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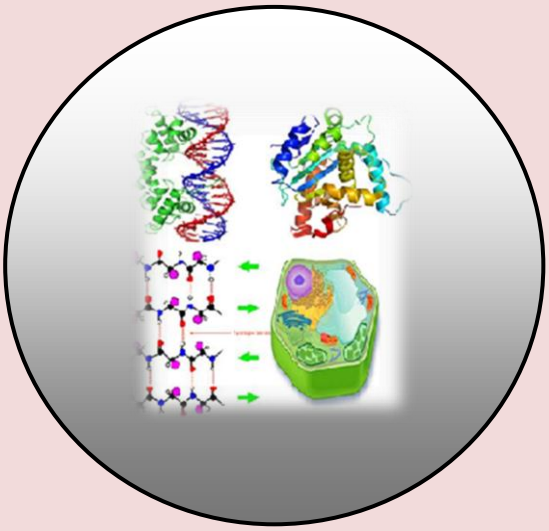

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Isolation of Gaddi Sheep Milk Oligosaccharides as Blood Platelet Enhancers

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ABSTRACT

Oligosaccharides are present in all the living organisms including plants, bacteria, fungi and milk of different origin. Different milk oligosaccharides have varied biological activities like immunostimulant, ant-cancer, anti-inflamatory, anti-coagulant, hypoglycemic and anti-viral. They are made up of Glucose, Galactose, GlcNHAc, GalNHAc, sometimes they are facilitated by the presence of Fucose and Sialic acid. The oligosaccharides with added Fucose are named as Fucosilated oligosaccharides while the presence of Sialic acid is sialated or acidic oligosaccharides. Both of these are present in sheep milk. Sheep milk oligosaccharides have reported blood platelets enhancer activities. It also aggravates hiccups and dysponea. It eliminates Pitta, Kapha and fat. Keeping in mind the above biological activities, we have studied the oligosaccharide contents of Gaddi sheep milk. For this purpose we have processed the Gaddi sheep milk by modified method of Kobata and Ginsburg and the oligosaccharide mixture was further assessed for its homogeneity by HPLC and for its isolation and purification it was acetylated and purified by column chromatography. Compounds so obtained were analysed by 1H and 13C NMR spectrometry and Mass spectrometry and data generated out of these experiments are also compiled and given in this paper.

Keywords: Oligosaccharides, Gaddi sheep, Milk, Isolation, Blood platelet enhancer, NMR and Mass spectrometry.

INTRODUCTION

Free oligosaccharides are natural constituents of all bacteria, fungi, plants and placental mammals' milk. The milk is a rich source of bioactive oligosaccharides (Cipalla et. al. 2010) which contain number of novel oligosaccharides depending on the nature of their origin to which mammals belongs. Many oligosaccharides exhibit potent biological activities such as

anti-tumor (Wu et. al. 2020), immunological (Triantis et. al. 2018), anti-complimentary (Clement et. al. 2010), anti-cancer (Azuma et. al. 2015), anti-inflammatory (Azuma et. al. 2015), anti-coagulant, hypoglycemic (Hsieh et. al. 2015) and antiviral activities (Van Hooijdonk et. al. 2000). Various developments on medicinal and pharmaceutical researches have unrevealed the importance of these oligosaccharide and their sources, which are either used in folk medicine, or their importance is reported in ancient medicinal systems (Ayurveda and Unani) (Mana et. al. 2021). The oligosaccharides isolated from various milk sources are categorized in two classes i.e. sialylated oligosaccharide and non-sialylated oligosaccharide. Both these class of oligosaccharide have been tested for their varied biological activities. The Elephant milk oligosaccharide (Osthoff et. al. 2007) contains a high ratio of sialyl oligosaccharide; this may be significant with respect to the formation of brain components, such as gangliosides of the suckling calves. N-acetyl-neuramin lactose sulphate, which is the dominant oligosaccharide in the dog milk play an important role in the nutrition of the rat pups. Buffalo milk oligosaccharides have ability to stimulate non-specific immunological resistance of the host against parasitic infections (Saxena et. al. 1999). Donkey milk oligosaccharides have ability to stimulate non-specific and specific immunological resistance (Deepak et. al. 1998). Goat milk oligosaccharides play an important roles in intestinal protection and repair after a damage caused by DSS (Dextron sodium sulphate)-induced colitis and their implication in human intestinal inflammation (Federico et. al. 2006). Goat milk oligosaccharides have anti-inflammatory effects in rats with trinitrobenzenesulfonic (T) acid induced colitis and may be useful in the management of inflammatory bowel disease (Hakkaraimen et. al. 2005). The cows' milk oligosaccharides reduce the adhesion of enterotoxic *Escherichia coli* (Hakkaraimen et. al. 2005) strains of the calf. Sheep (*Ovis aries*) milk aggravates hiccup and dyspnoea. It also eliminates pitta, Kapha and fat (Coni et. al. 1996). It is also reported that sheep milk enhances the blood platelet count which are lost during the dengue fever or other infectious diseases (The biochemical, biological and immunological results have opened up new possibilities for the diagnosis and specific treatment of various diseases. It is an established fact that all the biological activities residing in particular milk is due to their oligosaccharide contents, which have a low percentage as content of particular milk. It is therefore a challenging task for a natural product chemist to isolate these oligosaccharides in their purest form and elucidate their stereoscopic structures and relate them with their medicinal values.

Keeping in mind the biological activity of milk of Gaddi sheep (*Ovis aries orientalis*), it was collected in bulk from Kangara district of Himanchal Pradesh and was processed by modified method of Kobata and Ginsburg and was further purified by gel permeation chromatography for the separation of glycoproteins, proteins with oligosaccharides. A total amount of (263g) oligosaccharide mixture was obtained. The oligosaccharide mixture so obtained was analyzed by HPLC for confirming its homogeneity. Further this oligosaccharide mixture was acetylated by acetic anhydride and pyridine which was resolved nicely on TLC plates which showed eleven spots and was further purified on silica column chromatography which resulted into the isolation of eleven oligosaccharides. The method of their isolation and purification has been discussed in this paper. We have also described the physical constraints spot test for their identification and physicochemical data i.e. NMR [^{1}H, ^{13}C] and mass spectroscopy.

ZOOLOGICAL DESCRIPTION OF GADDI SHEEP

Gaddi sheep (*Ovis aries orientalis*) is a native of high altitude of Himalayan region i.e. Mountains of Bhadarvah of Jammu, Kullu, Kangara, Chamba and Mandi, Kinnour, Shimla, Nainital, Dehradun, Chamoli, Tehari- Garwal etc. Its life time is 10-15 years and gestation period is about 142-152 days (147±3 days). It is mainly source of high quality of wool but their faeces are used as high quality fertilizer for plants. Ewes' milk is also used in the production of cheese and yogurt in many upland parts of the world. Well known sheep's milk cheeses include the Roquefort of France, the brocciu of Corsica, the pecorino of Italy and the feta cheese of Greece. Sheep's milk does not trigger lactose intolerance in humans.

Zoological description of Gaddi sheep is as follows-

Kingdom: Animalia
Phylum: Chordata
Subphylum: Vertebrata
Class: Mammalia
Order: Artiodactyla
Family: Bovidae
Sub-family: Caprinae
Genus: Ovis
Species: aries
Sub-species: orientalis (Gaddi)

Gaddi sheep's milk is sweet and hot in nature. It has the highest amount of calcium and phosphate. Currently sheep's milk is used to produce milk skin bars (as a cleanser and moisturizer). Sheep's milk has the natural ability to moisturize and nourish the skin and is safe on even most delicate skin. According to 'Aurveda and Unani' system of medicine, the sheep's milk has various medicinal importance, which are as follows (Kay et. al. 2001, Sharon et al. 2000, Newburg et. al. 2004).

"It aggravates hiccup and dyspoea. It also elevates pitta and kapha. It decreases the fat."

Table 1. Milk oligosaccharides isolated from Sheep Colostrums.

Neutral oligosaccharide		
Gal (α1-3) Gal (β1-4) Glc	Sheep colostrum	Urashima et. al. (1989a)
3'-GL Gal (β1-3) Gal (β1-4) Glc	Sheep colostrum	Urashima et. al. (1989a)
6´-GL Gal (β1-6) Gal (β1-4) Glc	Sheep colostrum	Urashima et. al. (1989a)
Acidic milk oligosaccharides		
3'-SL Neu5Ac (α2-3) Gal (β1-4) Glc	Sheep colostrum	Nakamura et. al. (1998)
Neu5Gc (α2-3) Gal (β1-4) Glc	Sheep colostrum	Nakamura et. al. (1998)
Neu5Gc (α2-6) Gal (β1-4) Glc	Sheep colostrum	Nakamura et. al. (1998)

Table 2. Composition of sheep Milk.

Solids (%)	19.30
Fat (%)	7.0
Protein	5.98
Calcium (mg)	193
Calories (kcal)	108

The structures of oligosaccharides isolated from *Ovis aries* milk are as-

1- Galβ(1→6)Galβ(1→4)Glc
↓ ↓
Fucα(1→2) Galβ(1→3)

2- GalNAcβ(1→6)Galβ(1→4)Glc

3- Galβ(1→4)Glc
↓
Galβ(1→3)

4- Galβ(1→3)GalNAcβ(1→6)Galβ(1→4)Glc

5- Galβ(1→3)GalNAcβ(1→6)Galβ(1→4)Glc
↓
αGal(1→3)

6- GlcNAcβ(1→3) Galβ(1→3)GlcNAcβ(1→3)Galβ(1→4)Glc
↓ ↓
Fucα(1→3) Fucα(1→3)

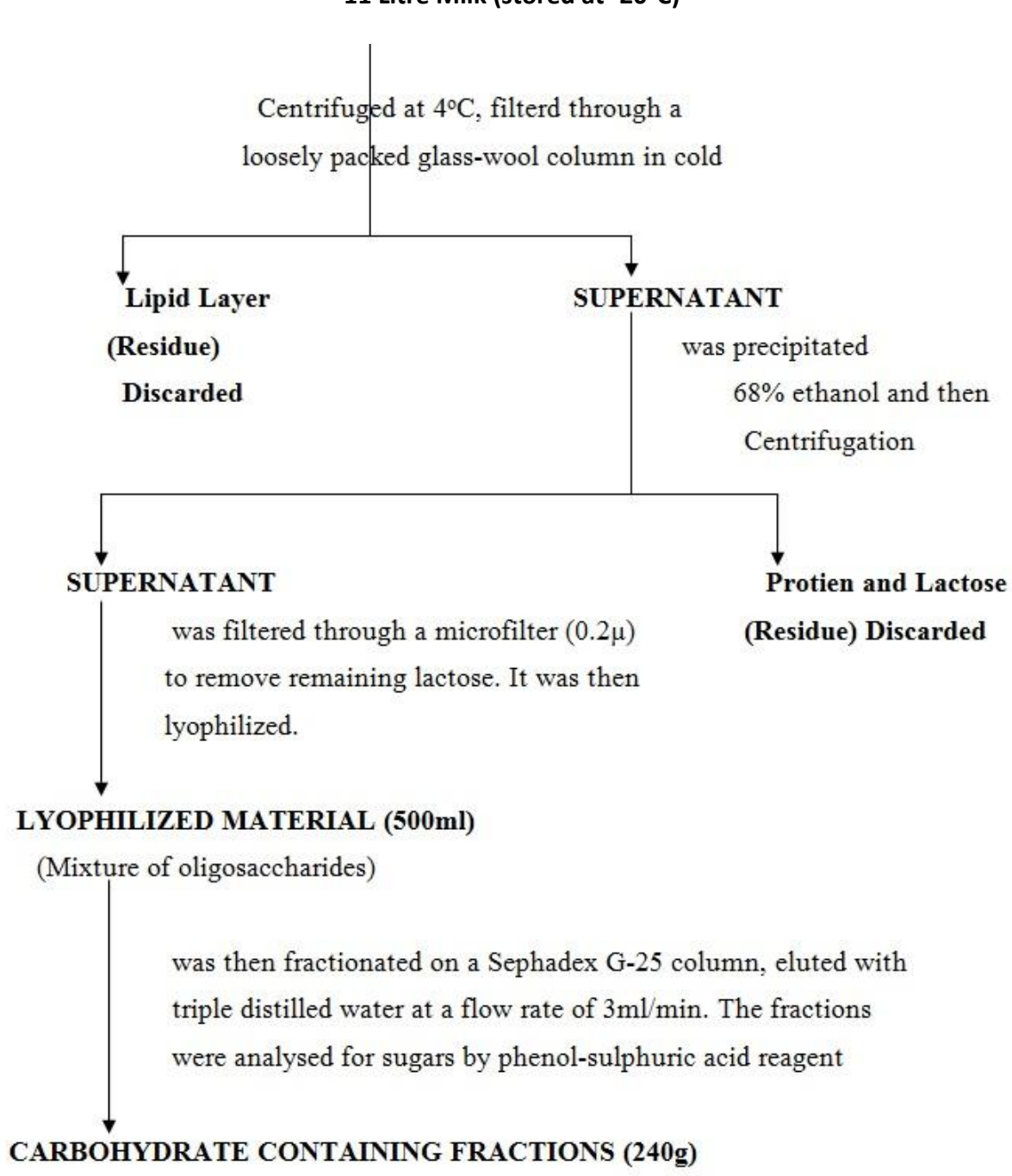
7- Galα(1→4)Glcβ(1→6)Galβ(1→3)GlcNAcβ(1→6)Galβ(1→3)GlcNAcβ(1→6)Galβ(1→4)Glc
↓ ↓
Galα(1→4) Galα(1→4)

Oligosaccharide mixture was isolated from Gaddi sheep's milk by Kobata and Ginsberg method and then this mixture was further purified by Sephadex G-25 Gel filtration.

Isolation of Gaddi Sheep Milk Oligosaccharides by Modified Method of Kobata and Ginsburg (Kumar et. al. 2018)

11 liter Gaddi Sheep milk was collected was stored at -20^{o}C. It was centrifuged for 15 min at 5000 rpm at -4^{o}C. The solidified lipid layer was removed by filtration through glass wool column in cold, Ethanol was added to the clear filtrate to a final concentration of 68% and the resulting solution was left overnight at 0^{o}C. The white precipitate formed, mainly of lactose and protein was removed by centrifugation and washed twice with 68% ethanol at 0^{o}C. The supernatant and washing were combined and filtered through a microfilter (0.24 µm) (to remove remaining lactose) and lyophilized affording crude oligosaccharide mixture (11.41 g). The lyophilized material responded positively to Phenol sulphuric acid, Feigl and Morgan-Elson test (Kumar et. al. 2018) and in the oligosaccharide mixture. This lyophilized material (mixture of oligosaccharides) was further purified by fractionating it on Sephadex G-25 column using glass double distilled water as elutant at a flow rate of 3ml/m. Each fraction was analysed for sugars by phenol-sulphuric acid reagent for presence of neutral sugar.

ISOLATION OF GADDI SHEEP'S MILK OLIGOSACCHARIDES
by
MODIFIED KOBATA AND GINSBURG METHOD



Purification of Oligosaccharide fractions

Fractions were pooled, lyophilized and finally analysed by HPLC

Analytical HPLC

eluted with triple distilled water (containing 90% TDW and 10% ACN) at a flow rate 1 ml/min, to check the homogeneity of the milk oligosaccharides.

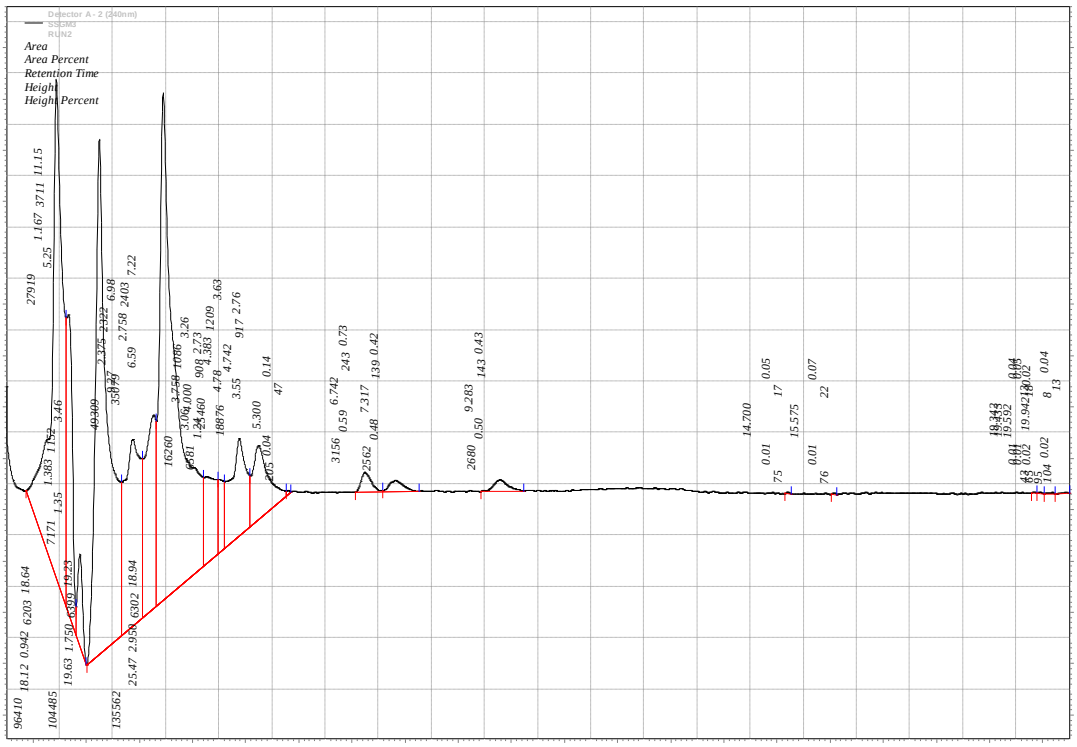
Chemical Transformations (Acetylation)

The fractions (50.00g)which gave positive phenol- sulphuric test was acetylated with pyridine and acetic anhydride converting the free sugar into their non- polar acetyl derivatives (55.50g) which were separated and purified by column chromatography over silica gel which resulted in the isolation of eleven chromatographically pure acetylated oligosaccharides

Confirmation of Homogenity of Gaddi's 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 plotters. The cyano column used for this purpose was a binary gradient system. The eluents were detected at 240 nm. Twenty one peaks were noticed in the sample at the varied retention times from 00.942 min. to 19.942 min. for convenience the peaks were numbered in their increasing order of retention time i.e. 19.942 min. (R_1),19.592 min. (R_2),19.435 min. (R_3),19.342 min. (R_4),15.575 min. (R_5), 14.700 min. (R_6),09.283 min. (R_7),07.317 min. (R_8),06.742 min. (R_9),05.300 min. (R_{10}),04.742 min. (R_{11}),04.383 min. (R_{12}),04.000 min. (R_{13}),03.758 min. (R_{14}),02.950 min. (R_{15}),02.758 min. (R_{16}),02.375 min. (R_{17}),01.750 min. (R_{18}),01.383 min. (R_{19}),01.167 min. (R_{20}) & 00.942 min. (R_{21}).

90% TDW: 10% ACN in water 240 nm—

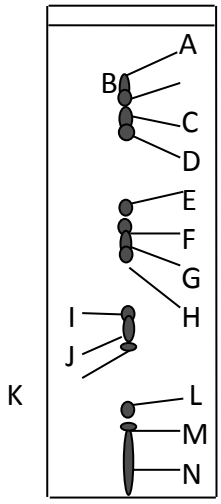


Acetylation of Oligosaccharide Mixture

21 gm of crude oligosaccharide mixture was acetylated with pyridine (20 ml) and acetic anhydride (20 ml) at 60^0 C and solution was stirred overnight. The mixture was evaporated under reduced pressure and the viscous residue was taken in $CHCl_3$ (500 ml) 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 (24.50 gm). The acetylation converted the free sugars into their nonpolar acetyl derivatives which were resolved nicely on TLC, giving fourteen spots on TLC i.e. A, B, C, D, E, F, G, H, I, J, K, L, M & N of which eleven compounds were finally separated by column chromatography over silica gel using hexane: chloroform, chloroform and $CHCl_3$:MeOH as eluents. Thin Layer Chromatography (TLC) of acetylated Gaddi Sheep's milk oligosaccharides.

Table 3. HPLC Table of Crude Gaddi Sheep Milk Oligosaccharides.

PK	Retention time	Area	Area %	Height	Height%
1	19.942	0013	00.04	000104	00.02
2	19.592	0008	00.02	000095	00.02
3	19.435	0018	00.05	000065	00.01
4	19.342	0013	00.01	000043	00.01
5	15.575	0022	00.07	000076	00.01
6	14.700	0017	00.05	000075	00.01
7	09.283	0143	00.43	002680	00.50
8	07.317	0139	00.42	002562	00.48
9	06.742	0243	00.73	003156	00.59
10	05.300	0047	00.14	000205	00.04
11	04.742	0917	02.76	018876	03.55
12	04.383	1209	03.63	025460	04.78
13	04.000	0908	02.73	006581	01.24
14	03.758	1086	03.26	016260	03.06
15	02.950	6302	18.94	135562	25.47
16	02.758	2403	07.22	035079	06.59
17	02.375	2322	06.98	049309	09.27
18	01.750	6399	19.23	104485	19.63
19	01.383	1152	03.46	007171	01.35
20	01.167	3711	11.15	027919	05.25
21	00.942	6204	18.64	096410	18.12



$CHCl_3$: MeOH (95:5)

The details of column chromatography are discussed as follows–

Purification of Acetylated Milk Oligosaccharides on Silica Gel

Different oligosaccharides were separated from crude (mixture of oligosaccharides 20 g) by column chromatography. Column was packed by silica gel and different proportion of Hexane, $CHCl_3$ and MeOH was used as solvent.

Chromatography-1

Purification of acetylated oligosaccharide mixture (20 g) was carried over silica gel (1000 g) using varying proportion of Hex:$CHCl_3$ $CHCl_3$ and $CHCl_3$: MeOH as eluents, collecting fractions of 1000 ml each. All these fractions were checked on TLC and those showing similar spots were taken together for further investigations. The chromatography was controlled on TLC and details are given in table- 4.

Table 4.

Fraction No.	Solvent	Eluted Residue Amorphous (in g)	Spots on TLC	Further I Investigation
1-18	10 Hex : 90 $CHCl_3$	0.504	No spot on TLC	-
19-25	Pure $CHCl_3$	1.045	a,b,c	C.C. – 2,3,4
26-46	Pure $CHCl_3$	0.154	c with streaking	C.C. – 5
47-53	Pure $CHCl_3$	0.220	c,d with streaking	-
54-72	Pure $CHCl_3$	0.280	c,d	-
73-107	.5 MeOH:99.5 $CHCl_3$	1.280	c,d with streaking	C.C. – 6
108-128	1 MeOH: 99 $CHCl_3$	0.350	d with streaking	C.C. - 7
129-147	1 MeOH: 99 $CHCl_3$	0.520	e,f,g,h with	-
148-165	2 MeOH: 98 $CHCl_3$	4.510	streaking	C.C. –8 to11
166-191	3 MeOH: 97 $CHCl_3$	4.850	e,f,g,h	C.C – 12-14
192-212	5 MeOH: 95 $CHCl_3$	2.250	h,i,j,k,l	C.C.- 15,16
213-227	10 MeOH: 90 $CHCl_3$	0.100	l,m with streaking	-
228-233	10 MeOH: 90 $CHCl_3$	2.915	m,n with streaking	-
234-245	15MeOH: 85$CHCl_3$+ washing	2.800	m,n,o m,n,o, washing	-

Chromatography-2

Combined fractions 19-25 (1.045 g) from chromatography 1 containing compounds a, b and c was chromatographed over 100 g silica gel. The elution was carried out using Hex: $CHCl_3$ and $CHCl_3$: MeOH as eluents, collecting fractions of 100 ml each and discussed in table -5 given below.

Table 5.

Fraction No.	Solvent	Eluted Residue Amorphous(in g)	Spots On TLC	Further Investigation
1-27	10 Hex : 90 $CHCl_3$	0.0760	a with streaking	-
28-42	Pure $CHCl_3$	0.0649	a with streaking	C.C. -3
43-49	Pure $CHCl_3$	0.0341	a,b	-
50-68	Pure $CHCl_3$	0.0855	b with streaking	C.C. -4
69-76	.5 MeOH: 99.5 $CHCl_3$.	0.0497	b,c with streaking	-
77-83	5 MeOH: 99.5 $CHCl_3$	0.3425	b,c	-
84-95	.5 MeOH: 99.5 $CHCl_3$	0.1041	b,c with streaking	-
96-113	.5 MeOH: 99.5 $CHCl_3$+washing	0.1537	b,c with streaking	-

Chromatography-3

Combined fractions 28-42 (0.0649 g) from chromatography 2 containing compound a with streaking was chromatographed over 10 g silica gel. The elution was carried out using Hex: $CHCl_3$ $CHCl_3$ and $CHCl_3$: MeOH as eluents, collecting fractions of 10ml each and discussed in table -6 given below.

Table 6.

Fraction No.	Solvent	Eluted Residue Amorphous(in g)	Spots On TLC	Further Investigation
1 - 17	10 Hex : 90 $CHCl_3$	0.0011	a with streaking	-
18-48	Pure $CHCl_3$	0.0095	a with streaking	-
49-56	Pure $CHCl_3$	0.0349	a	Physico- Chemical Investigation
57-65	.5 MeOH: 99.5 $CHCl_3$	0.0141	a,b with streaking	-
66-73	.5 MeOH: 99.5 $CHCl_3$	0.0 014	b with streaking	-
74-88	.5 MeOH: 99.5 $CHCl_3$ + washing	0.0007		-

0.0349g pure compound (compound a) was obtained from column chromatography 3.

Chromatography-4

Combined fractions 50-68 (0.0855 g) from chromatography 2 containing compound b with streaking was chromatographed over 10 g silica gel. The elution was carried out using Hex:$CHCl_3$ $CHCl_3$ and $CHCl_3$: MeOH as eluents, collecting fractions of 10 ml each and discussed in table - 7 given below.

Table 7.

Fraction No.	Solvent	Eluted Residue Amorphous(in g)	Spots On TLC	Further Investigation
1 - 23	10 Hex : 90 $CHCl_3$	0.0023	b with streaking	-
24-47	Pure $CHCl_3$	0.0049	b with streaking	-
48-51	Pure $CHCl_3$	0.0358	b	Physico-Chemical
52-74	.5 MeOH: 99.5 $CHCl_3$	0.0135	b with streaking	Investigation
75-83	.5 MeOH: 99.5 $CHCl_3$	0.0121	b,c	-
84-98	.5 MeOH: 99.5$CHCl_3$+washing	0.0133	b,c with streaking	- -

0.0358g pure compound (compound b) was obtained from column chromatography 4.

Chromatography-5

Combined fractions 24-46 (0.154 g) from chromatography 1 containing compound c with streaking was chromatographed over 20g silica gel. The elution was carried out using Hex: $CHCl_3$ $CHCl_3$ and $CHCl_3$: MeOH as eluents, collecting fractions of 20 ml each and discussed in table -8 given below.

Table 8.

Fraction No.	Solvent	Eluted Residue Amorphous(in g)	Spots On TLC	Further Investigation
1-13	10 Hex : 90 $CHCl_3$	0.0022	c with streaking	-
14-25	Pure $CHCl_3$	0.0109	c with streaking	-
26-57	Pure $CHCl_3$	0.1140	c	Physico-Chemical
58-63	Pure CHCl3	0.0067	c with streaking	Investigation
64-79	.5 MeOH: 99.5 $CHCl_3$	0.0084	c,d with streaking	-
80-97	.5 MeOH: 99.5$CHCl_3$+ washing	0.0103	c,d with streaking	- -

0.1140g pure compound (compound c) was obtained from column chromatography 5.

Chromatography-6

Combined fractions 73-107 (1.280 g) from chromatography 1 containing compounds c,d with streaking was chromatographed over 100 g silica gel. The elution was carried out using $CHCl_3$ and $CHCl_3$: MeOH as eluents, collecting fractions of 100ml each and discussed in table-9 given below.

Table 9.

Fraction No.	Solvent	Eluted Residue Amorphous (in g)	Spots On TLC	Further Investigation
1-25	Pure $CHCl_3$	0.0156	c,d with streaking	-
26-51	.5 MeOH: 99.5 $CHCl_3$	0.0279	c	Physico-Chemical Investigation
52-78	.5 MeOH: 99.5 $CHCl_3$	0.0390		
79-85	.5 MeOH: 99.5 $CHCl_3$	0.5421	c,d with streaking	-
86-91	.5MeOH: 99.5$CHCl_3$	0.2561	d	Physico-Chemical Investigation
92-115	.5MeOH: 99.5$CHCl_3$	0.2375		
116-125	1MeOH:99$CHCl_3$ +washing	0.1389	d with streaking	-
			d with streaking	-
			d with streaking	-

0.5421g pure compound (compound d) was obtained from column chromatography 6.

Chromatography-7

Combined fractions 19-25 (0.350 g) from chromatography 1 containing compound d with streaking was chromatographed over 50 g silica gel. The elution was carried out using $CHCl_3$ and $CHCl_3$: MeOH as eluents, collecting fractions of 50ml each and discussed in table-10 given below.

Table 10.

Fraction No.	Solvent	Eluted Residue Amorphous(in g)	Spots On TLC	Further Investigation
1-8	Pure $CHCl_3$	0.0183	d with streaking	-
9-23	.5MeOH:	0.1168	d with streaking	-
24-46	99.5$CHCl_3$	0.0380	d	Physico-Chemical Investigation
	.5MeOH:			
47-55	99.5$CHCl_3$	0.0671	d with streaking	
56-63		0.3750	d with streaking	-
64-78	.5 MeOH: 99.5$CHCl_3$	0.1200	d with streaking	-
	1 MeOH: 99$CHCl_3$			-
	1 MeOH: 99$CHCl_3$ +washing			

0.0380g pure compound (compound d) was obtained from column chromatography 7.

Chromatography-8

Combined fractions 148-165 (4.045 g) from chromatography 1 containing compounds e, f, g, and h was chromatographed over 300 g silica gel. The elution was carried out using $CHCl_3$ and $CHCl_3$: MeOH as eluents, collecting fractions of 300ml each and discussed in table-11 given below.

Table 11.

Fraction No.	Solvent	Eluted Residue Amorphous(in g)	Spots On TLC	Further Investigation
1-19	Pure $CHCl_3$	0.0083	e with streaking	-
20-34	1MeOH: 99 $CHCl_3$	0.0168	e with streaking	-
35-43	2 MeOH: 98 $CHCl_3$	0.2680	e with streaking	C.C.-9
44-57	2 MeOH: 98 $CHCl_3$	0.0671	e,f with streaking	-
58-76	2 MeOH: 98 $CHCl_3$	1.0501	f,g with streaking	-
77-89	2 MeOH: 98 $CHCl_3$	1.3450	g with streaking	C.C.-10
90-112	2 MeOH: 98 $CHCl_3$	0.0318	g,h with streaking	-
113-141	3 MeOH: 97 $CHCl_3$	1.1470	g,h with streaking	C.C.-11
142-156	3 MeOH: 97 $CHCl_3$	0.0128	g,h with streaking	-
157-189	3 MeOH:97$CHCl_3$ +washing	0.0047	h with streaking h with streaking	-

Chromatography-9

Combined fractions 35-43 (0.2680) from chromatography 8 containing compound e with streaking was chromatographed over 20 g silica gel. The elution was carried out using $CHCl_3$ and $CHCl_3$: MeOH as eluents, collecting fractions of 20 ml each and discussed in table- 12 given below.

Table 12.

Fraction No.	Solvent	Eluted Residue Amorphous(in g)	Spots On TLC	Further Investigation
1-8	Pure $CHCl_3$	0.0003	No spot on TLC	-
9-23	.5MeOH: 99.5$CHCl_3$	0.0068	e with streaking	-
24-46	1 MeOH: 99 $CHCl_3$	0.0480	e with streaking	-
47-55	2 MeOH: 98 $CHCl_3$	0.1820	e	Physico-Chemical
56-63	2 MeOH: 98 $CHCl_3$	0.0050	e with streaking	Investigation
64-78	2 MeOH:98$CHCl_3$ +washing	0.0200	e with streaking	- -

0.1820g pure compound (compound e) was obtained from column chromatography 9.

Chromatography-10

Combined fractions 77-89 (1.345 g) from chromatography 8 containing compound g with streaking was chromatographed over 100 g silica gel. The elution was carried out using $CHCl_3$ and $CHCl_3$: MeOH as eluents, collecting fractions of 100 ml each and discussed in table-13 given below.

Table 13.

Fraction No.	Solvent	Eluted Residue Amorphous(in g)	Spots On TLC	Further Investigation
1-8	Pure $CHCl_3$	0.0008	No spot on TLC	-
9-23	.5MeOH: 99.5$CHCl_3$	0.0138	g with streaking	-
24-46	1 MeOH: 99 $CHCl_3$	0.7460	gwith streaking	-
47-55	2 MeOH: 98 $CHCl_3$	0.0890	g	Physico-Chemical Investigation
56-63	2 MeOH: 98 $CHCl_3$	0.2250	g with streaking	-
64-78	2 MeOH: 98$CHCl_3$ +washing	0.1350	g with streaking	-

0.0890g pure compound (compound g) was obtained from column chromatography 10.

Chromatography-11

Combined fractions 120-141 (1.1470 g) from chromatography 8 containing compounds g,h with streaking was chromatographed over 100 g silica gel. The elution was carried out using $CHCl_3$ and $CHCl_3$: MeOH as eluents, collecting fractions of 100ml each and discussed in table-14 given below.

Table 14.

Fraction No.	Solvent	Eluted Residue Amorphous(in g)	Spots On TLC	Further Investigation
1-25	Pure $CHCl_3$	0.0004	No spot on TLC	-
26-45	.5MeOH: 99.5$CHCl_3$	0.0358	g with streaking	-
46-71	1 MeOH: 99 $CHCl_3$	0.1360	g with streaking	-
72-85	2 MeOH: 98 $CHCl_3$	0.1740	g,h with streaking	-
86-99	3MeOH: 97$CHCl_3$	0.5632	h with streaking	-
100-126	3 MeOH: 97 $CHCl_3$	0.3160	h	Physico-Chemical Investigation
127-158	3 MeOH: 97$CHCl_3$ +washing	0.0468	h with streaking	-

0.3160g pure compound (compound h) was obtained from column chromatography 11.

Chromatography-12

Combined fractions 166-191 (4.850 g) from chromatography 1 containing compounds h,i,j,k,l was chromatographed over 300 g silica gel. The elution was carried out using $CHCl_3$ and $CHCl_3$: MeOH as eluents, collecting fractions of 300 ml each and discussed in table-15 given below.

Table 15.

Fraction No.	Solvent	Eluted Residue Amorphous(in g)	Spots On TLC	Further Investigation
1-16	Pure $CHCl_3$	0.0012	No spot on TLC	-
17-34	1MeOH: 99 $CHCl_3$	0.0360	h with streaking	-
35-43	2 MeOH: 98 $CHCl_3$	0.9650	h,i with streaking	-
44-59	3MeOH: 97 $CHCl_3$	0.1120	j with streaking	-
60-73	5 MeOH: 95 $CHCl_3$	1.2584	j	Physico-Chemical
74-88	5 MeOH: 95 $CHCl_3$	0.1130	j ,k with streaking	Investigation
89-97	5 MeOH: 95 $CHCl_3$	1.4750	j,k with streaking	-
98-114	5 MeOH: 95 $CHCl_3$	0.0329	k,l with streaking	C.C.-14
115-136	5 MeOH: 95 $CHCl_3$	0.2925	l with streaking	-
137-154	5 MeOH: 95$CHCl_3$ +washing	0.0614	l with streaking	C.C.-13 -

1.2584 g pure compound (compound j) was obtained from column chromatography 12.

Chromatography-13

Combined fractions 115-136 (0.2925) from chromatography 12 containing compound l with streaking was chromatographed over 50 g silica gel. The elution was carried out using $CHCl_3$ and $CHCl_3$: MeOH as eluents, collecting fractions of 50ml each and discussed in table-16 given below:

Table 16.

Fraction No.	Solvent	Eluted Residue Amorphous (in g)	Spots On TLC	Further Investigation
1-21	1 MeOH: 99 $CHCl_3$	0.0012	No spot on TLC	-
22-56	2 MeOH: 98 $CHCl_3$	0.0135	l with streaking	-
57-78	3MeOH: 97$CHCl_3$	0.0230	l with streaking	-
79-94	5 MeOH: 95 $CHCl_3$	0.0740	l with streaking	-
95-117	5 MeOH: 95 $CHCl_3$	0.1700	l	Physico-Chemical
118-136	5 MeOH: 95$CHCl_3$ +washing	0.0048	l with streaking	Investigation -

0.1700g pure compound (compound l) was obtained from column chromatography 13.

Chromatography-14

Combined fractions 89-97 (1.4750 g) from chromatography 12 containing compounds j,k with streaking was chromatographed over 100 g silica gel. The elution was carried out using $CHCl_3$ and $CHCl_3$: MeOH as eluents, collecting fractions of 100 ml each and discussed in table- 17 given below.

Table 17.

Fraction No.	Solvent	Eluted Residue Amorphous(in g)	Spots On TLC	Further Investigation
1-25	1 MeOH: 99 $CHCl_3$	0.0002	No spot on TLC	-
26-45	2 MeOH: 98 $CHCl_3$	0.0278	j with streaking	-
46-71	3MeOH: 97$CHCl_3$	0.0860	j with streaking	-
72-85	5MeOH: 95$CHCl_3$	0.2760	j	Physico-Chemical
86-99	5MeOH: 95$CHCl_3$	0.5632	j, k with	Investigation
100-126	5MeOH: 95$CHCl_3$	0.3260	streaking k	-
126-178	5 MeOH:95$CHCl_3$ + washing	0.0512	k with streaking	Physico-Chemical Investigation -

0.2760 g pure compound (compound j) and 0.3260 g pure compound (compound k) was obtained from column chromatography 14.

Chromatography-15

Combined fractions 192-212 (2.2500 g) from chromatography 1 containing compounds l,m with streaking was chromatographed over 200 g silica gel. The elution was carried out using $CHCl_3$ and $CHCl_3$: MeOH as eluents, collecting fractions of 200ml each and discussed in table-18 given below.

Table 18.

Fraction No.	Solvent	Eluted Residue Amorphous(in g)	Spots On TLC	Further Investigation
1-18	1MeOH: 99 $CHCl_3$	0.0236	No spot on TLC	-
19-38	2MeOH: 98 $CHCl_3$	0.0230	l with streaking	-
39-53	3MeOH: 97$CHCl_3$	0.4390	l,m with streaking	-
54-77	5MeOH: 95 $CHCl_3$	0.3275	l,m with streaking	-
78-84	7MeOH: 93 $CHCl_3$	1.0980	m with streaking	C.C.-16
85-102	7MeOH: 93 $CHCl_3$	0.0274	m with streaking	-
103-120	10MeOH: 90$CHCl_3$ +washing	0.0890	m with streaking	-

Chromatography-16

Combined fractions 78-84 (1.0980 g) from chromatography 15 containing compound m with streaking was chromatographed over 100 g silica gel. The elution was carried out using $CHCl_3$ and $CHCl_3$: MeOH as eluents, collecting fractions of 100ml each and discussed in table-19 given below.

Table 19.

Fraction No.	Solvent	Eluted Residue Amorphous(in g)	Spots On TLC	Further Investigation
1-56	1MeOH: 99 $CHCl_3$	0.0006	No spot on	-
57-78	2MeOH: 98 $CHCl_3$	0.0379	TLC	-
79-97	3MeOH: 97$CHCl_3$	0.5872	m with	-
98-123	5MeOH: 95 $CHCl_3$	0.0828	streaking	-
124-156	7MeOH: 93 $CHCl_3$	0.2364	m with streaking	Physico-Chemical
157-173	7MeOH: 93 $CHCl_3$	0.0121	m with	Investigation
174-195	10MeOH: 90$CHCl_3$ +washing	0.0078	streaking m	- -
			m with streaking	
			m with streaking	

0.2364 g pure compound (compound m) was obtained from column chromatography 4.

Method of Deacetylaion of Isolated Compounds-

The pure acetylated compounds (a, b, c, d, e, g, h, j, k, l, and m) were isolated by column chromatography which on deacetylation gave native oligosaccharides (A, B, C, E, G, H, J, K, L, and m).

Table 20. Deacetylatd and Acetylated Oligosaccharides.

Acetylated Compound			Deacetylated Compound	
Alphabetical Notation	Quantity		**Alphabetical Notation**	**Quantity**
	Obtained from column	**Taken for deacetylation**		
a	34.9mg	28.0mg	A	24.0mg
b	35.8 mg	27.0mg	B	23.5 mg
c	141.9mg	25.0mg	C	21.0mg
d	580.1mg	34.0mg	D	30.0mg
e	182.0mg	24.0mg	E	21.0mg
g	89.0mg	26.4mg	G	20.5mg
h	316.0mg	20.4mg	H	16.2mg
j	1258.4mg	28.4mg	J	25.0mg
k	326.0mg	29.2mg	K	23.4mg
l	170.0mg	26.0mg	L	23.6mg
m	236.4mg	22.5mg	M	20.0mg

DESCRIPTION OF ISOLATED SUBSTANCES

SUBSTANCE C Substance C (141.90 mg) obtained from fraction 26-57 of chromatography–5. On deacetylation of 25mg of substance c with NH_3 / acetone it afford substance C (21.0mg) as a viscous mass, $[\alpha]_D$ +138.72^0(c, 4, H_2O) .

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

$C_{60}H_{101}N_3O_{46}$	%C	%H	%N
Calcd.	45.03	6.32	2.63
Found	45.02	6.31	2.61

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

δ in D_2O: ^{1}H NMR

δ5.194 [d, 1H, J=3.8Hz, αGlc (S_1), H-1], δ5.085 [d, 1H, J=3.6Hz, αGal (S_7), H-1], δ5.045 [d, 1H, J=3.6Hz, αGal (S_6), H-1], δ5.031 [d, 1H, J=4.8 Hz, αGal (S_8), H-1], δ5.011 [d, 1H, J=4.8Hz, αGal (S_9), H-1], δ4.655 [d, 1H, J=7.8Hz, βGlc (S_1'), H-1], δ4.594 [d, 1H, J=7.8Hz, βGlcNAc (S_3), H-1], δ4.519 [d, 2H, J=7.8Hz, βGal (S_2) & βGal (S_4), H-1], and δ4.502 [d, 1H, J=6.9Hz, βGal (S_5), H-1], δ4.059 [t, 1H, J=6.1, βGlcNAc (S_3), H-2], δ3.544 [t, 1H, J=5.9, βGlc (S_1'), H-2], δ2.042 [s, 6H, αGalNAc (S_8) & αGalNAc (S_9), NHCO$\underline{CH_3}$] and δ1.956 [s, 3H, βGlcNAc (S_3), NHCO$\underline{CH_3}$].

δ in D_2O: ^{13}C NMR

δ172.06[βGlcNAc (S_3), NH$\underline{CO}CH_3$], δ171.34 [αGalNAc (S_8) & αGalNAc (S_9), NH$\underline{CO}CH_3$], δ102.10 [βGlcNAc (S_3), C-1], δ101.60 [βGal (S_2) & βGal (S_4), C-1], δ97.40[β Gal (S_5), C-1], δ95.80 [αGalNAc(S_9)], δ94.20 [αGalNAc(S_8), C-1], δ91.50[αGal (S_6), C-1], δ90.80[αGal (S_7), C-1], δ89.90[βGlc(S_1'), C-1] and δ88.20 [αGlc (S_1), C-1], δ20.76 [βGlcNAc (S_3), NHCO$\underline{CH_3}$] and δ20.56 [αGalNAc (S_8) & αGalNAc (S_9), NHCO$\underline{CH_3}$].

δ in $CDCl_3$: ^{1}H NMR (Acetylated)-

δ6.255 [d, 1H, J=3.8Hz, αGlc (S_1), H-1], δ5.678[d, 1H, J=7.8Hz, βGlc (S_1'), H-1], δ5.087 [d, 1H, J=3.6Hz, αGal (S_7), H-1], δ5.058 [d, 1H, J=3.6Hz, αGal (S_6), H-1], δ5.037 [d, 1H, J=4.8 Hz, αGal (S_8), H-1], δ5.017 [d, 1H, J=4.8Hz, αGal (S_9), H-1], δ4.604 [d, 1H, J=7.8Hz, βGlcNAc (S_3), H-1], δ4.577 [d, 2H, J=7.8Hz, βGal (S_2) & βGal (S_4), H-1], and δ4.536 [d, 1H, J=6.9Hz, βGal (S_5), H-1], δ2.069[s, 3H, αGalNAc (S_8), NHCO$\underline{CH_3}$], δ2.054 [s, 3H, αGalNAc (S_9), NHCO$\underline{CH_3}$] and δ1.998 [s, 3H, βGlcNAc (S_3), NHCO$\underline{CH_3}$].

δ in $CDCl_3$: ^{13}C NMR (Acetylated)-

δ173.08 [αGalNAc (S_8), NH$\underline{CO}CH_3$], δ172.86[βGlcNAc (S_3), NH$\underline{CO}CH_3$], δ173.00 [αGalNAc (S_9), NH$\underline{CO}CH_3$], δ102.15 [βGlcNAc (S_3), C-1], δ101.89 [βGal (S_2), βGal (S_4), αGalNAc(S_8)& αGalNAc(S_9), C-1], δ98.97 [β Gal (S_5), C-1], δ91.52[βGlc(S_1'), αGal (S_6)& αGal (S_7), C-1], and δ88.94 [αGlc (S_1), C-1], δ20.78 [βGlcNAc (S_3), NHCO$\underline{CH_3}$] and δ20.56 [αGalNAc (S_8) & αGalNAc (S_9), NHCO$\underline{CH_3}$].

ES Mass

m/z 1623[M+Na+H]$^+$, m/z 1599[M]$^+$, m/z 1437 , m/z 1401, m/z 1368 , m/z 1312, m/z 1275, m/z1210, m/z 1180, m/z1120, m/z1109, m/z 1102, m/z 1072, m/z 1063, m/z 1027, m/z

1010, m/z 945, m/z 910, m/z 945, m/z 884, m/z 880 , m/z 852 , m/z 824, m/z 805, m/z 802, m/z 745, m/z 742, m/z 707 , m/z 704, m/z 687, m/z 643, m/z 582, m/z 559, m/z 548 , m/z 504, m/z 499, m/z 455, m/z 422, m/z 393 , m/z 384, m/z 375 , m/z 364, m/z 357, m/z 345 , m/z 342, m/z 303, m/z 281, m/z 264, m/z 261 , m/z 222, m/z 203, m/z 183, m/z 180, m/z 156, m/z and 150 m/z 144.

SUBSTANCE E

Substance E (182.00 mg) obtained from fraction 47-55 of column chromatography-9. On deacetylation of 24mg of acetylated compound e with NH_3/ acetone it afforded substance E (21mg) as a viscous mass, $[\alpha]_D$ +42.41^0(c, 2, H_2O).

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

$C_{30}H_{51}N_3O_{21}$		%C	%H	%N
	Calcd.	45.63	6.46	5.32
	Found	45.61	6.45	5.30

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

δ in D_2O: ^{1}H NMR

δ5.191 [d, 1H, J=3.8Hz, αGlc (S_1), H-1], δ4.632 [d, 1H, J=7.8Hz, βGlc (S_1'), H-1], δ4.489 [d, 1H, J=7.8Hz, βGlcNAc (S_4), H-1] and δ4.420 [d, 1H, J=7.8Hz, βGalNAc (S_2) & βGalNAc (S_3), H-1], δ3.857 [t, 1H, J=6.1, βGlcNAc (S_4), H-2], δ3.210 [t, 1H, J=5.8, βGlc (S_1'), H-2], δ2.052 [s, 6H, βGalNAc (S_2) & βGalNAc (S_3), NHCO$\underline{CH_3}$] and δ2.044 [s, 3H, βGlcNAc (S_4), NHCO$\underline{CH_3}$].

δ in D_2O: ^{13}C NMR

δ171.47 [βGalNAc (S_2) & βGalNAc (S_3), NH$\underline{CO}CH_3$], δ169.92 [βGlcNAc (S_4), NH$\underline{CO}CH_3$], δ102.00 [βGlcNAc (S_4), C-1], δ101.60 [βGalNAc (S_2) & βGalNAc (S_3), C-1], δ89.90[βGlc(S_1'), C-1] and δ88.20 [αGlc (S_1), C-1], δ20.81 [βGalNAc (S_3), NHCO$\underline{CH_3}$], δ20.61 [βGalNAc (S_2), NHCO$\underline{CH_3}$] and δ20.37 [βGlcNAc (S_4), NHCO$\underline{CH_3}$].

δ in $CDCl_3$: ^{1}H NMR (Acetylated)-

δ6.261 [d, 1H, J=3.8Hz, αGlc (S_1), H-1], δ5.660 [d, 1H, J=7.8Hz, βGlc (S_1'), H-1], δ4.503 [d, 1H, J=7.8Hz, βGlcNAc (S_4), H-1], δ4.499 [d, 1H, J=7.8Hz, βGalNAc (S_2), H-1] and δ4.439 [d, 1H, J=7.8Hz, βGalNAc (S_3), H-1], δ2.052 [s, 6H, βGalNAc (S_2) & βGalNAc (S_3), NHCO$\underline{CH_3}$] and δ2.044 [s, 3H, βGlcNAc (S_4), NHCO$\underline{CH_3}$].

δ in $CDCl_3$: ^{13}C NMR (Acetylated)-

δ171.47 [βGalNAc (S_2) & βGalNAc (S_3), NH$\underline{CO}CH_3$], δ169.92 [βGlcNAc (S_4), NH$\underline{CO}CH_3$], δ104.34[βGalNAc (S_2) & βGalNAc (S_3), C-1], δ104.20 [βGlcNAc (S_4), C-1], δ91.54 [βGlc(S_1'), C-1] and δ89.20 [αGlc (S_1), C-1], δ20.81 [βGalNAc (S_3), NHCO$\underline{CH_3}$], δ20.61 [βGalNAc (S_2), NHCO$\underline{CH_3}$] and δ20.37 [βGlcNAc (S_4), NHCO$\underline{CH_3}$].

ES Mass

m/z 850[M+Na+K]$^+$, m/z 790[M+H]$^+$, m/z 789[M]$^+$, m/z 758, m/z 716, m/z 714, m/z 597, m/z 586, m/z 528 , m/z 510, m/z 468, m/z 461, m/z 449, m/z 430, m/z 390, m/z 387, m/z 372, m/z 352, m/z 330, m/z 310, m/z 294, m/z 276, m/z 271, m/z 211, m/z 180, m/z 162 and m/z 144.

SUBSTANCE K

Substance K (326.00 mg) obtained from fraction 100-126 of column chromatography 14. On deacetylation of 29.2 mg of acetylated compound k with NH_3/ acetone it afforded substance K (23.4 mg) as a viscous mass, $[\alpha]_D$ +115.39^0 (c, 4, H_2O).

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

$C_{66}H_{111}N_3O_{51}$	%C	%H	%N
Calcd.	44.97	6.31	2.38
Found	44.96	6.30	2.36

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

δ in D_2O: ^{1}H NMR

δ5.274 [d, 1H, J=3.6Hz, αGlc (S_1) & αGal (S_7), H-1], δ4.721 [d, 1H, J=7.8Hz, βGlc (S_1'), H-1], δ5.276 [d, 1H, J=4.2Hz, αGalNAc (S_{10}), H-1], δ4.660 [d, 1H, J=8.4Hz, βGlc (S_3), H-1], δ4.606 [d, 1H, J=7.8Hz, βGlcNAc (S_9), H-1] δ4.542 [d, 1H, J=7.8 Hz, βGlcNAc (S_6), H-1] δ4.507 [d, 1H, J=6.9Hz, βGal (S_5), H-1], δ4.497 [d, 1H, J=7.8Hz, βGal (S_4), H-1] and δ4.445 [d, 1H, J=7.8Hz, βGal (S_2) & βGal (S_8), H-1], δ3.340 [t, 2H, J=6.1, βGlc (S_1') & βGlc (S_3), H-2], δ2.051 [s, 6H, βGlcNAc (S_6) & βGlcNAc (S_9), NHCO$\underline{CH_3}$], and δ1.965 [s, 3H, αGalNAc (S_{10}), NHCO$\underline{CH_3}$].

δ in D_2O: ^{13}C NMR

δ171.20 [βGlcNAc (S_6) NH$\underline{CO}CH_3$], δ169.10[βGlcNAc (S_9), NH$\underline{CO}CH_3$], δ168.20 [αGalNAc (S_{10}), NH$\underline{CO}CH_3$], δ101.65 [βGal (S_2), βGal (S_4), βGal (S_5) & βGal (S_8), C-1], δ100.50 [βGlcNAc (S_6) & βGlcNAc (S_9), C-1], δ95.10 [βGlc (S_3), C-1], δ90.60 [αGalNAc (S_{10}), C-1], δ89.50 [αGal (S_7), C-1], δ88.20 [βGlc (S'_1), C-1] and δ86.50 [αGlc (S_1), C-1], δ20.05 [αGalNAc (S_{10}), NHCO$\underline{CH_3}$] and δ20.01 [βGlcNAc (S_6) & βGlcNAc (S_9), NHCO$\underline{CH_3}$].

δ in $CDCl_3$: ^{1}H NMR (Acetylated)-

δ6.225 [d, 1H, J=3.6Hz, αGlc (S_1), H-1], δ5.656 [d, 1H, J=7.8Hz, βGlc (S_1'), H-1], δ5.371 [αGal (S_7), H-1], δ5.311 [d, 1H, J=4.2Hz, αGalNAc (S_{10}), H-1], δ4.664 [d, 1H, J=8.4Hz, βGlc (S_3), H-1], δ4.621 [d, 1H, J=7.8Hz, βGlcNAc (S_9), H-1] δ4.583 [d, 1H, J=7.8 Hz, βGlcNAc (S_6), H-1], δ4.553 [βGal (S_8), H-1], δ4.528 [d, 1H, J=6.9Hz, βGal (S_5), H-1], δ4.498 [d, 1H, J=7.8Hz, βGal (S_4), H-1] and δ4.447 [d, 1H, J=7.8Hz, βGal (S_2), H-1], δ2.064 [s, 3H, βGlcNAc (S_9), NHCO$\underline{CH_3}$], δ2.057 [s, 3H, βGlcNAc (S_6), NHCO$\underline{CH_3}$] and δ1.987 [s, 3H, αGalNAc (S_{10}), NHCO$\underline{CH_3}$].

δ in $CDCl_3$: ^{13}C NMR (Acetylated)-

δ171.60 [βGlcNAc (S_6) NH$\underline{CO}CH_3$], δ171.20 [βGlcNAc (S_9), NH$\underline{CO}CH_3$], δ170.80 [αGalNAc (S_{10}), NH$\underline{CO}CH_3$],δ104.13 [βGal (S_2), βGal (S_4), βGal (S_5) & βGal (S_8), C-1], δ101.69 [βGlcNAc (S_6) & βGlcNAc (S_9), C-1], δ95.18 [βGlc (S_3), C-1], δ92.20 [αGalNAc (S_{10}), C-1], δ91.55 [αGal (S_7), C-1], δ90.14[βGlc (S'_1), C-1] and δ89.20 [αGlc (S_1), C-1], δ20.83 [βGlcNAc (S_6), NHCO$\underline{CH_3}$], δ20.68 [βGlcNAc (S_9), NHCO$\underline{CH_3}$] and δ20.59 [αGalNAc (S_{10}), NHCO$\underline{CH_3}$].

ES mass

m/z 1823$[M+Na+K]^+$, m/z 1784$[M+Na]^+$, m/z 1761$[M]^+$, m/z 1712, m/z 1700, m/z 1694,

1662, m/z 1623, m/z 1606 , m/z 1599, m/z 1588, m/z 1556, m/z 1519, m/z 1506, m/z 1483, m/z 1448, m/z 1440, m/z 1396, m/z1358 , m/z 1300 , m/z 1271, m/z 1266 , m/z 1234, m/z 1231, m/z 1203, m/z 1185, m/z 1182, m/z 1161, m/z 1105, m/z 1093, m/z 1086, m/z 1075, m/z 1031, m/z 1015, m/z 979, m/z 995, m/z 948, m/z 927, m/z 908 , m/z 905, m/z 869, m/z 790, m/z 772, m/z 742, m/z 722, m/z 707, m/z 692, m/z 670, m/z 650, m/z 640, m/z 618, m/z 590, m/z 568, m/z 550, m/z 512, m/z 480 , m/z 465, m/z 454, m/z 440 , m/z 406, m/z 357, m/z 342,m/z 261, m/z 202, m/z 162 and m/z 144.

SUBSTANCE L

Substance L (170.00 mg) obtained from fraction 95-117 of column chromatography 13. On deacetylation of 26 mg of acetylated compound with NH_3/ acetone it afforded substance B (23.6 mg) as a viscous mass, $[\alpha]_D$ +114.44^0(c, 2, H_2O).

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

$C_{60}H_{101}N_3O_{46}$	%C	%H	%N
Calcd.	45.03	5.36	2.63
Found	45.02	5.35	2.62

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

δ in D_2O: ^{1}H NMR

δ5.231 [d, 1H, J=3.0Hz, αGlc (S_1), H-1], δ4.726 [d, 1H, J=7.8Hz, βGlc (S_1'), H-1], δ4.706 [d, 1H, J=7.8Hz, βGlc (S_3), H-1], δ4.665 [d, 1H, J=8.6Hz, βGlcNAc (S_8), H-1], δ4.655 [d, 1H, J=7.6Hz, βGlc (S_6), H-1], δ4.530 [d, 1H, J=8.1 Hz, βGlcNAc (S_9), H-1], δ4.520[d, 1H, J=8.1Hz, βGalNAc (S_4), H-1], δ4.466 [d, 1H, J=8.4Hz, βGal (S_5), H-1], δ4.412 [d, 1H, J=9.1Hz, βGal (S_7), H-1] and δ4.399 [d, 1H, J=8.4Hz, βGal (S_2), H-1], δ3.290 [t, 3H, J=5.9, βGlc (S_1'), βGlc (S_3) & βGlc (S_6), H-2], δ1.921 [s, 3H, βGalNAc (S_4), NHCO$\underline{CH_3}$] and δ2.004 [s, 6H, βGlcNAc (S_8) & βGlcNAc (S_9), NHCO$\underline{CH_3}$].

δ in D_2O: ^{13}C NMR

δ170.50 [βGlcNAc (S_8) NH$\underline{CO}CH_3$], δ169.80 [βGlcNAc (S_9), NH$\underline{CO}CH_3$], δ168.20 [βGalNAc (S_4), NH$\underline{CO}CH_3$],δ103.11 [βGal (S_7), C-1], δ102.90 [βGal (S_2) & βGal (S_5), C-1], δ102.89 [βGalNAc (S_4), C-1], δ101.10 [βGlcNAc (S_9), C-1], δ100.80 [βGlcNAc (S_8), C-1], δ95.06 [βGlc (S_6), C-1], δ95.02 [βGlc (S_3), C-1], δ91.20 [βGlc(S_1'), C-1] and δ89.90 [αGlc (S_1), C-1], δ20.24 [βGlcNAc (S_8) & βGlcNAc (S_9), NHCO$\underline{CH_3}$] and δ20.03 [βGalNAc (S_4), NHCO$\underline{CH_3}$].

δ in $CDCl_3$: ^{1}H NMR (Acetylated)-

δ6.250 [d, 1H, J=3.0Hz, αGlc (S_1), H-1], δ5.650 [d, 1H, J=7.8Hz, βGlc (S_1'), H-1], δ4.730 [d, 1H, J=7.8Hz, βGlc (S_3), H-1], δ4.682 [d, 1H, J=8.6Hz, βGlcNAc (S_8), H-1], δ4.672 [d, 1H, J=7.6Hz, βGlc (S_6), H-1], δ4.549 [d, 1H, J=8.1 Hz, βGlcNAc (S_9), H-1], δ4.539 [d, 1H, J=8.1Hz, βGalNAc (S_4), H-1], δ4.471 [d, 1H, J=8.4Hz, βGal (S_5), H-1], δ4.436 [d, 1H, J=9.1Hz, βGal (S_7), H-1] and δ4.406 [d, 1H, J=8.4Hz, βGal (S_2), H-1], δ2.090 [s, 3H, βGalNAc (S_4), NHCO$\underline{CH_3}$], δ2.083 [s, 3H, βGlcNAc (S_8), NHCO$\underline{CH_3}$] and δ2.077 [s, 3H, βGlcNAc (S_9), NHCO$\underline{CH_3}$].

δ in $CDCl_3$: ^{13}C NMR (Acetylated)-

δ170.80 [βGlcNAc (S_8) NH<u>CO</u>CH_3], δ170.20 [βGlcNAc (S_9), NH<u>CO</u>CH_3], δ169.71 [βGalNAc (S_4), NH<u>CO</u>CH_3], δ104.29 [βGal (S_7), C-1], δ103.97 [βGal (S_2), C-1], δ103.96 [βGal (S_5), C-1], δ103.63 [βGalNAc (S_4), C-1], δ101.62 [βGlcNAc (S_8), C-1], δ101.61 [βGlcNAc (S_9), C-1], δ96.30[βGlc (S_6), C-1], δ95.12 [βGlc (S_3), C-1], δ92.02 [βGlc(S_1'), C-1] and δ90.13[αGlc (S_1), C-1], δ20.94 [βGalNAc (S_4), NHCO<u>CH_3</u>], δ20.86 [βGlcNAc (S_8), NHCO<u>CH_3</u>] and δ20.73 [βGlcNAc (S_9), NHCO<u>CH_3</u>].

ES mass

m/z 1661$[M+Na+K]^+$, m/z 1622$[M+Na]^+$, m/z 1599$[M]^+$, m/z 1563, m/z 1541, m/z 1525, m/z 1522, m/z 1509, m/z 1503, m/z 1490, m/z 1480, m/z 1472, m/z 1431, m/z 1422, m/z 1412, m/z 1396, m/z 1372, m/z 1365, m/z 1361, m/z 1315, m/z 1283, m/z 1275, m/z 1241, m/z 1193, m/z 1159, m/z 1151, m/z 1113, m/z 1111, m/z 1109, m/z 1091, m/z 1036, m/z 1031, m/z 992, m/z 971, m/z 941, m/z 910, m/z 905, m/z 887, m/z 869, m/z 851, m/z 829, m/z 813, m/z 798, m/z 790, m/z 771, m/z 748, m/z 730, m/z 707, m/z 692, m/z 660, m/z 670, m/z 629, m/z 610, m/z 608, m/z 603, m/z 593, m/z 586, m/z 545, m/z 551, m/z 523, m/z 509, m/z 504, m/z 468, m/z 465, m/z 437, m/z 408, m/z 406, m/z 395, m/z 372, m/z 364, m/z 342, m/z 333, m/z 306, m/z 295, m/z 293, m/z 277, m/z 275, m/z 261, m/z 224, m/z 191, m/z 180, m/z 162 and m/z 144.

SUBSTANCE M

Substance M (236.40mg) obtained from fraction 124-156 of column chromatography 16. On deacetylation of 22.5mg of acetylated compound m with NH_3/ acetone it afforded substance M (20.0mg) as a viscous mass, $[\alpha]_D$ -2.68^0(c, 4, H_2O)

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

$C_{70}H_{118}N_2O_{56}$	%C	%H	%N
Calad.	44.63	6.31	1.49
Found	44.62	6.29	1.48

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

δ in D_2O: ^1H NMR

δ5.271 [d, 1H, J=3.9Hz, αGlc (S_1) & αGal (S_9), H-1], δ5.235 [d, 1H, J=3.6Hz, αGal (S_{11}), H-1], δ5.185 [d, 1H, J=3.8Hz, αGal (S_8), H-1], δ5.155 [d, 1H, J=3.8Hz, αGal (S_{10}), H-1], δ4.725[d, 1H, J=7.8Hz, βGlc (S_1'), H-1], δ4.710 [d, 1H, J=7.6Hz, βGlcNAc (S_7), H-1], δ4.695 [d, 1H, J=7.8Hz, βGlc (S_3), H-1], δ4.610 [d, 1H, J=7.6Hz, βGlcNAc (S_6), H-1], and δ4.501 [d, 1H, J=8.4Hz, βGal (S_2) & βGal (S_5), H-1], δ4.100 [t, 1H, J=6.1, βGlcNAc (S_6), H-2], δ3.980 [t, 1H, J=5.8, βGlcNAc (S_7), H-2], δ3.405 [t, 2H, J=5.9, βGlc (S_1') & βGlc (S_3), H-2], δ1.960 [s, 3H, βGlcNAc (S_6), NHCO<u>CH_3</u>] and δ1.848 [s, 3H, βGlcNAc (S_7), NHCO<u>CH_3</u>].

δ in D_2O: ^{13}C NMR

δ166.68 [βGlcNAc (S_6) NH<u>CO</u>CH_3], δ165.98 [βGlcNAc (S_7) NH<u>CO</u>CH_3], δ103.40 [βGal (S_5), C-1],

δ101.50 [βGal (S_2), C-1], δ99.70[βGal (S_4) & βGlcNAc (S_6), C-1], δ97.80 [βGlcNAc (S_7), C-1], δ94.20 [βGlc (S_3), C-1], δ92.00 [αGal (S_9), C-1], δ89.50 [αGal (S_{11}), C-1], δ89.00 [αGal (S_{10}), C-1], δ88.50 [αGal (S_8), C-1], δ88.12 [βGlc(S_1'), C-1] and δ86.00 [αGlc (S_1), C-1], δ20.89 [βGlcNAc (S_6), NHCO$\underline{CH_3}$] and δ20.19 [βGlcNAc (S_7), NHCO$\underline{CH_3}$].

δ in $CDCl_3$: ^{1}H NMR (Acetylated)-

δ6.185 [d, 1H, J=3.9Hz, αGlc (S_1), H-1], δ5.655 [d, 1H, J=7.8Hz, βGlc (S_1'), H-1], δ5.351 [d, 1H, J=3.8, αGal (S_8) & αGal (S_{10}), H-1], δ5.328 [d, 1H, J=3.8Hz, αGal (S_9), H-1], δ5.273[d, 1H, J=3.6Hz, αGal (S_{11}), H-1], δ4.750 [d, 1H, J=7.8Hz, βGlc (S_3), H-1], δ4.725 [d, 1H, J=7.6Hz, βGlcNAc (S_7), H-1], δ4.623 [d, 1H, J=7.6Hz, βGlcNAc (S_6), H-1], δ4.555 [d, 1H, J=8.4Hz, βGal (S_5), H-1] and δ4.504 [d, 1H, J=8.4Hz, βGal (S_2), H-1], δ2.034 [s, 3H, βGlcNAc (S_6), NHCO$\underline{CH_3}$] and δ1.985 [s, 3H, βGlcNAc (S_7), NHCO$\underline{CH_3}$].

δ in $CDCl_3$: ^{13}C NMR (Acetylated)-

δ166.80 [βGlcNAc (S_6) NH$\underline{CO}CH_3$], δ166.00 [βGlcNAc (S_7) NH$\underline{CO}CH_3$], δ104.50 [βGal (S_5), C-1], δ104.04 [βGal (S_2) & βGal (S_4), C-1], δ103.50 [βGlcNAc (S_6), C-1], δ98.70 [βGlcNAc (S_7), C-1], δ95.00 [βGlc (S_3), C-1], δ92.20 [αGal (S_9), C-1], δ90.10 [αGal (S_{11}), C-1], δ89.90 [αGal (S_{10}), C-1], δ88.70 [αGal (S_8), C-1], δ88.20 [βGlc(S_1'), C-1] and δ86.20 [αGlc (S_1), C-1], δ21.00 [βGlcNAc (S_6), NHCO$\underline{CH_3}$] and δ20.99 [βGlcNAc (S_7), NHCO$\underline{CH_3}$]

ES Mass

m/z 1944$[M+Na+K]^+$, m/z 1906$[M+Na+H]^+$, m/z 1882$[M]^+$, m/z 1852, m/z 1822, m/z 1821, m/z 1789, m/z 1769, m/z 1720, m/z 1679, m/z 1666, m/z 1662, m/z 1623, m/z 1601, m/z 1591, m/z 1565, m/z 1554, m/z 1542, m/z 1508, m/z 1501, m/z 1476, m/z 1431, m/z 1396, m/z 1378, m/z 1354, m/z 1314, m/z 1298, m/z 1278, m/z 1256, m/z 1234, m/z 1214, m/z 1200, m/z 1172, m/z 1152, m/z 1119, m/z 1103, m/z 1072, m/z 1042, m/z 942, m/z 932, m/z 911, m/z 883, m/z 865, m/z 828, m/z 813, m/z 798, m/z 771, m/z 747, m/z 737, m/z 691, m/z 670, m/z 666, m/z 633, m/z 610, m/z 603, m/z 580, m/z 572, m/z 565, m/z 547, m/z 526, m/z 504, m/z 490, m/z 465, m/z 440, m/z 423, m/z 408, m/z 380, m/z 367, m/z 365, m/z 342, m/z 312, m/z 292, m/z 260, m/z 242, m/z 203, m/z 180, m/z 162 and m/z 144.

Table : 21- Description Of Isolated Oligosaccharides From Gaddi Sheep's Milk—

	Analytical notation	Name of compound	Physical state	Molecular formula[#]	$[\alpha]_D$ (25^0C)	ES mass (m/z)	Phenol sulphuric Acid test*[1]	Morgon -Elson test*[2]	Thiobarbituric acid test*[3]	Bromo-cresol green test*[4]
A	SSGM-0	-	-	-	-	-	-	-	-	-
B	SSGM-1	-	-	-	-	-	-	-	-	-
C	SSGM-2	Arieose	Syrup	$C_{60}H_{101}N_3O_{46}$	+138.72	1599	+ve	+ve	-ve	-ve
D	SSGM-3	Iesose	Syrup	-	+139.44	-	+ve	+ve	-ve	-ve
E	SSGM-4	Isose	Syrup	$C_{30}H_{51}N_3O_{21}$	+42.41	789	+ve	+ve	-ve	-ve
G	SSGM-5	Avsose	Syrup	-	+113.53	-	+ve	+ve	-ve	-ve
H	SSGM-6	Gaddiose	Syrup	-	+9.02	-	+ve	+ve	-ve	-ve
J	SSGM-7	Gadose	Syrup	-	+6.64	-	+ve	+ve	-ve	-ve
K	SSGM-8	Oviasose	Syrup	$C_{66}H_{111}N_3O_{51}$	+115.39	1761	+ve	+ve	-ve	-ve
L	SSGM-9	Rieose	Syrup	$C_{60}H_{101}N_3O_{46}$	+114.44	1599	+ve	+ve	-ve	-ve
M	SSGM-10	Riesose	Syrup	$C_{70}H_{118}N_2O_{56}$	-2.68	1882	+ve	+ve	-ve	-ve

[#]Molecular formula is based on C, H, N, O analysis of compound.
*1 Test of normal sugar.
*2 test of amino sugar
*3 Test of sialic acid
*4 Test of carboxylic acid.

Structural Characterization of Milk Oligosacchrides

Recently a number of oligosaccharides have been isolated from milk of various origin and showed varied biological activities viz. antitumor, anticancer, immunostimulant etc. A survey of literature showed that some common basic core units were present in most of the milk oligosaccharides which are as follows.

1. Lactose - βGal (1→4)Glc

2. Lacto-N-tetraose (LNT) -

βGal-(1→3)βGlcNAc(1→3)βGal(1→4)Glc

3. Lacto-N-neotetraose (LNnT)-

βGal(1→4)βGlcNAc(1→3)βGal(1→4)Glc

4. Lacto-N-hexaose (LNH) -

βGal(1→4)βGlcNAc(1→6)
↑
βGal(1→4)Glc
↓
βGal(1→3)βGlcNAc(1→3)

5. Lacto-N-neohexaose (LNneoH)-

βGal(1→4)βGlcNAc(1→6)
↑
βGal(1→4)Glc
↓
βGal(1→4)βGlcNAc(1→3)

6. Para-Lacto-N-hexaose (paraLNH) -

βGal(1→3)βGlcNAc(1→3)βGal(1→4)βGlcNAc(1→3)βGal(1→4)βGlcNAc(1→3)βGal(1→4)Glc

7. Para-Lacto-N-neohexaose (paraLNneoH) -

βGal(1→4)βGlcNAc(1→3)βGal(1→4)βGlcNAc(1→3)βGal(1→4)βGlcNAc(1→3)βGal(1→4)Glc

8. Lacto-N-octaose-

βGal(1→4)βGlcNAc(1→3)βGal(1→4)βGlcNAc(1→6)
↑
βGal(1→4)Glc
↓
βGal(1→3)βGlcNAc(1→3)

9. para-Lacto-N-octaose-

βGal(1→3)βGlcNAc(1→3)βGal(1→4)βGlcNAc(1→3)βGal(1→4)βGlcNAc(1→3)βGal-(1→4)Glc

10. Lacto-N-neooctaose-

βGal(1→4)βGlcNAc(1→3)βGal(1→4)βGlcNAc(1→6)

↑

βGal(1→4)Glc

↓

βGal(1→4)βGlcNAc(1→3)

11. Iso-Lacto-N-octaose-

βGal(1→3)βGlcNAc(1→3)βGal(1→4)βGlcNAc(1→6)

↑

βGal(1→4)Glc

↓

βGal(1→3)βGlcNAc(1→3)

12. Lacto-N-decaose-

βGal(1→4)βGlcNAc(1→6)

↑

βGal(1→4)βGlcNAc(1→6)

↓ ↑

βGal(1→3)βGlcNAc(1→3) βGal(1→4)Glc

↓

βGal(1→3)βGlcNAc(1→3)

13. Lacto-N-fucopentaose-1(LNF-I)-

αFuc(1→2)βGal(1→ 3)βGlcNAc(1→ 3)β Gal(1→ 4)Glc

14. Lacto-N-Fucopentaose-2 (LNF-II)-

βGal(1→3)βGlcNAc(1→3)βGal(1→4)Glc

↓

αFuc(1→4)

15. Lacto-N-Fucopentaose-3 (LNF-III)-

βGal(1→4)βGlcNAc(1→3)βGal(1→4)Glc

↓

αFuc(1→3)

The previous workers have elucidated the structure of milk oligosaccharides by chemical degradation and spectroscopic techniques (NMR and MASS). Keeping above mentioned basic core units in mind, structure of isolated novel milk oligosaccharides were established by comparing the chemical shift (^{1}H and ^{13} C NMR) of anomeric proton and carbon resonance signals and other important signals of unknown milk oligosaccharides with the chemical shifts of certain 'structural reporter group resonances' for structural assignments of the oligosaccharides. All chemical shifts of anomeric proton signals of milk oligosaccharides were further confirmed by 2D (^{1}H-^{1}H HOMO COSY, TOCSY) NMR experiments which were earlier assigned with the help of ^{1}H and ^{13}C NMR data, position of glycosidic linkages were compared by comparing the ^{1}H NMR data of acetylated and deacetylated compounds which were finally compared by 2D NMR techniques like COSY,

TOCSY, HSQC, etc. Other techniques like deacetylation, methylation, hydrolysis, chemical degradation and mass spectrometry were also helpful for elucidation of the structure of oligosaccharides.

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