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Ashok Kr. Ranjan and Desh Deepak

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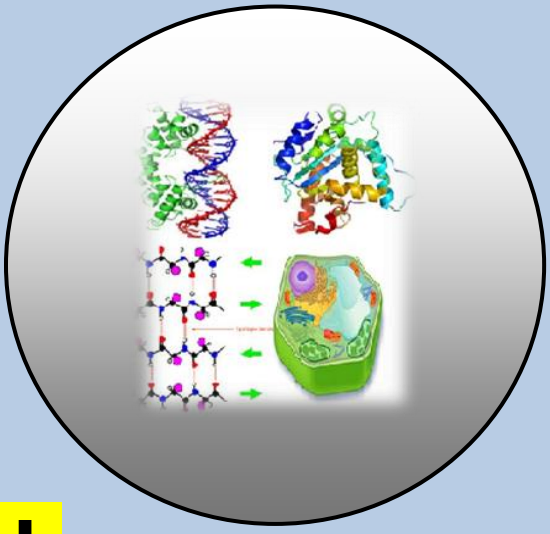

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Ashok Kr. Ranjan
http:// www.sasjournals.com
http:// www.jbcr.in
jbiolchemres@gmail.com
info@jbcr.in

RESEARCH PAPER

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Isolation and Purification of Sheep Milk Oligosaccharide as Therapeutic Agents

Ashok Kr. Ranjan and Desh Deepak

Chemistry Department, Lucknow University, Lucknow, U.P., India

ABSTRACT

The milk is a rich source of bioactive oligosaccharides which contain number of novel oligosaccharides. This depends on the nature of milk origin to which mammals it belongs. Milk helps in the development of the post natal immune system, defense against bacterial, viral infections, development of intestinal flora, and providing strength to the body. It contains proteins, fatty acids, minerals, vitamins and carbohydrates especially, lactose and large number of oligosaccharides. The milk oligosaccharides inhibit the adherence of pathogens to target cells, hence oligosaccharides and their derivatives are used as therapeutic agents and form the basis for the development of anti-tumor vaccines and act as effective drugs in the therapy of pathogenic diseases. In our endeavor to find novel oligosaccharides, sheep's milk was collected in bulk and processed by method of Kobata and Ginsburg. The milk was deproteinated, centrifuged and lyophilized, further it was subjected to gel filtration, while the homogeneity of oligosaccharides was confirmed by HPLC. The oligosaccharide fraction thus obtained was acetylated and purified by column chromatography. It was further deacetylated to obtain the milk oligosaccharides in their natural form. The physico-chemical data of isolated oligosaccharide is also given.
Keywords: Oligosaccharide, Chromatography, Lactose, Kobata and Ginsburg method.

INTRODUCTION

Milk is nature's food for the development of neonates of any mammal. It provides not only energy but develops immunological system, brain, neurological system and also provides strength against any infection (Tafaro et al., 2007). Milk of various origins has proven themselves as a source for biologically active oligosaccharides which are major constituents of every milk. The Elephant milk oligosaccharides fraction contained a high ratio of sialyl oligosaccharide; which is significant with respect to the formation of brain components, such as gangliosides of the suckling calves (Osthoff et al., 2007). N-acetylneuraminlactose sulphate is the dominant oligosaccharide in the Dog milk (Bubb et al., 1999) which plays an

important role in the nutrition of the rat pups. Buffalo Milk oligosaccharides have ability to stimulate non-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 important roles in intestinal protection and repair after damage caused by DSS (Dextron sodium sulphate) induced colitis and their implication in human intestinal inflammation (Villosladaa 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 (Hakkarainen et al., 2005). Cow milk oligosaccharides reduce the adhesion of enterotoxic Eschererchia coli strains of the calf (Johansson et al., 2005). Mare's milk has shown anti oxidant, lipid lowering and post heparin lipolytic activity (Srivastava et al., 2012). In search for more biologically active oligosaccharides Sheep milk was taken for the present studies. It is established from recent researches that Goat and Sheep milk is a perfect food as possible in the nature. It is a well balanced nutrient and exhibit varied process, i.e. absorption of nutrients, digestion, growth and development of various organs and also plays a definite role for providing the resistance for outer infection/ diseases (Egito et al., 2002). It contains amino acids (Davis et al., 1994 and Srwag et al., 1998), proteins and low fat content. Sheep milk protein is an important source of bioactive inhibitory and hypertensive defence and control of microbial infection. The peptides present in sheep milk have their affect in cardiovascular, nervous and immune system, besides these effects due to proteins and peptides present in sheep milk are enormous. Sheep milk has some other biological effects which is due to oligosaccharides (Herrera et al., 2012) present there in. Sheep milk is a rich source of fucosylated oligosaccharides which has definite biological effects like α1, 2-linked fucosylated oligosaccharides, probably in conjugation with other families of oligosaccharide, constitute a powerful innate immune system of human milk (Sharon and Ofek, 2000). In search for some novel biologically active oligosaccharides sheep milk was collected from Gausabad, Ghazipur (U.P.) and their oligosaccharides were isolated and purified by following methods.

MATERIAL AND METHODS

Isolation of Sheep Milk oligosaccharide by Method of Kobata and Ginsburg (Kobata and Ginsburg, 1999)

10 litre sheep milk was collected from domestic sheep and 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 ppt. 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 micro-filter (0.24 µm) (to remove remaining lactose) and was lyophilized affording crude oligosaccharide mixture (10.78 gm).

Sephadex G-25 Gel Filtaration of Sheep Milk Oligosaccharide Mixture

The crude oligosaccharide mixture obtained after Kobata and Ginsburg method may still contain some glycoprotein and protein in it. So for separating the glycoprotein and oligosaccharides. It was subjected to gel filtration on Sephadex G-25.

The repeated gel filtration was performed by Sephadex G-25 chromatography of crude sheep milk oligosaccharide mixture. Sheep milk oligosaccharide mixture was packed in a column (1.6x40 cm) (void volume = 25 ml) equilibrated with glass triple distilled water and it was left for 10 to 12 hrs to settle down. The material was applied on a sephadex G-25 column and was eluted for separation of protein and glycoprotein from oligosaccharide (low molecular weight component). Presence of neutral sugars was monitored in all eluted fractions by phenol-sulhuric acid test. In this U.V. monitored sephadex-G25 chromatography of sheep milk oligosaccharide mixture showed four peaks i.e. I, II, III, and IV. A substantial amount of proteins, glycoprotein's and serum albumin were eluted in the void volume which was confirmed by positive colouration with p-dimethyl aminobenzaldehyde reagent (Frehden and Goldschmidt, 1937) and phenol sulphuric acid reagent (Dubois et al., 1956) fractions under peaks II and III gave a positive phenol sulphuric acid test for sugars which showed the presence of oligosaccharide mixture in Sheep milk. These fractions (peak II and III) were pooled and lyophilized together to obtain 6.35 gm of Sheep milk oligosaccharides.

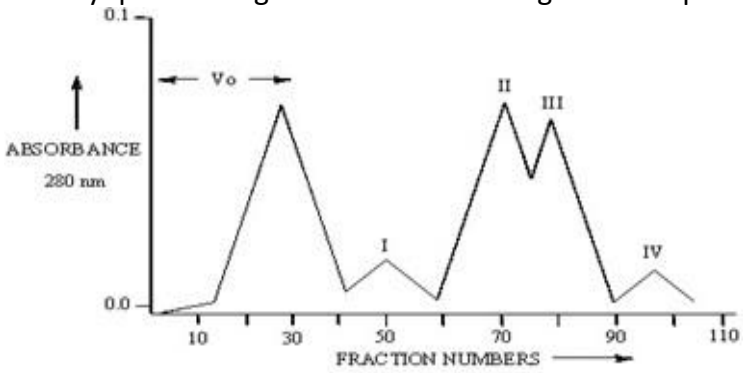


Fig 1. Sephadex G-25 gel filtaration of sheep milk oligosaccharide mixture

RT	% compound
7.21	11.11
7.63	12.07
8.45	5.29
17.21	8.81
17.41	3.32
20.58	20.92
21.39	10.79
21.78	3.21
24.69	12.33
25.42	8.99
25.97	3.22
27.51	2.88

Fig-2: Reverse phase HPLC of Sheep milk oligosaccharides

Confirmation of homogeneity of sheep milk oligosaccharide by reverse phase HPLC

Pooled fractions (peak II and III) obtained from sephadex G-25 column, containing oligosaccharide mixture were qualitatively analyzed by reverse phase HPLC.

The HPLC system was equipped with Perkin-Elmer 250 solvent delivering system, 235 diode array detector and G.P. 100 printer plotter. The column used for this purpose was C (Srivastava et al., 2014) purosphere 25cm x 5µ. A binary gradient system of acetinitrile: 0.5% trifluro acetic acid (5:95) in triple distilled water to CH_3CN: 0.5% TFA (60:40) within 25 min at a flow rate of 1 ml/min was used. The eluants were detected at 220 nm. Twelve peaks I to XII were noticed in the sample (pooled fractions II and III) at the varied retention times from 7.21 min to 27.51 min, for convenience the peaks were numbered in their increasing order of retention time i.e. 7.21 min (R_1), 7.63 min (R_2), 8.45 min (R_3), 17.21 min (R_4), 17.41 min (R_5), 20.58 min (R_6), 21.39 min (R_7), 21.78 min (R_8), 24.69 min (R_9), 25.42 min (R_{10}), 25.97 min (R_{11}), 27.51 min (R_{12}).

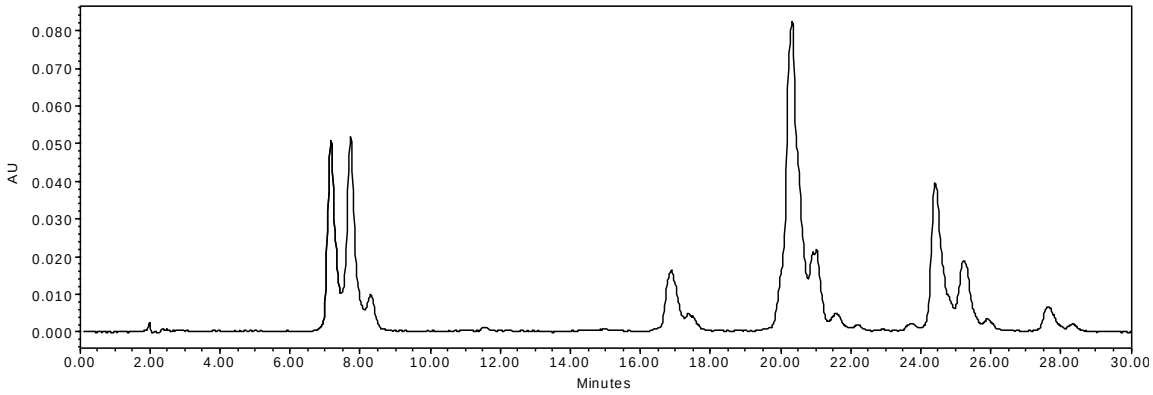


Fig 3. Table given retention time and % of compounds

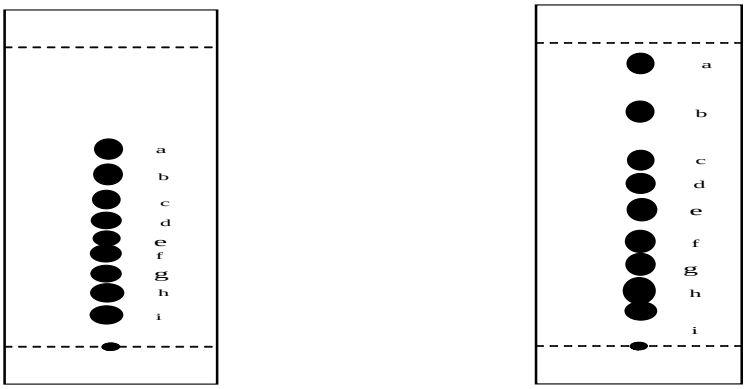
Acetylation of oligosaccharide mixture

4.45 gm of pooled fractions (peak II and III) which gave positive phenol-sulphuric acid test were acetylated with pyridine (5 ml) and acetic anhydride (5 ml) at 60^oC and the solution was stirred overnight. The mixture was evaporated under reduced pressure and the viscous residue was taken in $CHCl_3$ (450 ml) and washed in sequence with 2N- HCl (1 x 25 ml), ice cold 2N- $NaHCO_3$ (2 x 25 ml) and finally with H_2O (2 x 25 ml). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding the acetylated mixture (4.5 gm). The acetylation converted the free sugars into their non-polar acetyl derivatives which were resolved nicely on TLC, giving nine spot on TLC i.e. a, b, c, d, e, f, g, h and i respectively. to obtain the oligosaccharides in their purified form the acetylated oligosaccharides mixture was subjected to column chromatography over silica gel into compound: silica ratio of 1:100 i.e. (450 gm silica gel) using various proportions of $CHCl_3$:CH_3OH mixture which resulted into 8 fractions namely 1 (354mg), 2 (351gm), 3 (446mg), 4 (303mg), 5 (1.11gm), 6 (221mg), 7 (367mg) and 8 (352mg) respectively. These fractions were containing mixture of 2 to 3 compounds TLC enclosed (Fig-4). These fractions 1-6 were re-chromatographed over silica gel at the compound: silica gel ratio 1:100, and varied proportion of $CHCl_3$:CH_3OH that resulted into the isolation of four novel oligosaccharides a, b, c and d.

Deacetylation of isolated compounds a, b, c and d

Compounds a, b, c and d obtained from column chromatography of the acetylated oligosaccharides mixture was dissolved separately in acetone and ammonia and left overnight in a stoppered hydrolysis flask.

Ammonia was removed under reduced pressure and the compound was washed with $CHCl_3$ and was finally freeze-dried giving the natural oligosaccharide A, B, C and D.



$CHCl_3$: MeOH (98:2) **$CHCl_3$: MeOH (95:5)**

Fig 4. TLC of acetylated oligosaccharides mixture at different polarity proportions

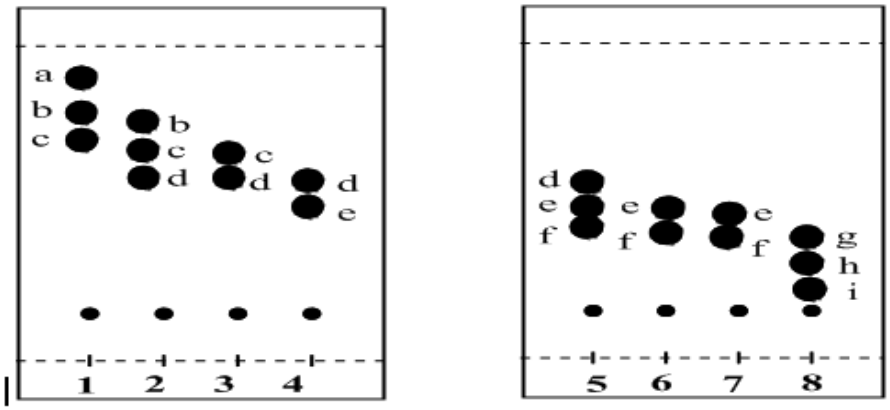


Fig 5. TLC of acetylated oligosaccharide fractions from 1 to 8

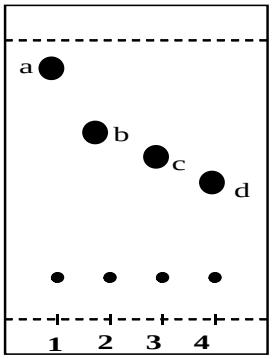


Fig 6. TLC of purified acetylated isolated compounds a, b, c and d

RESULT AND DISCUSSION

Milk of various origin has been found to an important source of oligosaccharides having antigenic, anti cancer, anti tumor, properties of different types of rare milks like camel, yak, Gaddi milk, Chouri cow milk etc has been studied for their oligosaccharides contents for their varied biological activities. It has been seen that, the milk contain di to undecasaccharides containing two to twelve monosaccharides unit in oligosaccharides. Although lactose a disaccharides is the most common constituents of the any milk.

Lactose is a basic core unit of the most of oligosaccharides obtained from any milk. The lactose plays multiple roles in the development of neonates by stimulating the growth of selected beneficial bacteria. The lactose is produced in various concentrations during lactation period in different mammals. In our previous communication, we have estimated the lactose concentration by HPTLC in milk of different mammals. Besides the lactose which is the most common core unit of any of the milk oligosaccharides, there are at least fifteen core units which are responsible for structure of different oligosaccharides. These core units are comprised of the Glc, Gal, GlcNAc and GalNAc. The structure of various core units found in different milk are given as under

Lactose :-

Gal-(β1→4)-Glc

Lacto-N-tetraose (LNT):-

Gal-(β 1→3)- GlcNAc-(β 1→3)- Gal-(β 1→4)-Glc

Lacto-N-neotetraose (LNnT):-

Gal-(β 1→4) - GlcNAc-(β 1→3) - Gal-(β 1→4)-Glc

Lacto-N-hexaose (LNH):-

Gal- β (1→4)-GlcNAc- (β 1→6)
Gal- (β 1→4)-Glc
Gal- (β 1→3)-GlcNAc- (β 1→3)

Lacto-N-neohexaose (LNneoH):-

Gal- (β 1→4)-GlcNAc- (β 1→6)
Gal- (β 1→4)-Glc
Gal- (β 1→4)-GlcNAc- (β 1→3)

Para-Lacto-N-neohexaose (paraLNH):-

Gal-(β1→4)-GlcNAc-(β1→3)-Gal-(β1→4)-GlcNAc-(β1→3)-Gal-(β1→4)-Glc

Para-Lacto-N-neohexaose (paraLNneoH):-

Gal- (β 1→3)-GlcNAc-(β 1→3) - Gal-(β 1→4)-GlcNAc-(β 1→3) - Gal- (β 1→4)-Glc

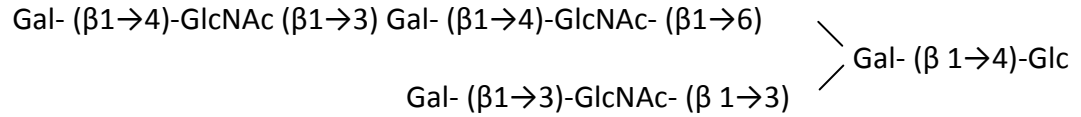
Lacto-N-octaose:-

Gal-(β1→4)-GlcNAc (β1→3) Gal-(β1→4)-GlcNAc- (β1→6)
Gal- (β 1→4)-Glc
Gal- (β1→4)-GlcNAc- (β 1→3)

Lacto-N-neooctaose:-

Gal- (β1→3)-GlcNAc (β1→3) Gal- (β1→4)-GlcNAc- (β1→6)
Gal- (β1→4)-Glc
Gal- (β1→4)-GlcNAc- (β 1→3)

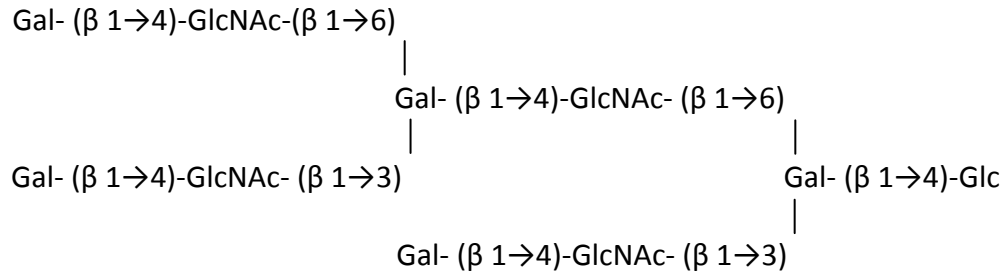
Iso-Lacto-N-octaose:-



Para-Lacto-N-octaose:-

Gal-(β1→3)-GlcNAc-(β1→3)-Gal-(β1→4)-GlcNAc-(β1→3)-Gal-(β1→4)-GlcNAc-(β 1→3)- Gal-(β 1→4)-Glc

Lacto-N-decaose:-



These core units are very help full for the structure elucidation of various oligosaccharide isolated from different milk. The structures of oligosaccharides are supported by these core units and structure reporter group (Dabrowski et al., 1983 and Wengang et al., 2005) of NMR. Although the number of modern analytical tools are available for isolation and identification of milk oligosaccharides and currently available analytical methods are fluorometry, online dialysis, colorimetric method, spectroscopic methods, gravimetric detection, spectrophotometry, HPLC, HPTLC and Apmerometric biosensor are available for the isolation and identification of milk oligosaccharides but it has been noted we cannot depend on any one technique for isolation of the milk oligosaccharides. It is advisable that a combination of 3-4 techniques gives better results. The most classical method of chromatography i.e. Thin layer chromatography and column chromatography cannot be ignored. A combination of modern techniques with classical methods provides better results. In the present study for isolation of sheep milk oligosaccharides we have combined the High performance liquid chromatography, gel filtration, Thin layer chromatography and Column chromatography for obtaining the novel sheep milk oligosaccharides.

In this process at the 1^{st} stage we obtained the crude sheep milk oligosaccharides by processing the milk by kobata and Ginsburg which resulted into the isolation of the mixture of protein, glycoprotein, oligosaccharides and lactose. With a view to isolate sheep milk oligosaccharides this crude mixture was subjected to sephadex G-25 chromatography which is a process of molecular sieving, Gel filtration separates the compounds present in mixture on basis of their molecular weight. In this chromatography sequence of the compound is based on molecular weight, compounds with the higher mol. wt. comes first followed by the compounds with lesser mol. wt. i.e. in the present case the glycoprotein come first followed by proteins then the oligosaccharides fallowed by lactose and finally the monosaccharides. For this purpose a total amount of (9.86gm.) crude oligosaccharide mixture was charged on a sephadex G-25 column which resulted into the isolation of four fractions (1, 2, 3 and 4) comprising of glycoprotein, protein and oligosaccharides having the 1.11gm, 1.21gm, 1.06gm and 6.15gm respectively.

The pooled fractions III and IV comprising of oligosaccharides which was confirmed by +Ve phenol sulphuric acid reagent and p-dimethyl aminobenzaldehyde reagent were taken for their further studies. These pooled mixtures were subjected to HPLC for checking their homogeneity into the oligosaccharide mixture. The results obtained from HPLC showed twelve peaks R_1 to R_{12} (Fig-2) with their respective retention times confirming the presence of twelve oligosaccharide in the sheep milk. These oligosaccharide were very polar in nature so it was not possible to isolate these oligosaccharide in good amount for their structure elucidation from routine chromatographic techniques therefore this oligosaccharides mixture was subjected to acetylation by AC_2O/pyridine at 60^oC for obtaining them in their acetylated form. These acetylated oligosaccharides were less polar in nature therefore it could be easily separated by classical chromatographic techniques i.e. Thin layer chromatography and Column chromatography.

The acetylated oligosaccharides mixture obtained after acetylation was 6.15 gm which was examined and analysed over TLC showing eight (8) spots (a to i) they were resolved by using various proportion of $CHCl_3$:CH_3OH. These resolved mixtures were further subjected to repeated silica gel chromatography as shown in our experimental part which resulted into the isolation of four novel sheep milk oligosaccharides in their acetylated form a, b, c and d which was further deacetylated to obtained compounds A, B, C and D as natural oligosaccharide of Sheep milk.

1. Compound-A

^{13}C NMR of Comound-A in D_2O at 300 MHz

89.68[α-Glc (S-1) C-1], 91.66[β-Glc (S-1) & α-FucNAc (S-3), C-1], 101.05[β-Gal (S-2), β-Gal (S-4) & β-GalNAc (S-5) C-1].

^{1}H NMR of Comound-A in D_2O at 300 MHz

5.57[d, 1H, J=3.0Hz, α-Glc (S-1) H-1], 4.64[d,1H, J=8.0Hz, β-Glc (S-1) H-1], 5.3[d, 1H, J=3.0Hz, α-FucNAc (S-3), H-1], 4.35[d,1H, J=8.0Hz, β-Gal (S_4) H-1 and β-GalNac(S-5)], 1.91[S, 3H, 2(NHCO$\underline{CH_3}$), β-GalNAc(S-5)], 1.2[d, 3H, α-FucNAc(S-3)].

ES Mass

879 [894-CH_3], 862[879-OH], 833[862-CHO], 747[833-$NHCOCH_3$], 691[894-S-5], 633[691-$NHCOCH_3$], 616[633-OH], 585[616-CH_2OH], 567[585-H_2O], 674[691-OH], 657[674-OH], 641[674-H_2O, CH_3], 609[641-CH_2OH, H^+], 529[691-S4], 342[488-S3], 324[342-H_2O], 180[342-S2].

2. Compound-B

^{13}C NMR of Compound-B in D_2O at 300 MHz

89.10[α-Glc (S-1) C-1], 91.56[β-Glc (S-1) C-1, β-Gal(S-3), C-1)], 101.97[β-GlcNAc (S-5) C-1], 101.84[β-Gal(S-2), C-1, β-GalNAc (S-4) C-1].

^{1}H NMR of Compound-B in D_2O at 300 MHz

δ 5.2[d, 1H, J= 4.0 Hz, α-Glc (S-1) H-1], 4.6[d,1H, J=8.0 Hz, β-Glc(S-1), H-1)], 4.49[d, 1H, β-Glc (S-3), H-1)], 4.46[d, 1H, β-Gal(S-2), H-1)], 4.39[d, 2H, β-GalNAc(S-4), H-1 and β-Gal (S-5) H-1], 1.94[s,3H, NHCO$\underline{CH_3}$ β-GalNAc(S-4)].

ES Mass

850[910-CH_2OHCHO], 819[850-CH_2OH], 761[819-$NHCOCH_3$], 743[761-H_2O], 726[743-OH], 707[H_2O, H^+], 879[910-CH_2OH], 861[879-H_2O], 821[879-$NHCOCH_3$], 789[821-CH_3, OH], 803[821-H_2O], 689[707-H_2O], 672[689-OH], 614[672-$NHCOCH_3$], 597[614-OH], 566[597-CH_2OH], 535[566-CH_2OH], 517[535-H_2O], 504[707-S-4], 342[504-S-3],180[342-S-2].

3. Compound-C

^{13}C NMR of Compound-C in D_2O at 300 MHz

89.13[α-Glc(S-1), C-1], 91.57[β-Glc(S-1), C-1], 101.88[β-Glc(S-2), C-1], 102.62[β-GalNAc(S-5), C-1], 100.92[β-GalNAc(S-3), C-1], 101.19[β-GalNAc(S-4), C-1].

^{1}H NMR of Compound-C in D_2O at 300 MHz

5.15[d, 1H, J=4.0Hz, α-Glc (S1), H-1], 4.59[d, 1H, J=8.0Hz, β-Glc (S1), H-1], 4.47 [d, 1H, J=8.0Hz, β-Gal(S-2), H-1], 4.45[d, 1H, J=8.0Hz, β-Gal(S-5), H-1], 4.38[d, 1H, J=8.0Hz, β-GalNAc(S-3), H-1 and β-GalNAc(S-4), H-1].

ES Mass:

892 [951- CH_2OHCHO (59)], 848 [892-CH_3CO,H (44),], 893 [951-$NHCOCH_3$ (58)], 916 [951-H_2O, OH (35)], 730 [748- H_2O (18), 687 [730-CH_3O (43)], 686 [687- H (1)], 668 [686-H_2O (18)], 510 [545-H_2O, OH (35)], 467 [510-CH_3CO (43)], 487[545-$NHCOCH_3$ (58)], 405 [465-CH_2OHCHO (60)].

4. Compound-D

^{13}C NMR of Compound-D in D_2O at 300 MHz

90.24[1C, αGlc (S-1) C-1], 90.12[1C, βGlc (S-1) C-1], 90.12[1C, αGlcNAc (S-3) C-1], 95.26[1C, βGalNAc (S-5), C-1)], 101.90[1C, β-Gal (S-6), C-1)], 101.05[1C, βGal (S-4) C-1], 100.96[1C, βGal(S-2) C-1].

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

δ 5.373[d, 1H, J=4.0Hz, α-Glc(S-1), H-1], 5.373[d, 1H, α-GlcNAc(S-3), H-1)], 4.776[d, 1H, β-Glc(S-1), H-1)], 4.591[d, 1H, J=8.0 Hz, β-Gal, (S-5) H-1], 4.523 [d, 1H, J=8.0Hz, β-GalNAc(S-4), H-1)], 4.523[d,1H, J= 7.2Hz, β-Gal(S-2) H-1].

ES Mass

1014[1072-$NHCOCH_3$], 1055[1072-OH], 1024[1055-CH_2OH], 989[1014-OH], 956[989-CH_2OH, $2H^+$], 910[1072-S-6], 892[910-H_2O], 875[892-OH], 826[875-H_2O,CH_2OH], 789[826-$2H_2O$, H^+], 750[826-CH_2OCHO, OH], 709[CH_2OH, H_2O], 545[707-S-4], 527[545-H_2O], 487[545-$NHCOCH_3$], 427[487-CH_2OHCHO], 342[545-S-3], 180[342-S-2].

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Corresponding authors: Ashok Kr. Ranjan and Dr. Desh Deepak, Chemistry Department, Lucknow University, Lucknow, U.P., India
Email: **deshdeepakraju@rediffmail.com** **/** **ashokranjan@india.com**