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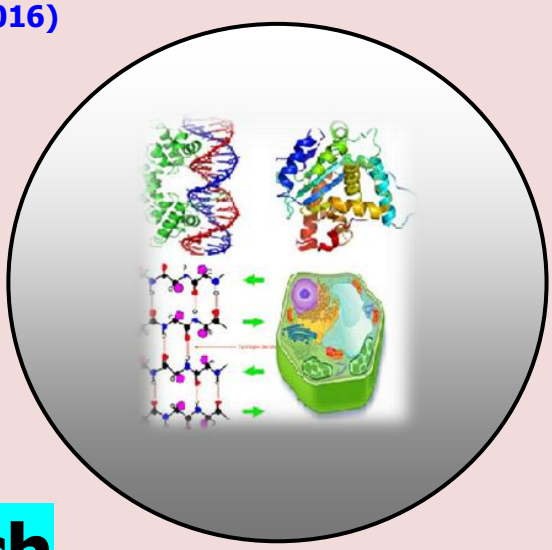

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RESEARCH PAPER

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Isolation of Novel Milk Oligosaccharide as Biologically Active Component

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ABSTRACT

Milk is rich source of biologically active oligosaccharides which exhibit the various inhibitory effects on certain virulence related abilities of monocytes, lymphocytes and nuetrophylls adhesion to endothelial cells. Milk oligosaccharides are known to be protected breast fed infants from host bacterial infections. A number of biologically active oligosaccharides have been isolated from Milk of various mammalian species, having numerous biological activities like anti-complementary, anti-inflammatory, anti-cancer, anti-coagulant, anti-microbial and immune-stimulant activity etc.Mare milk oligosaccharides are plentiful of fat resembling substances that contribute in transfer of certain nerve impulses and the regulation of blood pressure. Mare milk is also used as antioxidant, lipid lowering agent and mineral absorption regulating agent. In this present study, to find biologically active novel milk oligosaccharides, Mare milk was processed by the method of Kobata and Ginsburg followed by gel filtration HPLC and column chromatography which resulted in the isolation of a novel milk oligosaccharide named Labiose. The structure of isolated and purified milk oligosaccharide was elucidated with the help of chemical degradation, chemical transformation, spectroscopic techniques like NMR (^{1}H, ^{13}C and 2D NMR) and mass spectrometry. The isolated oligosaccharide (Labiose) was interpreted as heptasaccharide having branched structure as-

LABIOSE

$$\begin{array}{c} \alpha Gal(1\rightarrow4) \\ \uparrow \\ \alpha Gal(1\rightarrow4)\beta Glc(1\rightarrow6)\beta Gal(1\rightarrow3)\beta GlcNAc(1\rightarrow6)\beta Gal(1\rightarrow4)Glc \end{array}$$

Keywords: Biologically active oligosaccharides, monocytes, lymphocytes and nuetrophylls adhesion.

INTRODUCTION

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 of various origin has proven themselves as a source for

biologically active oligosaccharides which are major constituent of every milk are known to be protected breast fed infants from host bacterial infections (Millar et al 1994.,). Numerous oligosaccharides have been isolated from milk of many mammalian species including equine, bovine and marine mammals (Kunz et al., 2000 Urashima et al., 2001, Nakamura et al., 2004, and Urashima et al., 2008,). The milk oligosaccharides recognize cancer associated antigens, used as antimicrobial agents, tumour associated antigens and has physiological significance in infants (M.Singh et al 2016.,). 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.,). Camel milk has proven beneficial in lung ailments and treatment of tuberculosis (A.A Akhundov et al 1972). Sheep milk is very useful to aggravates 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). The oligosaccharide isolated from elephant milk contained a ratio of sialyl oligosaccharides; this may be significant with respect to the formation of brain components such as gangliosides of suckling calves (Ostho et al 2007). N-Acetylnueraminlactose sulphate is the dominant oligsaccharide in the dog milk (Bubb et al 1999,) play an important role in the nutrition of the rat pups. Mare milk oligosaccharide fractions are having multifold properties such as anti oxidant and lipid lowering activities (Srivastav et al 2012). We worked on isolation of novel milk oligosaccharides from Mare milk .In the present study, we are elucidating the structure of mare milk oligosaccharide named Labiose with the help of chemical degradation, chemical transformation, spectroscopic techniques like (^{1}H NMR,^{13}C NMR and 2D NMR).

MATERIAL AND METHODS

General procedure

Optical rotations were measured with a PERKIN-ELMER 241 automatic polarimeter in 1cm tube. ^{1}H and ^{13}C NMR spectra of oligosaccharides were recorded in D_2O and the spectra of acetylated oligosaccharides were recorded in $CDCl_3$ at 25^0C on a Bruker AM 300 FT NMR spectrometer. The electrospray mass spectra were recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. The sample (dissolved in suitable solvents such as methanol/acetonitrile/water) was introduced into the ESI source through a syringe pump at the rate 5µl per min. The ESI capillary was set at 3.5 KV and the cone voltage was 40 V. The spectra were collected in 6s scans and the print outs are averaged spectra of 6-8 scans. The C, H and N analysis were recorded on CARLO-ELBA 1108 an elemental analyzer. The sugars were visualized on TLC with 50% aqueous H_2SO_4 reagent and on Paper Chromatography 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). PC was performed on Whatman No.1 filter paper using solvent system ethyl acetate-pyridine (2:1) saturated with H_2O. Sephadex G –25 (PHARMACIA) was used in gel permeation chromatography. Freeze drying of the compound was done with the help of CT 60e (HETO) lyophylizer and centrifuged by a cooling centrifuged Remi instruments C-23 JJRCI 763. To check the homogeneity of the compounds reverse phase HPLC system was used equipped with Perkin Elmer 250 solvent delivering system, 235 diode array detector and G.P. 100 printer plotter. Authentic samples of glucosamine, galactosamine, galactose and glucose were purchased from Aldrich Chemicals.

Isolation of Mare milk oligosaccharide by Kobata and Ginsberg method

10 litres milk was collected from a Mare and then isolated by method of Kobata and Ginsberg method (Kobata 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 (S.M. Partridge 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 (M. Dubois et.al. 1956) for the presence of neutral sugar.

Acetylation of Mare milk oligosaccharide mixture

Dry oligosaccharides of pooled fractions (13gm) which gave positive phenol-sulphuric acid test were acetylated with pyridine (13ml) and acetic anhydride (13ml) at 60° C for 24 hr. The mixture was evaporated under reduced pressure and viscous residue was taken in $CHCl_3$ and washed in sequence with 2 N HCl, ice cold 2N $NaHCO_3$ and finally with H_2O. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding the acetylated mixture (16.5g). Non-polar acetyl derivative of oligosaccharides were resolved nicely on TLC using $CHCl_3$: MeOH as eluent. Detection of the spots was done by spraying with 50% H_2SO_4 and heat.

Purification of Acetylated milk oligosaccharide on Silica Gel Column

Separation and purification of acetylated derivative were carried over silica gel column chromatography into compounds : silica ratio of 1:100 using various proportion of Hexane: $CHCl_3$,$CHCl_3$, $CHCl_3$:MeOH mixture which was resolved into twelve fractions namely I(468mg), II(736mg), III(468mg), IV(2.008gm), V(280mg), VI(3.28gm), VII(1.06gm), VIII(467mg), IX(318mg), X(437mg), XI(767mg) and XII(324mg) respectively. These fractions were containing mixture of two to three compounds. Repeated column chromatography of fraction VI led to the isolation of one chromatographically pure compound "J" (61mg).

Deacetylation of Compound

Deacetylation of acetylated oligosaccharide "J" (61mg) was carried out in 2ml acetone and 1.3ml NH_3 for 24hrs in a stoppered hydrolysis flask. After 24hrs ammonia was removed under reduced pressure, equal volume of $CHCl_3$ and water were added and the compound was recovered in the aqueous phase and the water layer was finally freeze dried giving the deacetylated oligosaccharide Labiose (52mg).

Description of Isolated Compound Labiose

Substance j (61mg) obtained from fractions 63-68 of column chromatography-2, 41-46 of column chromatography-3 on deacetylation with NH_3/ Acetone, it afforded substance J (52mg) as a viscous mass. $[\alpha]_D$ + 68.6^0 (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.

$C_{44}H_{75}O_{26}N$	%C	%H	%N
Calad	44.26	6.29	1.17
Found	44.25	6.28	1.16

It gave positive Phenol-sulphuric acid test, Feigl test, morgon-Elson test

δ in D_2O (ppm) :^{1}H NMR

2.00 [s, 3H, $NHCOCH_3$, β-GlcNAc (S-3)], 3.324 [t, 2H, J=7.8 Hz, β-Glc(S-1, S-6), H-2], 3.941[d, 2H, J=2.7 Hz, β-Gal (S-2 and S-4), H-4], 4.4615 [d, 2H, J=7.8 Hz, β-Gal (S-2 and S-4), H-1], 4.5615[d, 1H, J=7.5 Hz, β-GlcNAc (S-3), H-1], 4.6745 [d, 2H, J=8.1 Hz, β-Glc (S1, S-6), H-1], 5.233[d, 2H, J=3.6 Hz, α-Glc (S-1), H-1 and α-Gal (S-7), H-1], 5.2501[d, 1H, J=2.7 Hz, α-Gal (S-5), H-1]

δ in D_2O (ppm) : ^{13}C NMR

21.2, 56.6, 60.5, 61, 68.5, 69.2, 69.9, 70.7, 70.9, 71.1, 71.3, 71.7, 71.8, 72.3, 72.5, 73.8, 74.3, 74.8, 75.3, 76.2, 78.1, 78.3, 78.4, 79.1, 80.8, 91.8, 92.01, 93.1, 95.7, 96.5, 100.9, 102.9, 173.

^1H NMR Values of acetylated compound J in $CDCl_3$

δ1.978-2.191 (NHCOC$\underline{H_3}$), δ 4.5395 [β-Gal (S-2) H-1], δ 4.653 [β-Glc (S1 and S-6), H-1], δ 4.731[β-GlcNAc (S-3), H-1], δ 4.7045[β-Gal (S-4) H-1], δ 5.387 [α-Glc (S-1), H-1 and α-Gal (S-5), H-1], δ 5.2521[α-Gal (S-7), H-1].

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

20.70, 20.83, 20.94, 21.08, 21.16, 60.57, 60.92, 61.90, 62.42, 67.06, 67.32, 67.58, 68.87, 69.45, 69.58, 70.95, 71.38, 71.53, 71.83, 72.08, 72.18, 72.86, 73.03, 73.28, 74.49, 74.67, 74.89, 75.05, 80.3, 81.3, 81.8, 82.2, 82.3, 82.9, 90.3, 92.1, 95.4, 95.5, 102.0, 102.1, 170.1, 170.3, 170.7, 170.8, 171.1, 171.5 and 171.7.

ES-MS PEAKS (m/z)

m/z 1232 $[M+K]^+$ and other fragments at m/z 1176,1175,1162, 1115, 1102, 1100, 1073, 1031, 1014, 1013, 995, 989, 983, 978, 973, 960, 958, 954, 942, 940, 922, 898, 869 , 851, 838, 833, 820, 803, 796, 791, 773, 707, 690, 689, 676, 671,658, 649, 647, 634, 630, 545, 509, 472, 467, 451, 342, 325,324, 300 ,293, 289, 240, 223 and 180.

RESULT AND DISCUSSION

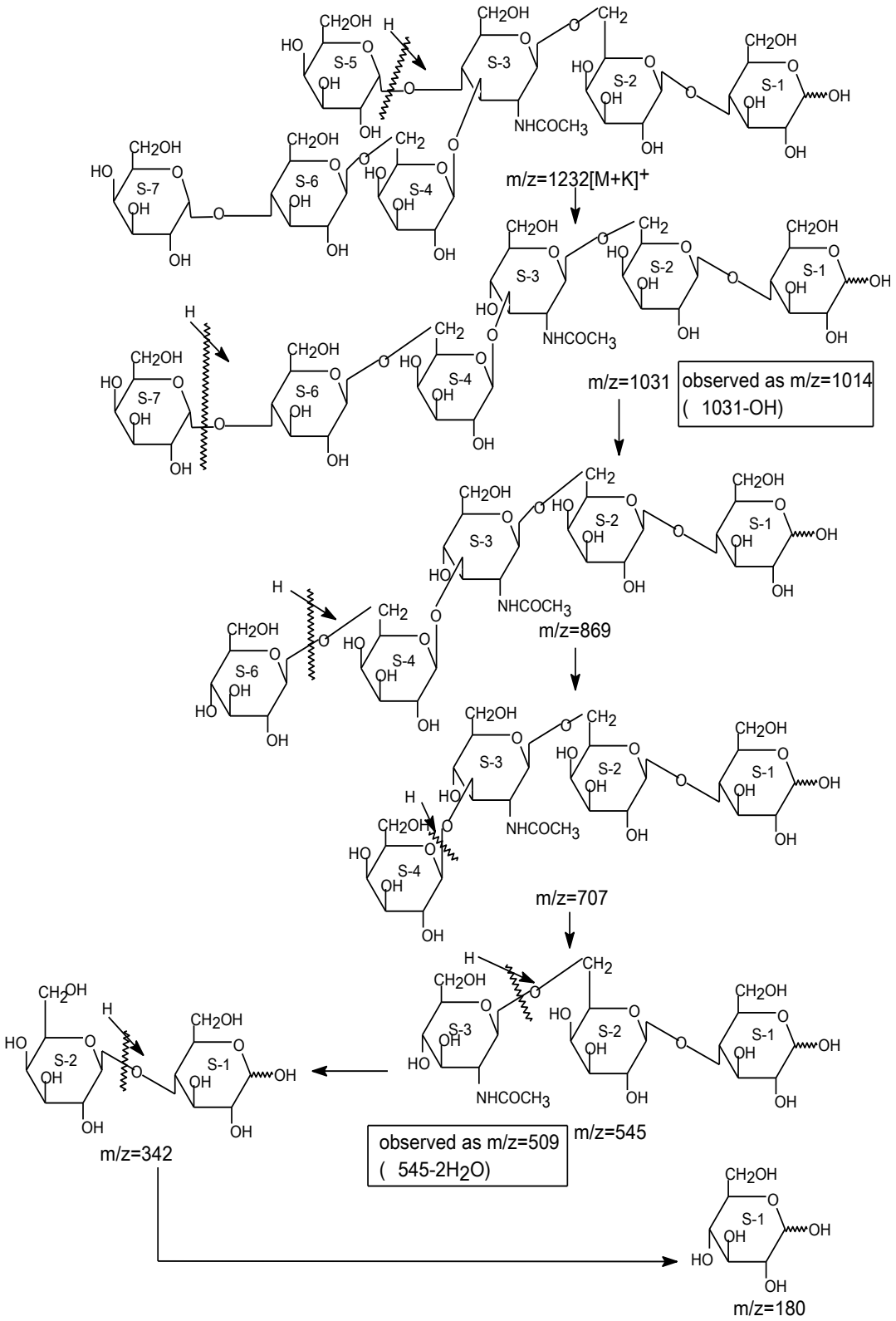
Compound J, $C_{44}H_{75}O_{36}N_1$ $[\alpha]_D$ $+68.6^0$ (c,4 ,H_2O)gave positive phenol sulphuric acid test, Fiegl test and Morgon-Elson test showing the presence of normal and amino sugar moiety in the compound J. ^1H NMR spectra of compound J showed eight anomeric proton signals appeared as five doublets at δ 5.2501(1H), δ5.233(2H), δ4.6745(2H), δ 4.5615(1H), δ4.4615(2H). These eight anomeric protons could be interpreted for presence of a heptasaccharide in its reducing form. The heptasaccharide nature of compound J was further confirmed by seven anomeric carbon signals for eight anomeric carbon at δ 91.8(1C), δ92.01(1C),93.1 (1C) 95.7(1C), 96.5(1C), 100.9(1C), 102.9(2C) in the ^{13}C NMR spectrum of compound J. The seven monosaccharides present in compound J have been designated as S-1, S-2, S-3, S-4, S-5, S-6, and S-7 for convenience. The monosaccharide constituents in compound J were confirmed by Killiani hydrolysis under strong acidic conditions, followed by paper chromatography and TLC. In this hydrolysis, three spots were found on TLC which were identical with Glucose, Galactose and GlcNAc by co-chromatography with authentic samples. Thus this heptasaccharide contained three types of sugar moieties. The reducing nature of compound J was confirmed by methylglycosylation of compound J by $MeOH/H^+$ followed by its acid hydrolysis which resulted into the formation of α and β methyl glucosides, suggesting that the Glucose was present in it's reducing form. The reducing nature of Glucose was further confirmed by the presence of two anomeric proton signals at δ5.233 (J=3.6Hz) and δ4.6745 (J=8.1Hz) for α and β-Glucose. Now the presence of anomeric doublet at δ4.4615 (J=7.8 Hz) in the ^{1} H NMR spectrum of compound J showed the presence of β–Gal residue as a 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) which appeared as a triplet at δ 3.324(J=7.8 Hz) in the ^{1} H NMR spectrum of compound J. Further third anomeric proton doublet which appeared at δ 4.5615 (1H) (J=7.5 Hz) was due to the presence of β–GlcNAc moiety linked to Lactose. This was supported by presence of a singlet of amide methyl of N-acetyl Glucosamine at δ2.00 Hz. The absence of the downfield shifted H-4 proton resonance of β–Gal (S-2) confirmed that this β–Gal(S-2) was not substituted at C-3 position by GlcNAc moiety (a structure reporter group). This implies that the β–GlcNAc may be 1→6 linked to β-Gal, this was assigned by ^{1}H NMR COSY and TOCSY spectrum of acetylated compound J which shows β–Gal (S-2) H-6 signal at δ 3.858 and chemical shift analogies of β–GlcNAc 1→6 as reported by Dua et al also supported this linkage. It was further confirmed by ^{13} C NMR spectrum of acetylated compound J which showed C-6 resonance of β–Gal (S-2) at 71.83. Thus this β-GlcNAc(S-3) was linked to β-Gal (S-2) by (1→6) linkage.

The next anomeric proton doublet present at δ 4.4615 (J=7.8Hz) reflects the presence of another β–Gal moiety (S-4) in the heptasaccharide. The linkage between S-3 and S-4 was established on the basis of ^{1}H NMR COSY spectrum of acetylated compound J, which showed the presence of β–GlcNAc(S-3) H-3 proton, appearing at δ3.941, this was also confirmed by ^{13}C NMR data of acetylated compound J which showed that the C-3 resonance of β–GlcNAc appeared at δ73.03. Further anomeric proton chemical shift of this β–Gal (S-4) was identical with lactosyl β– Gal (S-2) as structure reporter group, thus this β–Gal (S-4) was linked to β–GlcNAc (S-3) by (1→3) linkage. Another anomeric proton signal appearing at δ5.2501 as a doublet of 2.7 Hz was due to the presence of α-Gal (S-5) as a fifth monosaccharide present in the chain of heptasaccharide. This downfield shift of the α-Gal (S-5) H-1 was derived from the comparison with ^{1}H NMR data given by Prasoon et al. only difference of α–Gal instead of β–Gal which suggested that α-Gal (S-5) was linked to β-GlcNAc (S-3) by (1→4) linkage which was further confirmed by ^{1}H and ^{13}C NMR spectrum of acetylated compound J which showed β-GlcNAc (S-3) H-4 and C-4 at δ 3.6588 and δ 80.3 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 H-1 resonance. The next moiety in the compound J was another β–glucose moiety which give it's anomeric proton signal as doublet at δ4.6745 (J=8.1) in the ^{1}H NMR spectrum of compound J. The presence of H-6 and C-6 resonance of β–Gal (S-4) at δ3.6425 and δ73.03 in ^{1}H and ^{13}C NMR spectrum of acetylated compound J and absence of downfield shifted H-4 chemical shift of β–Gal (S-4) (a structure reporter group), suggested that this β–Glucose (S-6) was linked to β–Gal (S-4) by (1→6) linkage. The next another anomeric proton signal as doublet at δ 5.233 (J=3.6 Hz) was assigned for one more α-Gal (S-7) moiety in the heptasaccharide. The pattern of ^{1} H NMR signals of this α-Gal (S-7) and adjacent β–Glc(S-6) 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. This was further confirmed by β-Glc(S-6) H-4 and C-4 resonance at δ 3.886 and δ73.03 in ^{1}H and ^{13}C NMR spectrum of acetylated compound J, which confirmed that the β-Glc(S-6) was linked to α-Gal(S-7) by (1→4) linkage. The ^{13} C NMR data of compound J were also in confirmity with the derived structure. The compound J contains six anomeric signals for eight anomeric carbon at δ102.9[β Gal (S-4), β-GlcNAc (S-3)], δ100.9[β Gal (S-2), δ96.5[β-Glc (S-6)], δ95.7[β-Glc(S-1)], δ 93.1 [α-Gal,(S-5)] δ92.01 [α-Gal,(S-7)] and δ91.8[α-Gal, α-Glc (S-1)]. The Hetero nuclear single quantum-coherence (HSQC) spectrum of acetylated compound J confirmed anomeric assignments in ^{1}H and ^{13}C NMR spectra of compound J by showing the cross peaks for anomeric carbon with their proton as α-Glc (S-1) H-1&C-1 at [δ5.387 × δ90.3] and β-Glc(S-1) H-1&C-1 at [δ4.653 ×δ95.4]. It also contain cross peaks of β-Gal(S-2) H-1&C-1 at [δ4.5395 × δ102.1], β GlcNAc(S-3) H-1&C-1 at [δ 4.731 × δ102], β-Gal (S-4) H-1&C-1 at [δ 4.7045 × δ 102], α–Gal(S-5) H-1&C-1 at [δ5.387 × δ90.30], β –Glc(S-6) H-1&C-1 at [δ 4.653 × δ95.5], and α –Gal(S-7) H-1&C-1 at [δ 5.2521 × δ92.1] respectively.

^{13}C NMR data for compound J

Moieties	**C-1**	**C-2**	**C-3**	**C-4**	**C-5**	**C-6**
α-Glc (S-1)	δ91.8	δ71.8	δ72.3	δ78.3	δ71.1	δ61
β-Glc(S-1)	δ95.7	δ74.3	δ75.3	δ78.4	δ75.3	δ60.5
β -Gal (S-2)	δ100.9	δ 70.9	δ73.8	δ68.5	δ75.3	δ71.7
β GlcNAc(S-3)	δ102.9	δ56.6	δ78.1	δ73.8	δ75.3	δ60.5
β -Gal (S-4)	δ102.9	δ70.9	δ79.1	δ 69.2	δ 74.8	δ61.0
α–Gal(S-5)	δ93.10	δ73.8	δ72.5	δ70.7	δ71.3	δ61.0
β –Glc(S-6)	δ96.5	δ74.3	δ75.3	δ78.4	δ76.2	δ69.9
α –Gal(S-7)	δ92.01	δ74.8	δ72.5	δ68.5	δ71.3	δ61

SCHEME-2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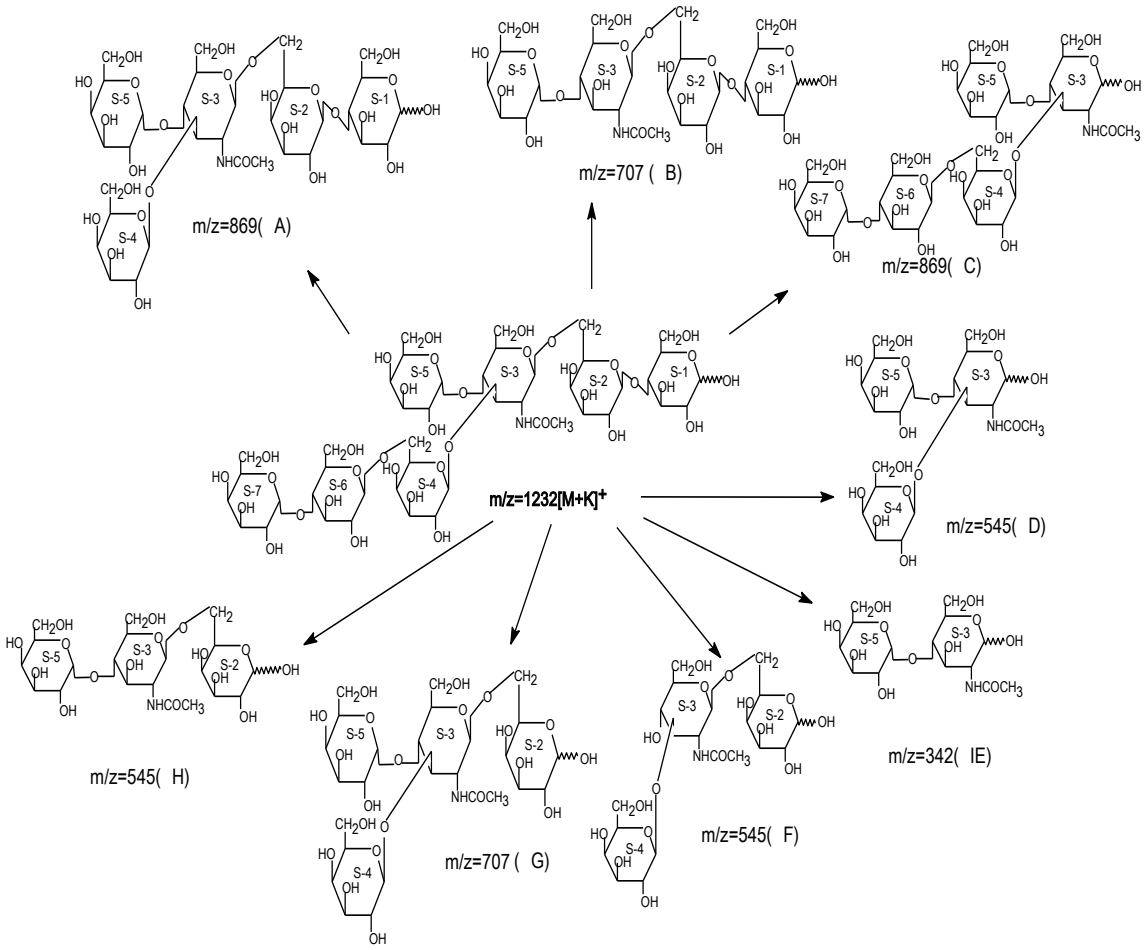
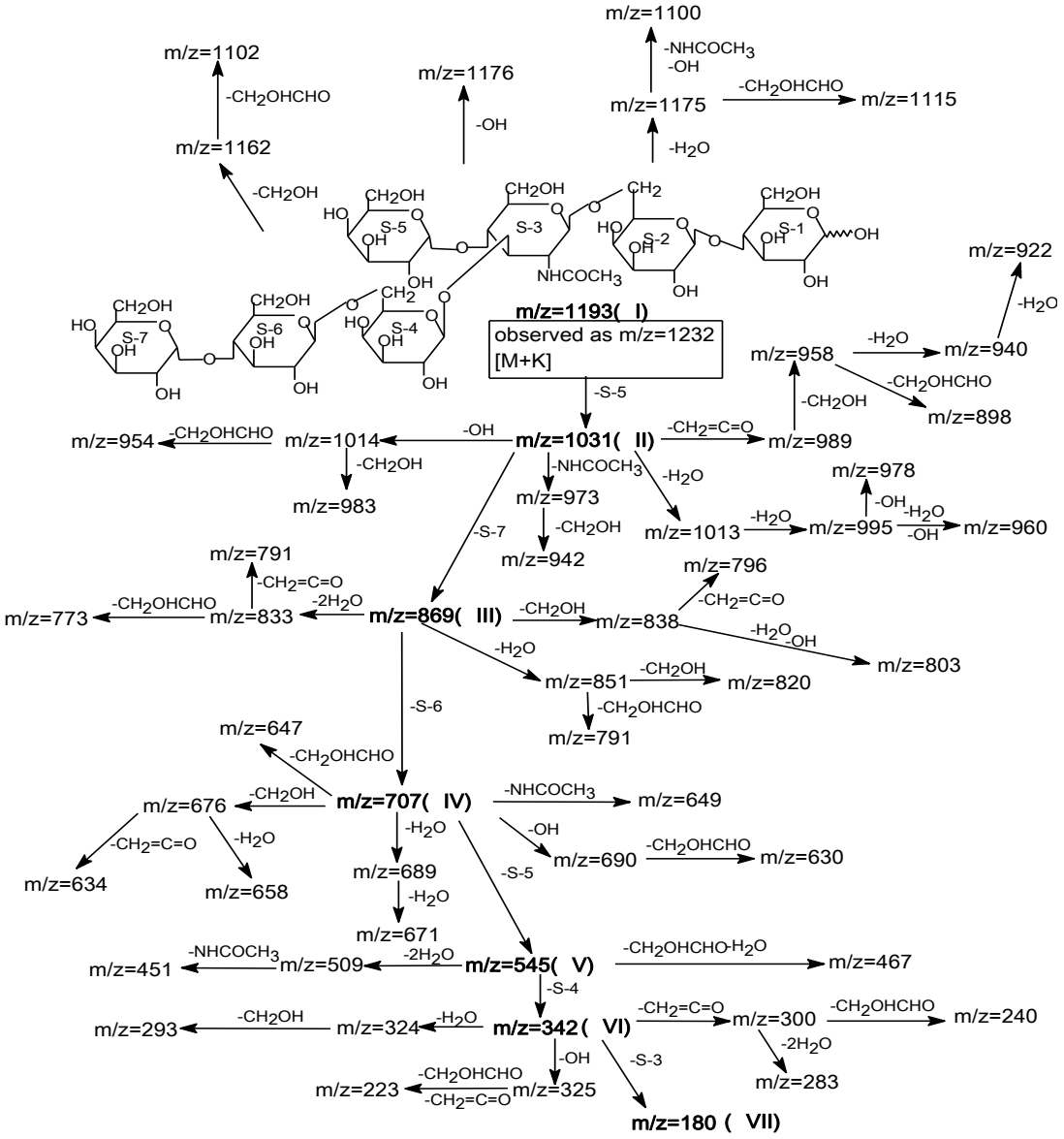


^{1}H and ^{13}C NMR anomeric assignments of compound J

Moieties	C-1	H-1
α-Glc (S-1)	δ91.8	δ 5.233 (J=3.6)
β-Glc(S-1)	δ95.7	δ4.6745 (J=8.1)
β -Gal (S-2)	δ100.9	δ 4.4615 (J=7.8)
β GlcNAc(S-3)	δ102.9	δ 4.5615 (J=7.5)
β -Gal (S-4)	δ102.9	δ 4.4615(J=7.8)
α–Gal(S-5)	δ93.10	δ 5.2501(J=2.7)
β –Glc(S-6)	δ96.5	δ4.6745(J=8.1)
α –Gal(S-7)	δ92.01	δ5.233(J=3.6)

The cross peaks for glycosidically linked carbons with their protons were also determined by HSQC. The values of these cross peaks are as- α-Glc(S-1) H-4 and C-4 at δ 3.6425× δ 80.3 shows (1→4) linkage, β –Glc(S-1) H-4 and C-4 at δ 3.7505× δ 81.3 shows(1→4) linkage, β-Gal (S-2) H-6 and C-6 at δ 3.858 × δ 71.83 shows (1→6) linkage, β GlcNAc H-3 and C-3at δ3.941× δ73.03 shows(1→3) linkage and also it's H-4 and C-4 at δ 3.6588× δ 80.3 shows(1→4) linkage , β-Gal (S-4) H-6 and C-6 at δ3.6425 and δ73.03shows (1→6) linkage, β –Glc(S-6) H-4 and C-4 at δ 3.886 and δ73.03 shows(1→4) linkage.

SCHEME-4



The sequence of the monosaccharide in compound 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 and TOCSY experiments. The results obtained from the Electrospray mass spectrometry further substained the structure of compound J, which was derived by its ^{1}H, ^{13}C NMR spectra. The composition of molecular size of compound J was confirmed by the mass ion peak at m/z=1232 which was due to $[M+K]^{+}$. Further the mass fragments were formed by repeated H transfer in the oligosaccharide and was accompanied by the elimination of terminal sugar less water. This fragmentation path way confirmed the sequence of monosaccharides in the oligosaccharide (scheme-1). The heptasaccharide fragmented to give mass –ion peak at m/z=1031, observed as m/z=1014 (1031-OH), which was due to the loss of S-5 sugar unit i.e. Gal(S-5) sugar unit. Supported by its respective fragment at m/z=180, that confirmed the presence of Gal (S-5) at the non-reducing end.

The hexasaccharide mass ion fragment m/z=1031 fragmented by the loss of other terminal sugar i.e. Gal (S-7), gave the corresponding pentasaccharide mass ion fragment (II) at m/z=869, which confirmed that two 'Gal' moieties were present in the heptasacchride moiety. The mass ion peak at m/z=869 further fragmented to give mass ion fragment for tetrasaccharide moiety which was arose by loss of sugar (S-6) [Glc]. It was accounted for the mass ion fragment at m/z=707 (IV). This tetrasaccharide mass ion fragment on further fragmentation gave an important trisaccharide segment (III) at m/z=545 observed as m/z=509 (545-2H_2O), by loss of sugar (S-4) [Gal]. The trisaccharide segment at m/z=545 was comprised of the LNT sequence which was suggested by NMR data. This on further fragmentation gave a disaccharide segment (V) at m/z= 342 by loss of sugar (S-3) [GlcNAc], which on further fragmentation give a monosaccharide fragment (VI) at 180 by loss of sugar (S-2) [Gal] (scheme-2). The mass spectrum of compound J also showed other mass ion peaks as heptasaccharide m/z=1193(I) fragment gave some other fragments on further fragmentation at m/z=1176 [M-OH], 1115[M-CH_2OHCHO,-H_2O], 1175 [M-H_2O], 1100 [M-H_2O, -OH,-$NHCOCH_3$], 1162 [M-CH_2OH], 1102 [M-CH_2OHCHO, - CH_2OH], 1073[M-H2O, -CH_2OHCHO, -CH_2=C=O], . The mass ion peak at m/z =1031(II) further fragmented to give important mass ion fragment at m/z 1014 [1031-OH], 954[1031-OH, - CH_3OHCHO], 983[1031- CH_2OH,-OH], 989[1031-CH_2=C=O], 958[1031- CH_2OH,-CH_2=C=O], 922[1031- CH_2OH,-CH_2=C=O,-2H_2O], 995 [1031-2 H_2O],978[1031-2 H_2O,-OH], 960[1031- 3 H_2O, -OH], 973 [1031-$NHCOCH_3$], 942 [1031- $NHCOCH_3$, - CH_2OH], 1013[1031-H_2O], 940[1031-CH_2=C=O, CH_2OH, -H_2O], 922[1031-CH_2=C=O,-CH_2OH, -2H_2O], 898[1031-CH_2=C=O,-CH_2OH, CH_2OHCHO]. The next ion fragment corresponds to the pentasaccharide mass ion fragment at m/z=869(III) appeared at 833[869-2 H_2O], 791[869- CH_2=C=O,-2H_2O], 773[869-2H_2O, - CH_2OHCHO], 820[869-H_2O,- CH_2OH], 838[869-CH_2OH], 803[869-CH_2OH, - H_2O, -OH], 796 [869- CH_2OH,-CH_2=C=O]. The tetrasaccharide mass ion m/z=707(IV) fragmented to give mass ion peaks at 697 [707-H_2O], 690[707-OH], 689[707-H_2O], 676[707-CH_2OH], 658[707-CH_2OH, -H_2O], 671 [707- H_2O], 647 [707-CH_2OHCHO], 649 [707- $NHCOCH_3$]. This tetrasaccharide mass ion fragment on further fragmentation gave an important trisaccharide segment m/z=545(V) which on further fragmentation give following fragments as- 509[545-2H_2O], 451[545-2H_2O, -$NHCOCH_3$], 467[M- H_2O, - CH_2OHCHO], which further gives other fragment 472[545- CH_2OH, - CH2=C=O]. The trisaccharide segment (V) on further fragmentation give next mass ion fragments at m/z=342(VI) which give following peaks as- m/z=325[342-OH], 289[342-2H_2O,-OH], 223[342-OH, -CH_2OHCHO], 324[342-H_2O], 293[342-H_2O,-CH_2OH,-CH_2=C=O], 300[342-CH_2=C=O], 240[342-CH_2=C=O, -CH_2OHCHO] (scheme-4). Further the mass ion fragment at m/z=869 which was due to the fragment A (S-5,S-4,S-3,S-2 and S-1) and Fragment C (S-3, S-4, S-5, S-6,S-7) and the next mass ion fragment at m/z=707 which was due to the fragment B (S-1,S-2,S-3 S-5) and Fragment at m/z=545 was due to the presence of fragment D(S-3,S-4, S-5) in scheme-3.All these fragments supported the sequence of sugar moieties in the heptasaccharide. The anchoring nature of Sugar-3 (GlcNAc) in the compound was confirmed by the mass ion fragment E (S-3,S-5)at m/z 342, fragment G (S-2, S-3, S-4, S-5) at m/z 707 & other mass ion fragment at m/z 545 due to fragment F (S-2,S-3,S-4) and Fragment H (S-2, S-3, S-5) also supported the anchoring nature of sugar-3 (scheme-3). Based on the results obtained from chemical degradation and chemical, transformation, mass spectrometry and ^{1}H,^{13}C NMR and 2D ^{1}H-^{1}H COSY , the structure and sequence of isolated oligosaccharide is deduced as-

LABIOSE

$$\begin{array}{c} \alpha Gal(1\rightarrow4) \\ \uparrow \\ \alpha Gal(1\rightarrow4)\beta Glc(1\rightarrow6)\beta Gal(1\rightarrow3)\beta GlcNAc(1\rightarrow6)\beta Gal(1\rightarrow4)Glc \end{array}$$

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