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Isolation, Structure Elucidation and NMR Studies of novel Nonasaccharide Taurose from Lal-Muha Cow's milk

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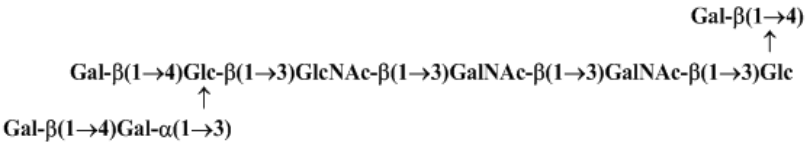
Abstract

Milk is one of the most important source of biologically active oligosaccharides. Many oligosaccharides has been isolated from various milk which have exhibited potent biological activities such as anti-tumor, anti-viral, anti cancer, anti-inflammatory, anti-coagulant, immunostimulant and hypoglycemic activities and they have also been used as certain biomarkers. These biological activities of milk oligosaccharides were based on different constituent monosaccharides and linkages which normally contain six monosaccharides namely, glucose, galactose, *N*-acetyl-glucosamine, *N*-acetyl-galactosamine, fucose, and *N*-acetyl-neuraminic acid. These monosaccharides combine in different order to form a large number of oligosaccharides and show different biological activities.Presence as galactose and sialic acid are in milk oligosaccharides were reported to develop the brain of infants. Milk oligosaccharides have a unique structure to act as a soluble ligand against infective bacteria to protect the gastrointestinal, respiratory, and urinary tracts. It is recently discovered that a trace amount of milk oligosaccharides are absorbed through the gut and interact directly with the immune system to modulate the immune response. We have chosen Lal- Muha cow (a rare species of cow found in north eastern part of India at high altitude) milk in search of novel milk oligosaccharides which have importance in folk medicine. A number of studies supported the constructive effects of supplementation of cow milk in diarrhea in human with immune-deficiency syndrome, NSAID-induced gastrointestinal disturbances. For this Lal- Muha cow's milk was collected in bulk and processed by the modified method of Kobata and Ginsburg followed by gel filtration, HPLC and column chromatography which resulted in the isolation of novel oligosaccharide namely Taurose. The structure of isolated oligosaccharides was elucidated by chemical transformations, chemical degradation, NMR (^{1}H, ^{13}C and 2D COSY, TOCSY, HSQC and HMBC) and mass spectrometry.



Keywords: Isolation; Milk Oligosaccharide; Nonasaccharide; 2D NMR and Taurose. efflux.

1. Introduction

Carbohydrates are organic compounds found in living organisms which participate in various biological processes. They are ubiquitous and play important roles in cellular recognition, bacterial and viral infections, structural material in cell walls and signalling processes, offering scope for development of carbohydrate-mimetic diagnostics and drug candidates.[1] Carbohydrates are present as free monosaccharides, oligosaccharides, polysaccharides and as essential components of glycoconjugates such as glycolipids, glycoproteins, glycopeptides and glycosides. Of these carbohydrates, oligosaccharides are most important class of carbohydrate which found as natural constituents of milk, fruits, vegetables, blood, bacteria and fungus etc. Milk oligosaccharide has shown various physiological functions such as the improvement of intestinal microflora, stimulation of mineral absorption, anticariogenicity and the improvement of plasma cholesterol and blood glucose level.[1-3] Biological activities of oligosaccharides depend on constituent of oligosaccharide i.e. which type of monosaccharide form oligosaccharide. Milk oligosaccharides generally contain glucose (Glc), galactose (Gal), *N*-acetyl-glucosamine (GlcNAc), *N*-acetyl-galactosamine (GalNAc), fucose (Fuc), and *N*-acetyl-neuraminic acid. These components combine in different ways to form a large chain of oligosaccharides carrying varied glycosidic linkages (α & β) at different position in milk oligosaccharide. The presence of α- and β-linkages in milk oligosaccharides reach the intestine

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intact and play an important role in the prevention of adhesion of potentially pathogenic bacteria to the intestinal epithelium by acting as decoy receptors for lectins and sugars, thus preventing their infectivity. [4, 5] The breakdown of milk oligosaccharides gives rise to short chain fatty acids which inhibits the growth of harmful bacteria by bringing down the pH.[6] Milk oligosaccharides have a unique structure to act as a soluble ligand against infective bacteria to protect the gastrointestinal, respiratory, and urinary tracts. It is recently discovered that a trace amount of milk oligosaccharides are absorbed through the gut and interact directly with the immune system to modulate the immune response.[7]

The basic unit of milk oligosaccharides is a galacto-oligosaccharides unit which improves intestinal micro-flora and fermentation in term of infants. [8] β-glycosidic linkages present in Milk oligosaccharides plays an important role for their prebiotic activity because it is non-digestible oligosaccharides. [9, 10] Fucose and galactose containing milk oligosaccharide mimics tumour cells surfaces and block cell adhesion to fibronectin.[11] Galactose and sialic acid are present in milk oligosaccharides which are required to develop the brain of infants.[12, 13, 14] While *N*-and *O*-linked oligosaccharides release of histamine and other mediators of the allergenic response which are responsible for the development of allergenic symptoms.[15] 1, 2- α linked fucose present in human milk containing glycoconjugate oligosaccharides inhibits campylobacter jejuni binding in vitro and it's in vivo activity and it also inhibit calciviruses in vitro.[16, 17, 18] Some specific fucosyl oligosaccharides present in human milk has been a potent inhibitor of specific pathogens.[16, 17, 18] Sialic acid present in human milk has shown its importance as anti-inflammatory components and it successfully reduces platelet-neutrophill complex formation which leads to a decrease in neutrophill B_2 integrin expression.[16, 17, 18] The binding of pathogenic strains of Escherichia coli and ulcer-causing human pathogens H. pylori is inhibited by sialylated human milk oligosaccharid. [19, 20, 21] Neutral human milk oligosaccharide behaves as a protecting agent for the intestinal tract of neonates from vibriocholera.[16,17,18] 1, 2- α linked fucosylated oligosaccharide conjugates with other oligosaccharides to make strong innate immune system of human to combat against some enteric pathogens e.g. rotavirus.[16,22,23]

Milk oligosaccharide has many biological activities due to its constitution that's why we chose lal-muha cow's milk. It was collected in bulk and was processed by modified method of Kobata and Ginsburg [24] followed by different chromatographic techniques like gel filtration, TLC, CC, HPLC etc. which resulted into the isolation of a novel milk oligosaccharides namely Taurose (D). The structures of purified milk oligosaccharides were elucidated by the using the data generated from spectroscopic techniques like NMR (^{1}H, ^{13}C, COSY, TOCSY, HSQC and HMBC) mass spectrometry and chemical degradation, chemical transformation. The present paper deals with structure elucidates of novel oligosaccharide from Lal-Muha cow milk namely Taurose (D). The name Taurose was generated from the name of animal *Bos Taurus*.

2.1 Experimental:

General procedures, isolation of lal-muha cow's milk oligosaccharides and acetylation of oligosaccharide mixture were described same as in our previous articles.[25]

2.2. Purification of acetylated milk oligosaccharide

Separation of the acetylated products (11.0 g) was purified by column chromatography. The silica was used in the ratio of 1:100 using various proportions of hexane $CHCl_3$, $CHCl_3$ and $CHCl_3$: MeOH mixture which resolved into eight fractions namely I (1.45 g), II (1.10 g), III (1.15 g), IV (1.20 g), V (1.30 g), VI (1.10 g), VII (1.40 g) and VIII (800 mg) respectively. These fractions were containing a mixture of three to four compounds. Repeated column chromatography of fractions IV and V, led to the isolation of chromatographically pure compound D (85 mg).

2.3. Deacetylation of compound d

85 mg compound d was obtained from column chromatography-3 of acetylated oligosaccharide mixture. 50 mg compound d was dissolved in acetone (3 mL) and 4 mL of NH_3 was added and left overnight in a stoppered hydrolysis flask. After 24h ammonia was removed under reduced pressure and the compound was washed with (3 x 5 mL) $CHCl_3$ (to remove acetamide) and the water layer was finally freeze dried giving the deacetylated oligosaccharide D (42.0 mg).

2.4. Methylglycosidation/ Acid Hydrolysis of compound D

6 mg compound D was ref1uxed 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 methylglucosides of D 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 ˚C and solution was left over night. The hydrolysis was complete after 24 h. The hydrolysate were neutralized with freshly prepared

$BaCO_3$ filtered and concentrated under reduced pressure to afford α-and β-methylglucosides along with the Glc, Gal, GalNAc and GlcNAc. Their identification was confirmed by comparison with authentic samples (TLC, PC).

2.5. Killiani Hydrolysis:

4 mg compound D was dissolved in 2 mL Kiliani mixture (AcOH-H_2O-HCI, 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, to it and was evaporated under reduced pressure to afford glucose, galactose, GalNAc and GlcNAc on comparison with authentic samples of glucose, galactose, GalNAc and GlcNAc, given in **scheme-1** (Supplementary File).

2.5. Description of compound

2.5.1 Compound D, Taurose

85 mg compound d was obtained from fraction 51-65 of chromatography-3 from acetylated oligosaccharide mixture. On deacetylation of 50 mg of substance d with NH_3/ acetone it afford substance D (42 mg) as a viscous syrup, $[\alpha]_D^{25}$ = -17 (c, 4, H_2O). For experimental analysis, this compound was dried over P_2O_5 at 100 ºC and 0.1 mm pressure for 8 hr. Compound Taurose gave positive phenol-sulphuric acid test,[26] Feigl test[27] and Morgan-Elson test.[28]

$C_{60}H_{101}O_{46}N_3$	%C	%H	%N
Calcd.	45.05	6.33	2.64
Found	45.04	6.33	2.63

^{1}H NMR of Compound d, in $CDCl_3$ at 300 MHz

δ 6.22 [1H, d, *J* = 3.9 Hz, -Glc (S-1) H-1], δ 5.36 [1H, d, *J* = 3.9 Hz, -Glc (S-8) H-1], δ 5.34 [1H, d, *J* = 8.1 Hz, β-Glc (S-1) H-1], δ 4.74 [1H, d, *J* = 7.8 Hz, β-GlcNAc (S-5) H-1], δ 4.67 [1H, d, *J* = 8.1 Hz, β-Glc (S-6) H-1], δ 4.58 [3H, d, *J* = 8.1 Hz, β-Gal (S-2, S-7 & S-9) H-1], δ 4.50 [2H, d, *J* = 7.8 Hz, β-GalNAc (S-3 & S-4) H-1], δ 3.80 [5H, m, β-Glc (S-1 & S-6) & β-GalNAc (S-3 & S-4) H-3 & -Glc (S-8) H-4], δ 3.60 [1H, m, β-GlcNAc (S-5) H-3], δ 3.57 [1H, m, β-Glc (S-1) H-4], δ 3.55 [1H, m, β-Glc (S-6) H-4].

^{13}C NMR of Compound d in $CDCl_3$ at 75 MHz

δ 89.40 [1C, -Glc (S-1) C-1], δ 90.17 [1C, -Glc (S-8) C-1], δ 90.30 [1C, β-Glc (S-1) C-1], δ 95.29 [1C, β-Glc (S-6) C-1], δ 95.40 [1C, β-GlcNAc (S-5) C-1], δ 101.11 [1C, β-GalNAc (S-3) C-1], δ 101.21 [1C, β-GalNAc (S-4) C-1], δ102.02 [2C, β-Gal (S-7 & S-9) C-1], δ 102.09 [1C, β-Gal (S-2) C-1].

^{1}H NMR of compound D in D_2O at 300 MHz

δ 5.27 [1H, d, *J* = 3.6 Hz, -Glc (S-1) H-1], δ 5.23 [1H, d, *J* = 3.6 Hz, -Glc (S-8) H-1], δ 4.67 [1H, d, *J* = 8.0 Hz, β-Glc (S-1) H-1], δ 4.58 [1H, d, *J* = 7.8 Hz, β-Gal (S-2) H-1], δ 4.55 [2H, d, *J* = 7.8 Hz, β-Gal (S-7 & S-9) H-1], δ 4.53 [1H, d, *J* = 7.8 Hz, β-Glc (S-6) H-1], δ 4.47 [3H, d, *J* = 7.5 Hz, β-GalNAc (S-3 & S-4) & β-GlcNAc (S-5) H-1], δ 3.27 [m, 1H, β-Glc(S-1), H-2] δ 2.09 [s, 3H, $NHCOC\underline{H}_3$, β-GlcNAc (S-5)], δ 2.01 [3H, s, $NHCOC\underline{H}_3$, β-GalNAc (S-3)], δ 1.99 [3H, s, $NHCOC\underline{H}_3$, β-GalNAc(S-4)].

ES Mass of Compound D

1662 [M+Na+K+H],1638 [M+K], 1622 [M+Na], 1599 [M^+], 1537 [1599-CH_3OH,-HCHO], 1518 [1537-H_3O^+], 1410 [1599-$CH_2OHCOCO$,-HOCHCHOH,-CH_2CO], 1383 [1437-$3H_2O$], 1219 [1275-$2H_3O^+$, -H_2O], 1186 [1275-CH_3CHO,-H^+], 1010 [1113-HOCHCHOH,-CH_2CHO], 956 [1010-$3H_2O$], 838 [951-$NHCOCH_3$,-$3H_2O$-H^+], 819 [838-H_3O^+], 722 [951-$CH_2OHCOCO$,-HOCHCHOH,-CH_3CHO,-$2H_3O^+$], 685 [748-CH_3CHO,-H_3O^+], 680 [748-CH_3OH,-$2H_2O$], 663 [680-OH], 560 [663-$NHCOCH_3$,-CH_3CHO,-H^+], 485 [545-HOCHCHOH,-H_3O^+], 466 [485-H_3O^+], 465 [466-H^+], 365 [545-$NHCOCH_3$,-$CH_2OHCOCO$,-H_2O,-OH], 318 [485-H_2O,-$2CH_2OHCHO$,-CHO], 274 [342 -CH_3OH,-$2H_2O$], 190 [174-$2CH_2CO$] and 180 [342-S_2].

3. Result and discussion

3.1. Structure elucidations of Taurose

3.1.1. NMR spectroscopy

Compound D, Taurose $C_{60}H_{101}O_{46}N_3$ $[\alpha]_D^{25}$ = -17º gave positive Phenol-sulphuric acid test.[26] Feigl test[27] and Morgan-Elson test[28] showing the presence of normal and amino sugars in Taurose. The HSQC spectrum **Fig-1** (S.F.) of acetylated Taurose indicated the presence of nine cross peaks of ten anomeric protons and carbons in their respective region at δ 6.22x89.40, δ 5.36x90.17, δ 5.34x90.30, δ 4.74x95.40, δ 4.67x95.29, δ 4.58x102.02, δ 4.58x102.09, δ 4.50x101.11 and δ 4.50x101.21. These cross peaks suggested that Taurose may be a nonasaccharide in its reducing form. Cross peaks at δ 6.22x89.40 and δ 5.34x90.30 suggested presence of and β anomers of glucose at its reducing end of oligosaccharide. Further the ^{1}H NMR spectrum **Fig-4** (S.F.) of Taurose in D_2O at 300 MHz showed seven anomeric proton doublet for ten anomeric protons at δ 5.27 (1H), δ 5.23 (1H), δ 4.67 (1H), δ 4.58 (1H), δ 4.55 (2H), δ 4.53 (1H) and δ 4.47 (3H) supporting the nonasaccharide nature of Taurose in its reducing form. The anomeric proton signals of Taurose showed two

doublet at δ 5.27 (J = 3.6 Hz) and δ 4.67 (J = 8.0 Hz) in for its and anomers of glucose suggesting that Taurose was a nonasaccharide in its reducing form, containing glucose at the reducing end. The reducing nature of Taurose was further confirmed by its methylglycosylation MeOH/H^+ followed by its acid hydrolysis **Scheme-1** (S.F.), this led to the isolation of α and β-methylglucosides, along with glucose (Glc), galactose (Gal), *N*-acetyl-glucosamine (GlcNAc) and *N*-acetyl-galactosamine (GalNAc suggesting the presence of glucose at the reducing end and presence of Glc, Gal, GlcNAc and GalNAc moieties in Taurose. For convenience, the nine monosaccharides present in compound have been assigned as S-1, S-2, S-3, S-4, S-5, S-6, S-7, S-8, and S-9 starting from reducing end. The nonasaccharide nature of acetylated Taurose was also confirmed by the presence of seven doublet for ten anomeric protons at δ 6.22 (1H), δ 5.36 (1H), δ 5.34 (1H), δ 4.74 (1H), δ 4.67 (1H), δ 4.58 (3H) and δ 4.50 (2H) in the ^{1}H NMR spectrum **Fig-2** (S.F.) of acetylated Taurose in $CDCl_3$ at 300 MHz. Further nonasaccharide nature of Taurose was also confirmed by the presence of nine anomeric carbon signals for ten anomeric carbon at δ 89.40 (1C), δ 90.17 (1C), δ 90.30 (1C), δ 95.29 (1C), δ 95.40 (1C), δ 101.11 (1C), δ 101.21 (1C), δ 102.02 (2C) and δ 102.09 (1C) in the ^{13}C NMR spectrum **Fig-3** (S.F.) of acetylated Taurose in $CDCl_3$ at 75 MHz. The monosaccharides constituents in Taurose were confirmed by its Killiani hydrolysis[29] **Scheme-1** (S.F.) under strong acidic condition, followed by its paper chromatography and TLC. In this hydrolysis four spots of oligosaccharide were found identical with the authentic samples of Glc, Gal, GlcNAc and GalNAc by co-chromatography (PC, TLC), which confirmed that Taurose contained four types of monosaccharides units i.e. Glc, Gal, GlcNAc and GalNAc. The ^{1}H and ^{13}C NMR spectra of Taurose justify the ten anomeric signals for nonasaccharide with total integral intensity of seven anomeric protons and carbons. Further the mass ion peak at 1599 $[M]^+$ present in ES-MS **Fig-8** (S.F.) of Taurose was in agreement with molecular formula $C_{60}H_{101}O_{46}N_3$.

Table 1: ^{1}H NMR of anomeric proton values of Taurose in D_2O and $CDCl_3$ at 300 MHz.

Moieties	In D_2O		In $CDCl_3$	
	^{1}H NMR(δ)	**Coupling constant(*J*)**	**^{1}H NMR(δ)**	**Coupling constant(*J*)**
α-Glc (S-1)	5.27	3.6Hz	6.22	3.9Hz
β-Glc (S-1)	4.67	8.0Hz	5.34	8.1Hz
β-Gal (S-2)	4.58	7.8Hz	4.58	8.1Hz
β-GalNAc (S-3)	4.47	7.5Hz	4.50	7.8Hz
β-GalNAc (S-4)	4.47	7.5Hz	4.50	7.8Hz
β-GlcNAc (S-5)	4.47	7.5Hz	4.74	7.8Hz
β-Glc (S-6)	4.53	7.8Hz	4.67	8.1Hz
β-Gal (S-7)	4.55	7.8Hz	4.58	8.1Hz
α-Glc (S-8)	5.23	3.6Hz	5.36	3.9Hz
β –Gal (S-9)	4.55	7.8Hz	4.58	8.1Hz

Table 2: ^{1}H NMR values of acetylated Taurose in $CDCl_3$ at 300 MHz.

Moieties	H-1	H-2	H-3	H-4	H-5	H-6	$NHCOCH_3$
S-1	5.34	4.80	3.80	3.57	-	-	-
S-2	4.58	5.25	5.05	5.40	-	-	-
S-3	4.50	4.12	3.80	4.80	-	-	2.01
S-4	4.50	4.12	3.80	4.80	-	-	1.99
S-5	4.74	3.80	3.60	4.25	-	-	2.09
S-6	4.67	4.80	3.80	3.55	-	-	-
S-7	4.58	5.25	5.05	5.40	-	-	-
S-8	5.36	4.85	5.56	3.80	-	-	-
S-9	4.58	5.25	5.05	5.40	-	-	-

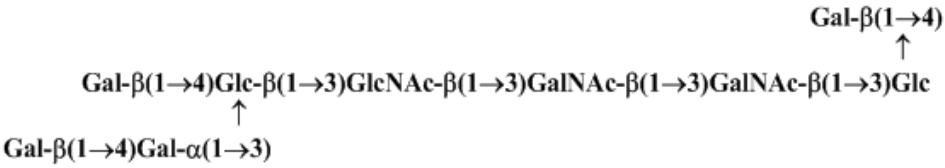
The ^{1}H NMR spectrum **Fig-4** (S.F.) of Taurose in D_2O at 300 MHz contain two anomeric proton doublet at δ 5.27 (J = 3.6 Hz) and δ4.67 (J=8.0Hz) which confirmed the presence of glucose at the reducing end in the nonasaccharide. [30, 31-33] Further the presence of another anomeric doublet at δ4.58 (J = 7.8 Hz) in ^{1}H NMR spectrum of D_2O suggested the presence of β-Gal (S-2) [33,34] residue as the next monosaccharide unit. In addition to anomeric signals of Glc and Gal, presence of a triplet at δ3.27 which was due to H-2 of β-Glc (S-1) suggested the presence of Lactose structure (structure reporter group)[35, 56] i.e. β-Gal (1-4)→Glc at the reducing end of Taurose. Simultaneously ^{1}H NMR and ^{13}C NMR spectrum of Taurose acetate **Fig-2 & 3** (S.F.) also showed downfield shifted α and β anomeric proton and carbon of reducing glucose (S-1) i.e. Glc (S-1) at δ 6.22 (J = 3.9 Hz), δ 5.34 (J = 8.1 Hz) and δ 89.40, δ 90.30 respectively.[37] Further the anomeric protons signal present at δ 5.34 in ^{1}H NMR spectrum of Taurose acetate assigned to β-Glc (S-1) gave three cross peaks at δ 5.34x3.57, δ 5.34x3.80 and δ 5.34x4.80 in TOCSY spectrum **Fig-5** (S.F.), which were later identified as H-4, H-3 and H-2 of reducing Glc respectively by COSY spectrum **Fig-6** (S.F.) of Taurose acetate. The chemical shift of the cross peak at δ 5.34x3.80 and δ 5.34x3.57 suggested that in glucose S-1, two positions were available for glycosidic linkage by next monosaccharide unit i.e. at H-3 and H-4 of S-1 respectively. The earlier suggested (1→4) linkage between β-Glc (S-1) and β-Gal (S-2) was further confirmed by HMBC spectrum **Fig-7** (S.F.) of Taurose acetate at 300 MHz which contain a cross peak at δ 3.57x102.09 between H-4 of β-Glc (S-1) and anomeric carbon of next monosaccharide (S-2) i.e. (S-2). The anomeric carbon of β-Gal (S-2) at δ 102.09 gave its complimentary anomeric proton signal at δ4.58 in the HSQC spectrum of Taurose acetate. The chemical shift of δ 102.09 and δ 4.58 of anomeric carbon and anomeric proton were having resemblance with literature value of anomeric chemical shift value of β-Gal (S-2), hence S-2 monosaccharide was confirmed as β-Gal (S-2).[30, 38] The anomeric proton signal present at δ4.58 for (S-2) had a J value of 8.1 Hz confirming β glycosidic linkage between S-2→S-1. Further the anomeric proton signal at δ 4.58 with J value 7.8 Hz in the ^{1}H NMR of Taurose in D_2O at 300 MHz also confirmed β glycosidically linked Gal as S-2. The (1→4) linkage between β-Glc (S-1) and β-Gal (S-2) was also supported by the presence of H-4 signal of S-1 at δ 3.57 in upfield region of ^{1}H NMR spectrum and a cross peak at δ 3.57x82.90 in glycosidic region of HSQC spectrum of Taurose acetate in $CDCl_3$. The anomeric proton signal at δ 4.58 in ^{1}H NMR spectrum of Taurose acetate in $CDCl_3$ assigned for β-Gal (S-2) gave three cross peaks at δ 4.58x5.05, δ 4.58x5.25 and δ 4.58x5.40 in its TOCSY spectrum **Fig-5** (S.F.) which was later identified as H-3, H-2 and H-4 by COSY spectrum **Fig-6** (S.F.) of Taurose acetate. This anomeric proton did not have any cross peak in the linkage region and hence confirmed that β-Gal (S-2) was present at non-reducing end and none of its hydroxyl groups were involved in glycosidic linkage. Since it was ascertained by the COSY and TOCSY spectrum of Taurose acetate that the β-Glc (S-1) had two vacant positions i.e. H-3 and H-4, and it was already confirmed that H-4 of S-1 was linked with β-Gal (S-2) whereas H-3 position of β-Glc (S-1) was still lying vacant for glycosidic linkage by next monosaccharide unit. The ^{1}H NMR signal of H-3 (S-1) at δ 3.80 gave a

cross peak at δ 3.80xδ101.11 in HMBC spectrum **Fig-7** (S.F.) of Taurose acetate confirming a (1→3) linkage between S-3 and S-1. The anomeric carbon signal at δ101.11 showed its complimentary signal at δ 4.50 in HSQC spectrum **Fig-1** (S.F.) of Taurose acetate in $CDCl_3$ at 300 MHz. The chemical shift of δ 101.11 and δ 4.50 of anomeric carbon and anomeric proton of S-3 were having resemblance with literature value of anomeric chemical shift value of GalNAc (S-3), hence S-3 monosaccharide was confirmed as β-GalNAc (S-3).[39] The anomeric proton signal present at δ4.50 (S-3) with *J* value of 7.8 Hz in $CDCl_3$ at 300 MHz confirmed a *β* glycosidic linkage between S-3→S-1. Simultaneously the ^{1}H NMR spectrum **Fig-4** (S.F.) of Taurose in D_2O contain a anomeric proton signal at δ 4.47 (*J* = 7.5 Hz) along with a singlet of three proton of amide methyl at δ 2.01 suggested that S-3 was GalNAc.[40] The anomeric proton signal at δ4.47 with *J* value of 7.5 Hz in the ^{1}H NMR of Taurose in D_2O at 300 MHz also supported a *β* configuration of β-GalNAc (S-3). The (1→3) linkage between *β*-Glc (S-1) and *β*-GalNAc (S-3) was also supported by the presence of H-3 signal of S-1 at δ 3.80 in upfield region of ^{1}H NMR spectrum and a cross peak at δ 3.80x76.51 in glycosidic region of HSQC spectrum **Fig-1** (S.F.) of Taurose acetate in $CDCl_3$. The next anomeric proton signal at δ 4.50 ^{1}H NMR spectrum of Taurose acetate assigned for β-GalNAc (S-3) gave three cross peaks at δ 4.50x3.80, δ 4.50x4.12 and δ 4.50x4.80 in its TOCSY spectrum **Fig-5** (S.F.) in $CDCl_3$ at 300 MHz. which was later identified as H-3, H-2 (containing NHAc group) and H-4 by COSY spectrum **Fig-6** (S.F.) of Taurose acetate. Out of these signals one proton signal at δ 4.12 corresponded to H-2 position of *β*-GalNAc (S-3) while the other ring proton signal of H-3 of *β*-GalNAc (S-3) observed at δ 3.80 was available for (1→3) glycosidic linkage with the next monosaccharide unit. The ^{1}H NMR signal at δ 3.80 assigned to of H-3 (S-3) gave a cross peak at δ 3.80xδ101.21 in HMBC spectrum **Fig-7** (S.F.) of Taurose acetate suggesting a (1→3) linkage between S-4 and S-3. The anomeric carbon signal at δ1 01.21 showed its complimentary signal at δ 4.50 in HSQC spectrum **Fig-1** (S.F.) of Taurose acetate in $CDCl_3$ at 300 MHz. The chemical shift value of anomeric carbon and anomeric proton at δ 101.21 and δ 4.50 assigned to S-4 were having resemblance with literature value of anomeric chemical shift value of β-GalNAc (S-4), hence S-4 monosaccharide was confirmed as *β*-GalNAc (S-4).[39] In addition to above information it was also supported by the anomeric proton signal at δ 4.47 (*J* = 7.5 Hz) along with a singlet of three proton of amide methyl at δ 1.99 in ^{1}H NMR spectrum **Fig-4** (S.F.) of Taurose in D_2O at 300 MHz confirmed that the S-4 was *β*-GalNAc.[40] The anomeric proton signal present at δ 4.50 assigned (S-4) with *J* value 7.8 Hz in $CDCl_3$ at 300 MHz confirmed a β glycosidic linkage between S-4→S-3. Simultaneously the anomeric proton signal present at δ 4.47 with *J* value 7.5 Hz in the ^{1}H NMR of Taurose in D_2O at 300 MHz also supported a *β* configuration of GalNAc (S-4). The (1→3) linkage between *β*-GalNAc (S-3) and β-GalNAc (S-4) was also supported by the presence of H-3 signal of S-3 at δ3.80 in upfield region of ^{1}H NMR spectrum and a cross peak at δ 3.80x76.51 in glycosidic region of HSQC spectrum of Taurose acetate in $CDCl_3$. The anomeric protons signal present at δ 4.50 assigned for *β*-GalNAc (S-4) in the ^{1}H NMR spectrum of Taurose acetate contains three cross peaks at δ 4.50x3.80, δ 4.50x4.12 and δ 4.50x4.80 in its TOCSY Spectrum **Fig-5** (S.F.) in $CDCl_3$ at 300 MHz, which was later identified as H-3, H-2 (containing NHAc group) and H-4 by COSY spectrum **Fig-6** (S.F.) of Taurose acetate. Out of these signals one proton signal at δ 4.12 corresponded to H-2 position of *β*-GalNAc (S-4) where as the other proton signal H-3 of *β*-GalNAc (S-4) observed at δ 3.80 showed the availability of H-3 of S-4 for glycosidic linkages by the next monosaccharide unit. Further the H-3 position of *β*-GalNAc (S-4) at 3.80 showed its complementary signal at δ 73.11 in HSQC spectra of Taurose acetate which showed a long range coupling with anomeric proton of next monosaccharide. The cross peak between H-1 of S-5 and C-3 of S-4 at δ 4.74x73.11 in Reverse HMBC **Fig-7** (S.F.) suggested the (1→3) linkage between S-5 and S-4. The anomeric proton signal present at δ 4.74 had its complimentary anomeric carbon signal at δ 95.40 in HSQC spectrum of Taurose acetate in $CDCl_3$ at 300 MHz. The chemical shift value of anomeric carbon and anomeric proton at δ 95.40 and δ 4.74 assigned to S-5 were having resemblance with literature value of anomeric chemical shift value of *β*-GlcNAc (S-5), hence S-5 monosaccharide was confirmed as *β*-GlcNAc (S-5).[30, 38] The anomeric proton signal present at δ 4.74 (S-5) with *J* value of 7.8 Hz in $CDCl_3$ at 300 MHz confirmed *β* glycosidic linkage between S-5→S-4. Further the anomeric proton signal at δ4.47 (*J* = 7.5 Hz) along with a singlet of three proton of amide methyl at δ 2.09 in ^{1}H NMR spectrum of Taurose in D_2O at 300 MHz supported that S-5 was GlcNAc [15,32,41]. The anomeric proton signal at δ 4.47 with *J* value 7.5 Hz in the ^{1}H NMR of Taurose in D_2O at 300 MHz also suggested a *β* configuration of β-GlcNAc (S-5). The (1→3) linkage between *β*-GalNAc (S-4) and β-GlcNAc (S-5) was also supported by the presence of H-3 signal of S-4 at δ 3.80 in upfield region of ^{1}H NMR spectrum and a cross peak at δ 3.80x73.11 in glycosidic region of HSQC spectrum of Taurose acetate in $CDCl_3$. The anomeric proton signal present at δ 4.74 in ^{1}H NMR spectrum of Taurose acetate in $CDCl_3$ at 300 MHz.

assigned to *β*-GlcNAc (S-5) contain three cross peaks at δ 4.74x3.60, δ 4.74x3.80 and δ 4.74x4.25 in its TOCSY spectrum Taurose acetate, which were later identified as H-3, H-2 (containing NHAc group) and H-4 by COSY spectrum of Taurose acetate. The chemical shifts of assigned to H-3 at δ 3.60 suggested that the H-3 of S-5 was available for glycosidic linkage by next monosaccharide unit i.e. (S-6). The H-3 position of *β*-GlcNAc (S-5) at δ 3.60 showed its complementary signal at δ 72.12 in HSQC spectra of Taurose acetate which showed a long range coupling with anomeric proton of next monosaccharide S-6 i.e. H-1 of S-6 and C-3 of S-5 at δ 4.67x72.12 in Reverse HMBC spectrum of acetylated Tausose in $CDCl_3$ at 300 MHz suggested the (1→3) linkage between S-6 and S-5. The anomeric proton signal present at δ 4.67 gave its complimentary anomeric carbon signal at δ 95.29 in HSQC spectrum of Taurose acetate in $CDCl_3$ at 300 MHz. The coupling constant of anomeric signal of (S-6) with *J* value of 8.1 Hz confirmed a *β* glycosidic linkage between S-6 and S-5. The anomeric proton signal at δ 4.53 with *J* value 7.8 Hz in the ^{1}H NMR spectrum of Taurose in D_2O at 300 MHz also suggested a *β* configuration of (S-6). The chemical shift value of anomeric carbon and anomeric proton at δ 95.29 and δ 4.67 assigned to S-6 were having resemblance with literature value of anomeric chemical shift value of *β*-Glc (S-6), hence S-6 monosaccharide was confirmed as *β*-Glc (S-6). The (1→3) linkage between *β*-GlcNAc (S-5) and *β*-Glc (S-6) was also supported by the presence of H-3 signal of S-5 at δ 3.60 in upfield region of ^{1}H NMR spectrum and a cross peak at δ 3.60x72.12 in glycosidic region of HSQC spectrum of Taurose acetate in $CDCl_3$. The anomeric proton signal present at δ 4.67 in TOCSY Spectrum of Taurose acetate assigned to β-Glc (S-6) gave three cross peaks at δ 4.67x3.55, δ 4.67x3.80, and δ 4.67x4.80, which was later identified as H-4, H-3 and H-2 respectively by COSY spectrum of Taurose acetate. The chemical shift of the cross peak at δ 4.67x3.55 and δ 4.67x3.80 suggested that in sugar S-6, two positions i.e. H-4 and H-3 were available for glycosidic linkage by next monosaccharide unit. Further the ^{1}H NMR signal of H-4 (S-6) at δ 3.55 gave a cross peak at δ 3.55x102.02 in HMBC spectrum of Taurose acetate confirming a (1→4) linkage between (S-7) and (S-6). The anomeric carbon signal at δ 102.02 showed its complimentary signal at δ 4.58 in HSQC spectrum of Tausose acetate in $CDCl_3$ at 300 MHz. The chemical shift value of anomeric carbon and anomeric proton at δ 102.02 and δ 4.58 assigned to S-7 were having resemblance with literature value of anomeric chemical shift value of *β*-Gal (S-7), hence S-7 monosaccharide was confirmed as *β*-Gal (S-7). [30,38] The anomeric proton signal present at δ 4.58 (S-7) had a *J* value of 8.1 Hz confirmed a *β* glycosidic linkage between S-7→S-6. The anomeric proton signal at δ4.55 with J value 7.8 Hz in the ^{1}H NMR of Taurose in D_2O at 300 MHz also confirming a *β* configuration of *β*-Gal (S-7). The (1→4) linkage between *β*-Gal (S-7) and *β*-Glc (S-6) was also supported by the presence of H-4 signal of S-6 at δ 3.55 in upfield region of ^{1}H NMR spectrum and a cross peak at δ 3.55x82.88 in glycosidic region of HSQC spectrum of Taurose acetate in $CDCl_3$. The anomeric proton signal at δ 4.58 in ^{1}H NMR spectrum of Taurose acetate in $CDCl_3$ assigned to *β*-Gal (S-7) gave three cross peaks at δ 4.58x5.05, δ 4.58x5.25 and δ 4.58x5.40 in their TOCSY spectrums, which were later identified as H-3, H-2 and H-4 respectively by COSY spectrum of Tausose acetate. This anomeric proton did not have any cross peak in the linkage region, hence confirmed that *β*-Gal (S-7) was present at non-reducing end and none of its hydroxyl groups were involved in glycosidic linkage. Since it was ascertained by the COSY and TOCSY spectrum of Taurose acetate that the *β*-Glc (S-6) has two vacant positions i.e. H-3 and H-4, and it was already confirmed that H-4 of S-6 was linked with *β*-Gal (S-7) whereas the left over H-3 position of *β*-Glc (S-6) must be linked with another monosaccharide unit. The ^{1}H NMR signal of H-3 position of *β*-Glc (S-6) at 3.80 showed its C-3 position at δ 71.02 in HSQC spectra of Taurose acetate which showed a long range coupling with anomeric proton of next monosaccharide S-8 i.e. H-1 of S-8 and C-3 of S-6 at δ 5.36x71.02 in Reverse HMBC spectrum of acetylated Taurose in $CDCl_3$ at 300 MHz confirming the (1→3) linkage between S-8 and S-6. The anomeric proton signal at δ 5.36 showed its complimentary carbon signal at δ 90.37 in HSQC spectrum of Taurose acetate in $CDCl_3$ at 300 MHz. The chemical shift value of anomeric proton and carbon at δ 5.36 and δ 90.37 assigned to S-8 were having resemblance with literature value of anomeric chemical shift value of *α*-Glc hence S-8 monosaccharide was confirmed as *α*-Glc (S-8). [33] The coupling constant of anomeric proton signal present at δ5.36 for (S-8) had a *J* value of 3.9 Hz in $CDCl_3$ which confirmed *α* glycosidic linkage between S-8→S-6. The anomeric proton signal at δ 5.23 with *J* value 3.6 Hz in the ^{1}H NMR of Taurose in D_2O at 300 MHz also suggested *α* configuration of *α*-Glc (S-8). Further the presence of *α*-Glc (S-8) as next monosaccharide in Taurose was also supported by appearance of anomeric proton signal at δ 5.23 (*J* = 3.6 Hz) in the ^{1}H NMR spectrum of Taurose in D_2O at 300 MHz. The (1→3) linkage between *α*-Glc (S-8) and *β*-Glc (S-6) was supported by the presence of H-3 signal of S-6 at δ 3.80 in upfield region of ^{1}H NMR spectrum and a cross peak at δ 3.80x71.02 in glycosidic region of HSQC spectrum of Taurose acetate in $CDCl_3$. The anomeric protons signal present at δ 5.36 in ^{1}H NMR

Spectrum of Taurose acetate assigned to α-Glc (S-8) gave three cross peaks at δ 5.36x3.80, δ 5.36x4.85 and δ 5.36x5.56 in TOCSY Spectrum of Taurose acetate which was later identified as H-4, H-2 and H-3 respectively by COSY spectrum of Taurose acetate. The chemical shift of the cross peak at δ 5.36x3.80 suggested that in sugar S-8, one position was available for glycosidic linkage by next monosaccharide unit. The ^{1}H NMR signal of H-4 (S-8) at δ 3.80 gave a cross peak at δ 3.80x102.02 in HMBC spectrum of Taurose acetate suggested a (1→4) linkage between S-9 and S-8. The anomeric carbon of β-Gal (S-9) at δ 102.02 gave its complimentary anomeric proton signal at δ 4.58 in the HSQC spectrum of Taurose acetate. The chemical shift value of anomeric carbon and anomeric proton at δ 102.02and δ 4.58 assigned to S-5 were having resemblance with literature value of anomeric chemical shift value of β-Gal (S-9), hence S-9 monosaccharide was confirmed as β-Gal (S-9). The anomeric proton signal present at δ 4.58 (S-9) had a *J* value of 8.1 Hz confirmed a β glycosidic linkage between S-9→S-8. The anomeric proton signal at δ 4.55 with *J* value 7.8 Hz in the ^{1}H NMR of Taurose in D_2O at 300 MHz also suggested that β configuration of β-Gal (S-9). The (1→4) linkage between α-Glc (S-8) and β-Gal (S-9) was supported by the presence of H-4 signal of S-8 at δ 3.80 in upfield region of ^{1}H NMR spectrum and a cross peak at δ 3.80x76.51 in glycosidic region of HSQC spectrum of Taurose acetate in $CDCl_3$. The anomeric proton signal at δ 4.58 in ^{1}H NMR spectrum of Taurose acetate in $CDCl_3$ assigned for β-Gal (S-9) gave three cross peaks at δ 4.58x5.05, δ 4.58x5.25 and δ 4.58x5.40 in their TOCSY spectrums, which were later identified as H-3, H-2 and H-4 respectively by COSY spectrum of Tausose acetate. This anomeric proton did not have any cross peak in the linkage region, hence confirmed that β-Gal (S-9) was present at non-reducing end and none of its -OH group were involved in glycosidic linkage.

All signals obtained in ^{1}H and ^{13}C NMR of compound D were in conformity with the assigned structure and their position which were confirmed by 2D NMR viz. COSY, TOCSY, HSQC and HMBC experiments of Taurose acetate. Thus based on the pattern of chemical shifts of ^{1}H NMR, ^{13}C NMR, COSY, TOCSY, HSQC and HMBC experiments, it was interpreted that the compound D, Taurose was a nonasaccharide having structure as:



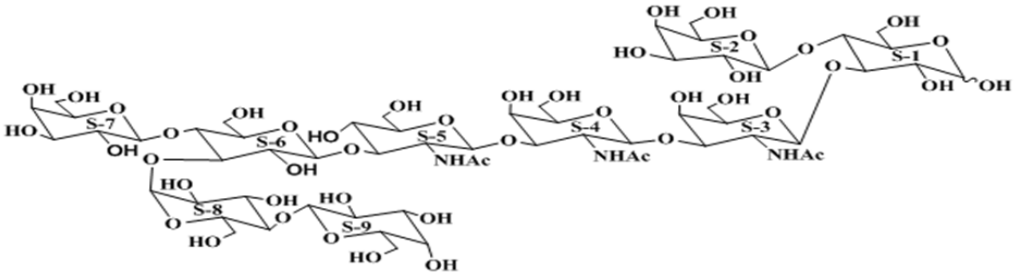
Taurose

ES Mass

The result obtained from the ES mass spectrum **Fig-8** (S.F.) further substantiated the structure of Taurose which was derived by its ^{1}H and ^{13}C NMR spectra. The highest mass ion peaks were recorded at m/z 1662, 1638 and 1622 which were due to [M+Na+K+H], [M+K] and [M+Na] respectively. It also contains the molecular ion peak at *m/z* 1599 confirming the molecular weight of Taurose as 1599 [M^+] which was in agreement with its molecular formula $C_{60}H_{101}O_{46}N_3$. 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 fragmentation pathway confirmed the sequence of monosaccharide units in the nonasaccharide (scheme 3.2, 3.3, 3.4).The nonasaccharide Taurose *m/z* 1599 (I) fragmented to give mass ion peak at *m/z* 1437 (II) [1599-S_9], this fragment was arisen due to the loss of terminal β-Gal (S_9) moiety from nonasaccharide indicating the presence of β-Gal (S_9) at the non-reducing end. It further fragmented to give mass ion peak at m/z 1275 (III) [1437-S_8] which was due to loss of α-Glc (S_8) moiety from octasaccharide.

This fragment of 1275 further fragmented to give mass ion peak at m/z 1113(IV) [1275-S_7] which was due to loss of β-Gal (S_7) moiety from the heptasaccharide. This hexasaccharide unit fragented to give mass ion peak at *m/z* 951 (V) [1113-S_6], which was due to loss of β-Glc (S_6) moiety from hexasaccharide. The pentasaccharide *m/z* 951 (V) fragmented to give mass ion at *m/z* 748 (VI) [951-S_5], this fragment was arisen due to the loss of β-GlcNAc (S_5) moiety from pentasaccharide indicating the presence of β-GlcNAc (S_5). It further fragmented to give mass ion peak at *m/z* 545(VII) [748-S_4] which was due to loss of β-GalNAc (S_4) moiety from tetrasaccharide. This trisaccharide unit fragmented to give mass ion peak at *m/z* 342 (VIII) [545-S_3], which was due to loss of β-GalNAc (S_3) moiety from trisaccharide. Further peak at fragment of 342 was fragmented to give mass ion peak at *m/z* 180 (IX) [342-S_2] which was due to loss of β-Gal (S_2) moiety from the disaccharide. The other fragmentation pathway in ES Mass spectrum **Fig-8** (S.F.) of D,

Taurose, *m/z* 1599 shows the mass ion peak at 1537 [1599-CH_3OH,-HCHO], 1518 [1537-H_3O^+], 1410 [1599-$CH_2OHCOCO$,-HOCHCHOH,-CH_2CO], 1383 [1437-$3H_2O$], 1219 [1275-$2H_3O^+$, -H_2O], 1186 [1275-CH_3CHO,-H^+], 1010 [1113-HOCHCHOH,-CH_2CHO], 956[1010-$3H_2O$], 838 [951-$NHCOCH_3$,-$3H_2O$-H^+], 819 [838-H_3O^+], 722 [951-$CH_2OHCOCO$,-HOCHCHOH,-CH_3CHO,-$2H_3O^+$], 685 [748-CH_3CHO,-H_3O^+], 680 [748-CH_3OH,-$2H_2O$], 663 [680-OH], 560 [663-$NHCOCH_3$,-CH_3CHO,-H^+], 485 [545-HOCHCHOH,-H_3O^+], 466 [485-H_3O^+], 465 [466-H^+], 365 [545-$NHCOCH_3$,-$CH_2OHCOCO$,-H_2O,-OH], 318 [485-H_2O,-$2CH_2OHCHO$,-CHO], 274 [342 -CH_3OH,-$2H_2O$] and 190 [174-$2CH_2CO$]. Based on result obtained from chemical degradation/acid hydrolysis, Chemical transformation, Electro spray mass spectrometry, structure reporter group and 1D NMR viz. ^{1}H NMR, ^{13}C NMR, and 2D NMR viz. COSY, TOCSY, HMBC and HSQC spectra of Taurose acetate and Taurose, the structure and sequence of isolated Novel oligosaccharide Taurose was deduced as-



TAUROSE

4. Conclusion

In summary, the novel milk oligosaccharides namely as D (Taurose) has been isolated from Lal-Muha cow's milk and structure was elucidated with the help of ^{1}H, ^{13}C, 2D NMR spectroscopy and mass spectrometry.

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