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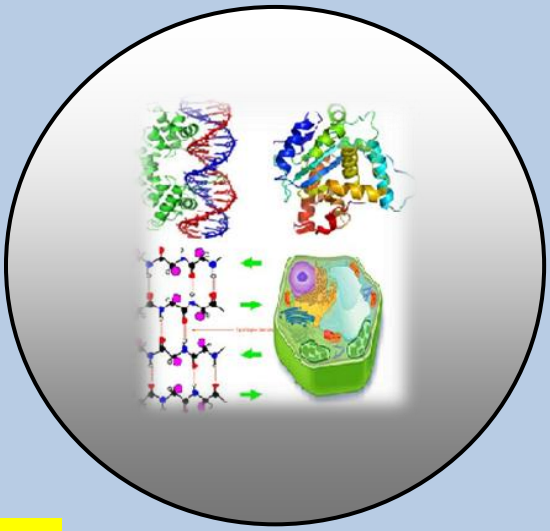

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RESEARCH PAPER

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Isolation of Novel Pentasaccharide Murtiose from Buffalo Colostrum

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ABSTRACT

Colostrum is a fluid produced by mammary glands of any mammal in early stage of lactation. It protects any newborn against infections and provides nutrients.It is a rich source of carbohydrate which includes oligosaccharides and glycoconjugates. The buffalo colostrum was collected in bulk and processed by modified method of Kobata and Ginsburg. The novel oligosaccharide was isolated from buffalo colostrum by a combination of gel filtration chromatography, silica gel column chromatography of derivatized oligosaccharides while their homogeneity was confirmed by HPLC.The structure elucidation of purified oligosaccharide was performed by chemical degradation, chemical transformation and physicochemical techniques like ^{1}H, ^{13}C, and 2D NMR (COSY, TOCSY, HSQC and HMBC) and ES mass spectroscopy. The structure of novel oligosaccharide was elucidated as-

β-Gal (1→3) -β-Gal(1→4)- Glc
α-GalNAc (1→3)-α-GalNAc(1→2)

Key words: Carbohydrate, Buffalo colostrum, Spectroscopy, Pentasaccharide and Murtiose.

INTRODUCTION

Colostrum is vital food secreted from mammary glands for the newborn of all mammals within the first 5-7 days after parturition. Colostrum is a rich source of various nutrients such as protein, fat, carbohydrate, water- and fat-soluble vitamins and minerals. It also contains various biologically active substances such as immunoglobulins, antimicrobial

factors, growth factors and others (Singh and Pathak, 2013, Elfstrand et al., 2002). Numbers of oligosaccharides have been isolated from the milk of many mammalians including equine, bovine, and marine mammals (Kunz, et al., 2000 and Urashima, et al., 2001, 2004, 2008). But no report for isolation of colostrum oligosaccharides is reported till date. Milk oligosaccharides serve as prebiotics to stimulate growth of beneficial intestinal bacteria such as bifidobacteria in neonates. Modulation of the postnatal immune system is a beneficial consequence because of the development of a balanced intestinal microbiota. Milk oligosaccharides have shown diverse biological activities such as anticancer immunostimulant, antitumor, anticancer, anti-inflammatory, hypoglycemic, anticomplementary, antiviral, and antimicrobial (Chihara, et al., 1969 and Liu, et al., 2007). Additionally, milk oligosaccharides have been reported to bind to certain pathogenic microorganisms thereby limiting their virulence. This behavior lowers the risk of diseases such as diarrhoea, meningitis, and otitis media in infants (Charles, et al., 2012). The distribution of different types of milk oligosaccharides (fucosyl oligosaccharides, sialyated oligosaccharides, fucosyl–sialyated oligosaccharides, and other normal lactosecontaining oligosaccharides) varies dynamically during different phases of lactation (Angelika, et al., 2009 and Kunz, et al., 2000). These milk oligosaccharides are responsible for immunologic effect and play potent role in inhibition of microorganism (Kunz, and Rudloff, 1993).

A board range of oligosaccharides and their derivatives act as an effective drug against most of acute and chronic disease. Oligosaccharides are essentially indigestible by the infant gut mucosa; therefore, their component sugars are not available for use as a macronutrient (Newburg and Neubauer 1995). Donkey milk oligosaccharides have ability to stimulant non - specific and specific immunological resistance (Deepak et al., 1998). Buffalo milk oligosaccharides has shown immunostimulant activity by using mouse model as revealed by increase in the hemagglutination titer, delayed type hypersensitivity reaction, and plaque-forming cell counts in mice (Saxena et al., 1999).

The enormous structural variability possible in oligosaccharides structure is the probable reason for their wide range of distribution and function, but structural diversity of oligosaccharides present a challenging analytical task because oligosaccharides of biological importance often exihibit branched covalent structure and dynamic conformational multiplicities. The information regarding structure and characteristics of the milk oligosaccharides of many mammalian species are well reported but no work on oligosaccharides of colostrum of buffalo is reported. Keeping in the mind, the biological activity of buffalo milk and importance of colostrum present research work includes isolation of novel oligosaccharides from colostrum of buffalo. The three dimensional structure of isolated novel oligosaccharide elucidated by chemical degradation, chemical transformation, and spectroscopic technique like ^{1}H, ^{13}C, and 2D NMR (COSY, TOCSY and HSQC, HMBC) and ES mass spectrometry.

MATERIAL AND METHODS

Collection and storage of buffalo colostrum - Twelve (12) liter of buffalo colostrum (1 to 5 day) was collected from a domestic buffalo (*Bubalus bubalis*) from the Kharika village near Telibagh of district Lucknow, Uttar Pradesh. The colostrum was fixed by addition of equal amount of ethanol (12 liter).

Isolation of colostrum oligosaccharides by modified method of Kobata and Ginsburg - Isolation of colostrum oligosaccharide was done by modified Kobata and Ginsburg method (Kobata and Ginsburg, 1970). The colostrum was fixed by addition of equal amount of ethanol. The preserved milk was taken to laboratory and there it was centrifuged for 30 min at 5000 rpm at - 4^{o}C. 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 ^{o}C. The white precipitate formed, mainly of lactose and protein was removed by centrifugation and washed twice with 68% ethanol at 0 ^{o}C. The supernatant and washing were combined and filtered through a microfilter and lyophilized affording crude oligosaccharides mixture.

Acetylation of oligosaccharide mixture- The oligosaccharides are very polar in nature hence there isolation is not an easy task therefore it was acetylated by acetic anhydride and pyridine for getting the oligosaccharides as its acetyl derivative. The acetylated oligosaccharide mixtures were purified by repeated column chromatography affording Compound (78 mg).

Deacetylation of compound - 50 mg compound was dissolved in acetone (2 ml) and NH_3 (3ml) was added to it and was left overnight in a stoppered hydrolysis flask. After 24h ammonia was removed under reduced pressure and the reaction was monitored on TLC after the completion of reaction. It was lyophilized compound was washed thrice with $CHCl_3$ (5 ml) (to remove acetamide) and theand water layer was finally freeze dried giving the deacetylated oligosaccharide (35 mg).

Methyl glycosidation/Acid hydrolysis of compound - Compound (8 mg) was refluxed 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. To the reaction concentrate 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^{o}C. The hydrolysis was completed after 24 h. The hydrolysate was neutralized with freshly prepared $BaCO_3$ and concentrated under reduced pressure to afford α-and β-methylglucosides along with the Glc, Gal, GalNAc and GlcNAc. Their identification was confirmed by comparison with authentic samples on TLC and PC.

Kiliani hydrolysis of compound -Compound (5 mg) was dissolved in 2 ml Kiliani mixture (AcOH-H_2O-HCl, 7:11:2) and heated at 100^{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 and was evaporated under reduced pressure to afford Glc, Gal, GalNAc and GlcNAc on comparison with authentic samples of Glc, Gal, GalNAc and GlcNAc (Killiani, 1930).

General procedure for structure elucidation -The ^{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^{0}C on a Bruker AM 300 and 400 FT NMR spectrometer. The electrospray mass spectra were recorded on a micromass quattro II triple quadruple 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 printouts are averaged spectra of 6-8 scans. The C, H and N analysis were recorded on elemental analyzer carlo-elba 1108.

The sugars were visualized on TLC with 30% 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. 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, glucose, fucose and sialic acid were purchased from Aldrich chemicals. The structure elucidation of established and purified novel oligosaccharide was performed by spectroscopic technique ^{1}H, ^{13}C, and 2D NMR (COSY, TOCSY and HSQC) and ES mass spectrometry.

RESULT AND DISCUSSION

Compound ($C_{34}H_{58}O_{26}N_2$) gave positive Phenol-sulphuric acid test (Dubois et.al., 1956), Fiegl test (Fiegl, F., 1975) and Morgon-Elson test (Gey, et. al., 1996) showing the presence of normal and amino sugar moieties in the compound Murtiose. The HSQC spectrum of acetylated Murtiose showed the presence of six cross peaks of anomeric protons and carbons in their respective region at δ 6.08 x 90.93, δ 5.62 x 91.29, δ 5.26 x 89.61, δ 4.70 x 94.69, δ 4.45 x 100.70, δ 4.37 x 100.63 suggesting the presence of six anomeric protons and carbons in it. The presence of six anomeric protons were further confirmed by the presence of six anomeric doublets at δ 6.08(1H), δ 5.62 (1H), δ 5.26 (1H), δ 4.70 (1H), δ 4.45 (1H), δ 4.37(1H) in the ^{1}H NMR spectrum of acetylated Murtiose in $CDCl_3$ at 400 MHz .The presence of six anomeric carbons were also confirmed by six anomeric carbon signals at δ 89.61 (1C), δ 90.93 (1C), δ 91.29 (1C), δ 94.69(1C), δ 100.63(1C) and δ 100.70 (1C) in the ^{13}C NMR spectrum of acetylated Murtiose in $CDCl_3$ at 400 MHz .The ^{1}H NMR spectrum of Murtiose in D_2O at 300 MHz showed anomeric proton signals as doublets at δ 5.69 (1H), δ 5.20(1H), δ 4.63 (1H), δ 4.49 and δ 4.42 (1H). Since all these NMR spectrum of Murtiose and Murtiose acetate contained downfield shifted α and β anomeric proton and carbon signals suggested that the compound Murtiose may be a pentasaccharide in its reducing form. The reducing nature of compound Murtiose was further confirmed by methylglycosylation of compound Murtiose by $MeOH/H^+$ followed by its acid hydrolysis which led to the isolation of α and β-methyl glucosides, suggesting the presence of glucose at the reducing end. For convenience, the five monosaccharides present in compound Murtiose have been designated as S-1, S-2, S-3, S-4 and S-5, respectively, starting from glucose (S-1) the reducing end. The monosaccharide constituents in compound Murtiose were confirmed by its Killiani hydrolysis (Killiani, 1930) under strong acidic conditions, followed by PC and TLC. In its hydrolysis four spots were detected on PC and TLC which were found identical with glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) by co-chromatography with authentic samples. Thus, confirming that the pentasaccharide contained four types of monosaccharide units i.e., Glc, Gal, GalNAc and GlcNAc in it. The chemical shifts values of anomeric protons and carbon observed in ^{1}H and ^{13}C NMR spectrum of Murtiose were also in agreement with the reported values of ^{1}H and ^{13}C anomeric chemical shifts of Glc, Gal, GalNAc and GlcNAc confirming the presence of these monosaccharides in the compound Murtiose.

The presence of two anomeric proton signals at δ 5.69 (J = 4.0 Hz) and δ 4.63 (J = 8.0 Hz) in the ^{1}H NMR spectrum of Murtiose in D_2O at 300 MHz were assigned for α and β anomers of glucose (S-1) confirming the presence of Glc (S-1) at the reducing end. In the ^{1}H NMR of Murtiose in D_2O another anomeric proton doublet at δ 4.42 (J = 8.0 Hz) showed the presence of β-Gal (S-2) residue as the next monosaccharide. The ^{1}H NMR of Murtiose also contain a triplet δ 3.27 (Stucture Reporter Groups) (Dua, and Bush, 1983) suggested the presence of lactose type of structure at reducing end confirming the 1→4 linkage between β-Gal (S-2) and β-Glc (S-1), confirming the presence of lactosyl moiety (Dua, and Bush, 1983) at the reducing end. Since, the HSQC spectrum of Murtiose acetate shows six anomeric proton and carbon signal. The anomeric signal for α and β anomers were detected at δ 6.08 and δ 5.62, respectively, which were assigned for reducing glucose. In the TOCSY spectrum of Murtiose acetate the anomeric signal of β-Glc(S-1) at δ 5.62 gave cross peak at 5.62 x 3.72, 5.62 x 5.00, 5.62 x5.20, out of which one cross peak at 5.62 x δ 3.72 suggested that only one position in glucose (S-1) was available for glycosidic linkage by the next monosaccharide unit (Fig. 3.15), which was later assigned as H-4 of β-Glc(S-1) by the COSY spectrum of Murtiose acetate (Fig. 3.16). This shows that β-Glc(S-1) was 1→4 linked with the next monosaccharide unit. Further another anomeric proton signal at δ 4.37 in the ^{1}H NMR of Murtiose acetate assigned for β-Gal(S-2), in the TOCSY spectrum of Murtiose acetate showed four cross peaks at 4.37 x 3.74, 4.37 x 4.05, 4.37 x 4.58 , 4.37 x 5.10, out of which two cross peaks at δ 4.37 x δ 3.74 and δ 4.37 x 4.05 in the ^{1}H NMR of Murtiose acetate in $CDCl_3$ showed that the two position in S-2 were available for glycosidic linkages, confirming that the two hydroxyl groups of β-Gal (S-2) were involved in glycosidic linkages by other monosaccharide moieties. These signals were identified for H-2 and H-3 of β-Gal (S-2) by the COSY spectrum of Murtiose acetate suggesting that H-2 and H-3 of β-Gal (S-2) were available for glycosidic linkages by the next monosaccharide units. The coupling constant of anomeric signal β-Gal (S-2) with J value of 8.0 Hz confirmed the β-configuration of the β-Gal (S-2) moiety and hence β 1→4 glycosidic linkage between S-2 and S-1 were confirmed. The next anomeric proton signal appeared as doublet at δ 4.45(J = 8.0 Hz) in the ^{1}H NMR spectrum of Murtiose in $CDCl_3$ at 400 MHz was due to the presence of β Gal (S-3) moiety. The anomeric proton signal at δ 4.45 in the ^{1}H NMR spectrum of Murtiose acetate showed its complementary ^{13}C anomeric signal at δ 100.70 in the HSQC spectrum, later this ^{13}C anomeric signal at δ 100.70 showed its cross peak at 100.70 x 3.74 in the HMBC spectrum of Murtiose acetate showing the glycosidic linkages between S-3 and S-2 (Fig. 3.17). The signal of δ 3.74 was assigned as H-3 of S-2 by COSY spectrum confirming the 1→3 glycosidic linkage between S-3→S-2. The coupling constant of anomeric signal of (S-3) with J value 8.0 Hz confirmed β-configuration of the β-Gal(S-3) moiety. Therefore the glycosidic linkage between S-3 and S-2 was confirmed as β 1→3. Since, the anomeric proton signal at δ 4.45 in ^{1}H NMR of Murtiose acetate does not have any cross peak in the TOCSY spectrum of Murtiose acetate in the linkage region and none of methine proton of β-Gal (S-3) was found in the linkage region which confirms that β-Gal (S-3) linked at the non reducing end. Another anomeric proton signal which appeared as a doublet at δ 4.70 (J = 2.0 Hz), along with a singlet of amide methyl (-$NHCOCH_3$) at δ 1.96 in $CDCl_3$ at 400 MHz, was assigned for the presence of α-GalNAc (S-4) moiety. Since it was ascertained by COSY and TOCSY spectrum of Murtiose acetate that the positions 2 and 3 of β-Gal (S-2) were available for glycosidic linkages and position 3 of β-Gal (S-2) was already linked with β-Gal (S-3).

Hence the leftover H-2 position of S-2 must be linked by α-GalNAc (S-4). The position of linkage between GalNAc (S-4) and β-Gal (S-2) was further confirmed by the appearance of H-2 signal of β-Gal δ 4.05 (S-2) in the ^{1}H NMR spectrum of Murtiose acetate which was also confirmed by COSY and TOCSY spectrum at 400 MHz in $CDCl_3$. The small coupling constant of α-GalNAc (S-4) (J = 2.0 Hz) confirmed the α-glycosidic linkage between GalNAc (S-4) and β-Gal (S-2). Further another anomeric proton signal which appeared as a doublet at δ 4.70 (J = 2.0 Hz) in the ^{1}H NMR of Murtiose acetate at 400 MHz gave signal in the TOCSY spectrum of Murtiose acetate, the anomeric proton of α-GalNAc (S-4) at δ 4.70 showed cross peak at 4.70 x 3.70, 4.70 x 4.07, 4.70 x 5.10, out of which one cross peak 4.70 x 3.70 suggested that one position of α-GalNAc (S-4) was available for glycosidic linkage by the next monosaccharide. Later this signal of δ 4.70 x 3.70 was ascertained as H-3 of α-GalNAc (S-4) by COSY spectrum of Murtiose acetate showing that H-3 of α-GalNAc (S-4) was glycosidically linked by H-1 of next monosaccharide unit while the other cross peak of 4.70 x 4.07 was assigned for H-2 of GalNAc (S-4). Further, the presence of another anomeric proton as doublet at δ 5.26 (J = 1.0 Hz) along with a singlet of amide methyl (-$NHCOCH_3$) at δ 1.90 in $CDCl_3$, was identified due to the presence of α-GalNAc (S-5) as the next monosaccharide unit. As ascertained by the COSY and TOCSY spectrum of Murtiose acetate the position of H-3 of S-4 was available for glycosidic linkage. The α-GalNAc (S-5) must be attached to H-3 of S-4. Hence 1→3 linkage between α-GalNAc (S-5) and α–GalNAc (S-4) was confirmed. The small coupling constant of α-GalNAc (S-5) of (J = 1.0 Hz) confirmed the α-glycosidic linkage between α-GalNAc (S-5) and α-GalNAc (S-4). Since the anomeric proton of α-GalNAc (S-5) at δ 5.26 does not contain any cross peak in the linkage region in TOCSY spectrum of Murtiose acetate does not contain any methine protons in glycosidic linkage region i.e., δ 3-4 ppm, confirmed that none of -OH group of α-GalNHAc (S-5) was involved in glycosidic linkages. Hence, confirming that α-GalNAc (S-5) were present at non-reducing end and none of their -OH group were available for glycosidic linkages.

All the ^{1}H NMR assignments for ring proton of monosaccharide units of Murtiose were confirmed by COSY (Bax and Morris, 1981) and TOCSY (Kover, et al., 1998) spectra. The positions of glycosidation in the oligosaccharide were confirmed by position of anomeric signals, and comparing the signals in ^{1}H and ^{13}C NMR of acetylated Murtiose. The glycosidic linkages in Murtiose were also confirmed by the cross peaks for glycosidically linked carbons with their protons in the HSQC (Strecker et al, 1992) spectrum of acetylated Murtiose. The values of these cross peaks appeared as Glc (S-1) H-4 x C-4 at δ 3.72 x 76 showed (1→4) linkage between S-2 and S-1, β-Gal (S-2) H-3 x C-3 at δ 3.74 x 73 showed (1→3) linkage between S-3 and S-2, α-GalNAc (S-4) H-3 x C-3 at δ 3.70 x 70 showed (1→3) linkage between S-5 and S-4, β-Gal (S-2) H-2 x C-2 at δ 4.05X 71 showed (1→2) linkage between S-4 and S-2.

All signals obtained in ^{1}H and ^{13}C NMR of compound was in conformity by 2D COSY, TOCSY and HSQC spectrum. Thus, based on the pattern of chemical shifts of ^{1}H, ^{13}C, COSY, TOCSY and HSQC NMR experiments it was interpreted that the compound is a pentasaccharide having the structure as shown in Figure 3.

The electrospray Mass spectrum of compound is shown in figure 1 and confirmed the derived structure but also supported the sequence of monosaccharide in compound. The highest mass ion peaks were recorded at m/z 972 assigned to $[M+Na+K]^{+}$ and m/z 949 assigned to $[M+K]^{+}$, it also contain the molecular ion peak at m/z 910 confirming the molecular weight as 910 which was in agreement with its molecular formula $C_{34}H_{58}O_{26}N_2$.

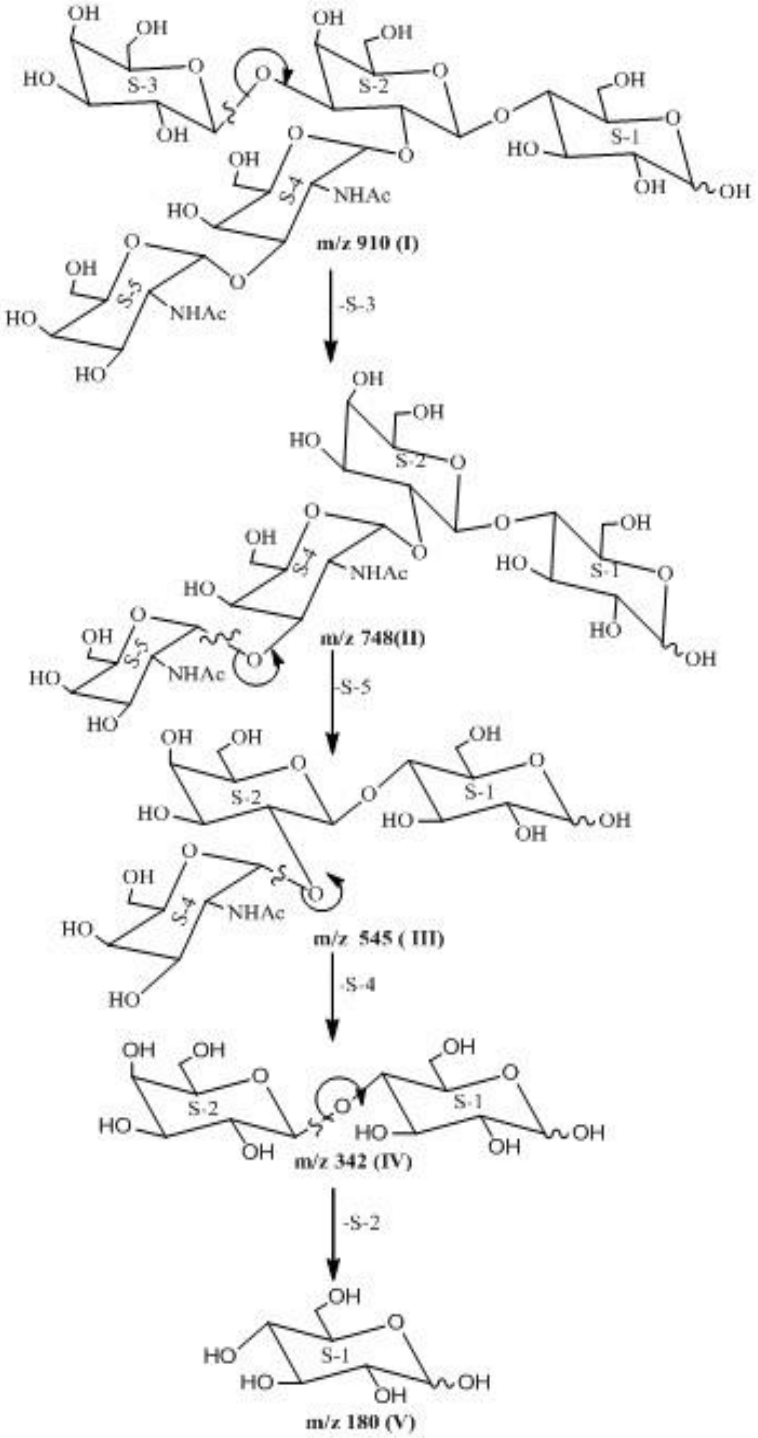

Figure1. Mass fragmentations of compound Murtiose.

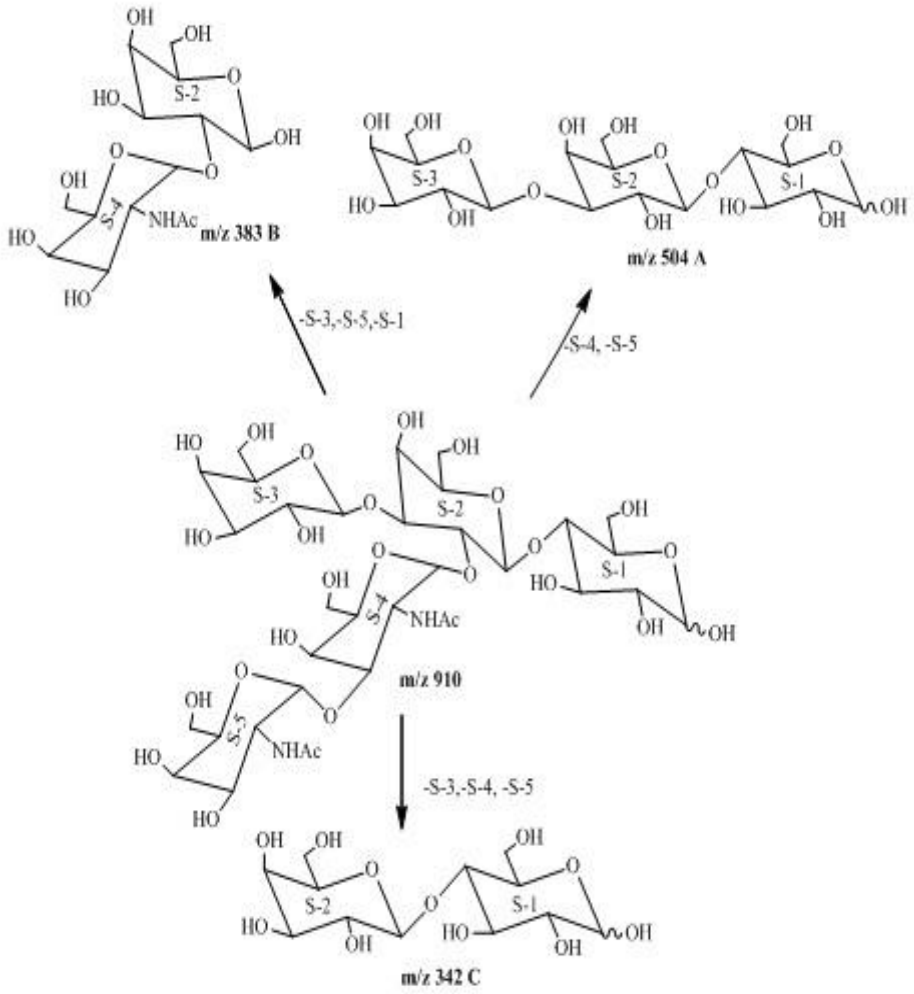

Figure 2. MS fragmentations of compound Murtiose.

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 pentasaccharide m/z 910 (I) fragmented to give mass ion at m/z 748(II) [910-S-3] which was tetrasaccharide (II), this fragment was arised due to the loss of 162 in Gal (S-3) moiety from pentasaccharide (I). It further fragmented to give mass ion peak at m/z 545 (III) which was a trisaccharide moiety (III) [748-S-5], due to loss of 203 in GalNAc (S-5) moiety from tetrasaccharide. This fragment of 545 further fragmented to give mass ion peak at m/z 342 (IV) [545-S-4] which was a disaccharide (IV), due to loss of 203 as GlcNAc (S-4) moiety from the tetrasaccharide. This disaccharide (IV) unit further fragmented to give mass ion peak at m/z 180 (V) [342-S-1], which was due to loss of 162 in Gal (S-1) moiety from disaccharide. These four mass ion peak II, III, IV, V were appeared due to the consequent loss of S-3, S-5, S-4, and S-1 from original molecule. The mass spectrum also contain the mass ion peak at m/z 748, 545, 586, corresponds to the mass ion fragment A, B, C which confirm the position of S-1, S-2, S-3 ,S-4, S-5 shown in figure 2.

The other fragmentation pathway in ES Mass spectrum of compound at m/z 910 shows the mass ion peaks at 875 [910-H_2O(18), -OH(17)], 851[910-CH_2OCHO(59)], 820 [851-CH_2OH(31)], 785 [820-OH(17), -H_2O(18)], 671 [748-CH_2OHCHO(60), OH(17)], 717 [748-CH_2OH(31)], 507 [545-2H_2O(36), $2H^+$(2)], 485 [545- CH_2OHCHO(60)], 487 [545-$NHCOCH_3$(58)], 342 [545-S-3], 484 [342-$NHCOCH_3$(58)], 325 [342-OH(17)], 281 [342-CH_2OHCHO(60), -H^+(1)].

Based on result obtained from chemical degradation/acid hydrolysis, chemical transformation, and ^1H, ^{13}C, and 2D NMR (COSY, TOCSY, HMBC and HSQC) technique of acetylated Murtiose shown is as in figure 3.

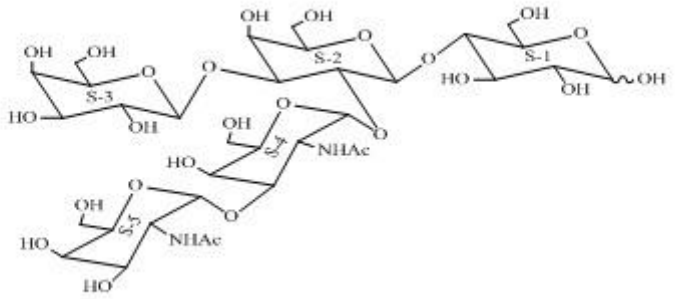

Figure 3. Structure of Murtiose.

β-Gal (1→3) -β-Gal(1→4)- Glc
|
α-GalNAc(1→3)-α-GalNAc(1→2)

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