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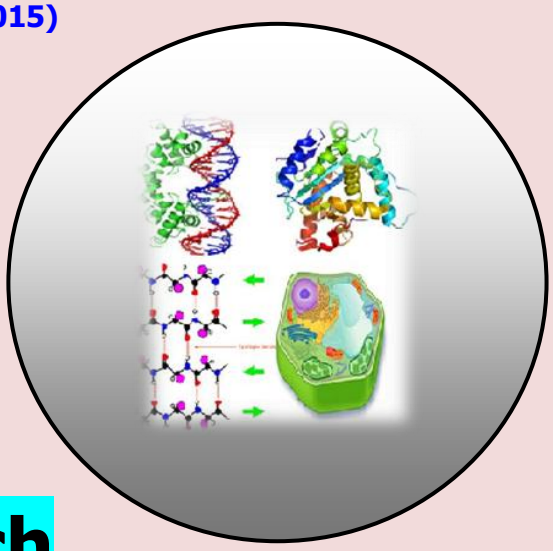

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RESEARCH PAPER

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Famiose: A Novel Hexasaccharide from Donkey's Milk

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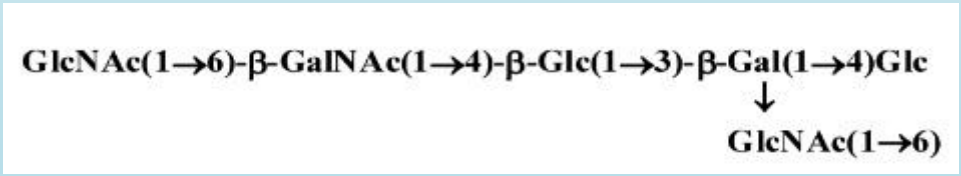
ABSTRACT

Milk oligosaccharides are complex carbohydrates that function as selective growth substrates for specific beneficial bacteria in the gastrointestinal system and supplements for the food and the pharmaceutical industries. Oligosaccharides are key components of milk and play multiple roles in the health of the neonate by stimulating growth of selected beneficial bacteria in the gut, participating in development of the brain and exerting antipathogenic activity. Donkey milk show a low lipid content and high lysozyme levels in comparision to human milk. The oligosaccharide mixture of Donkey's milk has shown significant stimulation of antibody, delayed type hypersensitivity response to sheep red blood cells in BALB/c mice. In our previous communication we have described the isolation and structure elucidation of immunostimulant oligosaccharide from Donkey's milk. In continuation to our previous studies we have isolated one more novel hexasaccharide Famiose. The structure of isolated and purified Donkey's milk oligosaccharide was elucidated by chemical degradation, chemical transformation, spectroscopic techniques like NMR (^{1}H, ^{13}C and 2D NMR), structure reporter group theory and ES mass spectrometry as-



Keywords: Donkey milk, Oligosaccharides, Famiose.

INTRODUCTION

The history of milk begins in the Neolithic period when hominids made the transition from nomadic hunting to gathering societies **(Yildiz 2010).** Over the many centuries, milk has become a desired and valuable source of nutrition. Milk is an excellent gift of nature to mankind that contains all necessary nutrients for growth and development of intestinal and immune system of any mammalian neonate **(Singh et al. 2015 and Srivastava et al. 2016).** Milk contains oligosaccharides with varied biological activities depending on the nature of their origin to which mammals they belongs **(Miller et al. 1994 and Singh et al. 2017).** Milk oligosaccharides are non-digested due to the presence of β-glycosidic linkage. So this β-glycosidic linkage plays an important role for its prebiotic activity **(Kim et al. 2005 and Ben et al. 2004).** Galactose and sialic acid present in milk oligosaccharide are required for optimal development of the infant's brain **(Urashima et al. 2001 and Sharon et al. 2000).** Human milk oligosaccharide containing α1,2-linked fucose inhibits the stable toxin-producing Escherichia coli in vitro, and its toxin induced secretory diarrhea in vitro and in vivo **(Kunz et al. 2000 and Newburg et al. 2004).** Specific fucosyl oligosaccharides of human milk have been observed to inhibit specific pathogens **(Guillermo et al. 2003).** Sialylated human milk oligosaccharide also inhibits binding of pathogenic strains of Escherichia coli and ulcer-causing human pathogen H. Pylori **(Kuhn et al. 1965).** Neutral human milk oligosaccharide may protect the intestinal tract of neonates from Vibrio cholerae. It was surprisingly found that oligosaccharides isolated from sheep milk strongly stimulate the immune system, and are used for treatment of immune system related disorders. In ancient literature, Hippocrates recommended donkey milk for healing infection and wounds, liver ailments, poisoning, fever, nose bleed and for clearing of edema from the body. Donkey milk is also helpful in raising the metabolism rate of human body which helps to deplete the excess triglyceride reserve of the body. Besides that, the presence of Omega-3 fats in its milk helps in driving away triglyceride from the body which also reduces low density lipoprotein (LDL) or in common parlance the bad cholestrol in the blood. Donkey milk oligosaccharides have ability to stimulate non-specific and specific immunological resistance **(Ranjan et al. 2016).** Keeping in mind, physiological, biological and medicinal importance of Donkey's milk oligosaccharides, Donkey's milk was collected in the bulk and processed by method of Kobata and Ginsburg yielding oligosaccharide mixture **(Kobata et al. 1970).** This oligosaccharide mixture on purification yielded a novel oligosaccharide namely **Famiose**. The structure was elucidated with the help of chemical degradation, chemical transformation and spectroscopic methods like ^{1}H NMR, ^{13}C NMR and 2D NMR i.e., COSY, TOCSY, HSQC technique as well as mass spectrometry.

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 $25^{0}C$ on a Bruker AM 300 FT NMR spectrometer. The electrospray mass spectra were recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. The samples (dissolved in suitable solvents such as methanol/acetonitrile/water) were 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 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 ethylacetate-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) lyophilized 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 diod array detector and G.P. 100 printer plotter. Authentic samples of glucosamine, galactosamine, galactose and glucose were purchased from Aldrich Chemicals.

Isolation of Donkey milk oligosaccharide

12 litres Donkey milk was collected and equal amount of ethanol was added and stored at -20^{0}C until used. 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^{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 (0.24 µ, to remove remaining lactose) and lyophilized affording crude oligosaccharide mixture (120gm). The lyophilized material which responded positively to phenol-sulphuric acid test and Morgon-Elson test were taken for further studies. The lyophilized material (mixture of oligosaccharides) of Donkey milk was further purified on Sephadex G-25 column chromatography for separation of glycoproteins, free proteins and oligosaccharide (low molecular weight component) by using glass distilled water as eluent at a flow rate of 5 ml/min. Donkey milk oligosaccharide mixture (24gm) was packed in a column (1.6x40 cm) (void volume = 25 ml) equilibrated with glass triple distilled water (TDW) and it was left for 10-12 hrs to settle down (19gm). Presences of neutral sugars were monitored in all eluted fractions by phenol-sulphuric acid test.

Acetylation of Donkey milk oligosaccharide mixture

12gm Donkey milk oligosaccharides of pooled fractions, which gave positive phenol-sulphuric acid test, were acetylated with pyridine (12ml) and acetic anhydride (12ml) at 60^{0}C and the reaction mixture was stirred overnight. The mixture was evaporated under reduced pressure and viscous residue was taken in $CHCl_3$ and washed in sequence with 2N 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 (14gm). The acetylation converted the free oligosaccharides into their non-polar derivatives which 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 heating.

Purification of Acetylated Donkey milk oligosaccharide on Silica Gel Column

Purification of acetylated derivative was 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 eleven fractions.

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 Famiose.

Deacetylation of Compound

The compound, Famiose (30mg) obtained from the column chromatography of acetylated oligosaccharide mixture was dissolved in acetone and NH_3 and left overnight in a stoppered hydrolysis flask. Ammonia was removed under reduced pressure and the compound was washed thrice with $CHCl_3$ (to remove acetamide) and was finally freeze dried giving the deacetylated oligosaccharide Famiose (26mg).

DESCRIPTION OF ISOLATED COMPOUND FAMIOSE

^{1}H NMR of Acetylated Famiose in $CDCl_3$

6.259 [d, 1H, J=3.6Hz, α-Glc (S_1), H-1], 5.509 [d, 1H, J=7.8Hz, β-Glc (S'_1), H-1], 4.731 [d, 1H, J=7.2Hz, β-Glc (S_3), H-1], 4.582 [d, 1H, J=9.6Hz, β-Gal (S_2), H-1], 4.517 [d, 1H, J=8.4Hz, β-GlcNAc (S_6), H-1], 4.508 [d, 1H, J=8.4Hz, β-GlcNAc (S_5), H-1], 4.494 [d, 1H, J=7.6Hz, β-GalNAc (S_4), H-1], 2.056 [s, 3H, $NHCOCH_3$, β-GlcNAc (S_6)], 2.063 [s, 3H, $NHCOCH_3$, β-GlcNAc, (S_5)], 2.045 [s, 3H, $NHCOCH_3$ (S_4)].

^{13}C NMR of Acetylated Famiose $CDCl_3$

89.19[□-Glc (S_1),C-1], 90.04[□-Glc (S'_1),C-1], 95.18[β-Glc (S_3),C-1], 100.93[□-Gal (S_2),C-1], 100.93[□-GlcNAc (S_5),C-1], 101.01[β-GlcNAc (S_6),C-1], 104.17[β-GlcNAc (S_6),C-1].

^{1}H NMR of Famiose in D_2O

5.194 [d, 1H, J=2.4Hz, α-Glc (S_1), H-1], 4.746 [d, 1H, J=7.8Hz, β-Glc (S'_1), H-1], 4.713 [d, 1H, J=3.0Hz, β-Glc (S_3), H-1], 4.520 [d, 1H, J=7.5Hz, β-Gal (S_2), H-1], 4.508 [d, 1H, J=8.4Hz, β-GlcNAc (S_6), H-1], 4.458 [d, 1H, J=7.6Hz, β-GalNAc (S_4), H-1], 4.458 [d, 1H, J=8.4Hz, β-Gal (S_5),H-1].

^{13}CNMR of Famiose D_2O

88.20[□-Glc (S_1),C-1], 89.00[□-Glc (S'_1),C-1], 94.50[β-Glc (S_3),C-1], 99.93[□-GlcNAc (S_6),C-1], 100.00[□-Gal (S_2),C-1], 100.00[□-GlcNAc (S_5),C-1], 102.89[β-GalNAc (S_4),C-1].

Mass spectral fragments of compound Famiose

ES-MS m/z; 1153 $[M+K+H]^+$, 1113 $[M]^+$, 1077, 1060, 1042, 1008, 979, 936, 910, 707, 689, 660, 617, 559, 504, 444, 402, 384, 342, 324, 288, 244, 180, 145, 127.

RESULT AND DISCUSSION

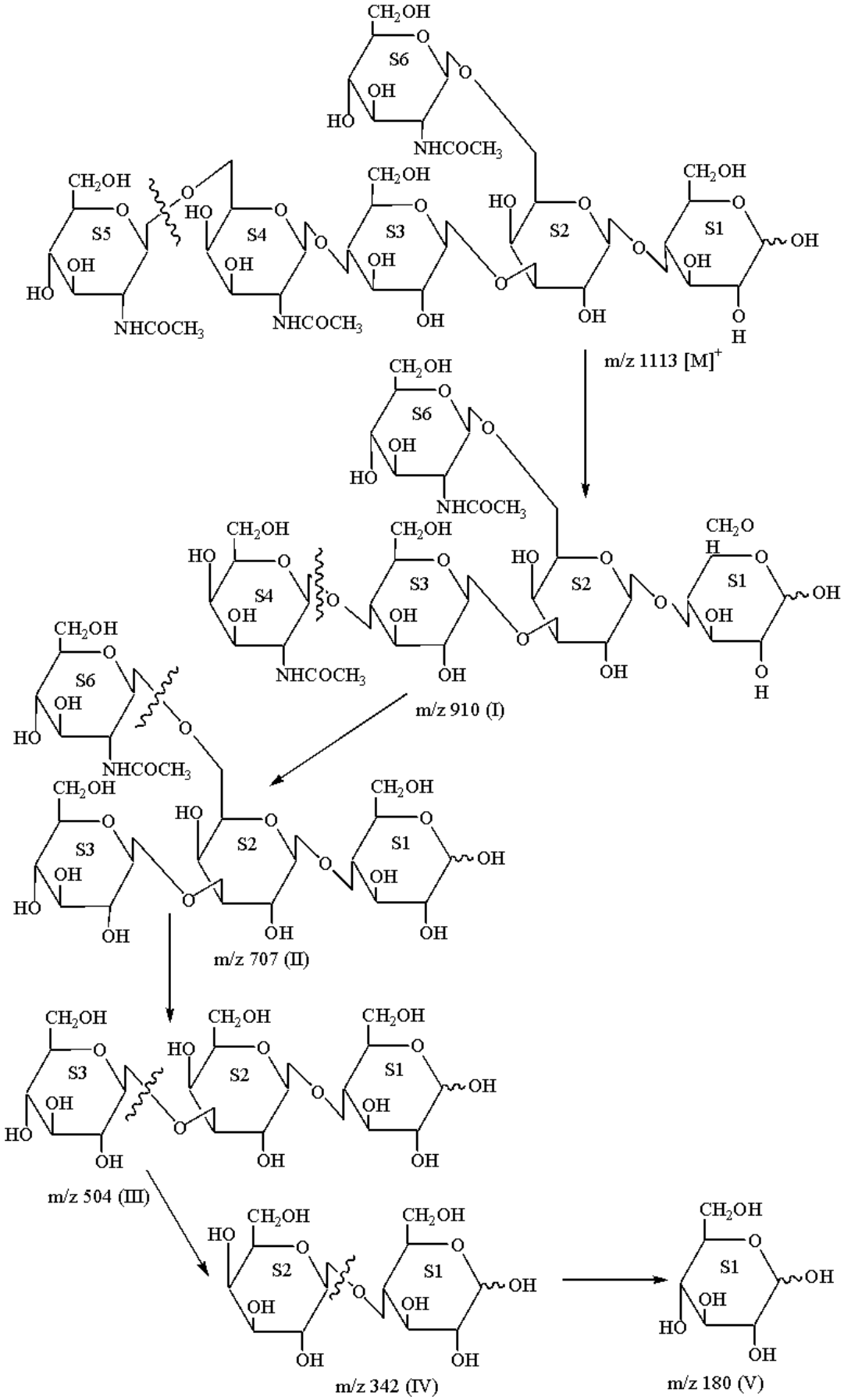
Compound Famiose, $C_{42}H_{71}N_3O_{31}$ $[\alpha]_D$ $+33^0$ gave positive phenol sulphuric acid test **(Dubois et al. 1956)**, Feigl test **(Feigl 1975)** and Morgon-Elson test **(Partridge et al. 1948)** showing the presence of normal and amino sugar(s) in the compound Famiose. The HSQC spectrum of acetylated compound at 400 MHz exhibited seven cross peaks for seven anomeric proton/carbon signals at δ6.259x□ 89.19, δ5.509x□ 90.04, δ4.731x□ 95.18, δ4.582x□ 100.93, δ4.517x□ 101.01, δ4.508x□ 100.93, and δ4.494x□ 104.17, indicating that the Famiose may be a hexasaccharide in its reducing form giving signals for α and β anomers of glucose at its reducing end. The hexasaccharide nature of acetylated Famiose was further confirmed by the presence of seven anomeric carbon and proton at δ89.19 (1C), δ90.04 (1C), δ95.18(1C), δ100.93(2C), δ101.01(1C) and δ104.17 (1C) in ^{13}CNMR and δ6.259 (1H), δ5.509(1H), δ4.731 (1H), δ4.582 (1H), δ4.517 (1H), δ4.508 (1H) and δ4.494 (1H) in ^{1}H NMR, respectively.

Methylglycosidation of Famiose by MeOH/H^+ followed by its acid hydrolysis led to the isolation of α and β Methylglucoside, which confirmed the presence of glucose at the reducing end of the oligosaccharide. It was also confirmed by the presence of two anomeric proton signals at δ5.194 and δ4.746 for α and β Glc in the ^{1}H NMR of Famiose in D_2O. For convenience the six monosaccharides in compound have been represented as S_1, S_2, S_3, S_4, S_5 and S_6 starting from reducing end. To confirm the monosaccharide constituents in compound Famiose, it was hydrolyzed under strong acidic conditions. In Killiani hydrolysis under strong acid condition, it gave four monosaccharides i.e. glucose, galactose, N-acetyl galactosamine and N-acetyl glucosamine, on paper chromatography and TLC. Since the glucose was present in its reducing form which was supported by ^{1}HNMR of Famiose in D_2O which contains two anomeric proton signals for α and β-Glc at δ5.194 (J=2.4 Hz) and at δ4.746 (J=7.2 Hz) **(Urashima et al. 2002).** It also contains signals for three Methyl groups at δ1.978, δ1.963 and δ1.920 suggesting the presence of three amino sugars in it. Further the presence of another anomeric proton signals at δ4.520 (J=7.5 Hz) was due to presence of β-Gal (S_2) moiety in the Famiose. The ^{1}HNMR also contains a triplet at δ3.225 for β-Gal (S_1) H-2 signal indicated that the equatorially oriented hydroxyl group at C-4 of the reducing β-Glc (S_1) was substituted and involved in glycosidation (SRG) **(Urashima et al. 2004)**, confirming the presence of a lactosyl moiety i.e. β-Gal (1→4) Glc in Famiose. The coupling constant of S_2 anomeric signal with J value of 7.5 Hz shows the β Configuration of anomeric linkage between S_2→S_1, in the lactosyl moiety. It was also supported by the presence of β-Glc H-4 proton resonance at δ3.824 in acetylated Famiose. Another anomeric proton signal, which appeared at δ4.713 (J=7.2 Hz) in the ^{1}HNMR of Famiose was due to the presence of another β-Glc moiety which was represented as S_3. The position of anomeric proton resonance at δ4.713 suggested that the β-Glc may be (1→3) linked (SRG) to β-Gal (S_2) along with the presence of H-3 and C-3 resonance of β-Gal (S_2), in ^{1}HNMR of Famiose acetate at δ4.078 & δ76.18, respectively. The coupling constant of anomeric signal with J value 7.2 Hz shows the β-configuration of anomeric linkage between S_3→S_2. The presence of next anomeric signal at δ4.458 with amide signal at δ1.978 was due to β-GlcNAc moiety S_6. The position of anomeric proton doublet at δ4.458 suggested the presence of β-Gal (S_2). The coupling constant of anomeric signal with J value 8.4 Hz shows the β configuration of anomeric linkage between S_6→S_2. It was further confirmed by the presence of H-6 & C-6 resonance of β-Gal (S_2) of Famiose acetate at δ3.870 & δ77.19, with upfield shifted H-4 value at δ3.933 (SRG). The next anomeric proton signal was appeared at δ4.458 with amide signal at δ1.920 was due to presence of β-GalNAc (S_4). The presence of H-2 triplet of Glc (S_3) at δ3.225 with amide signal at δ1.920 of S_4 suggested GalNAc may be (1→4) linked to Glc (S_3) like lactosyl moiety. It was further confirmed by the presence of H-4 and C-4 resonance of β-Glc S_3 at δ4.119 and δ76.67, respectively. The next anomeric signal appeared at δ4.508 with amide signal at δ1.963 was due to the presence of another β-GlcNAc (S_5). The position of anomeric proton at δ4.508 suggested it may be (1→6) linked to β-GlcNAc (S_4). This linkage was further confirmed by the presence of H-6 and C-6 proton resonance at δ3.824 and δ76.18 respectively. The hexasaccharide nature of compound was further confirmed by the spectral studies of acetylated derivative of compound Famiose. The heteronuclear single quantum coherence (HSQC) spectrum of acetylated compound confirmed linkages in ^{1}H and ^{13}C NMR spectra by showing cross peaks of an □-Glc (S_1) H-4 and C-4 at □ 3.854 x 81.81

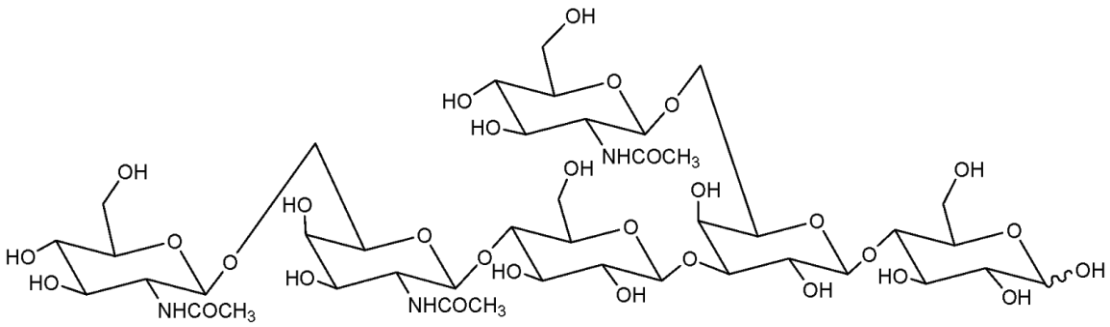
showed (1→4) linkage of S_2 and S_1, and also □-Glc(S_1) its H-4 and C-4 at □ 3.824 x 77.19 shows (1→4) linkage of S_2 and S_1. It also contain cross peak of H-3 and C-3 of □-Gal (S_2) at 4.078x76.18 showed (1→3) linkage of S_6 and S_2, H-6 and C-6 of □-Gal(S_2) at □ 3.870 x 77.19 showed (1→6) linkage of S_3 and S_2, H-4 and C-4 of □-Glc (S_3) at 4.119x76.67 showed (1→4) linkages of S_4 → S_3 and H-6 and C-6 □-GlcNAc (S_4) at □ 3.824 x 76.18 showed (1→6) linkages of S_5→ S_4, respectively showing in the same chemical shift region in acetylated and deacetylated spectra. It was further confirmed by the presence of same peaks in COSY and TOCSY spectrum. The structure of Famiose was elucidated as-

GlcNAc(1→6)-β-GalNAc(1→4)-β-Glc(1→3)-β-Gal(1→4)Glc
↓
GlcNAc(1→6)

The highest mass ion peak was recorded at m/z 1153 which was due to $[M+K+H]^+$, further mass ion peak at m/z 1113 for $[M]^+$ confirmed the molecular weight of compound was 1113. Further the mass fragments were formed by repeated H-transfer in the oligosaccharide and was accompained by the elimination of terminal sugar less water. The fragmentation pathway confirmed the sequence of monosaccharides in the oligosaccharide. The hexasaccharide m/z 1113 on fragmentation gave pentasaccharide at m/z 910 (I), which was due to loss of S_5 Sugar unit i.e. GlcNAc (S_5) sugar unit linked to the S_4 of hexasaccharide. It was supported by its respective fragments at m/z 180 that confirmed the presence of GlcNAc S_5 at non reducing end. The pentasaccharide m/z 910 (I) further fragmented to mass ion peak at m/z 707 (II) which obtained due to loss of GalNAc S_4 Sugar unit i.e. GalNAc S_4, its corresponding tetrasaccharide (II) moiety of pentasaccharide i.e. GalNAc (S_4) Sugar unit linked to the S-3 of pentasaccharide. The tetrasaccharide m/z 707 on fragmentation gave trisaccharide at m/z 504 (III) which was due to loss of S_6 sugar unit i.e. GlcNAc (S_6) Sugar unit is linked to the (S_2) of tetrasaccharide unit. The trisaccharide m/z 504 on fragmentation gave mass ion peak at m/z 342 (IV) which was due to loss of (S_3) Sugar unit i.e. Glc (S_3) Sugar unit linked to the (S_2) of trisaccharide unit. This disaccharide m/z 342 on further fragmentation gave a mass ion peak at m/z 180(V), which was due to loss of (S_2) Sugar unit i.e. Gal (S_2) Sugar unit linked to the (S_1) of disaccharide. The ES mass of compound Famiose showed other mass ion peaks at m/z 1077 [M - $2H_2O$], 1060[1077 - OH], 1042[1060 - H_2O], 1008[1042 - 2OH], 979[1008 - CHO], 936[979 - CH_3CO]. The mass ion at m/z 1113 fragmented by the loss of other terminal Sugars (S_5) gave the corresponding pentasaccharide mass ion fragment (I) at m/z 910 which was confirmed that two HexNAc moieties were presented at two non reducing ends of the hexasaccharide moiety. The mass ion peak at m/z 910 further fragmented to give mass ion fragment for tetrasaccharide moiety which obtained by the loss of Sugar (S_4) other mass ion fragment corresponds to the moiety m/z 707 appeared at m/z 689[707 - H_2O], 660[689 - CHO], 617[660 - $COCH_3$], 559[617 - $NHCOCH_3$]. Tetrasaccharide mass ion fragment on further fragmentation gave an important trisaccharide fragment at m/z 504. The trisaccharide fragment at m/z 504 on further fragmentation gave at m/z 444[504 - CH_2OHCHO], 402[444 - CH_2CO], 384[402 - H_2O]. The trisaccharide fragment at m/z 504 on further fragmentation gave a disaccharide fragment at m/z 342. The disaccharide further fragmented to give mass ion at m/z 324[342 - H_2O], 288[324 - 2 H_2O], 244[288 - CH_2=CHOH], 182[244 - CH_3CHOH - OH]. The disaccharide fragment at m/z 342 on further fragmentation gave a mass ion peak at m/z 180(V), it was supported by m/z at 145[180 - H_2O - OH], 127[145 - H_2O].


m/z 1113 [M]+
m/z 910 (I)
m/z 707 (II)
m/z 504 (III)
m/z 342 (IV)
m/z 180 (V)

Based on the results obtained from chemical degradation, chemical transformation, mass spectrometry and 1H , 13C, HOMOCOSY, TOCSY , HSQC NMR, the structure of the isolated novel hexasaccharide, Famiose was deduced as-



Compound Famiose

CONCLUSION

From the above informations, we conclude the structure of isolated Donkey milk oligosaccharide, **Famiose**. This oligosaccharide was reported for the first time from any natural source or any milk and elucidated with the help of spectroscopic technique like ^{1}H, ^{13}C, 2 DNMR (COSY, TOCSY and HSQC) spectroscopy and mass spectrometry.

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