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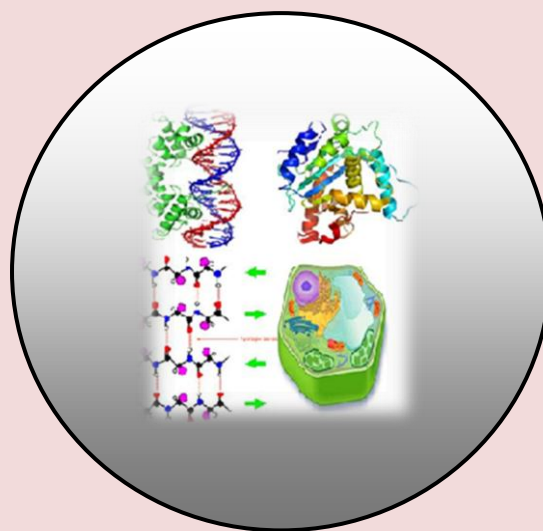
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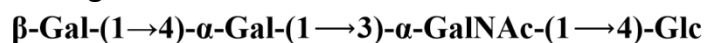
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ABSTRACT

Milk is a food supplement for any neonate for their proper growth and development due to not having any side effect. It is a source of many bioactive components, which not only help meeting the nutritional requirements of the consumers, but also play a relevant role in preventing various disorders. The animal milk contains up to 10% carbohydrate, of which the disaccharide, lactose (Gal(β 1-4) Glc), is usually a prominent component. Milk or colostrum contain lesser amounts of other saccharides, referred to as milk oligosaccharides, nearly all of which have a lactose unit at their reducing end to which GlcNAc, Gal, Fuc and/or Neu5Ac or Neu5Gc residues can be attached. Oligosaccharides have established themselves as an effective class of organic biomolecules impacting various physiological and pathological processes such as molecular recognition, signal transaction, differentiation and developmental events and exhibit varied biological activities such as antitumor, immunostimulant, anticancer, anticomplementary, anticoagulant, antiinflammatory, hypoglycemic, antiviral & immunological activities. Characterization of various animal milk oligosaccharides structures have been reported earlier. With respect to these characterization and their varied biological activities, in search on novel milk oligosaccharide we have chosen camel milk which emerged as a potent therapeutic alternative which can help in reducing insulin doses in diabetic patients. Epidemiological surveys also strongly indicate low prevalence of diabetes in communities consuming camel milk. So keeping in mind this entire thing we collected Camel milk in 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.

The structure elucidation of purified oligosaccharide was performed by chemical degradation, chemical transformation and physicochemical techniques like ¹H, ¹³C and 2D NMR (COSY, TOCSY, HSQC and HMBC). The spectroscopic data suggested that it was a tetrasaccharide in its reducing form.

The structure of novel oligosaccharide was elucidated as-



CAMMYOSE

Keywords: Oligosaccharide, camel milk oligosaccharide and NMR.

INTRODUCTION

Carbohydrates are an important energy source and play numerous key roles in all living organisms. Carbohydrates chemistry involved in diagnosis and treatment of diseases has been attracting increasing attention. Carbohydrates could be one of the major focuses of new drug discovery (Jiang, et al, 2021). In nature, carbohydrates (sugars) are created in plants, where they function as building blocks and energy suppliers. Then, carbohydrates are one of the major components of our food. These carbohydrates (glycans) play most important roles in human life (Gerwig, G.J., 2021). Mammalian milk contains up to 10% carbohydrate, of which the disaccharide, lactose (Gal(β1-4)Glc), is usually a prominent component. This milk is secreted by all species of mammals to supply nutrition and immunological protection to their young one. Irrespective of containing protein, fat, milk and colostrum also contain lesser amounts of other saccharides, referred to as milk oligosaccharides, nearly all of which have a lactose unit at their reducing end to which GlcNAc, Gal, Fuc and/or Neu5Ac or Neu5Gc residues can be attached (Urashima *et al.*, 2009). Human and animal milk contains a rich variety of oligosaccharides (OSs), which are of great interest due to a similar biological efficacy. Pronounced heterogeneity as well as homology of milk oligosaccharide structures among different mammalian species has been documented earlier (Messer and Urashima, 2002). The oligosaccharides present therein have varied biological activities i.e. anti-viral (Gao et al, 2020), anti-cancer (Zhao et al, 2017), anti-tumor (Wu et al 2020), anti-fungal (Gonia et al, 2015), anti-bacterial (Craft et al, 2019), immunostimulant (Singh et al, 2016), anti-inflammatory (Sahi et al, 2020) etc. which is the basic natural support system for the development of infants. Various animal's milk oligosaccharide having different biological activities like Donkey milk oligosaccharide having immunostimulant activity (Deepak et al, 1998), Goat milk containing galacto-oligosaccharides could be recommended to decrease most of infant allergy and diseases. Goat milk shows therapeutic virtues for individuals with certain dietetic problems (Bulgaru et al, 2016). The cow's milk oligosaccharides reduce the adhesion of enterotoxigenic *E. Coli* strains of the calf (Martín et al, 2002). Camel milk is more similar to human milk than any other milk and differs from other ruminant milk because it contains low cholesterol, low sugar, high minerals (sodium, potassium, iron, copper, zinc and magnesium), high vitamin C, protective proteins like as lactoferrin, lactoperoxidase, Immunoglobulins, lysozyme. It has been reported that camel milk contains the low quantity of β-casein and the lack of β-lactoglobulin which cause allergic reaction in lactose intolerant person (Kula, 2016). Camel milk oligosaccharides play a vital part in our digestive health by decreasing gut permeability. They help to nourish and support a healthy microbiome and play a vital role in immune function.

Camel milk's oligosaccharide benefits in infant's guts as well as promote brain development. Since, Camel milk has various biological and medicinal properties so in search of novel milk oligosaccharide and its biological properties from it we have chosen camel milk in bulk and isolated it by the modified method of Kobata and Ginsburg followed by chemical degradation, chemical transformation and various spectroscopic techniques like ^1H , ^{13}C , COSY, TOCSY, HSQC and Mass spectrometry.

MATERIAL AND METHODS

GENERAL PROCEDURE

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

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

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

Acetylation of camel milk oligosaccharide mixture

10 gm of crude oligosaccharide mixture was acetylated with pyridine (10ml) and acetic anhydride (10 ml) at 60°C and solution was stirred overnight. The mixture was evaporated under reduced pressure and the viscous residue was taken in CHCl_3 (250ml) and washed in with ice cold water and evaporated to dryness yielding the acetylated mixture (10 g). The acetylation converted the free sugars into their nonpolar acetyl derivatives which were resolved nicely on TLC, giving six spots on TLC i.e. a, b, c, d, e and f of which four compounds were finally isolated and purified by column chromatography over silica gel using hexane: CHCl_3 and MeOH: CHCl_3 as eluents. Detection of the spots was done by spraying with 50% H_2SO_4 and heat.

Deacetylation of Compound Acetate (c)

Compound c (15 mg) obtained from column chromatography 2 of acetylated oligosaccharide mixture was dissolved in acetone (2 ml) and 2 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 C (12.5 mg).

Methylglycosidation/ acid hydrolysis of Compound C (Gangwar et al, 2018)

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. The reaction mixture was filtered while hot and filtrate was concentrated. To this amorphous reaction mixture, 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°C . 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 C (Singh et al, 2016)

Compound C (5 mg) was dissolved in 1.5 ml Kiliani mixture ($\text{AcOH-H}_2\text{O-HCl}$, 7:11:2) and heated at 100°C for 1 h followed by evaporation under reduced pressure. It was dissolved in 2 ml of 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, it was evaporated under reduced pressure to afford glucose, galactose and GalNAc on comparison with authentic samples (TLC, PC).

Description of Isolated Compound C, Cammyose

For elemental analysis, this compound was dried over P₂O₅ at 100° C and 0.1 mm pressure for 8 h.

C ₂₆ H ₄₅ O ₂₁ N		%C	%H	%N
	Calculated	44.13	6.41	1.98
	Found	44.11	6.41	1.98

¹H NMR of Compound c, Cammyose Acetate in CDCl₃ at 300 MHz

6.24 [d, 1H, J=3.3 Hz, α-Glc(S-1) H-1], 5.68 [d, 1H, J=8.4Hz, β-Glc(S-1) H-1], 4.49 [d, 1H, J=3.9 Hz, α-GalNAc(S-2) H-1], 4.48[d, 1H, J=3.8, α-Gal(S-3) H-1], 4.46 [d, 1H, J=7.8Hz, β-Gal(S-4) H-1], 3.89[m, 1H, β-Glc(S-1) H-4], 3.80[m, 1H, α-GalNAc(S-2) H-3] and 3.85 [m, 1H, α-Gal(S-3) H-4].

¹³C NMR of Compound c, Cammyose Acetate in CDCl₃ at 300 MHz

88.96 [1C,α-Glc(S-1) C-1], 91.52 [1C, β-Glc(S-1) C-1], 101.21 [2C, α-GalNAc(S-2) C-1 & α - Gal(S-3) C-1] and 100.96 [1C, β-Gal(S-4) C-1].

¹H NMR of Compound C, Cammyose in D₂O at 300 MHz

5.59 [d, 1H, J= 3.1 Hz, α-Glc(S-1) H-1], 4.53 [d, 1H, J= 8.2 Hz, β-Glc(S-1) H-1], 4.38 [d, 2H, J= 3.6 Hz, α-GalNAc(S-2) H-1 & α-Gal(S-3) H-1], 4.31 [d, 1H, J= 7.2 Hz, β-Gal(S-4) H-1]& 1.98 [s, 3H, NHCOCH₃, α-GalNAc(S-4)]

ES Mass

746[M+K]⁺, 730[M+Na]⁺, 707[M]⁺, 690[707-2H₂O]⁺, 652[669-2H₂O], 567[652--2CH₂CO], 545[707-S₄], 448[465-OH], 487[545-NHCOCH₃], 438[487-CH₃OH+OH]421[438-OH],383[545-S₃], 341[383-CH₂CO], 345[383-2H₃O⁺], 299[345-CHO,OH] , 281[341-CH₂OH,CHO], 299[345-CHO-HO], 263[299-2H₂O] and 180[383-S₂].

RESULT AND DISCUSSION

Compound C, Cammyose, C₂₆H₄₅O₂₁N, was isolated from camel milk as its acetate and the name of novel oligosaccharide was originated from the name of camel as Cammyose. Since the compound was isolated in acetylated form was designated as **compound c**, and its natural form was specified as **compound C**. It gave positive Phenol-sulphuric acid test (DuBois et al, 1956), Feigl test (Fiegel F, 1924), Morgon-Elson test (Hartmann, 1991) showing the presence of normal and amino sugar(s) in the compound. The ¹H NMR spectrum in D₂O at 300 MHz exhibited three anomeric proton doublets for five anomeric protons at δ5.59 (1H), δ4.53 (1H), δ4.38 (2H), and δ4.31(1H) in ¹H NMR spectrum of compound Cammyose in D₂O at 300 MHz suggesting that compound C may be a tetrasaccharide in its reducing form. Further, the ¹H NMR spectrum of acetylated Cammyose in CDCl₃ at 300 MHz exhibited four doublets for five anomeric protons at δ6.24(1H), δ5.68(1H), δ4.49(1H) δ4.48(1H) and δ4.46(1H) indicating that Cammyose may be a tetrasaccharide in its reducing form. The HSQC spectrum of acetylated compound Cammyose at 300 MHz in CDCl₃ showed the presence of four cross peaks of five anomeric protons and carbons in their respective region at δ6.24x88.96, δ5.68x91.52, δ4.49x101.21, δ4.48x101.21& 4.46x100.96 suggesting that compound Cammyose must be a tetrasaccharide in its reducing form.

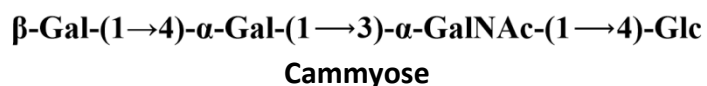
The reducing nature of compound Cammyose was again confirmed by its methylglycosylation followed by its acid hydrolysis (MeOH/H⁺), which led to the isolation of α and β -methylglucosides, along with Glc, Gal & GalNHAc suggesting the presence of glucose at the reducing end and presence of Glc, Gal and GalNAc moieties in the Cammyose. Thus ¹H and ¹³C NMR spectra of acetylated Cammyose justify the five anomeric signals for tetrasaccharide with total integral intensity of four anomeric protons/carbons. The tetrasaccharide nature of Cammyose acetate was further confirmed by the presence of four signals for five anomeric carbons at δ 88.96(1C), δ 91.52(1C), δ 101.21 (2C) and δ 100.96 (1C) in ¹³C NMR of acetylated Cammyose at 300 MHz in CDCl₃. To confirm the monosaccharide constituents in compound Cammyose, it was hydrolyzed under strong acidic conditions of Kiliani hydrolysis which gave three spots i.e. Glc, Gal and GalNAc which were found identical with the mobility of authentic samples of Glc, Gal and GalNAc by co-chromatography(PC), confirming that the tetrasaccharide compound Cammyose consisted three monosaccharide unit i.e. Glc, Gal and GalNAc. For convenience, starting from reducing end, the monosaccharides present in compound Cammyose have been designated as S-1, S-2, S-3 and S-4. The ¹H NMR spectrum of Cammyose acetate in CDCl₃ at 300 MHz contain two anomeric proton doublets at δ 6.24 (d, J=3.3 Hz) and δ 5.68 (d, J= 8.4Hz) for α and β -anomers of reducing monosaccharides S-1 i.e. Glc. The anomeric carbon of S-1 at δ 91.52 gave its complimentary anomeric proton signal at δ 5.68 (8.4Hz) in the HSQC spectrum of Cammyose acetate. The anomeric proton signal present at δ 5.68 of Cammyose acetate assigned to Glc(S-1) gave three cross peaks at δ 5.68x3.89, δ 5.68x5.00 and δ 5.68x5.50 in TOCSY Spectrum, which was later identified as H-4, H-2 and H-3 respectively by COSY spectrum of acetylated Cammyose at 300 MHz in CDCl₃. The chemical shift of H-4 of S-1 at δ 3.89 suggested that H-4 of S-1 was available for glycosidic linkage by next monosaccharide unit i.e. S-2. Further the ¹H NMR signal present at δ 3.89 assigned to H-4 of reducing Glc (S-1) gave a cross peak at δ 3.89x101.21 in HMBC spectrum of Cammyose acetate which was H-4 of reducing Glc and C-1 of S-2, confirmed a (1 \rightarrow 4) linkage between Glc (S-1) and S-2. The anomeric carbon of S-2 at δ 101.21 gave its complimentary cross peak signal at δ 4.49x δ 101.21 in the HSQC spectrum of Cammyose acetate. The chemical shift values of anomeric carbon at δ 101.21 and anomeric proton at δ 4.49 were having resemblance with literature value of anomeric chemical shift value of GalNAc hence S-2 was confirmed as GalNAc. Further the coupling constant of anomeric signal (S-2) at δ 4.49 had J value of 3.9 Hz confirmed the α -configuration of the glycosidic linkage between (S2 \rightarrow S1) in Cammyose acetate. Moreover the presence of α -GalNAc as next monosaccharide in Cammyose was confirmed by appearance of anomeric proton signal at δ 4.38(J=3.6 Hz) along with a singlet of three protons at δ 1.98 in ¹H NMR spectrum of Cammyose in D₂O at 300 MHz. Further the anomeric proton signal at δ 4.49 assigned to S-2 gave three cross peaks at δ 4.49x3.80, δ 4.49x4.12 and δ 4.49x5.25 in the TOCSY spectrum of Cammyose acetate at 300 MHz which was later identified as H-3, H-2 and H-4 of α -GalNAc (S-2) respectively by COSY spectrum of acetylated Cammyose in CDCl₃ at 300 MHz. The cross peak at δ 4.49x3.80, and δ 4.49x4.12 suggested that in sugar (S-2) there may be two position available for glycosidic linkage with the next monosaccharide unit which was later identified as H-3 and H-2 of (S-2) respectively by COSY spectrum of acetylated Cammyose in CDCl₃ at 300 MHz.

Table 1. Anomeric Proton Values of Compound (C) (Cammyose) in D₂O and Cammyose Acetate (c) in CDCl₃ at 300 MHz.

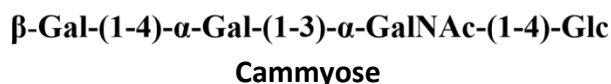
Moieties	In D ₂ O		In CDCl ₃	
	¹ HNMR (δ)	Coupling constant (J) Hz	¹ HNMR (δ)	Coupling constant (J)Hz
α-Glc(S-1)	5.59	3.1Hz	6.24	3.3Hz
β-Glc(S-1)	4.53	8.2Hz	5.68	8.4Hz
α-GalNAc(S-2)	4.38	3.6Hz	4.49	3.9Hz
α-Gal(S-3)	4.38	3.6Hz	4.48	3.8Hz
β-Gal(S-4)	4.31	7.2Hz	4.46	7.8Hz

Since it was already ascertained that S-2 was GalNAc and hence the chemical shift value of δ4.12 was due to presence H-2 of S-2 alongwith a singlet of three protons of amide methyl at δ1.98 in ¹H NMR spectrum of Cammyose in D₂O and hence the leftover position i.e. H-3 of S-2 at δ3.80 showed the availability for glycosidic linkage by next monosaccharide unit i.e. S-3. Further the ¹H NMR signal present at δ3.80 assigned to H-3 of α-GalNAc (S-2) gave a cross peak at δ3.80x101.21 in HMBC spectrum of Cammyose acetate which was between H-3 of α-GalNAc (S-2) and C-1 of S-3 confirmed a 1→3 linkage between S-3 and S-2. The anomeric carbon of S-3 at δ101.21 gave its complimentary anomeric cross peak at δ4.48x δ101.21 in the HSQC spectrum of Cammyose acetate. The chemical shift values of anomeric carbon at δ101.21 and anomeric proton at δ4.48 were having resemblance with literature value of anomeric chemical shift value of Gal, confirming that S-3 was Gal. Further the coupling constant of anomeric signals of S-3 at δ4.48 had J value of 3.8 Hz confirmed the α-configuration of the glycosidic linkage between (S3→S2) in Cammyose acetate. Further, the presence of α-Gal as next monosaccharide in Cammyose was also confirmed by appearance of anomeric proton doublet at δ4.38(J=3.6 Hz) in ¹H NMR spectrum of Cammyose in D₂O at 300 MHz. Further the anomeric proton signal at δ4.48 assigned for α-Gal (S-3) showed three cross peaks at δ4.48x3.85, δ4.48x5.20 and δ4.48x5.25 of acetylated Cammyose which was later identified as H-4, H-2 and H-3 of α-Gal (S-3) respectively by COSY spectrum of acetylated Cammyose in CDCl₃ at 300 MHz. The chemical shift at δ3.85 assigned to H-4 of sugar S-3 showed the availability of OH group for glycosidic linkage by next monosaccharide unit i.e. S-4. Further the HMBC spectrum of Cammyose acetate at 300 MHz showed a cross peak signal of H-4 of β-Gal (S-3) and anomeric carbon of next monosaccharide C-1of S-4 at δ3.85x100.96 confirmed a (1→4) linkage between S-4 and S-3. The anomeric carbon at δ100.96 gave its complimentary cross peak at δ4.46 x δ100.96 in the HSQC spectrum of acetylated Cammyose. The chemical shift values of anomeric carbon at δ100.96 and anomeric proton at δ4.46 were having resemblance with literature value of anomeric chemical shift value of Gal confirming that S-4 was Gal. The coupling constant of anomeric signal (S-4) at δ4.46 with larger J value of 7.8 Hz confirmed the β-configuration of the glycosidic linkage between (S4→S3) in Cammyose acetate. The anomeric proton signal at δ4.46(7.8 Hz) assigned to β-Gal (S-4) gave three cross peaks at δ4.46x5.10, δ4.46x5.20and δ4.46x5.30in the TOCSY spectrum of Cammyose acetate which was later identified as H-2, H-4 and H-3 of β-Gal (S-4) respectively by COSY spectrum of acetylated Cammyose in CDCl₃ at 300 MHz.

Since The chemical shift values of ring protons of S-4 at δ 5.30, δ 5.20 and δ 5.10 does not reside in the linkage region and hence they did not show any cross peak in the linkage region i.e. δ 3.5-4.2ppm confirming that β -Gal (S-4) was present at non-reducing end and none of its -OH group were involved in glycosidic linkage by any monosaccharide which was confirmed by TOCSY and COSY spectra of acetylated Cammyose in CDCl_3 at 300MHz. All signals obtained in ^1H and ^{13}C NMR of compound Cammyose were in confirmity with the assigned structure and their positions were confirmed by 2D NMR viz. COSY, TOCSY, HSQC and HMBC experiments of Cammyose acetate. Thus based on the pattern of chemical shifts of ^1H NMR, ^{13}C NMR, COSY, TOCSY, HSQC and HMBC experiments, it was interpreted that the compound C Cammyose, was a tetrasaccharide having the following structure :



The Electrospray Mass Spectrometry data of compound Cammyose not only confirmed the derived structure of Cammyose but also confirmed the sequences of monosaccharides in this compound. The highest mass ion peak were recorded at m/z 746 and at m/z 730 which were due to $[\text{M}+\text{K}]^+$ and $[\text{M}+\text{Na}]^+$. It also contains the molecular ion peak at m/z 707 confirming the molecular weight of Cammyose as $707[\text{M}]^+$ and was in agreement with its molecular formula $\text{C}_{26}\text{H}_{44}\text{O}_{21}\text{N}$. 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 tetrasaccharide Cammyose m/z 707(I) fragmented to give mass ion peak at m/z 545(II) $[707-\text{S}_4]$, this fragment was arised due to the loss of terminal β -Gal (S_4) (163) moiety from tetrasaccharide indicating the presence of β -Gal(S_4) at the non-reducing end. It was further fragmented to give mass ion peak at m/z 383(III) $[545-\text{S}_3]$ which was due to loss of α -Gal (S_3) (162) moiety from trisaccharide. This fragment of 383 was further fragmented to give mass ion peak at m/z 180(IV) $[383-\text{S}_2]$ which was due to loss of β -GalNAc (S_2) (203) moiety from the disaccharide. In other fragmentation pathway in ES Mass spectrum of Cammyose, m/z 707 shows the mass ion peak at 690 $[707-2\text{H}_2\text{O}^+]$, 652 $[669-2\text{H}_2\text{O}]$, 567 $[652-2\text{CH}_2\text{CO}]$, 545 $[707-\text{S}_4]$, 448 $[465-\text{OH}]$, 487 $[545-\text{NHCOCH}_3]$, 438 $[487-\text{CH}_3\text{OH}+\text{OH}]$, 421 $[438-\text{OH}]$, 383 $[545-\text{S}_3]$, 341 $[383-\text{CH}_2\text{CO}]$, 345 $[383-2\text{H}_3\text{O}^+]$, 299 $[345-\text{CHO}, \text{OH}]$, 281 $[341-\text{CH}_2\text{OH}, \text{CHO}]$, 299 $[345-\text{CHO}-\text{HO}]$, 263 $[299-2\text{H}_2\text{O}]$ and 180 $[383-\text{S}_2]$. Based on result obtained from chemical degradation/acid hydrolysis, Chemical transformation, Electro spray mass spectrometry and 1D-NMR viz. ^1H NMR, ^{13}C NMR and 2D-NMR viz. COSY, TOCSY, HMBC and HSQC spectra of Cammyose acetate and Cammyose, the structure and sequence of isolated a novel oligosaccharide was deduced as:



CONCLUSION

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

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