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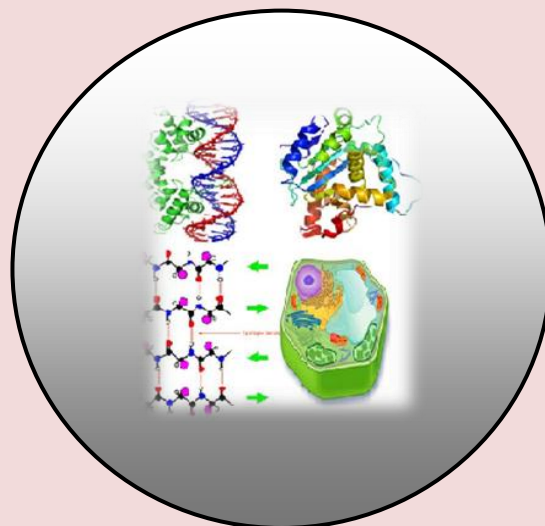
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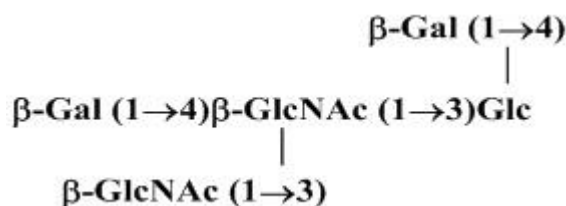
Structure Elucidation of Novel Oligosaccharide Orientose from Buffalo Milk

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ABSTRACT

Oligosaccharides have been isolated from milk of many mammalian species including equine, bovine and marine mammals. Oligosaccharides are important components of milk with bioefficacy as prebiotics, anti-infective, and immune system modulators and as a possible source of sialic acid for neural function. In all mammals buffalo is the major milk cattle of north India. Buffalo milk presents physicochemical features different from that of other ruminant species, such as a higher content of fatty acids and proteins. Buffalo milk oligosaccharide induces significant stimulation of antibody, delayed-type hypersensitivity response to sheep red blood cells in BALB/c mice. This also stimulates non-specific immune response of the animals measured in terms of macrophage migration index. In continuation to our previous work on buffalo milk oligosaccharides we have isolated a novel pentasaccharide from the buffalo milk having immunostimulant activity. In the present process buffalo milk was collected and processed by the modified method of Kobata and Ginsburg followed by gel filtration, HPLC and CC techniques, which led to isolation of a novel pentasaccharide Orientose. The result obtained from chemical degradation, chemical transformation along with spectroscopic data suggested that it was a pentasaccharide. Further the structure was confirmed by ^1H , ^{13}C and 2D NMR studies involving COSY, TOCSY and HSQC techniques along with mass spectrometry. The structure of Orientose was deduced as:



Keywords: Milk oligosaccharide, modified method of Kobata and Ginsburg and Orientose.

INTRODUCTION

Human milk and animal milk are rich source of oligosaccharides. Both human milk oligosaccharides and bovine milk oligosaccharides have received a lot of interest, mainly for their biological efficacy as prebiotics, anti-infective, and immune modulators and because they are a possible source of sialic acid for the nervous system (Fong et al., 2011).

Many biological activities have also been reported for certain milk oligosaccharides including anti-adhesion effects, anti inflammatory properties, glycome modifying activity, and a role in brain development and growth related characteristics of intestinal cells. Milk oligosaccharides are typically composed of three to ten monosaccharide units, including glucose (Glc), galactose (Gal) and N-acetylglucosamine (GlcNAc) as well as fucose and sialic acid. A large number of biologically active oligosaccharides have been isolated from human, buffalo (Gangwar et al., 2017), donkey, cow (Gunjan et al., 2016), mare (Maurya R.K. et al., 2017), sheep (Ranjan et al., 2015) and goat milk (Kumar et al., 2016). Milk is one of the most abundant sources of bioactive oligosaccharides, with human milk being the richest (Gangwar et al., 2017). In all mammals' buffalo milk, being the second largest global source of milk, differ from its closely related ruminant species with higher proportions of proteins, carbohydrate and fat (Abd El-Salam et al., 2011). Some components may only be present in buffalo milk such as specific classes of gangliosides. In addition, a recent study indicated that subjects with cow milk allergies are capable of tolerating buffalo milk, thus adding to the nutritional benefits of buffalo milk (Sheehan et al., 2009). A processed oligosaccharide mixture of buffalo milk induced significant stimulation of antibody, delayed type hypersensitivity response to sheep red blood cells in BALB/c mice. This also stimulates non-specific immune response of the animals measured in terms of macrophage migration index. Since buffalo milk is commonly used in Northern India, it was of interest to analyse buffalo milk for its oligosaccharide contents having immunostimulant activity (Saxena et al., 1999). Keeping in mind the biological activity of buffalo milk oligosaccharides we have worked on buffalo milk oligosaccharides and isolated a novel pentasaccharide, Orientose. Structure elucidation of this oligosaccharide was done with the help of chemical degradation, chemical transformation and by the modern spectroscopic techniques (^1H , ^{13}C , HSQC, COSY and TOCSY) and mass spectrometry.

MATERIAL AND METHODS

General procedure

General procedures were same as described in our previous communication (Singh et al., 2016).

Isolation of buffalo milk oligosaccharide by modified method of Kobata and Ginsburg

Isolation of buffalo milk oligosaccharide was done by the modified method of Kobata and Ginsburg (Kumar et al., 2016) which was described in our previous communication (Singh et al., 2016) except the isolation, was done from 10 litre of buffalo milk and the yield of oligosaccharide mixture was 315 gm.

Acetylation of buffalo milk oligosaccharide mixture

For acetylation 10.0 gm of pooled oligosaccharide fractions which gave positive phenol-sulphuric acid test (Dubois et al., 1956) were acetylated with pyridine (10 ml) and acetic anhydride (10 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 it was 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 acetylated mixture (12.60 g). TLC of this oligosaccharide mixture showed eight spots namely A, B, C, D, E, F, G and H.

Deacetylation of Compound D (Orientose)

Compound D (57 mg) was obtained from column chromatography 8 of acetylated oligosaccharide mixture. 35 mg of compound D was dissolved in acetone (3 ml) and 3.5 ml of NH_4OH was added in it and was left overnight in a stoppered hydrolysis flask. After 24 hrs ammonia was removed under reduced pressure and the compound was washed thrice with CHCl_3 (5 ml) (to remove acetamide) and water layer was finally freeze dried giving the natural oligosaccharide D (29 mg).

Methyl glycosidation/Acid hydrolysis of compound D

Compound D (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. In the reaction mixture, 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 °C. The hydrolysis was completed after 24 h. The hydrolysate was neutralized with freshly prepared BaCO_3 , filtered and concentrated under reduced pressure to afford α - and β -methylglucosides along with the Glc, Gal and GlcNAc. Their identification was confirmed by comparison with authentic samples (TLC, PC).

Kiliani hydrolysis of compound D

Compound D (5 mg) was dissolved in 2 ml Kiliani mixture (AcOH-H₂O-HCl, 7:11:2) and heated at 100°C for 1 h followed by evaporation under reduced pressure. It was dissolved in 2 ml of H₂O and extracted twice with 3 ml CHCl₃. The aqueous residual solution was made neutral by addition of 1-2 drops of 2N NaOH and was evaporated under reduced pressure which afforded glucose, Gal and GlcNAc which were identified by comparison with authentic samples of glucose, Gal and GlcNAc.

Description of Isolated Compound D (Orientose)

Compound 'D' (57 mg) was obtained from fraction 79- 85 of column chromatography 8. Deacetylation of its 35 mg was done by using NH₄OH/ acetone and it gave approx. 29 mg of compound D as a viscous mass, $[\alpha]_D + 5.10^0$. For experimental analysis, this compound was dried over P₂O₅ at 100 °C and 0.1 mm pressure for 8 hrs.

C₃₄H₅₈O₂₆N₂	%C	%H	%N
Calculated	44.83	6.37	3.07
Found	44.82	6.36	3.07

It gave positive Phenol sulphuric acid test (Dubois et al., 1956), Feigl test (Fiegl et al., 1975) and Morgan Elson test (Patridge et al., 1948).

δ in CDCl₃: ¹H NMR (Acetylated)

δ 6.22 [d, 1H, J=3.6 Hz, α -Glc (S₁)], δ 5.66 [d, 2H, J=8.4 Hz, β -Glc (S₁), β -GlcNAc (S₃)], δ 4.60 [d, 1H, J=7.5 Hz, β -GalNAc (S₄)], δ 4.57 [d, 2H, J=7.8 Hz, β -Gal (S₅), β -Gal (S₂)], δ 4.05 [m, 1H, J=8.4 Hz, β -Glc (S₁), H-3], δ 3.82 [m, 1H, J=8.4 Hz, β -GlcNAc (S₃), H-3], δ 3.70 [m, 1H, J=8.4 Hz, β -Glc (S₁), H-4], δ 3.60 [m, 1H, J=8.4 Hz, β -GlcNAc (S₃), H-4].

δ in CDCl₃: ¹³C NMR (Acetylated)

δ 101.83 [2C, β -Gal (S₂), β -Gal (S₅), C-1], δ 101.69 [1C, β -GalNAc (S₄), C-1], δ 91.39 [2C, β -Glc (S₁), β -GlcNAc (S₃), C-1], δ 88.96 [1C, α -Glc (S₁), C-1].

δ in D₂O: ¹H NMR (Deacetylated)

δ 5.17 [d, 1H, J=4Hz, α -Glc (S₁)], δ 4.90 [d, 1H, J=8Hz, β -GlcNAc (S₃)], δ 4.59 [d, 1H, J=8Hz, β -Glc (S₁)], δ 4.42 [d, 1H, J=8Hz, β -GalNAc (S₄)], δ 4.38 [d, 1H, J=8Hz, β -Gal (S₅)], δ 4.36 [d, 1H, J=8Hz, β -Gal(S₂)], δ 3.18 [t, 1H, β -Glc(S₁), H-2], δ 1.92 [s, 3H, β -GlcNAc(S₃), NHCOCH₃], δ 1.91 [s, 3H, β -GalNAc(S₄), NHCOCH₃].

δ in D₂O: ¹³C NMR (Deacetylated)

δ 101.66 [3C, β -Gal (S₅), β -Gal (S₂) and β -GalNAc (S₄), C-1], δ 99.72 [1C, β -GlcNAc (S₃), C-1], δ 94.52 [1C, β -Glc (S₁), C-1], δ 90.56 [1C, α -Glc (S₁), C-1].

ES-mass

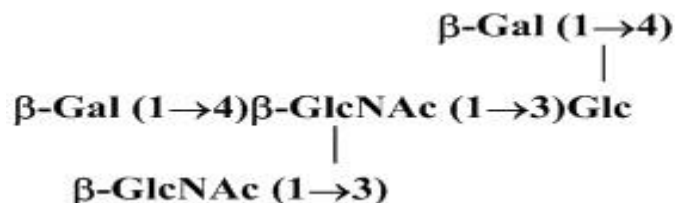
m/z 972 [M+Na+K]⁺, m/z 933 [M+Na]⁺, m/z 910 [M]⁺, m/z 748, m/z 730, m/z 712, m/z 699, m/z 681, m/z 664, m/z 663, m/z 545, m/z 527, m/z 511, m/z 498, m/z 482, m/z 467, m/z 465, m/z 342, m/z 325, m/z 307, m/z 278, m/z 260, m/z 180.

RESULT AND DISCUSSION

Compound D, Orientose, C₃₄H₅₈O₂₆N₂, $[\alpha]_D +5.10^0$, gave positive Phenol-sulphuric acid test (Dubois et al., 1956), Fiegl test (Fiegl et al., 1975) and Morgon-Elson test (Patridge et al., 1948) showing the presence of normal and amino sugar moiety(s) in the compound Orientose. The HSQC spectrum of acetylated Orientose showed the presence of six cross peaks of anomeric protons and carbons in the respective region at δ 6.22 x 88.96, δ 5.66 x 91.39, δ 5.66 x 91.39, δ 4.60 x 101.69, δ 4.57 x 101.83, δ 4.57 x 101.83 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 6.22 (1H), δ 5.66 (2H), δ 4.60 (1H) and δ 4.57 (2H) in the ¹H NMR spectrum of acetylated Orientose in CDCl₃ at 400 MHz. The presence of six anomeric carbons were also confirmed by six anomeric carbon signals at δ 101.83 (2C), δ 101.69 (1C), δ 91.39 (2C) and δ 88.96 (1C) in the ¹³C NMR spectrum of acetylated Orientose in CDCl₃ at 400 MHz. The ¹H NMR spectrum of Orientose in D₂O at 400 MHz showed six anomeric proton signals as doublets at δ 5.17 (1H), δ 4.90 (1H), δ 4.59 (1H), δ 4.42 (1H), δ 4.38 (1H) and δ 4.36 (1H). The pentasaccharide nature of Orientose was also confirmed by the appearance of six anomeric carbon signals at δ 101.66 (3C), δ 99.72 (1C), δ 94.52 (1C) and δ 90.56 (2C) in the ¹³C NMR spectrum of Orientose in D₂O at 400 MHz.

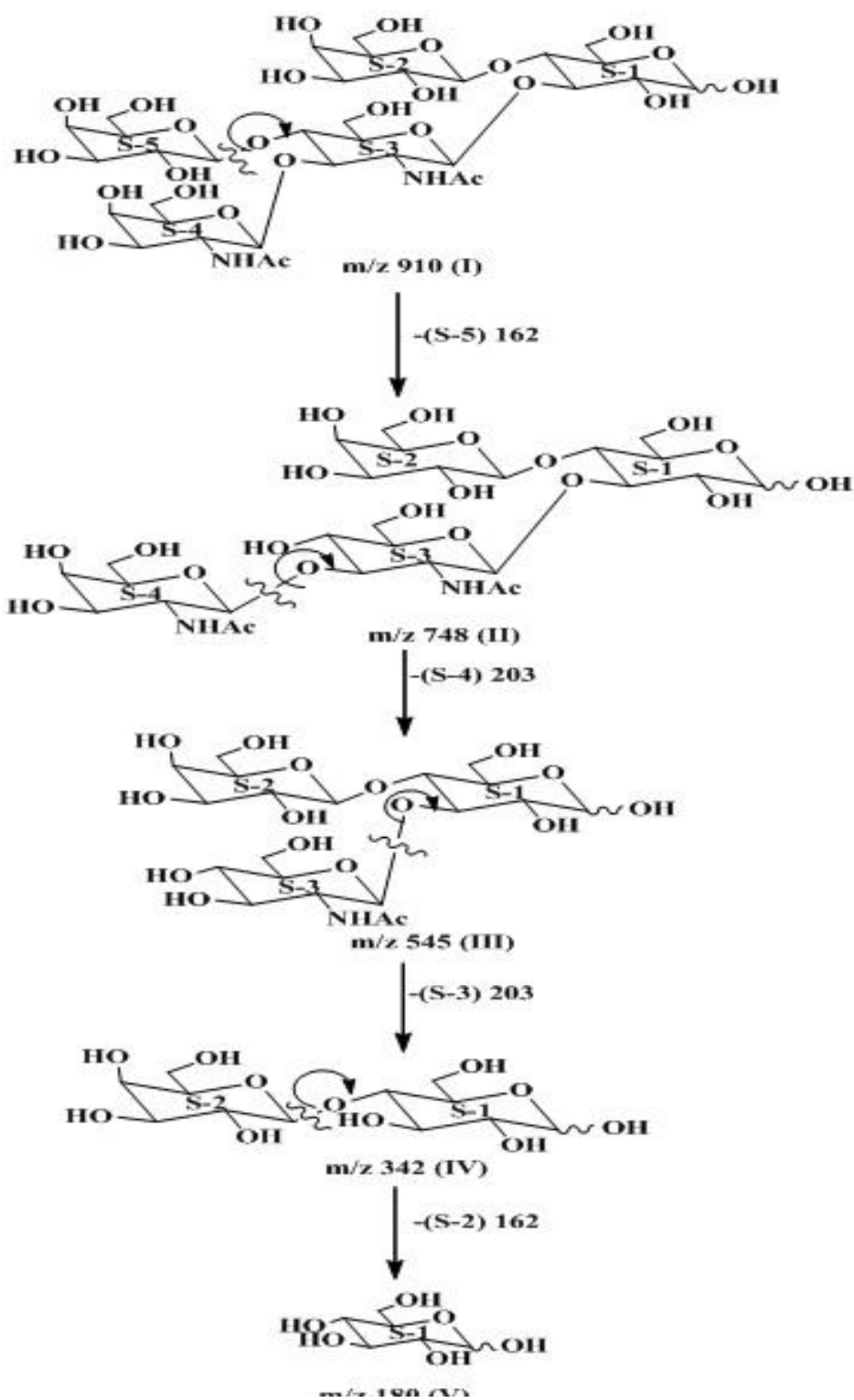
Since all these spectrums contained downfield shifted α and β anomeric protons and carbons suggested that compound Orientose may be a pentasaccharide in its reducing form. The reducing nature of compound Orientose was further confirmed by methylglycosylation of compound Orientose by MeOH/H⁺ followed 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 Orientose were designated as S-1, S-2, S-3, S-4 and S-5 respectively starting from glucose (S-1) at the reducing end. The monosaccharide constituents in compound Orientose were confirmed by its Killiani hydrolysis (Killiani et al., 1930) under strong acidic conditions, followed by Paper chromatography and TLC. In this hydrolysis four spots were found on TLC and PC which were found identical with the authentic samples of Glucose, Galactose, GlcNAc and GalNAc by co-chromatography. 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 ¹H NMR and ¹³C NMR spectrum of Orientose were also in agreement with the reported values of ¹H and ¹³C anomeric chemical shifts of Glc, Gal, GalNAc and GlcNAc confirming the presence of these monosaccharides in the compound Orientose. The presence of two anomeric proton signals at δ 5.17 (J=4.0 Hz) and δ 4.59 (J=8.0 Hz) in the ¹H NMR spectrum of Orientose in D₂O at 400 MHz were assigned for α and β anomers of glucose(S-1) confirming the presence of Glc(S-1) at the reducing end (Dorland et al., 1977, Chaturvedi et al., 1990) in compound Orientose. In the TOCSY spectrum of Orientose acetate, the anomeric signal of β -Glc(S-1) at δ 5.66 gave two cross peaks at δ 4.05 and δ 3.70 in the linkage region suggesting that two positions in S-1 were available for glycosidic linkages, which were later assigned as H-3 and H-4 respectively of β -Glc(S-1) by the COSY spectrum of Orientose acetate. This shows that β -Glc(S-1) was 1 $\rightarrow$ 3 and 1 $\rightarrow$ 4 linked with next monosaccharide units. Further the presence of another anomeric proton doublet at δ 4.36 (J=8.0 Hz) in the ¹H NMR of Orientose in D₂O showed the presence of β -Gal(S-2) residue as the next monosaccharide, which was 1 $\rightarrow$ 4 linked with β -Glc(S-1). The 1 $\rightarrow$ 4 linkage between β -Gal (S-2) and β -Glc (S-1) was further confirmed by the appearance of β -Glc(S-1) H-2 signal as triplet at δ 3.18 (SRG) (Gronberg et al., 1990, Dua et al., 1985) and hence the presence of lactosyl moiety (Uemura et al., 2006) was confirmed at the reducing end in Orientose. 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 $\rightarrow$ 4 glycosidic linkage between S-2 and S-1. Since none of the methine proton signal of β -Gal(S-2) was found in the linkage region in the TOCSY spectrum of Orientose acetate, it may be defined that Gal was present at the non reducing end. The next anomeric proton signal which appeared as doublet at δ 4.90 (J=8.0 Hz) in the ¹H NMR spectrum of Orientose in D₂O at 400 MHz along with a singlet of amide methyl (-NHCOCH₃) at δ 1.92 was due to the presence of β -GlcNAc(S-3) moiety (Urashima T. et al., 2004, Dua V.K. et al., 1985). Since by the TOCSY spectrum of Orientose acetate, it was confirmed that H-3 and H-4 of β -Glc(S-1) were available for glycosidic linkage, out of which H-4 of β -Glc(S-1) was already linked with β -Gal (S-2). Hence, β -GlcNAc(S-3) must be linked with H-3 of β -Glc(S-1). The 1 $\rightarrow$ 3 linkage between β -GlcNAc (S-3) and β -Glc (S-1) was further supported by the ¹H NMR spectrum of acetylated Orientose in which the signal for H-3 of β -Glc (S-1) appeared at δ 4.05 which was later confirmed by COSY, TOCSY and HSQC spectrum of acetylated Orientose at 400 MHz in CDCl₃. The coupling constant of anomeric signal of (S-3) with J value 8.0 Hz confirmed the β -configuration of the β -GlcNAc(S-3) moiety. Therefore the glycosidic linkage between S-3 and S-1 was confirmed as β 1 $\rightarrow$ 3. In the TOCSY spectrum of acetylated Orientose the β -GlcNAc(S-3) at δ 5.66 showed two consecutive complementary signals at δ 3.82 and δ 3.60, which shows that β -GlcNAc(S-3) also contains two -OH groups which were available for glycosidic linkage by next monosaccharide units. These cross peaks were further assigned as H-3 and H-4 of S-3 respectively by the COSY spectrum of acetylated Orientose suggesting that H-3 and H-4 of S-3 were available for glycosidic linkages. The next anomeric proton signal which appeared as a doublet at δ 4.42 (J=8.0 Hz) along with a singlet of amide methyl (-NHCOCH₃) at δ 1.91 in ¹H NMR spectrum of Orientose in D₂O was assigned for the presence of β -GalNAc(S-4) moiety which was linked with β -GlcNAc(S-3) by 1 $\rightarrow$ 3 linkage. The 1 $\rightarrow$ 3 linkage between S-3 and S-4 was further confirmed by the appearance of H-3 of S-3 at δ 3.82 in the TOCSY spectrum of Orientose acetate at 400 MHz.

The large coupling constant of β -GalNAc(S-4) of $J=8.4$ Hz confirmed the β linkage between β -GlcNAc(S-3) and β -GalNAc(S-4). Since it was ascertained by COSY and TOCSY spectrum of Orientose acetate that the positions 3 and 4 of β -GlcNAc (S-3) were available for glycosidic linkages and position 3 of β -GlcNAc (S-3) was already linked with β -GalNAc(S-4), the leftover H-4 position of S-3 must be linked by β -Gal (S-5). The position of linkage between β -Gal (S-5) and β -GlcNAc (S-3) was further confirmed by the appearance of H-4 signal of β -GlcNAc (S-3) at δ 3.60 in the ^1H NMR spectrum of Orientose acetate which was also confirmed by COSY and TOCSY spectrum of Orientose acetate at 400 MHz in CDCl_3 . The large coupling constant of β -Gal(S-5) of $J=8.0$ Hz confirmed the β -glycosidic linkage between β -Gal(S-5) and β -GlcNAc (S-3). Since in the NMR data of Orientose acetate, the anomeric signal of β -Gal(S-5) at δ 4.38 and β -GalNAc(S-4) at δ 4.42 present in the TOCSY spectrum of Orientose acetate does not contain any methine protons signal in glycosidic linkage region i.e., δ 3-4 ppm showed that none of -OH group of β -Gal(S-5) and of β -GalNAc(S-4) were involved in glycosidic linkages hence, confirmed that β -Gal(S-5) and β -GalNAc (S-4) were present at non-reducing end, which was confirmed by the TOCSY and COSY spectrum of acetylated Orientose. All the ^1H NMR assignments for ring proton of monosaccharide units of Orientose were confirmed by HOMOCOSY (Gronburg et al., 1989, Gronburg et al., 1992) and TOCSY (Kover et al., 2000) experiments. The positions of glycosidation in the oligosaccharide were confirmed by position of anomeric signals, S.R.G. and comparing the signals in ^1H and ^{13}C NMR of acetylated Orientose and Orientose. The glycosidic linkages in Orientose were also confirmed by the cross peaks for glycosidically linked carbons with their protons in the HSQC (Strecker et al., 1992, Bodenhausen et al., 1971) spectrum of acetylated Orientose. The values of these cross peaks appeared as β -Glc(S-1) H-4 $\times$ C-4 at δ 3.70 $\times$ 73.0 showed (1 $\rightarrow$ 4) linkage between S-2 and S-1, β -Glc(S-1) H-3 $\times$ C-3 at δ 4.05 $\times$ 71.5 showed (1 $\rightarrow$ 3) linkage between S-3 and S-1, β -GlcNAc(S-3) H-4 $\times$ C-4 at δ 3.60 $\times$ 72.5 showed (1 $\rightarrow$ 4) linkage between S-5 and S-3, and β -GlcNAc(S-3) H-3 $\times$ C-3 at δ 3.82 $\times$ 69.0 showed (1 $\rightarrow$ 3) linkage between S-4 and S-3. All signals obtained in ^1H and ^{13}C NMR of compound Orientose was in conformity by 2D ^1H - ^1H COSY, TOCSY and HSQC experiments. Thus based on the pattern of chemical shifts of ^1H , ^{13}C , COSY, TOCSY and HSQC NMR experiments it was interpreted that the compound 'D'Orientose was a pentasaccharide having the following structure as-

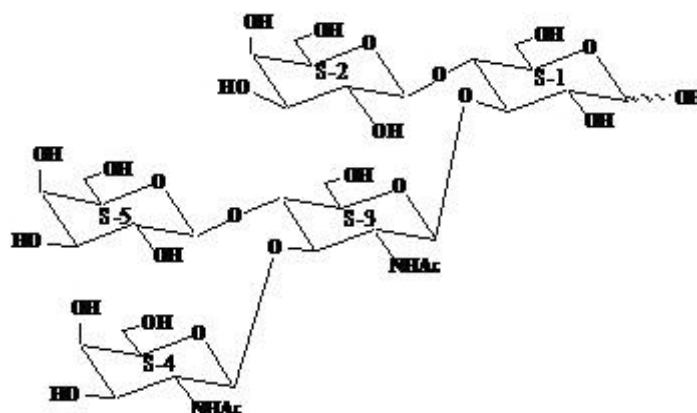


SCHEME: ES-MS FRAGMENTS OF COMPOUND (D) ORIENTOSE

The Electrospray Mass Spectrometry data of Orientose not only confirmed the derived structure but also supported the sequence of monosaccharide in Orientose. The highest mass ion peaks were recorded at m/z 972 assigned to $[\text{M}+\text{Na}+\text{K}]^+$ and m/z 949 assigned to $[\text{M}+\text{K}]^+$, 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 $\text{C}_{34}\text{H}_{58}\text{O}_{26}\text{N}_2$. 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 peak at m/z 748(II) [910-(S-5)], this fragment was arise due to the loss of β -Gal S-5(162) moiety from pentasaccharide. The tetrasaccharide m/z 748 fragmented to give mass ion peak at m/z 545(III) [748-(S-4)] of trisaccharide, which was due to loss of β -GalNAc S-4 (203). This fragment m/z 545 of trisaccharide, further fragmented to give mass ion peak at m/z 342 (IV) [545-(S-3)] which was a disaccharide (IV), due to loss of GlcNAc S-3 (203) moiety. This disaccharide unit fragmented to give mass ion peak at m/z 180 (V) [342-(S-2)], which was due to loss of Gal (S-2) moiety from disaccharide. These four mass ion peak II,III,IV,V were appeared due to the consequent loss of S-5, S-4, S-3, and S-2 from original molecule. The other fragmentation pathway in ES Mass spectrum of compound C m/z 910 shows the mass ion peak at 664[748- 36($2\text{H}_2\text{O}$), 31(CH_2OH), 17(OH)], 663[748-18(H_2O), 31(CH_2OH), 36($2\text{H}_2\text{O}$)], 467 [545-18(H_2O), 29(CHO), 31(CH_2OH)], 465 [748-34(2OH), 29(CHO), 17(OH)], 260 [342-17(OH)], 18(H_2O), 29(CHO), 18(H_2O).



Based on result obtained from chemical degradation/acid hydrolysis, Chemical transformation, Electro spray mass spectrometry and ^1H , ^{13}C NMR and 2D NMR technique (HOMOCOSY, TOCSY and HSQC) of Orientose acetate and Orientose, the structure and sequence of monosaccharides in isolated Novel oligosaccharide molecule Orientose was deduced as –



CONCLUSION

In summary, the novel milk oligosaccharide namely as D (Orientose) has been isolated from buffalo milk and its structure was elucidated with the help of ^1H , ^{13}C , 2D NMR spectroscopy and mass spectrometry.

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