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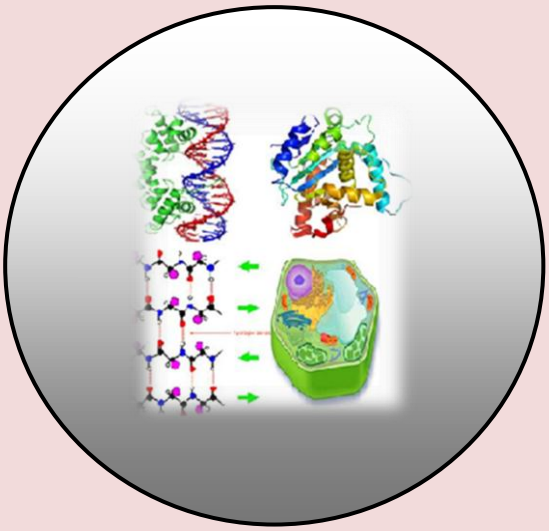
ISSN 2319-3077 Online/Electronic
ISSN 0970-4973 Print

Index Copernicus International Value
IC Value of Journal 82.43 Poland, Europe (2016)
Journal Impact Factor: 4.275
Global Impact factor of Journal: 0.876
Scientific Journals Impact Factor: 3.285
InfoBase Impact Factor: 3.66

J. Biol. Chem. Research
Volume 39 (2), 2022 Pages No. 1-10



**Journal of Biological and Chemical Research**

An International Peer Reviewed / Referred Journal of Life Sciences and Chemistry

Indexed, Abstracted and Cited: Index Copernicus International (Europe), World Science Index, Polish Ministry of Science and Higher Education (Poland, Europe) Research Bible (Japan), Scientific Journals Impact Factor Master List, Directory of Research Journals Indexing (DRJI), Indian Science. In, Database Electronic Journals Library (Germany), Open J-Gate, J Gate e-Journal Portal, Info Base Index, International Impact Factor Services (IIFS) (Singapore), Scientific Indexing Services (USA), International Institute of Organized Research (I2OR), Cosmos Science Foundation (Germany), Science Library Index (UAE), Eye Source, Swedish Scientific Publication, World Cat, International Innovative Journal Impact Factor, Einstein Institute for Scientific Information {EISI} and Impact Factor.pl - Kompendiumwiedzy o czasopismachnaukowych, Philadelphia citefactor.org journalsindexing Directory Indexing of International Research Journals

Published by Society for Advancement of Sciences®

J. Biol. Chem. Research. Vol. 39, No. 2, 1-10, 2022
(An International Peer Reviewed / Refereed Journal of Life Sciences and Chemistry)
Ms 39/02/090/2022

ISSN 2319-3077 (Online/Electronic)
ISSN 0970-4973 (Print)

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REVIEW PAPER

Received: 13/07/2022 Revised: 25/09/2022 Accepted: 26/09/2022

# Structure Elucidation of *Camelus dromedaries* Milk Oligosaccharide Romeose by Spectroscopic Techniques

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## ABSTRACT

***Oligosaccharides are amongst the most biologically diverse and important carbohydrate in biological system. Milk oligosaccharides are important class of complex carbohydrates which play an essential role in many molecular processes. Milk oligosaccharides exhibit varied biological activity such as immunostimulant, hypoglycemic, anti-tumor, antiviral, anticancer, anticoagulant, anti-complementary, immunological and anti-inflammatory activities. Camel milk contains vitamins, minerals and lactoferrin in which has antibacterial, antiviral and anti-tumor properties. Camel milk also contains disease-fighting immunoglobulin and is also a rich source of insulin. In India Camel milk is used in treatment of tuberculosis, asthama and spleen. Keeping in mind the biological activity of milk of Camel (Camelus dromedarius)] it was collected in bulk and processed by modified method of Kobata and Gingsburg. In this process the milk was deproteinated, centrifuged and lyophilized and then it was subjected to gel filtration, its homogeneity was confirmed by HPLC. The mixture of oligosaccharides was acetylated and purified by column chromatography resulting in the isolation of a novel oligosaccharide. The structure of novel isolated compound was confirmed by 1H, 13C and 2D NMR (COSY, TOCSY, HSQC, and HMBC) spectroscopy. The $^{1}$H NMR spectrum of acetylated compound in $CDCl_3$ at 300 MHz showed the presence of five cross peaks δ 6.20 x 89, δ 5.66 x 91.49, δ 4.48 x 100.95, δ 4.49 x 100.95, δ 4.49 x 101.17 in the anomeric proton region by which it could be interpreted for presence of four anomeric protons. Glycosidic linkages were confirmed by COSY, TOCSY and HMBC spectra. The stereoscopic structure of purified compound was elucidated with the help of chemical degradation, chemical transformation and stereoscopic techniques i.e. NMR and mass spectrometry. The isolated compound was optimized which indicates the stability of the compound. The structure of isolated compound was given as under-***

**-Gal(1→4) -GalNAc(1→2) β-Gal (1→4) Glc**

***Keywords: Camel milk, Oligosaccharides, Kobata and Gingsburg and 2D NMR.***

## INTRODUCTION

Milk is secreted by all families of mammals to supply nutrition and immunological protection to their mammalian neonates. It performs these functions with a large array of distinctive compound. It is a nutritious food containing numerous essential nutrients for the metabolism, growth, development and wellbeing of the young mammals. Milk supplies energy, amino acid, vitamins and minerals to the suckling during important phase of life [Kumar et al., 2016]. One of the major components of milk is carbohydrate which contains lactose and oligosaccharide. So, the oligosaccharides are a class of bioactive macromolecule found in mammalian milk that is receiving a lot of commercial attention. These complex carbohydrates (oligosaccharides) are known to be responsible for the beneficial effects of breast feeded newly borns and perform a number of bioactive functions including prebiotic enrichment of a protective micro biota, limiting the virulence of several pathogens and increasing postnatal neural development [Gunjan et al., 2016] and also inhibit the adhesion of pathogenic microorganism to the intestinal and urinary tract by acting receptor analogues to preventing gastric and urinary infections. So these oligosaccharide exhibits varied biological activities such as an anti-inflammatory, anti-tumor, antithrombotic, immune stimulant, anti-cancer, antiviral, antimicrobial and cardioprotective activities [Ehresmann et al., 1979, Piere, 1982]. Moreover, these oligosaccharides have been isolated from various mammalian milk of different origin e.g. buffalo, equine, caprine, elephant, donkey, rat, goat, camel and human etc. The bovine milk is a source of simple as well as complex oligosaccharides. Recent research has demonstrated that bovine milk contains similar protective and nutritional role in an immune system of infants and human being [Angela et al., 2011]. Donkey milk oligosaccharides have ability to nonspecific and specific immunological resistance [Deepak et al., 1998]. Goat milk oligosaccharides play roles in human intestinal inflammation [Lara et al., 2006]. Buffalo milk oligosaccharides has shown immune stimulant activity by increase in the haemagglutination titer, delayed type hypersensitivity reaction, and plague forming cell counts in mice [Saksena et al., 1999]. Sheep milk is a rich source of fucosylated oligosaccharides which has definite biological effects like α 1,2-linked fucosylated oligosaccharides probably in conjugation with other families of oligosaccharides, constitute of powerful innate immune system of human [Sharon and Ofek, 2000]. Camel milk is proposed as a precautionary intake in gastric ulcers. Regular intake of camel milk helps to control blood sugar levels. Camel milk also helps in reducing coronary heart diseases, in infection, gastroenteritis and cancer [Mani and Deepak 2016]. The medicinal importance of the camel milk in the ancient Indian medicinal system is enormous that it shows potent activity against tuberculosis, small pox, epilepsy consumption, gonorrhoea, septic and hysteria properties. Keeping this in mind, Camel milk was processed by Kobata and Ginsburg method which resulted followed by Gel filteration and HPLC and column chromatography in the isolation of a novel milk oligosaccharide [Gunjan et al., 2018] and then its structure was elucidated with the help of chemical degradation, chemical transformation and spectroscopic method like 1H NMR, 13C NMR and 2D-NMR i.e., COSY TOCSY, HSQC and HMBC technique.

## MATERIAL AND METHODS

### General procedure

General procedures were same as described in our previous articles [Singh et al., 2012].

**ISOLATION OF CAMEL MILK OLIGOSACCHARIDES** (modified method of Kobata and Ginsburg [Kobata and Ginsburg, 1970] Isolation of camel milk oligosaccharides was done by the modified method of Kobata and Ginsburg method, which was described in our previous communication except the isolation and optimization of structure. The isolation was done from 10 litre of camel milk and the yield of oligosaccharide mixture was 315 gm.

**Acetylation of Oligosaccharide Mixture**

12.0gm of pooled fractions which gave positive phenolsulphuric acid test were acetylated with pyridine (12 ml) and acetic anhydride (12 ml) at 60°C and the solution was stirred overnight. The mixture was evaporated under reduced pressure and the viscous residue was taken in CHCl3 (500 ml) and washed in sequence with 2N-HCl (I x 25 ml), ice cold 2N-NaHCO3 (2 x 25 ml) and finally with H2O (2 x 25 ml). The organic layer was dried over anhydrous Na2SO4, filtered and evaporated to dryness yielding the acetylated mixture (14 gm).

**Purification of Acetylated Milk Oligosaccharides on Silica Gel Column**

Separation or purification of acetylated oligosaccharides (14 gm) was carried over silica gel (700gm) using varying proportion of Hex: CHCl3, CHCl3 and CHCl3: MeOH as eluents, collecting fractions of 300ml each. So ten fractions namely I(4.28gm), II (736mg), III (3.29gm), IV (468mg), V (380mg) ,VI(2.007gm), VII(1.06gm), VIII (767mg), IX (319mg), X (137mg) respectively obtained. All these fractions were checked on TLC and those showing similar spots were taken together for further investigations. Substance (135mg) was obtained from fraction IX.

**Deacetylation of Compound ROMEOSE**

Compound ROMEOSE acetate (30 mg) obtained from column chromatography of acetylated oligosaccharide mixture was dissolved in acetone (2 ml) and 3 ml of NH3 was added and overnight in a stoppered hydrolysis flask. After 24 h ammonia was removed under reduced pressure and the compound was washed with (3 x 10 ml) CHCl3 and the water layer was finally freeze dried giving the deacetylated oligosaccharide R (16 mg).

**Kiliani Hydrolysis of Compound ROMEOSE**

Compound ROMEOSE (5 mg) was dissolved in 2 ml Kiliani mixture (AcOH-H2O-HCl, 7: 11:2) and heated at l00$^{o}$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 to it and was evaporated under reduced pressure to afford Glc, Gal and GalNAc identified by comparison with authentic samples of Glc, Gal and GalNAc.

**Methylglycosidation/Acid Hydrolysis of Compound ROMEOSE**

Compound ROMEOSE (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 a solution of methylglycoside of ROMEOSE in 1,4-dioxane (1 ml), 0.1 N H2SO4 (1 ml) was added and the solution was warmed for 30 minutes at 50°C. The hydrolysis was complete after 22 h. The hydrolysate were neutralized with freshly prepared BaCO3, filtered and concentrated under reduced pressure to afford α-and β-methylglucosides along with the Glc, GalNAc. Their identification were confirmed by comparison with authentic samples glucose, galactose and GalNAc

**Description of Compound Romeose**

**COMPOUND ROMEOSE**

Substanc obtained from fraction 31-55 of column chromatography 5. On deacetylation of 100mg of acetylated substance with $NH_3$/ acetone it afforded compound ROMEOSE (76.50mg) as a viscous mass.

For experimental analysis, this compound was dried over $P_2O_5$ at $100^0$ C and 0.1 mm pressure for 8 hr.

| **$C_{26}H_{45}O_{21}N$** | %C | %H | %N |
|---|---|---|---|
| Calcd. | 44.93 | 6.52 | 1.94 |
| Found | 44.92 | 6.51 | 1.94 |

It gave positive Phenol-sulphuric acid test and Morgon-Elson test.

**$^1$H NMR of ROMEOSE ACETATE: δ in $CDCl_3$ at 300 MHz**

δ 4.49 [d, 1H, J=3.0Hz α-GalNAc (S-3), H-1)], 4.49 [d, 1H, J=3.0Hz, α-Gal (S-4) H-1], 5.66 [d, 2H, J=9.0Hz, β-Glc (S-1), H-1], 4.48 [d, 2H, β-Gal (S-2), H-3), 3.90 [m, 2H, β-Glc (S-1).

**$^{13}$C NMR of ROMEOSE ACETATE: δ in $CDCl_3$ at 300 MHz**

δ 100.95 [1C, α-GalNAc (S-3), C-1], 91.49 [1C, α-Gal (S-4), C-1], 100.95 [1C, β-Gal (S-2), C-1], 89[1C, α-Glc (S-1), C-1], 91.49 [1C, β-Glc (S-1), C-1].

## RESULT AND DISCUSSION

**NMR spectroscopy**

Compound ROMEOSE $C_{26}H_{45}O_{21}N_D^{gave}$ positive phenol-sulphuric acid test, Feigl test and Morgon-Elson test [Partridge and Westall 1948, Dubois et al., 1956, Urashima et al., 2002] showing the presence of normal and amino sugar(s) in the moiety. The HSQC spectrum of acetylated ROMEOSE in $CDCl_3$ at 300 MHz showed the presence of five cross peaks of anomeric protons and carbons in the respective region at δ 6.20 x 89, δ 5.66 x 91.49, δ 4.48 x 100.95, δ 4.49 x 100.95, δ 4.49 x 101.17 suggesting the presence of five anomeric protons and carbons in it. The presence of five anomeric protons were further confirmed by the presence of five anomeric proton doublets at δ 6.20 (1H), δ 5.66 (1H), δ 4.48 (1H), δ 4.49 (2H) in the $^1$H NMR spectrum of acetylated ROMEOSE in $CDCl_3$ at 300 MHz. The $^{13}$C NMR spectrum of ROMEOSE acetate in $CDCl_3$ at 300 MHz also confirmed the presence of five anomeric carbon at δ 89 (1C), 91.49(1C), 100.95(2C) and 101.17(1C).

**Table for NMR data of compound ROMEOSE ACETATE**

**Table 1. Anomeric protons and carbons of compound ROMEOSE ACETATE.**

| Sugar | 1H(ppm) | 13C(ppm) |
|---|---|---|
| S1 | 6.20 | 89.00 |
| S1 | 5.66 | 91.49 |
| S2 | 4.48 | 100.95 |
| S3 | 4.49 | 100.95 |
| S4 | 4.49 | 101.17 |

**Table 2. Order of TOCSY proton of Compound ROMEOSE ACETATE.**

| S1 (ppm) | S2 (ppm) | S3(ppm) | S4(ppm) |
|---|---|---|---|
| 3.77 | 3.82 | 3.85 | 4.38 |
| 5.08 | 4.10 | 4.49 | 4.49 |
| 5.22 | 4.48 | 5.22 | 5.10 |
| 5.66 | 5.02 | 5.47 | 5.36 |

**Table 3. COSY spectra Of Compound ROMEOSE ACETATE.**

| Sugar | S1(ppm) | S2(ppm) | S3(ppm) | S4(ppm) |
|---|---|---|---|---|
| H1 | 5.66 | 4.48 | 4.49 | 4.49 |
| H2 | 5.08 | 3.82 | 4.10 | 5.10 |
| H3 | 5.22 | 5.02 | 5.47 | 5.36 |
| H4 | 3.77 | 4.10 | 3.85 | 4.38 |

**Table 4. HMBC linkages of compound ROMEOSE ACETATE.**

| Sugar | Linkage | Type of Linkage | Coupling constant |
|---|---|---|---|
| S1-S2 | 1-4 | β | J=9 Hz |
| S2-S3 | 1-2 | □ | J=3 Hz |
| S3-S4 | 1-4 | □ | J=3 Hz |

**Table 5. Linkage carbon and proton OF COMPOUND ROMEOSE ACETATE.**

| SUGAR | $C_4$ x $H_4$ | Cross peaks | Linkage |
|---|---|---|---|
| S1(Glc) | $C_4$ x $H_4$ | □ 3.77 x 70 | 1→4linkage |
| S2(Gal) | $C_4$ x $H_4$ | □ 3.80 x 76 | 1→2 linkage |
| S3(GalNHAc) | $C_4$ x $H_4$ | □ 3.85 x 73 | 1→4 linkage |

The reducing nature of compound ROMEOSE was further confirmed by its methylglycosidation by MeOH / $H^+$ followed by its acid hydrolysis which led to the isolation of □□and β -methyl glucoside leading to the presence of glucose at the reducing end in the oligosaccharide. The four monosaccharides present in ROMEOSE have been designated as $S_1$, $S_2$, $S_3$, $S_4$ for convenience from the reducing end. To confirm the monosaccharide constituents in ROMEOSE, it was hydrolyzed under strong acidic conditions (Kiliani Hydrolysis). In Kiliani hydrolysis the reducing tetrasaccharide gave spots on TLC and paper chromatography which were identified as Glc, Gal and GalNHAc by co-chromatography with authentic samples (paper chromatography) [Urashima et al., 2004]. Suggesting that the reducing tetrasaccharide was made up of these three monosaccharide units. The chemical shifts values of anomeric protons and carbons observed in $^1H$ and $^{13}C$ NMR spectrum of ROMEOSE were also in agreement with the reported values of $^1H$ and $^{13}C$ anomeric chemical shifts of Glc, Gal and GalNHAc confirming the presence of these monosaccharides in the compound ROMEOSE.

The 1H NMR spectrum of Romeose acetate in CDCl3 at 300MHz contain two anomeric proton doublets at δ6.20 and δ5.66 for □ and β anomers of reducing monosaccharide (S1). The presence of 1H NMR signal at δ 5.66 along with 13C NMR signal at δ 91.49 resembles with the chemical shift value of glucose hence the reducing monosaccharide may be glucose

(S1) which was later confirmed by its methylglycosidation by MeOH / $H^+$ followed by its acid hydrolysis of romeose. The anomeric protons signal present at δ5.66 in TOCSY Spectrum of Romeose acetate assigned to β-Glc (S-1) gave three cross peaks at δ5.67x3.77, δ5.67x5.08 and δ5.67x5.22. These cross peaks δ5.67x5.08, δ5.67x5.22 and δ5.67x3.77 were later identified as H-2, H-3 and H-4 of reducing Glc respectively by COSY spectrum of acetylated Romeose at 300 MHz in $CDCl_3$. The chemical shift of H-4 of S-1 at δ3.77 suggested that H-4of S-1 was available for glycosidic linkage by next monosaccharide unit. Further the $^1H$ signal present at δ3.77 assigned to H-4 of reducing Glc (S-1) gave a cross peak at δ3.77x100.95 in HMBC spectrum of Romeose acetate which was between H-4 of reducing Glc and C-1 of S-2,confirmed the (1→4) linkage between Glc (S-1) and S-2. Further the coupling constant of anomeric signal (S-2) at δ4.48 with larger J value of 9.0 Hz confirmed the β-configuration of the glycosidic linkage between (S2→S1) in Romeose acetate. The anomeric carbon of S-2 at δ100.95 gave its complimentary anomeric proton signal at δ4.48 (9 Hz) in the HSQC spectrum of Romeose acetate. The chemical shift values of anomeric carbon at δ100.95 and anomeric proton at δ4.48 were having resemblance with literature value of anomeric chemical shift value of Gal hence S-2 was confirmed as Gal. Further, the anomeric proton signal at δ4.48 assigned toS-2(β-Gal) showed three cross peaks at δ4.48x3.82, δ4.48x5.02 and δ4.48x4.10 in the TOCSY spectrum of Romeose acetate at 300 MHz which were later identified as H-2, H-3 and H-4 of β-Gal (S-2) respectively by COSY spectrum of acetylated Romeose in $CDCl_3$ at 300 MHz.

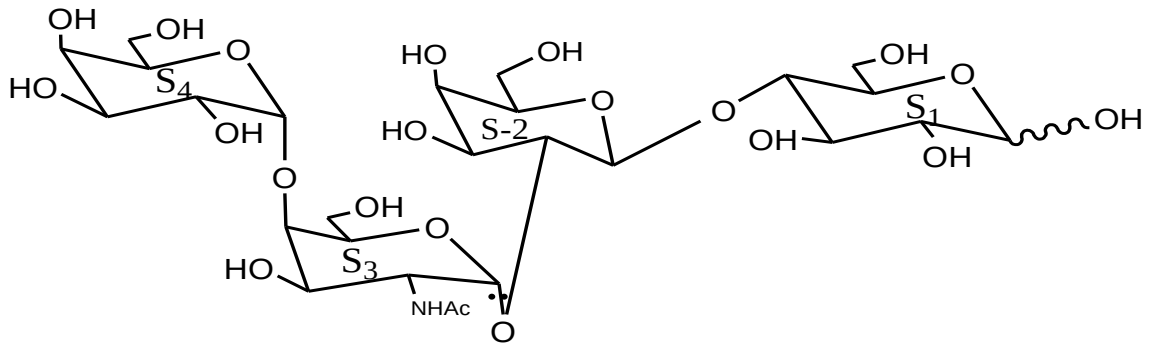
The chemical shift of H-2 of S-2 at δ3.82, suggested that H-2was available for glycosidic linkage by next monosaccharide unit i.e. S-3. Further the $^1H$ NMR signal present at δ3.82 assigned to H-2 of β-Gal (S-2) gave a cross peak at δ3.82x100.95 in HMBC spectra of Romeoseacetate which was between H-2of β-Gal (S-2) and C-1 of S-3 confirmed a 1→2 linkage between S-2 and S-3. The anomeric carbon of S-3 at δ100.95 gave its complimentary anomeric proton signal at δ4.49 in the HSQC spectrum of Romeose acetate. The chemical shift values of anomeric carbon at δ100.95 and anomeric proton at δ4.49 were having resemblance with literature value of anomeric chemical shift value of GalNAc, confirming that S-3 was GalNAc. Further the coupling constant of anomeric signal of S-3 at δ4.49 with larger J value of 3.0Hz confirmed the □-configuration of the glycosidic linkage between (S3→S2) in Romeose acetate.

Further the anomeric proton signal at δ4.49 (3.0 Hz) assigned for □-GalNAc (S-3) showed three cross peaks δ4.49x3.85, δ4.49x4.10 andδ4.49x5.47 in the TOCSY spectrum of acetylated Romeose. These peaks δ4.49x4.10, δ4.49x5.47 and δ4.49x3.85 were later identified as H-2, H-3 and H-4 of □-GalNAc (S-3) respectively by COSY spectrum of acetylated Romeose in $CDCl_3$ at 300 MHz. The chemical shift of the cross peak at δ4.10 was due to presence of $NHCOCH_3$ at C-2 of S-3 (□-GalNAc). Moreover the chemical shift of H-4 of S-3 at δ3.85 showed the availability of -OH group for glyosidic linkage by next monosaccharide unit i.e.S-4.Further the HMBC spectrum of Romeose acetate at 300 MHz showed a cross peak signal of H-4 of □-GalNAc (S-3) and anomeric carbon of next monosaccharide C-1of S-4 at δ3.85 x 101.17 confirmed a (1→4) linkage between S-4 and S-3.The anomeric carbon at δ101.17 gave its complimentary anomeric proton signal at δ4.49in the HSQC spectrum of acetylated Romeose. The chemical shift values of anomeric carbon atδ101.17 and anomeric proton at δ4.49 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 δ4.49 with larger J value of Hz 3.0 Hz confirmed the □-configuration of the glycosidic linkage between (S4→S3) in Romeose acetate. The anomeric proton signal at δ4.49 assigned to □-Gal (S-4) gave three cross peaks at δ4.49x4.38, δ4.49x5.10 and δ4.49x5.36 in the TOCSY spectrum of Romeose acetate. These crosspeaks δ4.49x5.10, δ4.49x5.36 and δ4.49x4.38 were later identified as H-2, H-3 and H-4 of β-Gal (S-4) respectively by COSY spectrum of acetylated Romeose in $CDCl_3$ at 300 MHz. Since The chemical shift values of ring protons of S-4 at δ5.10, δ5.36 and δ4.38 does not reside in the linkage region and hence they did not show any cross peak in the linkage region confirming that □-Gal (S-4) was present at non-reducing end and none of its -OH group were available for glycosidic linkage, which was confirmed by the TOCSY and COSY spectra of acetylated Romeose in $CDCl_3$ at 300 MHz. All the $^1H$ NMR assignments for ring protons of monosaccharide units of Romeose were confirmed by COSY and TOCSY [Khan et al., 2017, Singh et al., 2019] experiments. The positions of glycosidation in the oligosaccharide were confirmed by position of anomeric signals and comparing the signals in $^1H$ and $^{13}C$ NMR of acetylated and deacetylated oligosaccharide. The glycosidic linkages in Romeose were assigned by the cross peaks for glycosidically linked carbons with their protons in the HSQC and HMBC spectra of Romeose acetate. All signals obtained in $^1H$ and $^{13}C$ NMR of compound Romeose were in conformity with the assigned structure and their positions were confirmed by 2D NMR viz. COSY, TOCSY, HSQC and HMBC [Gangwar et al., 2018] experiments of Romeoseacetate. 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 (Romeose), was a tetrasaccharide having the following structure:

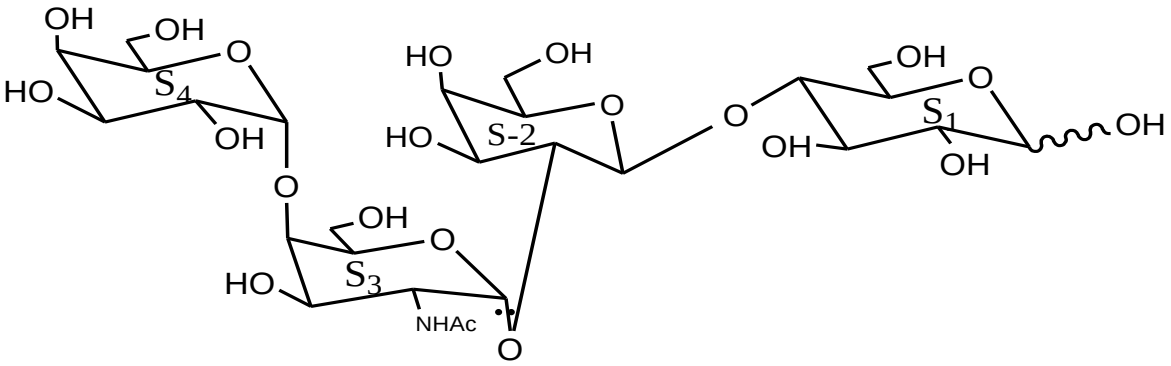
**□□□□□□□□□□□□□□□□-Gal(1→4) □-GalNAc(1→2) β-Gal (1→4) Glc**

The heteronuclear single quantum-coherence (HSQC) spectrum of ROMEOSE acetate confirmed the cross peaks of carbon atoms involved in glycosidation were also present in HSQC spectrum at □ 3.77 x 70 [Glc (S1) $C_4$ x $H_4$ showing 1→4linkage], □ 3.82 x 76 [□-Gal (S2) $C_2$ x $H_2$ showing 1→2 linkage], □ 3.85 x 73 [□-GalNHAc (S3) $C_4$ x $H_4$ showing 1→4 linkage]. Thus based on the pattern of chemical shifts of $^1H$, $^{13}C$, HOMOCOSY and TOCSY, HSQC NMR experiments, it was interpreted that the compound ROMEOSE was a tetrasaccharide comprised of one Glc, two Gal, one GalNAc moieties in it and having the following structure:




**Structure of Compound ROMEOSE**

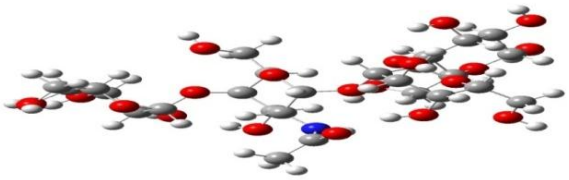
Based on result obtained from chemical degradation/acid hydrolysis, Chemical transformation, Electro spray mass spectrometry and $^1H$, $^{13}C$ NMR and HOMOCOSY, TOCSY and HSQC 2D NMR technique of ROMEOSE and acetylated ROMEOSE the structure and sequence of isolated Novel oligosaccharide molecule Romeose was deduced as-




**Structure of Compound ROMEOSE**

**Optimized Structure**

Structure of the compound ROMEOSE was optimized by Gaussian Software. The optimized structure was given as under-



Optimized structure of the compound ROMEOSE

## CONCLUSION

From the above information, we conclude the structure of isolated a novel Camel milk oligosaccharide, ROMEOSE. This oligosaccharide was reported for the first time from any natural source or any milk and elucidated with the help of spectroscopic technique like 1H, 13C, 2D NMR (COSY, TOCSY and HSQC) spectroscopy. Optimized structure of the compound showed that the isolated compound is found to be stable.

## ACKNOWLEDGMENTS

Authors are thankful to the Head, Department of Chemistry, University of Lucknow to provide lab, NMR and computational facilities.

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