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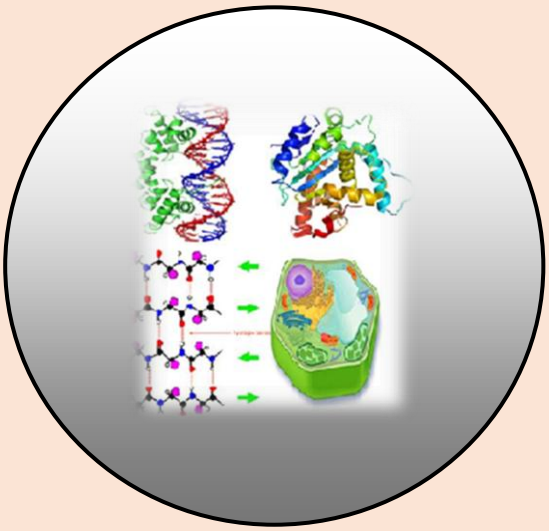

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Isolation and Structure Elucidation of Novel Tetrasaccharide from Mare Milk

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ABSTRACT

In addition to providing complete postnatal nutrition, milk is a complex biofluid that delivers bioactive components for the growth and development of the intestinal and immune system. Milk oligosaccharides have established themselves as an effective class of organic biomolecule impacting various physiological and pathological processes such as bimolecular recognition, signal transaction, differentiation and developmental events and exhibit various biological activities such as antitumor, immuno stimulant, anti-cancer, anti-complementary, anti-coagulant, anti-inflammatory, hypoglycemic, antiviral and immunological activities. Keeping in mind the physiological, biological and medicinal importance of milk oligosaccharide the mare milk was collected in bulk and processed by modified method of Kobata and Ginsburg. The novel oligosaccharide was isolated from its milk 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 spectrometry. The spectroscopic data confirmed that it was a tetrasaccharide in its reducing form. The structure of novel oligosaccharide marose was elucidated as under -

GalNAc-β-(1→6)-GalNAc-β-(1→6)-Gal-β-(1→4)-Glc
MAROSE

Keywords: Oligosaccharide, mare milk oligosaccharide and Marose.

INTRODUCTION

Milk is an excellent source of well-balanced nutrients responsible for development of neonates. The milk oligosaccharides recognize cancer associated antigens and has physiological significance in infants (Singh et al., 2016).

It contains all necessary nutrients for the growth and development of the newborn and also contains proteins, vitamins and carbohydrates especially lactose and large number of oligosaccharides. Oligosaccharides have established themselves as an effective class of organic biomolecules impacting various physiological and pathological processes such as molecular recognition, signal transaction and differentiation developmental events exhibit varied biological activities such as anti-tumour, immunostimulant, anti-cancer, anti-inflammatory, anti-viral and immunological activities (Singh et al., 2016).Milk oligosaccharides are made up of six monosaccharides namely, Glucose, Galactose, N-acetyl-glucosamine, N-acetyl-galactosamine, Fucose and N-acetyl-neuraminic acid.It consist of neutral and acidic oligosaccharides. The oligosaccharides isolated from various milk sources are categorized in two classes i.e. sialylated oligosaccharide and non sialylated. Both classes of oligosaccharides have been tested for their varied biological activities (Ranjan et al., 2016). Numerous oligosaccharides have been isolated from milk of many mammalian species including equine, bovine and marine mammals (Kunz et al., 2000, Urashima et al., 2008and Nakamura et al., 2004). Mare milk has shown anti-oxidant, lipid lowering and post heparin lipolytic activity (Srivastava et al., 2012). Mare milk is easily digestible, boost the immune system, strengthens the body and increase a person's energy and vitality. Mare's milk contains fourteen times the amount of Vitamin C found in cow's milk. It has valuable antioxidant properties, fights free radicals, which age our skin. Biological activity of mare's milk is well described in ancient medical literature of India and in some recent literature. Keeping in mind the biological activity of Mare milk and oligosaccharide present therein, it was collected in bulk and was processed by modified method of Kobata and Ginsburg followed by different chromatographic techniques like gel filtration, TLC, CC, HPLC etc. which resulted into the isolation of a new milk oligosaccharides namely MAROSE. The structures of purified milk oligosaccharides were elucidated by the using of the data generated from spectroscopic techniques like NMR (^{1}H, ^{13}C, COSY, TOCSY and HSQC) mass spectrometry and chemical degradation, chemical transformation.

MATERIAL AND METHODS

GENERAL PROCEDURE

General procedure was same as described in our previous communication (Maurya et al., 2017).

Isolation of mare milk oligosaccharide by the modified method of Kobata and Ginsburg-

Isolation of mare milk oligosaccharides was done by the modified method of Kobata and Ginsburg (Kumar et al., 2016), which was described in our previous communication (Maurya et al., 2017)except the isolation, was done from 10 litre of camel milk and the yield of oligosaccharide mixture was 315 gm.

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 (12.010gm). This acetylated mixtures gave tenspots on TLC namely a,b,c,d,e,i,j. Non-polar acetyl derivative of oligosaccharides were resolved nicely on TLC using CHCl3: MeOH as eluent. Detection of the spots was done by spraying with 50% H_2SO_4 and heat.

Column chromatography of acetylated oligosaccharide mixture

Acetylated Mare's milk oligosaccharides mixture (12.010gm)gave ten spots a,b,c,d,e,f,g,h,i,j on TLC which on repeated column chromatography by using various proportion of$CHCl_3$ and $CHCl_3$: MeOH resulted into isolation of of compound b (70mg) in pure form.

Deacetylation of Compound Marose Acetate (b)

Compound (70 mg) obtained from column chromatography 2 of acetylated oligosaccharide mixture was dissolved in acetone (4 ml) and 5 ml of NH_3 was added and left overnight in a stoppered hydrolysis flask. After 24 hr ammonia was removed under reduced pressure and the compound was washed with (3 x 8 ml) $CHCl_3$ and the water layer was finally freeze dried giving the deacetylated oligosaccharide B(38 mg) having retention time 2.025 min in HPLC.

Methylglycosidation/ acid hydrolysis of Compound B

Compound B Marose (8 mg) was refluxed with absolute MeOH (2 ml) at 70^0C for 18 h in the presence of cation exchange IR-120 (H^+) resin. The reaction mixture was filtered while hot and filtrate was concentrated. To a solution of methylglycoside of A in 1,4-dioxane (1 ml), 0.1 N H_2SO_4 (1 ml) was added and the solution was warmed for 30 minutes at 50^oC. The hydrolysis was completed after 26 hr. The hydrolyzates were neutralized with freshly prepared $BaCO_3$, filtered and concentrated under reduced pressure to afford α and β-methylglucosides along with the Gal and GalNAc. Their identification was confirmed by comparison with authentic samples (TLC, PC).

Kiliani Hydrolysis of Compound B

Compound B (5 mg) was dissolved in 1.5 ml Kiliani mixture (AcOH-H_2O-HCl, 7:11:2) and heated at 100^0C 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, to it and was evaporated under reduced pressure to afford glucose, galactose and galNAc on comparison with authentic samples (TLC, PC).

Description of Isolated Compound Marose

For elemental analysis, this compound was dried over P_2O_5 at 100^o C and 0.1 mm pressure for 8 h.

$C_{28}H_{48}N_2O_{21}$	-	%C	%H	%N
	Calcd.	**45.91**	**6.41**	**3.74**
	Found	**45.86**	**6.42**	**3.74**

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

δin D_2O (ppm) : ^{1}H NMR

δ 1.93 [s, 3H, NHCO$\underline{CH_3}$, β-GalNAc(S_3)], δ 2.00 [s, 3H, NHCO$\underline{CH_3}$, β-GalNAc(S_4)], δ3.274 (t, 1H, J = 8.4 Hz, β-Glc(S_1), H-2), δ 4.125 (m, 1H, J = 4.5 Hz, β-GalNAc(S_4), H-4), 4.166 (m, 1H, J = 4.5 Hz, β-GalNAc(S_3), H-4), δ4.45 (d, 1H, J = 7.8 Hz, β-Gal(S_2), H-1), 4.52 (d, 1H, J = 8.4 Hz, β-GalNAc (S_3), H-1),4.57 (d, 1H, J= 8.4 Hz, β-GalNAc (S_4)H-1), δ4.66 (d,1H, J=8.1Hz, (S_1)H-1), β-Glc δ 5.23 (d, 1H, J=3.0 Hz, α-Glc (S1) , H-1).

δin D_20 (ppm) : ^{13}C NMR

δ24.92[NHCO$\underline{CH_3}$,β-GalNAc(S_3)], 24.97[NHCO$\underline{CH_3}$,β-GalNAc(S_4)], 91.8, α Glc (S_1) C1, 95.7 β Glc (S_1) C-1, 100.9 β Gal (S_2) C-1, 102.9 β-GalNAc (S_3) & (S_4) C-1, 173.2 β-GlcNAc (S_3) & (S_4) NH$\underline{CO}CH_3$, 174.0 β-GalNAc(S_3) NH$\underline{CO}CH_3$, 176.0 β-GalNAc(S_4) NH$\underline{CO}CH_3$.

Mass Spectral Fragments of Compound Marose

ES-MS M/z : 810 $[M+Na+K]^+$, m/z 748$[M]^+$, m/z 545 [748-GalNAc(S_4)], m/z 342[545-S_3] and 180[342-S_2], and other fragments at m/z 705, 684, 652, 617, 602, 584, 545, 527, 522, 584, 487, 454, 442, 441, 440, 422, 391, 365, 360, 346, 325, 293, 291, 279, 278, 260

RESULT AND DISCUSSION

Compound B $[\alpha]_D$ +241(c, 0.3, H_2O), C_{28} H_{48} O_{21} N_2, gave positive Phenol-sulphuric acid test(Dubois et al., 1956), Feigl test (Fiegl, F., 1975)and Morgon-Elson test(Partrige et al., 1948), indicating the presence of normal and amino sugar(s) in the moiety. The ^1H NMR spectrum of Marose at 300 MHz exhibited five signals in the anomeric proton region as doublets at δ5.23 (1H), 4.66(1H), 4.57 (1H) and δ 4.521 (1H) and δ 4.453 (1H) for five protons indicating the presence of five anomeric protons in it. It was further supported by the appearance of five anomeric carbons at δ 91.8 (1C), 95.7 (1C), 102.9 (2C), 101.9 (1C) in the ^{13}C NMR spectrum of compound Marose. These data led to the suggestion that B may be a tetrasaccharide in its reducing form with a glucose unit at the reducing end. The molecular formula C_{28} H_{48} O_{21} N_2 was in agreement with mass ion peaks obtained from ES-MS spectrum of compound C which showed the highest mass ion peak at m/z 810$[M+K+Na]^+$ and 748$[M]^+$ for a tetrasaccharide. The four-monosaccharide units present in compound BMarose have been designated as S_1, S_2, S_3 and S_4 for convenience starting from the reducing end. The reducing nature of glucose was confirmed by the Methylglycosidation of Marose by MeOH /H^+ followed by its acid hydrolysis led to the isolation of α and β methyl glucoside. Further, for confirmation of the monosaccharide constituents in Marose, it was hydrolyzed under strong acidic condition (Kiliani hydrolysis) followed by paper chromatography showed the presence of GalNAc, Gal, Glc moieties, whose cleared that these moieties participate in the building of the compound B. The chemical shifts of anomeric carbons observed in ^{13}C NMR spectrum and anomeric protons observed in ^1H NMR spectrum of Marose are also in agreement with the reported values of ^1H and ^{13}C anomeric chemical shifts of Glc, Gal and GalNAc. The presence of four monosaccharides and its molecular formula suggested that there may be two GalNAc, one Gal and one moiety of Glc present in Marose.

The free anomeric nature of glucose was further supported by presence of two anomeric proton signals as doublets and their coupling constants for α-Glc δ 5.23 (1H) J =3.0 Hz and β-Glc δ 4.66 (1H) J=8.1 Hz. This also confirms the reducing and free nature of glucose sugar in Marose molecule. Further the presence of another anomeric signal at δ 4.45 (1H) J=7.8 Hz suggested the presence of β Gal moiety in Marose led to the suggestion for the presence of lactose molecule in it. This was further confirmed by β Glc (S_1) H-2 signal (a structural reporter group) which appeared as a triplet at δ3.274 (J=8.1 Hz). Further, another anomeric protons appeared at δ 4.52 (J=8.4Hz) along with signal of NHAc group at 1.934 was due to the presence of GalNAc which is present next in sequence with lactose moiety. The linkage of GalNAc to the lactose moiety GalNAc (S_3) [1→6] β-Gal (S_2) was confirmed by acetylated ^1H-^1H COSY and TOCSY spectrum of Marose, In which H-6 of Gal (S_2) appear at upfield region δ 3.71 ppm and H-2, H-3 and H-4 of Gal appear at δ 5.26, δ 5.03 and δ 5.40 respectively, these data confirms that C-2, C-3 and C-4 positions of Gal (S_2) are not involved in glycosidic linkages with β-GalNAc (S_3).

This was also confirmed with the HSQC spectrum of acetylated compound B.InHSQC spectrum the H-6 proton of Gal (S_2) present at δ 3.71 in ^{1}H axis and its cross peak with the ^{13}C axis present at δ 71.1 (δ3.7 x δ 71.1), confirmed the GalNAc (S_3) [1→6] β-Gal (S_2) linkage. The fourth anomeric proton of Marose appeared as a doublet at δ 4.57 (1H, J=8.4 Hz) along with a singlet of three protons at δ 2.00 showed the presence of another β-GalNAc (S_4) in Marose, which was glycosidically linked to the C-6 of GalNAc (S_3). This linkage GalNAc (S_4) [1→6] β-GalNAc (S_3) was confirmed by the acetylated ^{1}H-^{1}H COSY and TOCSY spectrum of Marose, which shows H-3 and H-4 proton signals at δ 5.02ppm and δ5.39 ppm respectively but H-6 proton present at δ3.75 ppm. The HSQC spectrum also confirms that the H-6 proton of S_3 was given cross peak at (δ 3.75x δ 72.5) which strongly indicated that (S_4) moiety β glycosidically linked to S_3 at C-6 position.

The chemical shifts of the anomeric carbons of compound B at δ 91.8 (1C, α-Glc), 95.7 (1C, β-Glc), 100.9 (1C, β-Gal) and δ 102.9 (2C, β-GalNAc) are in accordance with the anomeric carbon values of Glc, Gal and GalNAc. The comparison of chemical shifts of ring carbons of this tetrasaccharide with the reported values (structural reported groups) also supported the derived structure of compound B.

Table: ^{13}C NMR Values of Compound B Marose.

	C-1	C-2	C-3	C-4	C-5	C-6	-CO	-CH_3
α-Glc(S_1)	91.8	71.1	72.5	78.7	71.1	61.1		
β-Glc(S_1)	95.7	74.7	75.3	78.4	75.3	60.5		
β-Gal(S_2)	102.9	70.9	81.0	69.2	75.3	69.2		
β-GalNAc(S_3)	102.9	56.6	72.5	68.9	75.3	60.0	174	21.8
β-GalNAc(S_4)	102.9	56.6	72.7	68.6	76.2	62.9	176	21.8

The tetrasaccharide nature of Marose was further confirmed by spectral studies of acetylated derivative of Marose**.** The heteronuclear single quantum-coherence (HSQC) spectrum of acetylated product of compound Bconfirms the anomeric assignments in ^{1}H and ^{13}C NMR spectra of B by showing the ^{1}H and ^{13}C cross peaks of α-Glc (S_1) at δ6.25 x δ 89.1 and β-Glc (S_1) at δ 5.6 x 91.5. It also contains three crosspeaks of one β- Gal (S_2) at δ 4.54 x δ 101.8, and two β-GalNAc moieties (S_3) & (S_4) present at δ4.56 x δ102.0 and δ4.594 x δ102.0 respectively. Based on the pattern of chemical shift of ^{1}H, ^{13}C, HOMOCOSY, TOCSY and HSQC NMR experiments, it was interpreted that the compound B was a tetrasaccharide having one Glc, one Gal and two GalNAc moieties and compound was interpreted as linear tetrasaccharide having following structure.

GalNAc-β- (1 → 6)-GalNAc-β-(1 → 6) Gal-β-(1 → 4) Glc

Table. ^{1}H AND ^{13}C NMR Values of Compound B.

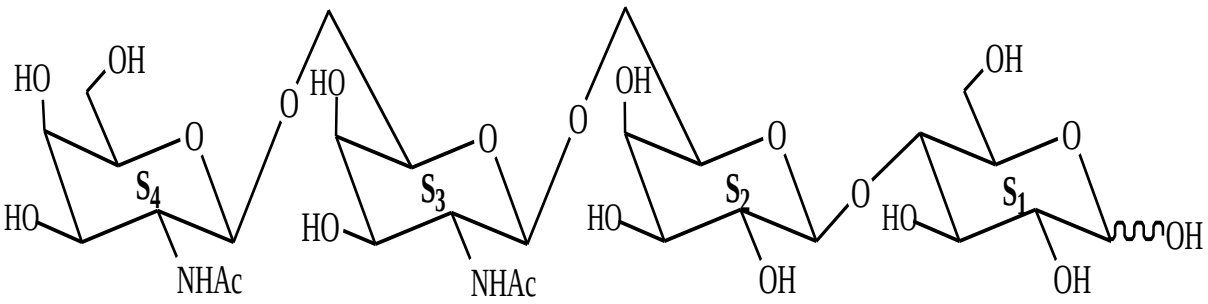
	αGlc(S_1)	**βGlc(S_1)**	**βGal(S_2)**	**βGalNAc (S_3)**	**βGalNAc(S_4)**
^{1}H	5.23	4.66	4.45	4.52	4.57
^{13}C	91.8	95.7	101.9	102.9	102.9

The Electrospray Mass Spectrometric data of compound B Marose helped in substantiating the sequence of monosaccharide units in it.

The highest mass ion peaks were recorded at m/z 810 and m/z 748 which were due to $[M+Na+K]^+$ and $[M]^+$ confirming the molecular weight of Marose as 748. 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 also confirmed the sequence of monosaccharides in the oligosaccharide. The tetrasaccharide m/z 748 (I) fragmented to give mass ion at m/z 545 (II), this fragmentation corresponded to the loss of terminal GalNAc (S_4) moiety from tetrasaccharide [707-S_4], indicating the presence of GalNAc (S_4) at the non-reducing end. It further fragmented to give mass ion peak at m/z 342 (III) with loss of another GalNAc moiety from Trisaccharide [342-S_3], this further fragmented to give mass ion peak at 180 (IV) These three mass ion peaks II, III, IV are appeared due to the consequent loss of GalNAc, GalNAc and Gal from original molecule.

The other fragmentation pathway in ES Mass spectrum of compound B of m/z 748 shows the mass ion peak at m/z at 684[M-2CH_3OH], 652[748-3H_2O-CH_2C=O], 617[652-H_2O-OH], 545[617-4H_2O], 601[652-3OH], 602[652-CH_3OH-H_2O], 585[602-OH], 501[585-CH_2=O], 443[501-$NHCOCH_3$], 385[443-$NHCOCH_3$] and 325[385-CH_2OHCHO], 545[603-$NHCOCH_3$]. The mass ion fragment at m/z 545 (II) was also supported by its respective fragment at m/z 527[545-H_2O]. In Second way 440[545-CH_2OH-CH_3OH-CH_2C=O], 365 [440-$NHCOCH_3$-OH],422 [440-H_2O],391[422-CH_2C=O],360[422-2CH_2OH], In third way of fragment pattern of m/z 545 given m/z 441[545-CH_2OH], 360[441-CH_3OH-CH_2OH-H_2O],342[360-H_2O], 323[342-OH] or 325[360-H_2O-OH], 346[441-CH_2OHCHO-H_2O-OH] and in fourth way m/z at 454[545-CH_2OH-CH_2OHCHO] obtained. On further fragmentation of m/z 545 gave disaccharide fragment m/z at 342 (III) with loss of second GalNAc moiety from II fragment ion m/z 545, it further fragmented to given 262[342-OH-CH_3OH-CH_2OH], 214[262-CH_2OH-OH], 163[214-3OH], 183[214-CH_2OH]. In other ways of fragmentation it gave m/z at 279[342-CH_3OH-CH_2OH], 261[279-H_2O] and m/z at 325[342-OH], 291[325-2OH], 214[291-CH_2OHCHO-OH], 260[291-CH_2OH]. In another way m/z 325 Fragmented to give mass ion peak at 293[325-CH_3OH], 203[293-CH_2OH,-CH_2=C=O,-OH], 143[203-CH_2=C=O-H_2O], 91[143-2OH-H_2O], 186 [203-OH], 151[186-H_2O-OH], 116[151-H_2O-OH]. The fourth sugar unit m/z 180 (IV) also fragmented to given mass ion peak at 116[180-2CH_3OH], 99[116-OH], 82[99-OH], and m/z at 85[116-CH_2OH].

Based on the results obtained from chemical degradation/acid Hydrolysis, Chemical transformation, Electro Spray Mass spectrometry and ^1H, ^{13}C NMR and HOMOCOSY, TOCSY and HSQC 2D NMR techniques of Marose and acetylated Marose, the structure and sequence of isolated Novel oligosaccharide molecule **MAROSE** was deduced as-



MAROSE

CONCLUSION

In summary, the novel milk oligosaccharide B, a tetrasaccharide namely Marose has been isolated from mare milk and elucidated with help of ^{1}H, ^{13}C, 2D NMR spectroscopy and mass spectrometry.

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