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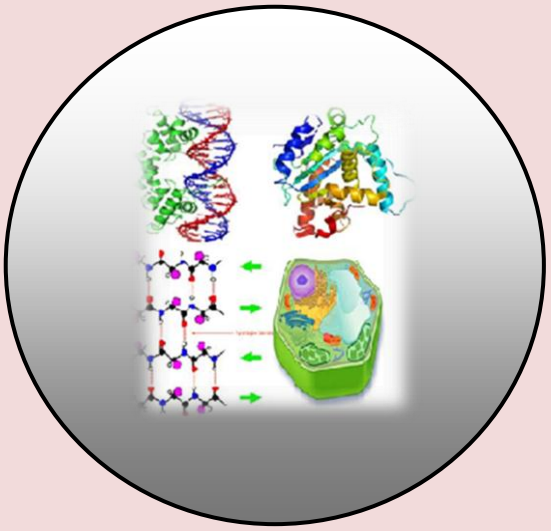

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RESEARCH PAPER
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Isolation and Structure Elucidation of Novel Oligosaccharide (BEBIOSE) from *Bubalus Bubalis* Colostrum by 2D NMR

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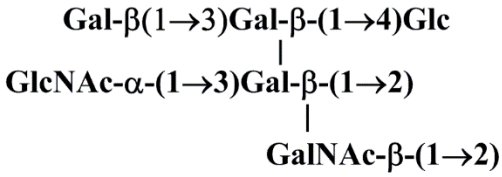
ABSTRACT

The carbohydrate and carbohydrate containing molecules like oligosaccharides, glycoproteins and glycolipids are major components of bovine milk and colostrum. A number of biologically active oligosaccharide have been isolated from milk of various mammalian species, having numerous biological activities like anti-microbial, anti-coagulant, anti-inflammatory, hypoglycaemic, and antiviral activities etc. Buffalo milk oligosaccharides have shown immuno-stimulant activity. Keeping in mind the biological activities of Buffalo's milk, the buffalo colostrums was collected in bulk and processed by modified method of Kobata and Ginsburg followed by gel filtration, affording crude oligosaccharide mixture which on acetylation and silica gel column chromatography afforded a purified novel compound Bebiose. The structure of isolated oligosaccharide was elucidated by chemical transformation, chemical degradation and NMR techniques. ^{1}H, ^{13}C NMR, 2D NMR and Mass spectrometry, which revealed three dimensional structure of novel compound Bebiose as under-



Keywords: Milk oligosaccharide, Colostrums, Bebiose, Hexasaccharide and NMR studies.

INTRODUCTION

Milk is a source for proteins, fatty acids, minerals, vitamins and carbohydrates especially, lactose and large number of oligosaccharides which are responsible for infant growth and child development.

Oligosaccharides which are major component of milk exert various bioactivities and modulate the immune system (Gangwar et al., 2018). Milk of various origins has proven themselves as a source for biologically active oligosaccharides which are major constituent of every milk are known for protection of breast feded infants from host bacterial infections (Millar et al., 1994). Both milk and colostrum are rich resource of oligosaccharides. Oligosaccharides are the effective class of bio- molecules impacting various physiological and pathological processes such as molecular recognition, signal transaction, differentiation and developmental events and exhibit varied biological activities such as antitumor (Schwonzen et al., 1992), immuno-stimulant (Abe et al., 1983) anticancer (Fang et al., 1985), and immunological activities (Srivastava et al., 1989). The Elephant milk oligosaccharides fraction contained a high ratio of sialyl oligosaccharide, this may be significant with respect to the formation brain components, such as gangliosides of the suckling calves (Osthoff et al., 2007). Donkey milk oligosaccharides have ability to non-specific and specific immunological resistance. Buffalo milk oligosaccharides have ability to stimulate non-immunological resistance of the host against parasitic infections (Saksena et al., 1999). The toxin binding properties of buffalo milk and anti-inflammatory activity of lipophilized gangilosides fraction is important for developing innovative food application, as well as subject of future research (Colarow et al., 2003). Keeping in mind various biological activities of buffalo milk oligosaccharide, we worked on isolation of some more novel milk oligosaccharides from buffalo colostrum. In the present study, we have isolated and elucidated the structure of a novel buffalo colostrum oligosaccharide named as Bebiose by chemical degradation, chemical transformation, spectroscopic techniques like (^{1}H NMR, ^{13}C NMR and 2D NMR).

EXPERIMENTAL

GENERAL PROCEDURES

For evaporation of alcohol from crude extract of milk oligosaccharides, Buchi Rotary evaporator was used. Freeze drying of the compounds was done with the help of CT 60e (HETO) lyophilizer and centrifuged by a cooling centrifuge Remi instruments C-23 JJRCI 763. Optical rotations were measured with a Buchi automatic Polarimeter in 1.2 cm tube. The C, H and N analyses were recorded on CARLO-ELBA 1108 elemental analyzer.^{1}H and ^{13}C NMR and 2D experiments were recorded in solvent $CDCl_3$ and D_2O at 25^{o}C on a Bruker AM 300 MHz FT NMR spectrometer. The Electrospray mass spectra were recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. The milk oligosaccharide sample (dissolved in water as solvent) was introduced into the ESI source through a syringe pump at the rate of 5ul per min. The ESI capillary was set at 3.5 kV and the cone voltage was 40 V. The spectra were collected in 6 s scans and the print outs are averaged spectra of 6-8 scans. Spectrum recorded in higher mass scale is computerized deconvoluted. Authentic samples of glucosamine, galactosamine, galactose, glucose, fucose and sialic acid were purchased from Aldrich Chemicals.

ISOLATION OF BUFFALO COLOSTRUM OLIGOSACCHARIDES BY MODIFIED METHOD OF KOBATA AND GINSBURG

Twelve (12) liter of buffalo colostrum (1 to 5 day) was collected from a domestic buffalo and equal amount of ethanol was added and stored until used.

The milk was processed by the modified method of Kobata and Ginsburg (Maurya et al 2017). In order to isolate milk oligosaccharide it was centrifuged for 15 min at 5000 rpm at 4^{o}C. The solidified lipid layer was removed by filtration through glass wool column in cold. Ethanol was added to the clear filtrate to a final concentration of 68% and the resulting solution was left overnight at 0^{0}C. The white precipitate formed, mainly of lactose and protein was removed by centrifugation and washed twice with 68% ethanol at 0^{0}C. The supernatant and washings were combined and filtered through a micro filter (0.24 mm) (to remove remaining lactose) was lyophilized affording crude oligosaccharide mixture 435gm. This lyophilized material was further purified by fractionating it on Sephadex G-25 chromatography using glass triple distilled water as eluent at a flow rate of 5 ml/m. Each fraction was analysed by phenol-sulphuric acid reagent for the presence of neutral sugar affording 185gm of oligosaccharide mixture.

ACETYLATION OF OLIGOSACCHARIDE MIXTURE

12.0 gm of crude oligosaccharide mixture obtained after sephadex chromatography was acetylated by adding pyridine (12.0 ml) and acetic anhydride (12.0 ml) at 60^{o}C with constant stirring and kept overnight. The mixture was evaporated under reduced pressure and the viscous residue was taken in $CHCl_3$ (250 ml) and washed with ice cold water. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding the acetylated mixture (12.6 gm). The acetylation converted the free sugars into their nonpolar acetyl derivatives which were resolved nicely on TLC, giving eight spots i.e, a, b, c, d, e, f, g and h, using $CHCl_3$: MeOH as eluent. Detection of the spots was done by spraying with 50% H_2SO_4 and heating.

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 eight fractions namely I(30mg), II(218mg), III(1.242g), IV(1.302g), V(504mg), VI(432mg), VII(650mg) and VIII(215mg).These fractions were containing mixture of two to three compounds. Repeated column chromatography of fraction III led to the isolation of one chromatographically pure compound “d” (73mg).

DEACETYLATION OF COMPOUND “d”

Compound “d” (30mg) was dissolved in acetone and NH_3 was added in it and was left over night in a stopper hydrolysis flask. After 24 h ammonia was removed under reduced pressure and the compound was washed thrice with $CHCl_3$ to remove acetamide and the water layer was finally freeze dried giving the deacetylated oligosaccharide D (16 mg).

METHYL GLYCOSIDATION/ACID HYDROLYSIS OF COMPOUND D (BEBIOSE)- Compound D (8 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 this solution 1, 4-dioxane (1 ml), and 0.1N H_2SO_4 (1 ml) was added and the solution was warmed for 30 minutes at 50^{o}C. The hydrolysis was complete 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 and GlcNAc. Their identification was confirmed by comparison with authentic samples on TLC and PC.

KILIANI HYDROLYSIS OF COMPOUND D (BEBIOSE) - Compound D (5 mg) was dissolved in 2 ml Kiliani 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.

DESCRIPTION OF ISOLATED COMPOUND D (BEBIOSE)- Compound D (16mg) was obtained from fraction 28-45 of third column chromatography. On deacetylation of 30 mg of substance with NH_3 / acetone, it afforded substance D (16mg). For experimental analysis, this compound was dried over P_2O_5at 100^oC and 0.1 mm pressure for 8 hr.

$C_{40}H_{68}O_{31}N_2$	%C	%H	%N
Calculated	44.77	6.34	2.60
Found	44.78	6.34	2.60

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

^{1}H NMR OF BEBIOSE: δ IN D_2O AT 300 MHZ

5.69 [d, 1H, J = 4.0, α- Glc(S-1), H-1], 5.20[d, 1H, β Glc(S-1), H-1], 4.52[d,1H, J = 7.8, β-Gal(S-2), H-1], 4.42 [d, 1H, J = 6.3, β-Gal(S-6), H-1], 1.97 [S, 3H, α-GlcNAc(S-5)NHCOCH_3], 1.96 [S, 3H, β-GalNAc (S-6)NHCOCH_3].

^{1}H NMR OF BEBIOSE ACETATE: δ IN $CDCl_3$ AT 400 MHZ

6.17 [d, 1H, J = 4.0 Hz, α-Glc (S-1) H-1], 5.69 [d, 1H, J = 5.2Hz, β-Glc (S-1) H-1], 5.40 [d, 1H, J = 2.8Hz, α-GlcNAc (S-5) H-1], 4.72 [d, 1H, J = 8.0Hz, β-Gal (S-4) H-1], 4.59 [d, 1H, J = 8.0Hz, β-Gal (S-3) H-1], 4.52 [d, 1H, J = 8.4, β-Gal (S-2) H-1], 4.44 [d, 1H, J = 6.3, β-GalNAc (S-6) H-1], 3.81 [d, 1H, β-Glc(S-1) H-4], 3.80 [d, 2H, β-Gal (S-4) H-2& β- Gal (S-2) H-3], 3.64 [d, 1H, β-Gal (S-4)H-3]

^{13}C NMR OF BEBIOSE ACETATE: δ **IN $CDCl_3$ AT 400 MHZ**

91.00[1C, α-Glc (S-1) C-1], 91.08[1C, β -Glc (S-1) C-1], 90.00 [1C, α-GlcNAc (S-5) C-1], 96.00[1C,β-Gal(S-4)C-1],102.00[1C,β-Gal(S-3),C-1],102.00[1C,β-Gal(S-2)C-1],102.00[1C,β GalNAc (S-6)C-1].

ES MASS OF COMPOUND BEBIOSE

1134[M+Na+K], 1095[M+Na], 1072[M+], 1054, 1013, 869, 833, 811, 666, 648, 606, 504, 473, 467, 342, 324, 180

RESULT AND DISCUSSION

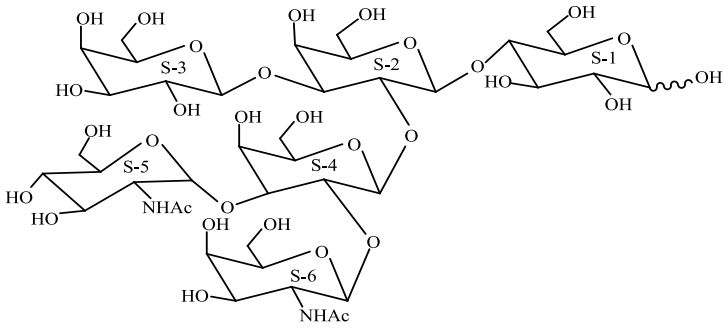
Compound D, $C_{40}H_{68}O_{31}N_2$, gave positive Phenol-sulphuric acid test (Dubois et al., 1956), Fiegl test (Fiegl et al., 1975) and Morgon-Elson test showing the presence of normal and amino sugar(s) moieties in the compound Bebiose.

The ^{1}H NMR spectrum of Bebiose at 300 MHz exhibited seven signals in the anomeric proton region as doublets at δ 6.17 (1H), δ 5.69 (1H), δ 5.40 (1H), δ 4.72 (1H), δ 4.59 (1H), δ 4.52 (1H), δ 4.44(1H) for seven anomeric protons indicating the presence of seven anomeric protons in it in. It was further supported by the appearance of seven anomeric carbons at δ 102.00 (3C), δ 96.00 (1C), δ 90.00 (1C), δ 91.08(1C) and δ 91.00 (1C) in the ^{13}C NMR spectrum of acetylated compound Bebiose in $CDCl_3$ at 400 MHz. These data led to the suggestion that "D" may be a hexasaccharide in its reducing form. The reducing nature of compound Bebiose was further confirmed by methylglycosylation of compound Bebiose by MeOH/H+ followed by its acid hydrolysis which led to the isolation of α and β-methyl glucosides. The presence of two anomeric proton signals at δ 5.69 (J = 4.0 Hz) and δ 5.20 in the ^{1}H NMR spectrum of Bebiose in D_2O at 300 MHz were assigned for α and β-anomers of glucose confirming the presence of glucose at the reducing end. Six monosaccharide units present in Bebiose has been designated as S-1, S-2, S-3, S-4, S-5 and S-6, respectively, starting from glucose (S-1) the reducing end.

The monosachharide constituents in compound Bebiose was confirmed by its Killiani hydrolysis under strong acidic conditions, followed by paper chromatography and TLC showed that hexasaccharide contained four types of monosaccharide units i.e., Glc, Gal, GalNAc and GlcNAc in it which are the building block of compound "D". The chemical shifts values of anomeric protons and carbon observed in ^{1}H NMR and ^{13}C NMR spectrum of Bebiose 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 Bebiose. The presence of six monosaccharides and its molecular formula suggested that there may be two GalNAc, three Gal and one Glc unit was present in Bebiose. In the ^{1}H NMR presence of another anomeric proton doublet at δ 4.52 (J = 7.8 Hz) along with a triplet δ 3.25 (Structure Reporter Groups) of Bebiose in D_2O showed the presence of β-Gal(S-2) residue as the next monosaccharide, for presence of lactose type of structure at reducing end confirming the 1→4 glycosidic linkage between β-Gal (S-2) and β-Glc (S-1), hence confirming the presence of lactosyl moiety (Dua, and Bush, 1983) at the reducing end, since the HSQC spectrum of Bebiose acetate showed seven anomeric proton and carbon signal. The anomeric signal for α and β anomers were detected at δ 6.17 and δ 5.69, respectively, which were assigned for reducing glucose. In the TOCSY spectrum of Bebiose acetate the anomeric signal of β-Glc (S-1) at δ 5.69 gave cross peaks at 5.69 x 3.81, 5.69 x 5.07, 5.69 x 5.28 out of which one cross peak at δ 5.69 x 3.81 suggested that only one position in S-1 was available for glycosidic linkage, which was assigned as H-4 of β-Glc (S-1) by the COSY spectrum of Bebiose acetate. This shows that reducing Glc (S-1) was 1→4 linked with as next monosaccharide unit i.e. galactose. Another anomeric proton signal present at δ 4.52 (J = 8.4 Hz) was assigned for β-Gal (S-2). The large coupling constant of anomeric signal β-Gal (S-2) with J value of 8.4 Hz confirmed the β-configuration of glycosidic linkage of β-Gal (S-2). Further the anomeric proton signal of β-Gal (S-2) at δ 4.52 (J = 8.4 Hz) in the TOCSY spectrum of Bebiose acetate showed cross peak at δ 4.52 x 3.80, 4.52 x 4.13, 4.52 x 4.37, out of which two cross peaks at δ 4.52 x 3.80 and δ 4.52 x 4.13 that the two position in S-2 were available for glycosidic linkages, i.e. the two hydroxyl groups of β-Gal (S-2) were involved in glycosidic linkages were by other monosaccharide moieties, the position of these glycosidic linkages were confirmed by the COSY spectrum of Bebiose acetate confirmed that

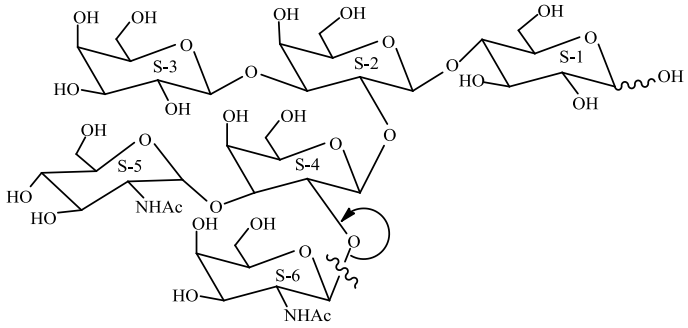
H-2 and H-3 of β-Gal (S-2) were available for glycosidic linkages by the next monosaccharide units. The next anomeric proton signal which appeared as a doublet at δ 4.59 (J = 8.0 Hz) in the ^{1}H NMR spectrum of Bebiose acetate in $CDCl_3$ at 400 MHz was assigned the presence of β Gal (S-3) moiety. The anomeric proton signal at δ 4.59 in the 1H NMR spectrum of Bebiose acetate showed its complementary ^{13}C anomeric signal at δ102.00 in the HSQC spectrum of Bebiose acetate later this ^{13}C anomeric signal at 102.00 showed its cross peak at 102.00 x 3.80 in the HMBC spectrum of Bebiose acetate showing the glycosidic linkages, between S-3 and S-2. The signal of δ 3.80 was assigned for H-3 of S-2 by COSY spectrum confirming the 1→3 glycosidic linkage between S-3→S-2. The large coupling constant of anomeric signal at δ 4.59 (J = 8.0 Hz) of (S-3) 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 of β-Gal (S-3) does not have any cross peak in to linkage region of TOCSY spectrum of Bebiose acetate hence confirming that none of methine proton of β-Gal (S-3) gave its signal into the linkage region confirming that β-Gal (S-3) was linked at the non-reducing end. Another anomeric proton signal which appeared as a doublet at δ 4.72 (J = 8.0 Hz), in the ^{1}H NMR Bebiose acetate in $CDCl_3$ at 400 MHz, was assigned for the presence of β-Gal (S-4) moiety. Since it was ascertained by COSY and TOCSY spectrum of Bebiose 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 β-Gal (S-4). The position of linkage between β-Gal (S-4) and β-Gal (S-2) was further confirmed by the appearance of H-2 signal of β-Gal δ 4.13 (S-2) in the ^{1}H NMR spectrum of Bebiose acetate which was also confirmed by COSY and TOCSY spectrum of Bebiose acetate at 400 MHz in $CDCl_3$. The large coupling constant of β-Gal (S-4) at δ 4.72 (J = 8.0 Hz) showed β-glycosidic linkage between β-Gal (S-4) and β-Gal (S-2). Further, anomeric proton signal which appeared as a doublet at δ 4.72 (J = 8.0 Hz) in the ^{1}H NMR of Bebiose acetate cross peaks at δ 4.72 x 3.64, 4.72 x 3.80, in the linkage region showed that the two position in S-4 were available for glycosidic linkages, showing that the two hydroxyl groups of β-Gal (S-4) were involved in glycosidic linkages by other monosaccharide moieties by the COSY spectrum of Bebiose acetate suggested that H-2 at δ 3.80 and H-3 at δ 3.64 of β-Gal (S-4) were available for glycosidic linkages by the next monosaccharide units. The next anomeric proton signal which appeared as doublet at δ 5.40 (J = 2.8 Hz) along with singlet at δ 1.97 for $NHCOCH_3$ in the ^{1}H NMR spectrum of Bebiose in $CDCl_3$ at 400 MHz was suggested for the presence α-GlcNAc (S-5) moiety. The signal of δ 4.72 x 3.64 was assigned for H-3 of S-4 by COSY spectrum suggesting that the H-3 of S-4 was available for glycosidic linkage, which may be linked to S-5. The ^{1}H NMR signal of H-3 at δ 3.64 along with anomeric proton signal of S-5 at δ 5.40 with the small coupling constant of anomeric signal of S-5 (J = 2.8 Hz) confirmed 1→3 glycosidic linkage between S-5 and S-4 with α-configuration of the (S-5) moiety. Therefore the glycosidic linkage between S-5 and S-4 was confirmed as α-1→3. Since, none of methine proton of α-GlcNAc (S-5) came in the linkage region confirmed that α- GlcNAc (S-5) was linked at the non-reducing end. Another anomeric proton signal which appeared as a doublet at δ 4.44 (J = 6.3 Hz) along with a signal of NAc at 1.96 in $CDCl_3$ at 400 MHz, was assigned for the presence of β-GalNAc (S-6) moiety. Since it was ascertained by COSY and TOCSY spectrum of Bebiose acetate that the positions 2 and 3 of β-Gal (S-4) were available for glycosidic linkages and position 3 of β-Gal(S-4) was already linked with α-GlcNAc (S-5),

hence the leftover H-2 position of S-4 must be linked by β-GalNAc (S-6), which was confirmed by the chemical shift of H-2 of S-4 at δ 3.80 was assigned at H-2 of S-4 by COSY spectrum confirming the 1→2 glycosidic linkage between S-6 and S-4. The position of linkage between β-GalNAc (S-6) and β-Gal (S-4) was further confirmed by COSY and TOCSY spectrum of Bebiose acetate at 400 MHz in $CDCl_3$. The large coupling constant of β-GalNAc (S-6) J = 6.3 Hz confirmed β-glycosidic linkage between β-GalNAc (S-6) and β-Gal (S-4). Since the anomeric signal of β-GalNAc (S-6) present in the TOCSY spectrum of Bebiose acetate at δ 4.44 does not contain any methine protons in glycosidic linkage region i.e., δ 3-4 ppm, confirmed that none of -OH group of β- GalNAc (S-6) was involved in glycosidic linkages hence, confirming that β- GalNAc (S-6) were present at non-reducing end and none of their -OH group participated in glycosidic linkages. All the ^{1}H NMR assignments for ring proton of monosaccharide units of Bebiose were confirmed by COSY and TOCSY 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 Bebiose. The glycosidic linkages in Bebiose were also confirmed by the cross peaks for glycosidically linked carbons with their protons in the HSQC spectrum of acetylated Bebiose. The values of these cross peaks appeared as Glc (S-1) H-4 x C-4 at δ 3.81 x 75 showed (1→4) linkage between S-2 and S-1, β-Gal (S-2) H-3 x C-3 at δ 3.80 x 75 showed (1→3) linkage between S-3 and S-2, β-Gal (S-2) H-2 x C-2 at δ 4.13 x 70 showed (1→2) linkage between S-4 and S-2, β -Gal (S-4) H-3 x C-3 at δ 3.64 x 71 showed (1→3) linkage between S-5 and S-4, β -Gal (S-4) H-2 x C-2 at δ 3.80 x 75 showed (1→2) linkage between S-6 and S-4. All signals obtained in ^{1}H and ^{13}C NMR of compound Bebiose were in conformed by 2D COSY, TOCSY and HSQC spectra. 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 'D' Bebiose is a hexasaccharides having the following structure as-



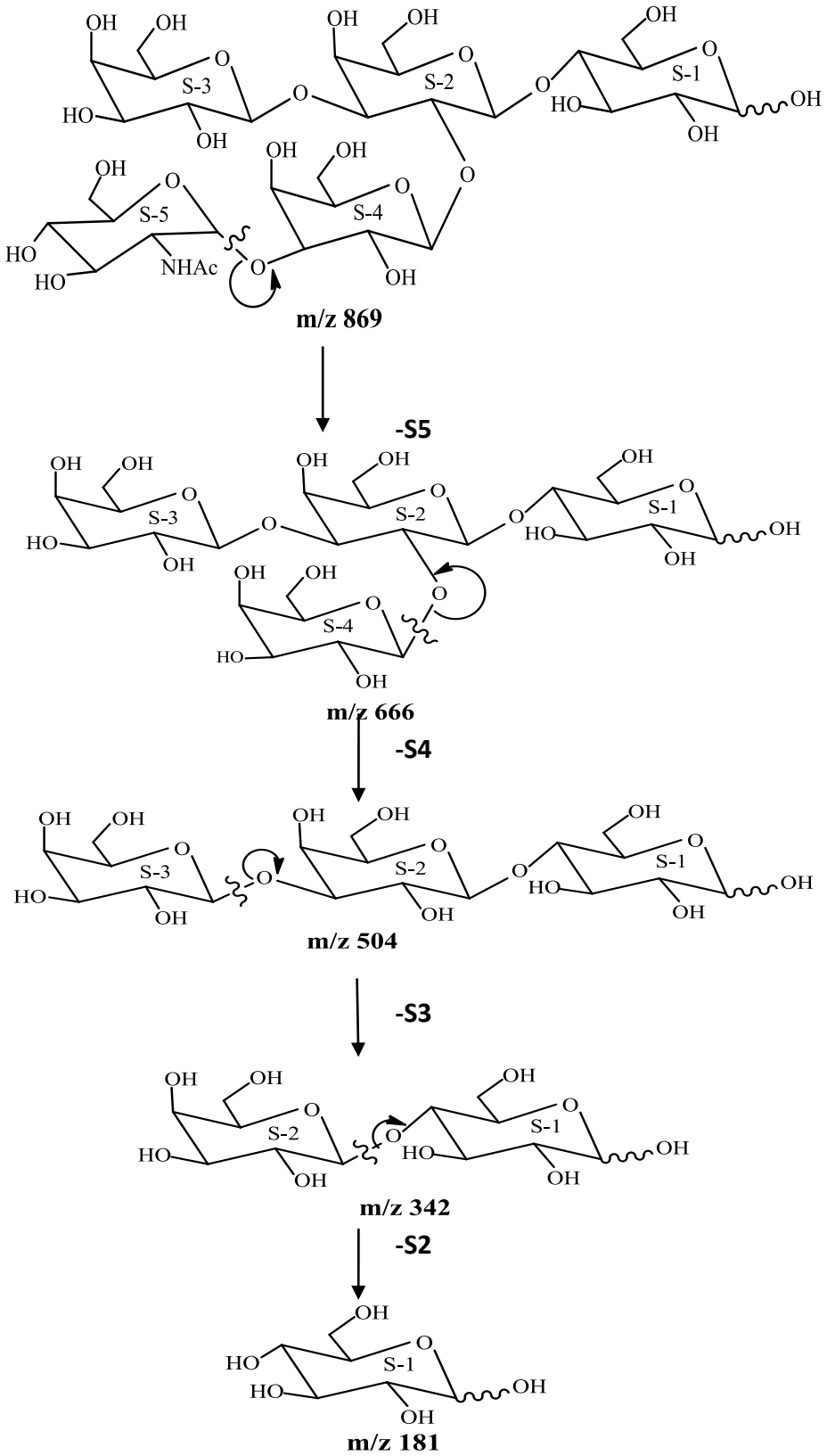
Compound D Bebiose

MASS SPECTRAL FRAGMENTS OF COMPOUND D (BEBIOSE)



m/z 1072

-S6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S-2
S-3
S-4
S-5
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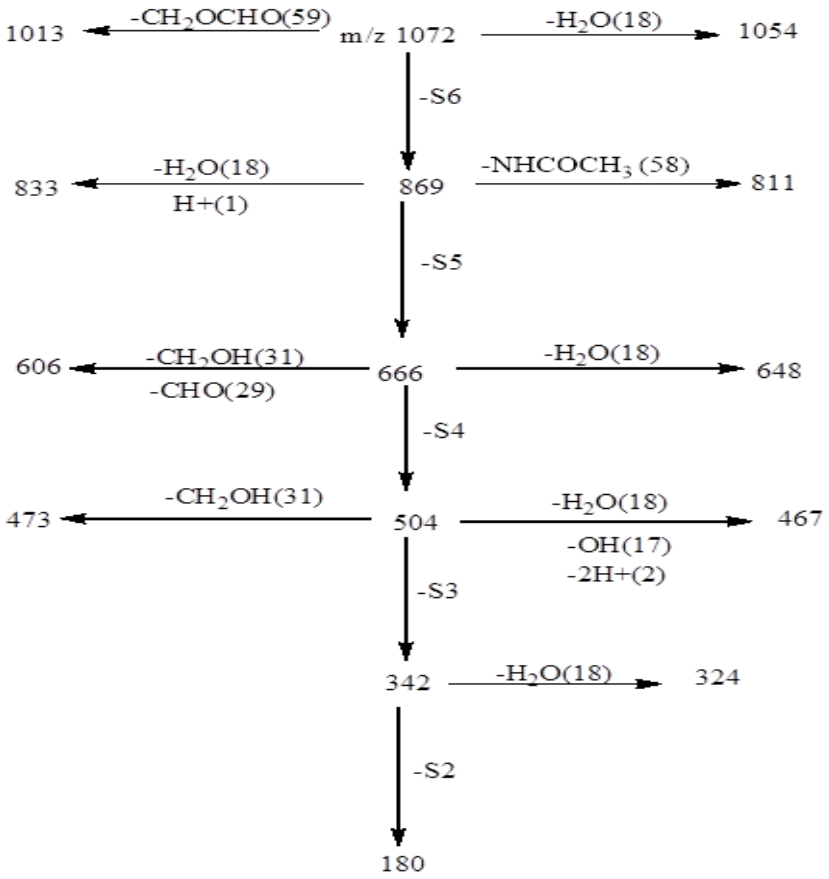

Line diagram of ES-MS fragmentations of compound D Bebiose

CONCLUSION

From the above information, we conclude the structure of isolated Buffalo milk oligosaccharide, Bebiose. This oligosaccharide was reported for the first time from any natural source or any milk and its structure was elucidated with the help of spectroscopic techniques like 1H, 13C, 2D-NMR (COSY, TOCSY and HSQC) spectroscopy and mass spectrometry.

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