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DFT Studies and Structure Elucidation of Novel oligosaccharide from Camel Milk

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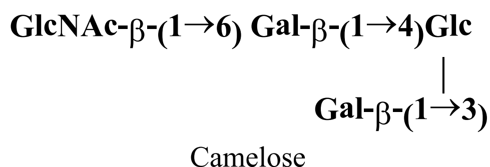
Abstract: Milk is an excellent source of well balanced nutrients to exhibit a range of biological activities that influence digestion, growth and development of specific organs. It is a complete natural source of metabolic responses to absorbed nutrients and resistance to disease. Camel milk contains higher concentration of sialyl oligosaccharides which have shown anti-adhesive effects against certain pathogens as well as important nutrients for brain development. The therapeutic value of camel milk to treat stomach ulcers, liver disorders, diarrhoea, constipation, wounds, and chronic diseases of gastro intestinal tract, chronic hepatitis and spleen inflammation is well established. In present work we have isolated a novel oligosaccharides "Camelose" from Camel milk by employing method of Kobata and Ginsburg followed by gel filtration, column chromatography and its subsequent HPLC. The structure of the isolated compound was established by result obtained from chemical transformation, degradation of sugar chains and spectroscopic data of ^{1}H NMR, ^{13}C NMR, 2D NMR (COSY, TOCSY and HSQC) and ES Mass Spectroscopy. The geometry of compound has been optimized at B3LYP method and 6-311G+ basic set. The structure of novel oligosaccharide was established as:



Camelose

Keywords: Anti-adhesive, Camel milk, Camelose, Kobata and Ginsburg and HPLC.

1. Introduction

Milk is the sole nourishment of the mammalian neonate which has a complete source of bioactive molecules that help to protect the new born against infectious disease and also promote the development of specific organs [1] [2]. Milk contains glycoprotein, glycopeptides

and oligosaccharides, which are found almost exclusively in this secretion at early stages of lactation and influence the digestion and resistance to disease [2] [3]. Recent studies have shown that milk furnishes a broad range of biologically active compounds that guard neonates and adult against pathogens and illness such as immunoglobins, antibacterial peptides, antimicrobial proteins lipids and oligosaccharides [4]. It is generally believed that milk oligosaccharides are biologically significant as receptors analogs that inhibit the attachment of pathogenic microorganism to the colonic mucosa, as prebiotics which stimulate the growth of bifidobacteria in the colon and as nerve growth factors [5]. More than 300 milk oligosaccharides have been isolated from milk of cow [6], buffalo, donkey, horse, sheep, goat [7], bear, mare, yak [8] [9], camel [10] and human [11]**.** The enormous biological activity of these oligosaccharide such as anti-tumour [12], immunostimulant [13], anti-cancer [14], anti-complementary, anti-inflammatory [15], hypoglycaemic, antiviral, antimicrobial, antioxidant, lipid lowering [16], immunological activity [17] and regulation of mineral absorption [18] are reported. Donkey milk oligosaccharides have ability to stimulate non-specific and specific immunological resistance [19]. A study by Deepak et al., established that buffalo milk oligosaccharides have ability to stimulate non-specific immunological resistance of the host against parasitic infections [20]. Camel milk oligosaccharides, which contain sialyl oligosaccharides and this sialic acid may exhibit a number of health benefits for human infants, including the promotion of infant brain development [21] [22]. Sialyl oligosaccharides possess anti-adhesive effects against certain pathogens as well as important nutrients for brain development [23] [24]. Camel milk and colostrum have medicinal and protective properties which may have a possible role for enhancing the immune defence mechanism [25] [26]. So keeping in the mind the anti adhesive effects of sialyl oligosaccharide present in the camel milk, we have isolated the novel milk oligosaccharide, which structure was elucidated with the help of chemical degradation, chemical transformation and spectroscopic technique like 1D NMR and 2D NMR as well as mass spectrometry and the geometry of compound has been optimized at B3LYP method and 6-311G+ basic set.

2- Theoretical study

The theoretical studies of the compound Camelose was performed by the DFT studies. DFT is an effective tool in quantum chemistry for evaluation of the molecular structure, electron density and optimized geometry etc. Geometries of compound C has been first optimized and the presence of positive wave numbers values for all the optimized geometry indicates stability of the compound. All computations were performed using the Gaussian 09 program package.

2.1. Materials and Methods

2.1.1 General procedures

Optical rotations were measured with a PERKIN-ELMER 241 automatic polarimeter in 1cm tube. 1 D and 2 D NMR spectra of compound C were recorded in D_2O and the spectra of acetylated oligosaccharides were recorded in $CDCl_3$ at 25^0C on a Bruker AM 300 FT NMR spectrometer. The electrospray mass spectra were recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. The C, H and N analysis were recorded on CARLO-ELBA 1108 an elemental analyzer. The sugars were visualized on thin layer chromatography (TLC) with 50% aqueous H_2SO_4 reagent and on Paper Chromatography (PC) with acetyl acetone and p-dimethyl amino benzaldehyde reagents. The absorbent for TLC and column chromatography (CC) was silica gel G (SRL) and silica gel (SRL, 60-120 mesh) respectively. 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) lyophylizer and centrifuged by a cooling centrifuged Remi instruments C-23 JJRCI 763. To check the homogeneity of the compound 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 glucose glucosamine and galactose were purchased from Aldrich Chemicals.

2.2 Isolation of camel milk oligosaccharide by Method of Kobata and Ginsberg method

12 liter Camel milk was collected from a domestic camel and then processed by method of Kobata and Ginsberg method [27]. For this method, it was stored at -20°C and centrifuged for 15 min at 5000 rpm at -4°C. The solidified lipid layer was removed by filtration through glass wool column in cold. Ethanol was added to clear filtrate to a final concentration of 68 % and the resulting solution was left overnight at 0°C. The white precipitate formed, mainly of lactose and protein was removed by centrifugation and washed twice with 68% ethanol at 0°C. The supernatant and washings were combined and filtered through a microfilter and lyophilized affording crude oligosaccharide mixture. The lyophilized material responded positively to Morgan-Elson test [28] and thiobarbituric acid assay [29] suggesting the presence of N-acetyl sugars and sialic acid in oligosaccharide mixture (315gm). This lyophilized material (mixture of oligosaccharide) was further purified by fraction it on sephadex G-25 chromatography using glass triple distilled water as eluant at a flow rate of 3 ml/m. each fraction was analyzed by phenol sulphuric acid reagent [30] for the presence of neutral sugar.

2.3. Confirmation of homogenity of camel milk oligosaccharide by reverse phase HPLC

Oligosaccharide mixture were quantitatively analysed by reverse phase HPLC. The HPLC system was equipped with Perkin-Elmer 250 solvent delivering system, 235 diode array detector and G.P. 100 printer plotter. The cyano column used for this purpose A binary gradient system 0.05% trifluroacetic acid (5:95) in triple distilled water (TDW) to acetonitrile : 0.05% TFA within 25 min. at a flow rate of 1 ml/min was used. The eluents were detected at 215 nm. Ten peaks were noticed in the sample at the varied retention times from 2.41min. to 17.63 min for convenience the peaks were numbered in their increasing order of retention time i.e. 2.432 min(R_1), 2.997 min(R_2), 3.349 min(R_3), 3.744 min(R_4), 4.235 min(R_5), 5.483 min(R_6), 8.203 min(R_7), 8.619 min(R_8), 10.667 min(R_9) and 17.579min(R_{10}). **(Fig. 1)**

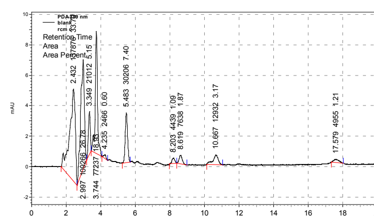


Fig 1. Reverse phase HPLC of crude oligosaccharide fraction. Sample was injected on a cyano column and eluted with TDW using binary gradient 0.05%TFA in acetonitrile at a flow rate of 1 ml/min. Elution was monitered by U.V absorbance at 215nm.

2.4. Acetylation of Oligosaccharide Mixture

12gm of crude oligosaccharide mixture was acetylated with pyridine (12ml) and acetic anhydride (12ml) at 60^0C and solution was stirred overnight. The mixture was evaporated under reduced pressure and the viscous residue was taken in $CHCl_3$ (250ml) 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 (14gm). The acetylation converted the free sugars into their non-polar acetyl derivatives which were resolved nicely on TLC, giving ten spots on TLC i.e. A, B, C, D, E, F, G, H, I and J of which coumpound C was finally separated by column chromatography over silica gel using hexane:chloroform and $CHCl_3$:MeOH as eluents.

2.5. Purification of acetylated milk oligosaccharide on silica gel column

Separation of acetylated product (12 gm) was carried over silica gel (300 gm) using varying proportion of Hex: $CHCl_3$, $CHCl_3$ and $CHCl_3$: MeOH as eluents, collecting fractions of 300ml each. All these fractions were checked on TLC. So ten fractions namely I (3.28gm), II (736mg), III (3.29gm), IV (468mg), V (380mg), VI (1.007gm), VII (1.06gm), VIII (767mg), IX (319mg) and X (137mg) respectively obtained. These fractions are mixtures of two and three compound of oligosaccharides which are further separated by repeated column chromatography. Fraction III was re-chromatographed on silica gel column (1.5 x 40 cm) and eluted with the same solvent system to yield chromatographically pure compound **'C'** (165 mg).

2.6. Deacetylation of Compound

Compound C (40 mg) obtained from column chromatography of acetylated oligosaccharide mixture was dissolved in acetone (2 ml) and 3 ml of NH_3 was added and left overnight in a stoppered hydrolysis flask. After 24 h ammonia was removed under reduced pressure and the compound were washed with $CHCl_3$ and the water layer were finally freeze dried giving the deacetylated oligosaccharide C (36 mg).

2.7. Methylglycosidation/Acid Hydrolysis of Compound

Compound C (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 separately. The reaction mixture was filtered while hot and filtrate was concentrated. To a solution of methylglycoside of C in 1, 4-dioxane (1 ml), 0.1 N H_2SO_4 (1ml) were added and the solution were warmed for 30 minutes at 50ºC. The hydrolysis was completed in 26 hr. The hydrolysate was neutralized with freshly prepared $BaCO_3$, filtered and concentrated under reduced pressure to afforded α and β-methylglucosides along with the Glc, Gal and GlcNAc. Their identification were confirmed by comparison with authentic samples glucose, galactose and glucosamine (Scheme 1).

2.8. Killiani Hydrolysis of Compound

Compound C (5 mg) was dissolved in 2 ml Killiani mixture (AcOH-H_2O-HCI, 7:11:2) and heated at 100°C for 1 h followed by evaporation under reduced pressure [31]. It was dissolved in 2 ml of H_20 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, galactose and glucosamine on comparison with authentic samples glucose, galactose and glucosamine (Scheme 1).

2.8. Description of compound

2.8.1 Compound C (Camelose)

Compound C, $C_{26}H_{45}O_{21}N$, $[\alpha]_D$= +70.22^0 (c, 2, H_2O), for elemental analysis compound C was dried over P_2O_5 at 100ºC and 0.1 mm pressure for 8 hr. Elemental analysis : Calcd. C- 44.13% ; H- 6.36% ; N- 1.98 % and Found . C- 44.08%; H- 6.35 %; N-1.95 %.

^{1}H NMR value of compound C in D_2O (δ value)

2.02[s, 3H, $NHCOCH_3$, β-GlcNAc (S-3)], 3.52[t, 1H, J=7.0 Hz, β-Glc(S-1), H-2], 3.95 [β-Gal (S-2), H-4], 4.48[d, 1H, J=6.9 Hz, β-Gal (S-2), H-1], 4.55[d, 1H, J=6.9 Hz, β-Gal (S-4), H-1], 4.58[d, 1H, J=8.4 Hz, β-Glc (S-1), H-1], 4.69[d, 1H, J=8.1 Hz, β-GlcNAc (S-3), H-1], 5.25[d, 1H, J=3.6 Hz, α-Glc (S-1), H-1]

^{13}C NMR value of compound C in D_2O (δ value)

77.2[α-Glc (S-1), C-3], 77.7[β-Glc (S-2), C-3], 80.7[α-Glc (S-1), C-4], 81.9[β-Glc (S-1), C-4], 91.8 [α-Glc (S-1), C-1], 95.7[β-Glc (S-1), C-1], 100.9[β-Gal (S-2), C-1], 102.8[β-Gal (S-4) & β-GlcNAc (S-3), C-1].

^{1}H NMR value of Acetylated compound C in $CDCl_3$ (δ value)

3.57[β-Glc (S-1), H-4], 3.60[α-Glc (S-1), H-4], 3.76[β-Gal, (S-2), H-6)], 3.79[β-Glc (S-1), H-3], 3.82[α-Glc (S-1), H-3], 4.55[d, 2H, J=6.9 Hz, β-Gal (S-2) & β-GlcNAc (S-3), H-1], 4.57 [d, 1H, J=8.4, β-Gal (S-4), H-1], 5.63[d, 1H, J=8.4 Hz, β-Glc (S-1), H-1], 6.21[d, 1H, J=3.6 Hz, α-Glc (S-1), H-1].

^{13}C NMR value of Acetylated compound C in $CDCl_3$ (δ value)

72.75[β-Gal (S-2), C-6], 73.47[α-Glc & β-Glc (S-1), C-3], 82.2[β-Glc (S-1), C-4], 82.35[α-Glc (S-1), C-4], 89.3[α-Glc (S-1), C-1], 91.7[β-Glc (S-1), C-1], 102.0 [β-Gal (S-4), C-1], 102.2 [β-Gal (S-2) & β-GlcNAc (S-3), C-1].

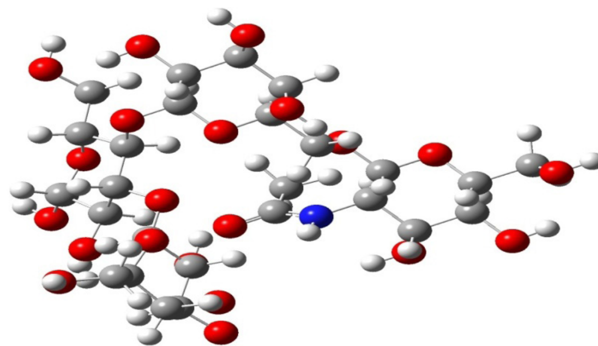
ES Mass of compound C

769[M+Na+k] $^+$, 746[M+K] $^+$, 730[M+Na] $^+$, 707[M] $^+$, 689, 649, 648, 647, 629, 618, 612, 601, 552, 547, 504, 473, 456, 421, 343, 342, 325, 300, 294, 288, 269, 251, 242, 233, 232, 197, 184, 180, 163.

3-Result and Discussion

3.1. Stability of Molecular geometries of the isolated compound

Density functional theory (DFT), a computational method, was employed to evaluate the structure-activity relationship. In this study, the geometry of compound C was optimized at B3LYP method and 6-311G(d,p) basis set using Gaussian 09 program package [32]. The molecular geometries can be determined by the quantum mechanical behaviour of the electrons and computed by ab-initio quantum chemistry methods to high accuracy. Molecular geometry represents the three-dimensional arrangement of the atoms that determines several properties of a substance including its reactivity, polarity, phase of matter, magnetism and biological activity. The optimized geometries of compound C show positive wave-number values indicated that they have been obtained at minima in potential surface scan and result the stability of compound C. Compound C has optimized energy -2652.97194185a.u and the optimized structure of compound C is given below:

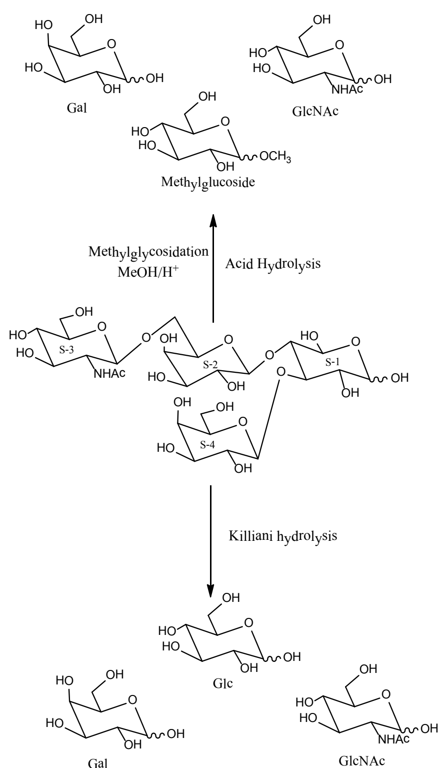


3.2. Structure elucidation of the isolated camel milk oligosaccharides

The structure of novel oligosaccharide compound C was defined with the help of different NMR spectroscopic method like ^{1}H, ^{13}C and 2D NMR and mass spectroscopy as well as chemical degradation, chemical transformation and structural reporter group theory were also

used in the structure elucidation of compound C.

Compound C, **Camelose**, $C_{26}H_{45}O_{21}N$, $[M]^+$ 707, $[\alpha]_D$ +70.22^0 gave positive Phenol-sulphuric acid test [30], Feigl test [33] and Morgon-Elson test [28] showed the nature of compound C as a oligosaccharide containing normal and amino sugar(s). ^{1}H NMR spectrum of compound C exhibited five anomeric proton signals at δ5.25 (1H), δ4.69 (1H), δ4.58 (1H), δ4.55 (1H), δ4.48 (1H) and five anomeric carbon at δ91.8 (1C), δ95.7 (1C), δ100.9 (1C) and δ102.8 (2C) in ^{13}C NMR spectrum having total integral intensity of four protons/carbons, concluded that compound C is tetrasaccharide in nature with their reducing form (Table 1). The reducing nature of compound C was also confirmed by the presence of *α* and *β*-methyl glucosides obtained from methyl glycosidation of compound C followed by its acid hydrolysis.(Scheme 1)



Scheme 1: Methylglycosidation/Acid Hydrolysis and Killiani Hydrolysis of Compound C

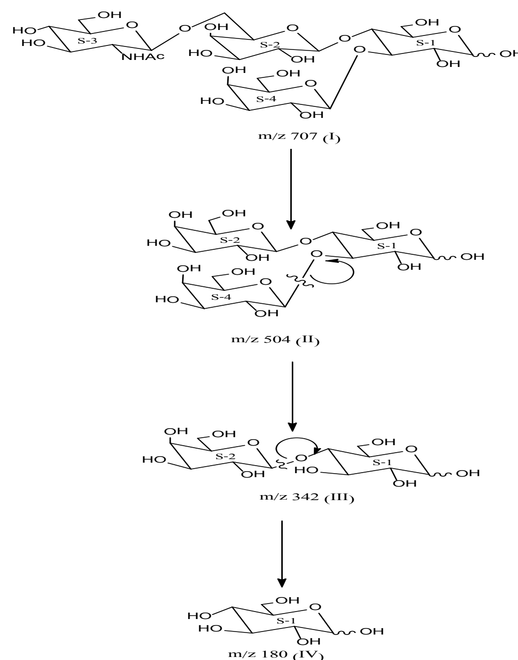
The tetrasaccharide nature of compound C was further supported by HSQC spectrum of acetylated compound C which showed four cross peak in the anomeric region at δ6.21 x 89.3, δ5.63 x 91.7, δ4.55 x 102.2 and δ4.57 x 102.0. For convenience, the four monosaccharide units in compound **C** are designated as S_1, S_2, S_3 and S_4 starting from reducing end. The monosaccharides constituents in compound C were confirmed by its Killiani hydrolysis under strong acidic condition, followed by paper chromatography and TLC. In this hydrolysis three spots were found identical with the authentic samples of Glc, Gal and GlcNac by co-chromatography. ^{1}H NMR spectrum of compound **C** containing two anomeric doublets, each of one proton, (J=3.6) at δ5.25 (1H) and (J=8.4) at δ4.58 for *α* and *β*-Glc respectively [34] [35]. Further other anomeric proton doublet (J=6.9) at δ4.48 was due to presence of *β*-Gal moiety in the compound C, also it showed H-2 signal of *β*-Glc(S_1) at 3.52 in the downfield region, which indicated that both the equatorially oriented hydroxyl groups at C-3 and C-4 of the reducing *β*-Glc (S_1) were substituted and involved in glycosidation.

Table 1: ^{1}H NMR and ^{13}C NMR values of Compound C in D_2O and $CDCl_3$

Moieties	In D_2O		In $CDCl_3$	
	^{1}H NMR (δ)	^{13}C NMR (δ)	^{1}HNMR (δ)	^{13}C NMR (δ)
α-Glc(S-1)	5.25 (3.6Hz)	91.8	6.21 (3.6Hz)	89.3
β-Glc(S-1)	4.58 (8.4Hz)	95.7	5.63 (8.4Hz)	91.7
β-Gal(S-2)	4.48 (6.9Hz)	100.9	4.55 (6.9Hz)	102.2
β-GlcNAc(S-3)	4.69 (8.1Hz)	102.8	4.55 (6.9Hz)	102.2
β-Gal(S-4)	4.55 (6.9Hz)	102.8	4.57 (8.4Hz)	102.0

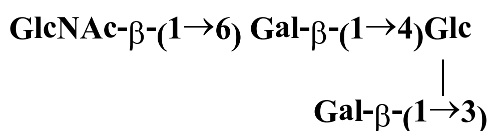
It was also supported by the presence of *β*-Glc H-3 proton at δ3.79 and H-4 proton at δ3.57 in

acetylated derivative of compound **C**. Another anomeric proton doublet which appeared at δ4.55 (J=6.9), was due to presence of *β*-Gal (S_4) moiety, linked to H-3 of Glc (S_1) which was confirmed by its TOCSY and COSY spectrum. This downfield shifting of *β*-Gal may be due to crowding and steric hindrance at the nonreducing end. The presence of another anomeric proton doublet (J=8.1) at δ4.69 (1H) was due to the presence of *β*-GlcNAc moiety which was supported by a singlet of amide methyl of N-acetyl glucosamine at δ2.02[36]. The H-4 proton of *β*-Gal (S_2), which appeared at δ3.95, implies that the *β*-GlcNAc (S_3) may be 1-6 linked to *β*-Gal (S_2) [37] [38]. This was confirmed on the downfield shifting of H-6 at δ3.76 and chemical shift analogies of *β*-GlcNAc(1→6)Gal-***β*****.** The glycosidic linkage were further assigned by the cross peaks for glycosidically linked carbons with their protons in HSQC spectrum of acetylaed compound C. The values of these cross peaks are as *α*-Glc (S_1) H-3 and C-3 at δ3.82 x 73.47 shows (1→3) linkage of S_4 and S_1, *α*-Glc (S_1) H-4 and C-4 at δ3.60 x 82.35 shows (14) linkage of S_2 and S_1, *β*-Glc (S_1) H-3 and C-3 at δ3.79 x 73.47 shows (1→3) linkage of S_4 and S_1, *β*-Glc (S_1) H-4 and C-4 at δ3.57 x 82.2 shows (1→4) linkage of S_3 and S_1 and *β*-Gal (S_2) H-6 and C-6 at δ3.763 x 72.75 shows (1→6) linkage of S_3 and S_2 respectively. COSY spectrum gave assignments of ring protons involved in linkage at δ3.82 (3-position, *α*-Glc), δ3.57 (4-position, *α*-Glc), δ3.79 (3-position, *β*-Glc), δ3.60 (4-position, *β*-Glc) and it was also confirmed by the presence of same peaks in TOCSY spectrum.



Scheme 2: Mass fragmentation of compound C

The ES mass spectrum of compound C showed highest mass ion peak at m/z 769 for $[M+Na+K]^+$. The $[M]^+$ of compound was also found at m/z 707 confirming the molecular weight of Compound C as 707 and was in agreement with its molecular formula i.e. $C_{26}H_{45}O_{21}N$. Other prominent mass fragments were obtained in the ES mass spectrum of compound C i.e. m/z 504[707-S_3], 342[504-S_4] and 180[342-S_2]. In the light of foregoing evidence the structure of the isolated tetrasaccharide C was deduced as



Camelose

4-Conclusion

In the summary, we conclude that compound **C** (Camelose) was reported for the first time from any mammalian milk and its structure

was elucidated with the help of spectroscopic technique like ^{1}H, ^{13}C and 2 D NMR spectroscopy along with mass spectroscopy. The geometry of compound has been optimized at B3LYP method and 6-311G+ basic set.

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