3 and C-4 position of β –GlcNAc(S-3) has caused the crowding and steric hindrance resulting in the downfield shift of α-Gal(S-5) H-1 resonance. Further the spectrum contained next anomeric proton doublet which appeared at δ 4.599(1H) (J=7.9 Hz) was due to the presence of β-GlcNAc(S-6) moiety which was next in sequence and was linked to galactose by glycosidic linkage. This was supported by presence of a singlet of amide methyl of N-acetyl Glucosamine at δ2.006 Hz. The chemical shift analogies of β GlcNAc 1→6 as reported by Dua et al suggested that this β -GlcNAc (S-6) was 1→6 linked to β-Gal (S-4).The presence of C-6 and H-6 signal of β -Gal(S-4) in ^{1}H and ^{13} C NMR spectrum of acetylated compound H at δ3.530 and δ72.069 also showed that this β –GlcNAc (S-6) was (1→6) linked to β -Gal (S-4).This was further confirmed by the absence of downfield shifted H-4 doublet of β -Gal (S-4), (a structure reporter group)**.** The next anomeric proton doublet present in the ^{1} H NMR spectrum of compound Ovisose at δ 4.462 (J=7.5Hz) reflects the presence of another β -Gal moiety (S-7) in the compound Ovisose.

The anomeric chemical shift of this β -Gal (S-7) was also identical with lactosyl β -Gal (S-2) as structure reporter group so this β -Gal (S-7) was linked to β-GlcNAc(S-6) at C-3 because β-GlcNAc (S-6) H-3 proton resonance appeared at δ3.876 in ^{1}H NMR spectrum of acetylated compound Ovisose i.e.in the linkage region. This was further supported by ^{13} C NMR data of acetylated compound Ovisose which shows C-3 resonance of β-GlcNAc (S-6) at δ73.77. Thus β -Gal (S-7) was linked to β -GlcNAc (S-6) by (1→3) linkage. The next anomeric proton signal in the spectrum of compound H appeared as doublet at δ 5.396 (J=3.7 Hz) was for α-Gal (S-8) present as eighth moiety in the compound Ovisose. The glycosidic linkage between this α-Gal (S-8) and β-GlcNAc (S-6) was established similarly as above linkage between α-Gal (S-5) and β-GlcNAc (S-3) was confirmed. This linkage was further confirmed by the ^{1}H and ^{13}C NMR spectrum of acetylated compound Ovisose which showed β-GlcNAc (S-6) H-4 and C-4 resonances at δ 3.505 and δ 81.972 respectively. So this α-Gal (S-8) was (1→4) linked to β-GlcNAc (S-6) .Further the spectrum contained anomeric proton doublet which appeared at δ4.679 (J=8.1) was due to the presence of β-Glc(S-9) moiety. The presence of H-6 proton resonance of β -Gal (S-7) in ^{1}H NMR spectrum of acetylated compound H at δ3.530and C-6 carbon resonance of β -Gal (S-7) in ^{13}C NMR spectrum of acetylated compound H at δ72.069 suggested that this Glucose (S-9) was linked to β -Gal (S-7) by (1→6) linkage. This was further confirmed by absence of downfield shifted H-4 doublet of β -Gal (S-7). Further another anomeric proton signal in ^{1}H NMR spectrum of compound H as doublet at δ 5.234 (J=3.9Hz) was assigned for one more α-Gal (S-10) moiety in the compound Ovisose.

^{13} C NMR data for compound Ovisose

Moieties	C-1	C-2	C-3	C-4	C-5	C-6
α-Glc (S-1)	δ90.50	δ72.46	δ72.70	δ79.79	δ71.39	δ61.28
β-Glc(S-1)	δ97.05	δ75.11	δ75.66	δ79.66	δ75.66	δ61.41
β-Gal (S-2)	δ99.36	δ72.25	δ73.83	δ71.39	δ76.08	δ72.05
β-GlcNAc(S-3)	δ104.21	δ57.01	δ76.63	δ75.66	δ76.08	δ61.41
β -Gal (S-4)	δ102.12	δ76.63	δ72.25	δ70.01	δ76.08	δ71.39
α–Gal(S-5)	δ92.01	δ73.83	δ72.46	δ71.39	δ72.70	δ61.28
β-GlcNAc(S-6)	δ104.21	δ57.01	δ79.66	δ76.08	δ76.00	δ6128
β-Gal (S-7)	δ104.21	δ76.08	δ72.05	δ71.39	δ76.08	δ69.85
α–Gal(S-8)	δ93.10	δ73.83	δ72.46	δ69.85	δ71.39	δ61.41
β-Glc(S-9)	δ97.05	δ75.11	δ75.66	δ79.66	δ76.08	δ61.28
α-Gal(S-10)	δ93.10	δ73.83	δ72.46	δ69.85	δ71.39	δ61.41

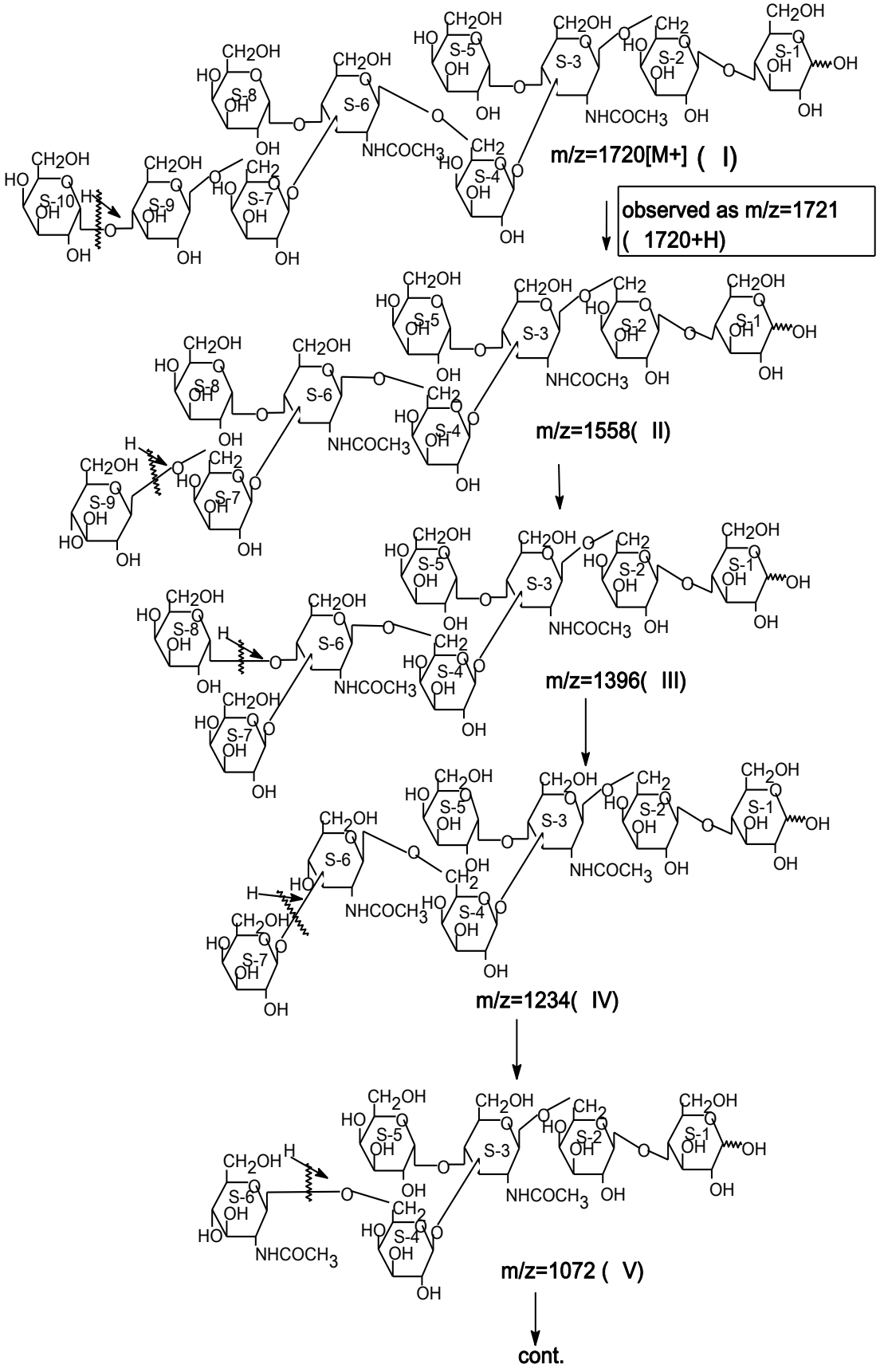
^{1}H NMR and^{13}C NMR values of anomeric carbons/protons of compound Ovisose

Moieties	C-1	H-1
α-Glc (S-1)	δ90.50	δ 5.234 (J=3.9)
β-Glc(S-1)	δ97.05	δ4.679 (J=8.1)
β-Gal (S-2)	δ99.36	δ 4.462 (J=7.5)
β-GlcNAc(S-3)	δ104.21	δ4.569 (J=8.0)
β -Gal (S-4)	δ102.12	δ 4.462 (J=7.5)
α–Gal(S-5)	δ92.01	δ 5.270 (J=3.4)
β GlcNAc(S-6)	δ104.21	δ4.599 (J=7.9)
β -Gal (S-7)	δ104.21	δ4.462 (J=7.5)
α–Gal(S-8)	δ93.10	δ5.396 (J=3.7)
β –Glc(S-9)	δ97.05	δ 4.679 (J=8.1)
α –Gal(S-10)	δ93.10	δ5.234 (J=3.9)

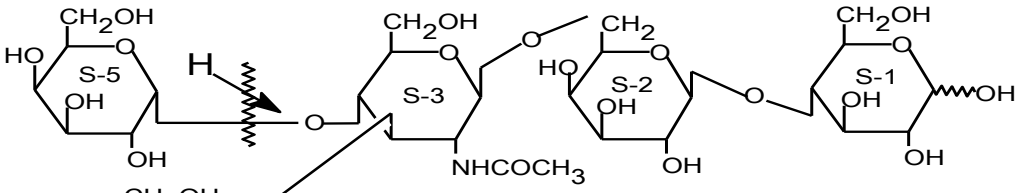
This downfield shift of α-Gal (S-10) anomeric proton signal suggested that this was present at non-reducing terminal of compound Ovisose. The pattern of ^{1} H NMR signals of this Gal (S-10) and adjacent Glc(S-9) sugar units were having the resemblance with ^{1} H NMR resonance of Lactose moiety differing only with α-glycosidic linkage of Gal moiety which was otherwise β. Further β-Glc(S-9) H-4 proton resonance at δ 3.698 and C-4 carbon resonance at δ75.014 in ^{1}H and ^{13}C spectra of acetylated compound H, confirmed that the β-Glc(S-9) was linked to α-Gal(S-10) by 1→4 linkage. The ^{13} C NMR′ data of compound Ovisose were also in confirmity with the derived structure. The compound Ovisose contains seven carbon signals for eleven anomeric carbon at δ 90.50(1C) [α-Glc(S-1)], δ 92.01(1C) [α-Gal(S-5)], 93.10(2C) [α-Gal(S-10) and (S-8)], δ 97.05(2C), [β-Glc(S-1), (S-9)], δ 99.36 (1C) [β Gal (S-2)], 102.12(1C) [β Gal (S-4)], 104.21(3C) [β Gal (S-7) β-GlcNAc (S-3), (S-6)], in the ^{13}C NMR of compound Ovisose.

The Hetero nuclear single quantum-coherence (HSQC) spectrum of acetylated compound Ovisose confirmed anomeric assignments in ^{1}H and ^{13}C NMR spectra of compound Ovisose by showing the cross peaks for anomeric carbon with their proton as α-Glc (S-1)H-1 & C1 at [δ5.387 **×** δ90.415] and β-Glc(S-1') H-1 & C-1 at [δ4.685 ×δ95.156]. It also contain cross peaks of β-Gal(S2) H-1 & C-1 at [δ4.506 × δ103.957], β GlcNAc(S-3) H-1 & C-1 at [δ 4.560 × δ101.56], β-Gal (S-4) C-1 & H1 at [δ 4.545 × δ 103.787], α–Gal(S-5) H-1 & C-1 at [δ 5.387 × δ90.415], β GlcNAc(S-6) H-1 & C-1 at [δ 4.748 × δ101.56], β-Gal (S-7) H-1 & C-1 at [δ 4.545 × δ 103.957], α–Gal(S-8) H-1 & C-1 at [δ 5.387 × δ90.415],β –Glc(S-9) H-1 & C-1 at [δ 4.685 × δ95.692], and α –Gal(S-10) H-1 & C-1 at [δ 5.300 × δ91.982] respectively. The glycosidic linkages were assigned by the cross peaks for glycosidically linked carbons with their protons in (HSQC). The values of these cross peaks are as- α-Glc(S-1) H-4 and C-4 at [δ 3.775 × δ 75.66] shows (1→4) linkage, β –Glc(S-1) H-4 and C-4 at [δ 3.698× δ 75.014] shows(1→4) linkage, β-Gal (S-2) H-6 and C-6 at [δ 3.876 × δ 71.886] shows (1→6) linkage, β GlcNAc(S-3) H-3 and C-3 at [δ3.530 × δ82.062]shows(1→3) linkage and also it's H-4 and C-4 at [δ 3.731 × δ 76.02] shows(1→4) linkage , β-Gal (S-4) H-6 and C-6 at [δ 3.530 × δ 72.069] shows (1→6) linkage, β GlcNAc(S-6) H-3 and C-3 at [δ3.876 × δ73.77] shows(1→3) linkage and also it's H-4 and C-4 at [δ 3.505 × δ 81.972] shows(1→4) linkage , β-Gal (S-7) H-6 and C-6 at [δ 3.530 × δ 72.069] shows (1→6) linkage, β –Glc(S-9) H-4 and C-4 at [δ 3.698× δ 75.014] shows(1→4) linkage. The sequence of the monosaccharide in compound Ovisose was confirmed by the results obtained from chemical transformation, chemical degradation, acid hydrolysis and comparative NMR study of compound with the literature value of known compound. All the assignment made in ^{1}H NMR was confirmed by ^{1}H-^{1}H HOMOCOSY, TOCSY and HSQC experiments. The results obtained from the Electrospray mass spectrometry further substained the structure of compound Ovisose, which was derived by its ^{1}H, ^{13}C NMR spectra. The composition of molecular size of compound Ovisose was confirmed by the highest mass ion peak recorded at m/z=1720(I) observed at m/z=1721 (M+H). Further the mass fragments were formed by repeated H transfer in the oligosaccharide and was accompanied by the elimination of terminal sugar less water (scheme-1). This fragmentation path way confirmed the sequence of monosaccharides in the oligosaccharide. The Decasaccharide fragmented to give mass-ion peak at m/z=1558(II), which was due to the loss of S-10 sugar unit i.e. Gal(S-10) sugar unit linked to S-9. Supported by its respective fragment at m/z=180, that confirmed the presence of Gal (S-10) at the non-reducing end.

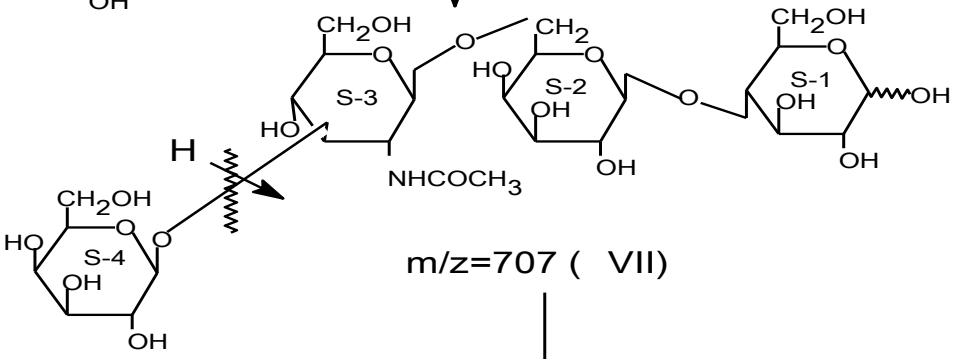
Scheme-8



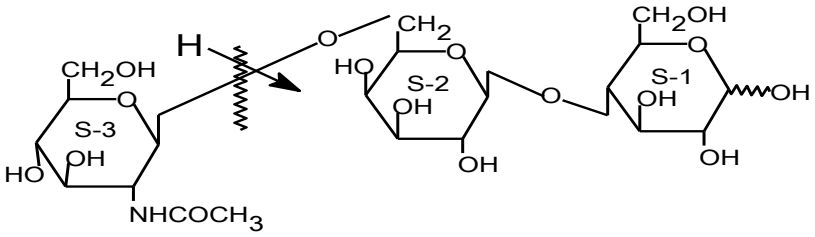
Scheme- 8 Cont.



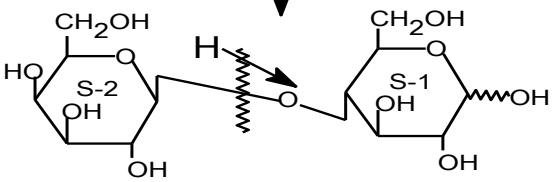
m/z=869 (VI)



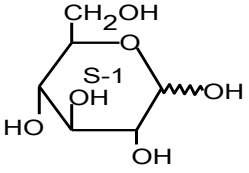
m/z=707 (VII)



m/z=545 (VIII)

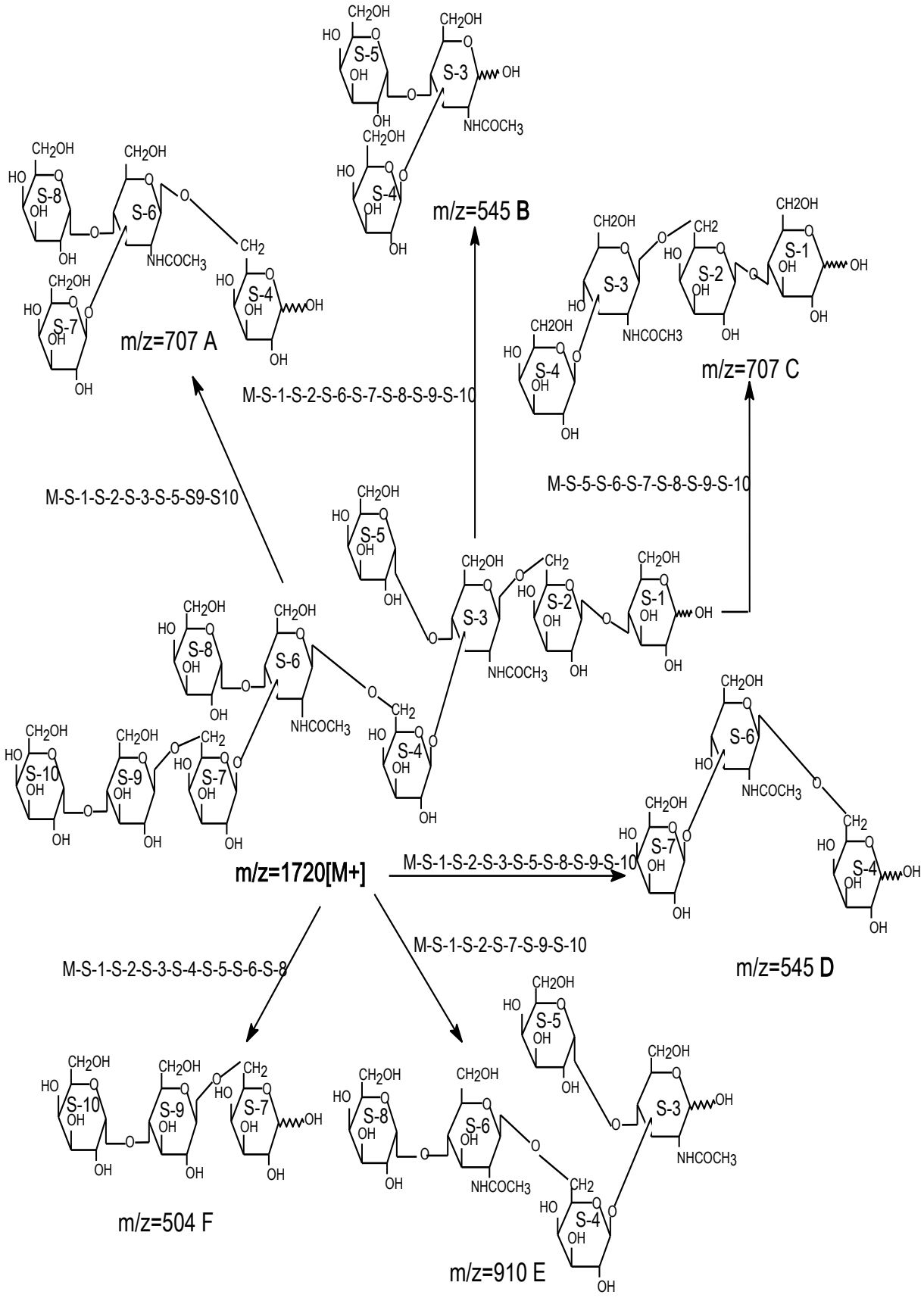


m/z=342 (IX)

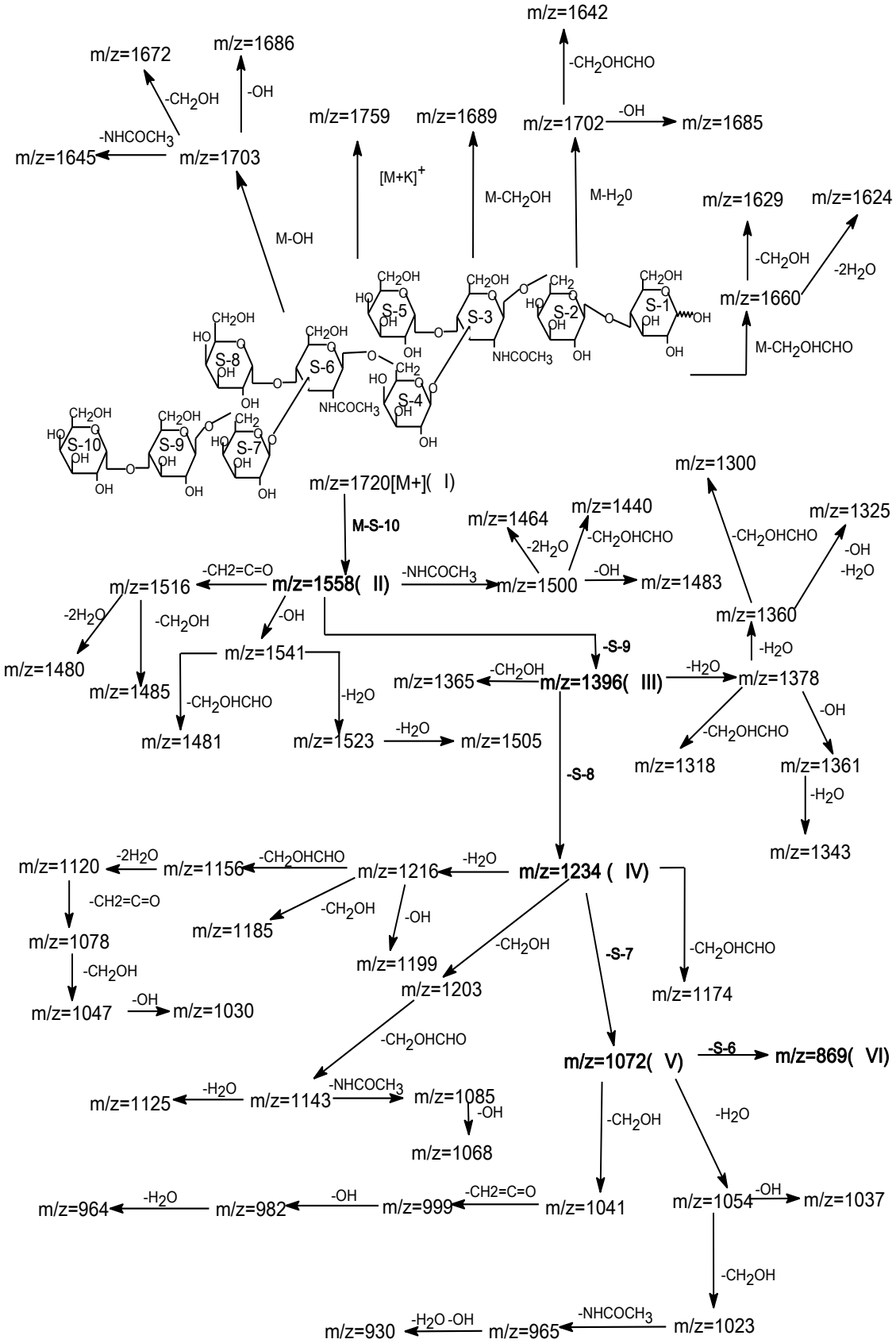


m/z=180 (X)

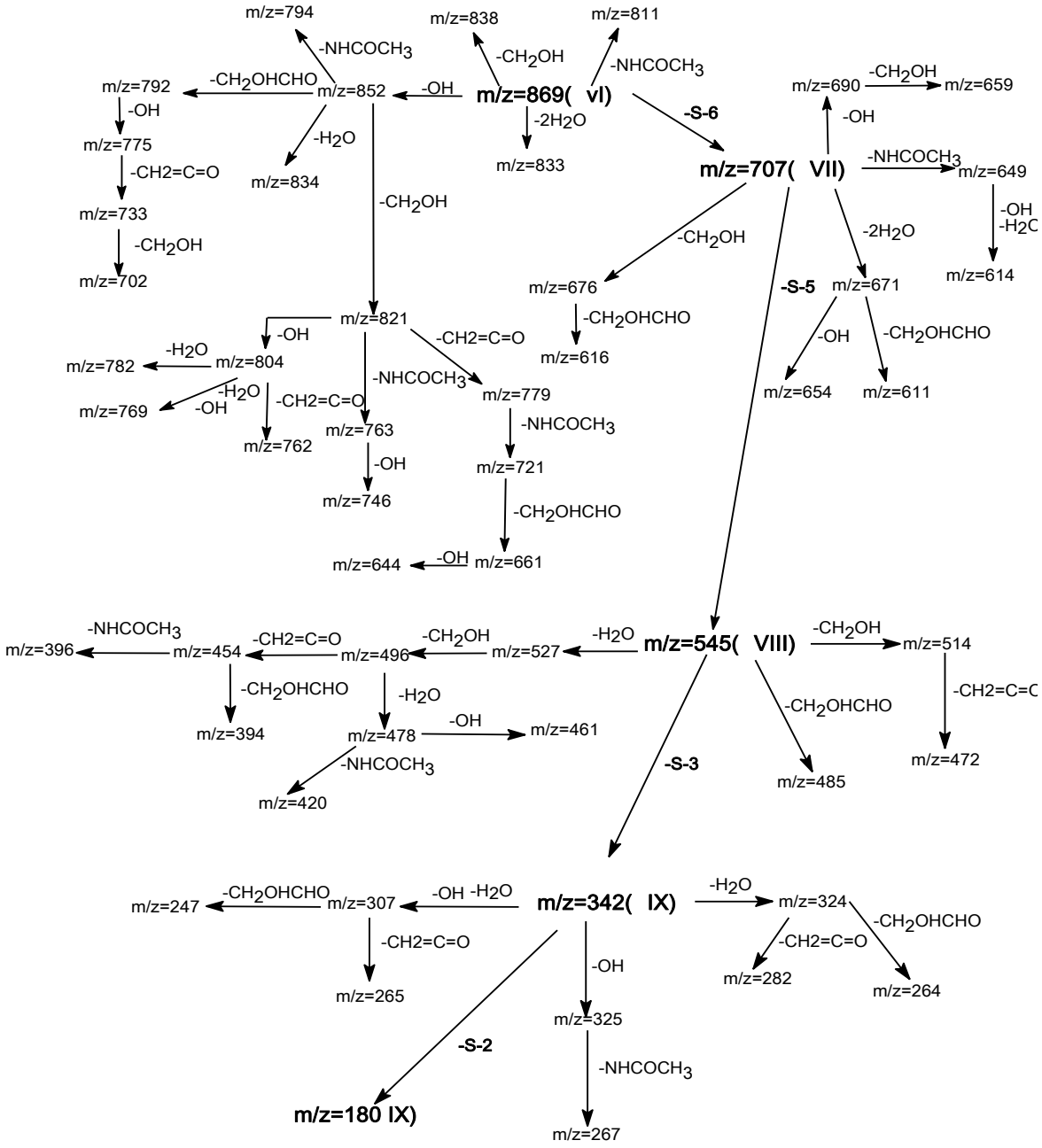
Scheme-9


m/z=545 B
m/z=707 A
m/z=707 C
m/z=1720[M+]
m/z=545 D
m/z=504 F
m/z=910 E
M-S-1-S-2-S-6-S-7-S-8-S-9-S-10
M-S-5-S-6-S-7-S-8-S-9-S-10
M-S-1-S-2-S-3-S-5-S9-S10
M-S-1-S-2-S-3-S-5-S-8-S-9-S-10
M-S-1-S-2-S-7-S-9-S-10
M-S-1-S-2-S-3-S-4-S-5-S-6-S-8

Scheme-10


m/z=1642
m/z=1672
m/z=1686
-CH2OHCHO
-CH2OH
-OH
m/z=1759
m/z=1689
m/z=1702
-OH
m/z=1685
-NHCOCH3
m/z=1645
m/z=1703
[M+K]+
M-CH2OH
M-H20
m/z=1629
m/z=1624
M-OH
-CH2OH
-2H2O
m/z=1660
M-CH2OHCHO
S-1
S-2
S-3
S-4
S-5
S-6
S-7
S-8
S-9
S-10
NHCOCH3
m/z=1720[M+](I)
M-S-10
m/z=1300
m/z=1464
m/z=1440
m/z=1325
-2H2O
-CH2OHCHO
-CH2OHCHO
-OH
-H2O
-CH2=C=O
m/z=1516
m/z=1558(II)
-NHCOCH3
m/z=1500
-OH
m/z=1483
m/z=1360
-2H2O
-CH2OH
-OH
-H2O
m/z=1480
m/z=1541
-S-9
m/z=1485
m/z=1365
-CH2OH
m/z=1396(III)
-H2O
m/z=1378
-CH2OHCHO
-H2O
-CH2OHCHO
-OH
m/z=1481
m/z=1523
-H2O
m/z=1505
m/z=1318
m/z=1361
-S-8
-H2O
m/z=1343
m/z=1120
-2H2O
m/z=1156
-CH2OHCHO
m/z=1216
-H2O
m/z=1234 (IV)
-CH2=C=O
m/z=1185
-CH2OH
-OH
-CH2OH
-S-7
-CH2OHCHO
m/z=1078
m/z=1199
m/z=1203
m/z=1174
-CH2OH
m/z=1047
-OH
m/z=1030
-CH2OHCHO
m/z=1072(V)
-S-6
m/z=869(VI)
m/z=1125
-H2O
m/z=1143
-NHCOCH3
m/z=1085
-OH
-CH2OH
-H2O
m/z=1068
m/z=964
-H2O
m/z=982
-OH
m/z=999
-CH2=C=O
m/z=1041
m/z=1054
-OH
m/z=1037
-CH2OH
m/z=930
-H2O -OH
m/z=965
-NHCOCH3
m/z=1023

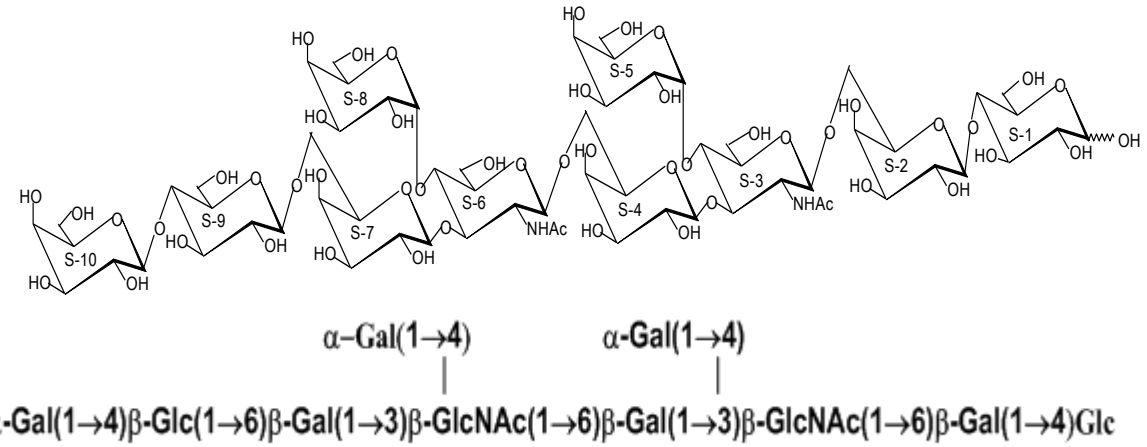
Scheme-10 Cont.



The nonasaccharide mass ion fragment at m/z=1558 fragmented by the loss of other terminal sugar i.e. Glc (S-9), gave the corresponding octasaccharide mass ion fragment (III) at m/z=1396. The mass ion peak at m/z=1396 further fragmented to give mass ion fragment for Heptasaccharide moiety which was arise by loss of sugar (S-8) [Gal]. It was accounted by the mass ion fragment at m/z=1234 (IV). This heptasaccharide mass ion fragment on further fragmentation gave an important Hexasaccharide segment (V) at m/z=1072, by loss of sugar (S-7) [Gal]. The hexasaccharide mass ion fragment m/z=1072 [(IV)-S-7] fragmented by the loss of next sugar i.e. GlcNAc (S-6), gave the corresponding pentasaccharide mass ion fragment (VI) at m/z=869.

The mass ion peak at m/z=869 further fragmented to give mass ion fragment for tetrasaccharide moiety which was arise by loss of sugar (S-5) [Gal]. It was accounted for the mass ion fragment at m/z=707 (VII). This tetrasaccharide mass ion fragment on further fragmentation gave an important trisaccharide segment (VIII) at m/z=545, by loss of sugar (S-4) [Gal]. The trisaccharide segment at m/z=545 was comprised of the LNT sequence. This on further fragmentation gave a disaccharide segment (IX) at m/z= 342 by loss of sugar (S-3) [β GlcNAc], which on further fragmentation give a monosaccharide fragment (X) at 180 by loss of sugar (S-2) [β-Gal].

The decasaccharide mass ion peak at m/z=1720 in the spectrum of compound Ovisose also showed other supporting mass ion peaks which are shown in sceme-10. The anchoring nature of Sugar-6 (GlcNAc) in the compound Ovisose was confirmed by the mass ion fragment A at m/z=707 due to (S-4, S-6, S-7, S-8). Further fragment B at m/z=545 (S-3,S-4,S-5), Fragment C at m/z=707(S-1.S-2,S-3,S-4) , Fragment D at m/z=545 (S-4,S-6,S-7), Fragment E at m/z=504 (S-7, S-9, S-10) and fragment F at m/z=895 [(S-8, S-6, S-5, S-4, S-3) $+H^+$] also supported the sequence of sugar moieties and the anchoring nature of sugar-9 (Scheme-9). Base on the results obtained from chemical degradation and chemical, transformation, mass spectrometry and ^1H,^{13}C NMR and 2D spectroscopy, the structure and sequence of isolated oligosaccharide was deduced as-



Ovisose

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