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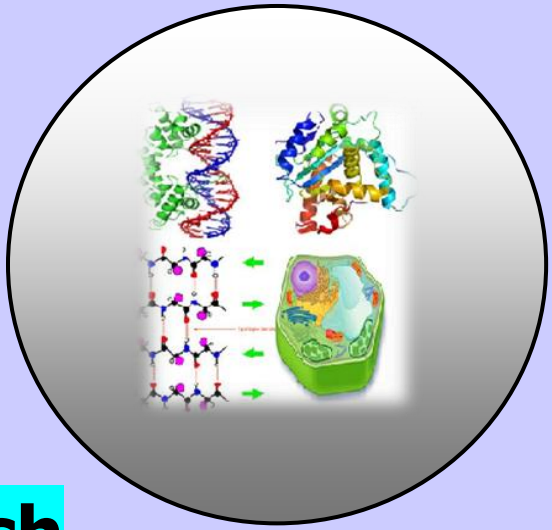

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RESEARCH PAPER

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Isolation and Purification of Camel Milk Oligosaccharides as Therapeutic Agent

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ABSTRACT

Oligosaccharides are amongst the most biologically diverse and important carbohydrate in biological system. Milk oligosaccharides are important class of complex carbohydrates which play an essential role in many molecular processes impacting eukaryotic biology and diseases and exhibit varied biological activity such as immunostimulant, hypoglycemic, anti-tumor, antiviral, anticancer, anticoagulant, anti-complementary, immunological and anti-inflammatory activities. Camel milk contains vitamins, minerals and lactoferrin in which has antibacterial, antiviral and anti-tumor properties. Camel milk also contains disease-fighting immunoglobulin and is also a rich source of insulin. In India Camel milk is used in treatment of tuberculosis, asthama and spleen. Keeping in mind the biological activity of milk of Camel (Camelus dromedarius)] it was collected and processed by modified method of Kobata and Gingsburg. Further the milk was deproteinated, centrifuged and lyophilized and then it was subjected to gel filtration, its homogeneity was confirmed by HPLC. The mixture of oligosaccharides was acetylated and purified by column chromatography. Later the oligosaccharide was deacetylated to get the oligosaccharides in their natural form. The physicochemical data of isolated oligosaccharides are also given.
Keywords: Oligosaccharides, HPLC, chromatography, Kobata and Gingsburg.

INTRODUCTION

Milk oligosaccharides are the important class of complex carbohydrates as supplements for the food and the pharmaceutical industries. More than 250 milk oligosaccharides have been isolated from mammalian (Urashima et al, 2001) milk of different origin eg., Cow, Buffalo, Donkey, Horse, Sheep, Goat, Bear etc. Human milk oligosaccharides are known to protect breast fed infants from a host of bacterial infection. Milk and colostrums contain more than 80 different oligosaccharides (e.g. fucosyl and sialyl –lactose and lacto-N-tetraose etc.)

constituting over 20% of total milk carbohydrate. Human milk oligosaccharide binds to a wide range of lectins on the surface of epithelial cells lining the mouth, oesophagus and stomach and throughout the gastrointestinal system in the new born baby. This in turn prevents opportunistic infection whilst the baby's immune system is developing (Velupillai etal, 1994). Oligosaccharides are abundant components of mammalian milk & have primary roles in immune defense and in brain development (Wang et al, 2009). Oligosaccharides in association with lactose play an important role in postnatal brain development. Breast milk provides an important source of Neuraminic acid for the neonate in context of the low capacity for the neonatal brain. Sialic acids, due to their negative charge & hydrophilic nature, help to modulate the cell-cell interactions(Lo casio et al, 2007).It is also believed that sialic acid serve as ligands for lectin binding involved in regulating the immune response (Rutishauser et al, 1996). SHMOs are therefore believed to play a key role in postnatal brain development (Wang et al, 2009). The elephant milk oligosaccharide fraction contained a high ratio of sialyl oligosaccharide, this may be significant with respect to the formation of brain components such as gangliosides of suckling claves (Ostho et al., 2007). Donkey milk oligosaccharides have ability to stimulate non-specific and specific immunological resistance (Deepak et al, 1998). The cow's milk oligosaccharides reduce the adhesion of enterotoxic *E.Coli* strains of the calf (Johansson et al, 2005). Goat milk containing galacto-oligosaccharides could be recommended to decrease most of infant allergy and diseases. Goat milk oligosaccharides play an important role in intestinal protection and repair after a damage caused by DSS (dextran sodium sulphate) – induced colitis and their implication in human intestinal inflammation (Villosladaa et al., 2006). Goat milk shows therapeutic virtues for individuals with certain diebetic problems (Ben XM et al, 2004). N-acetylneuramin lactose sulphate may play an important role in the nutrition of rat pups, which is the dominant oligosaccharide in the dog milk (Bubb et al, 1999). Bovine milk oligosaccharides have several potentially important biological activities including the prevention of pathogen binding to the intestinal epithelial and as nutrients for beneficial bacteria. Mare's milk oligosaccharide fractions are having multifold properties such as antioxidant and lipid lowering activities (Srivastava et al, 2012). The milk oligosaccharides isolated from camel milk shows anti-tuberculosis activity. Camel milk contains disease-fighting immunoglobulins which are small in size, allowing penetration of antigens and boosting the effectiveness of the immune system. It is a rich source of insulin which makes it a great treatment option for Type1 and Type2 diabetics as well as gestational diabetes. Camel milk is supposed a precautionary in ulcers. Regular intake of camel milk helps to control blood sugar levels. Camel milk helps in reducing coronary heart diseases. Camel milk also benefits in infection, gastroenteritis and cancer (Mullaicharan, 2014). In search of novel oligosaccharide, Camel milk was collected from a domestic Camel from rural area of Ujjain city in Madhya Pradesh and the oligosaccharide were isolated and purified.

MATERIAL AND METHODS

Isolation of camel milk oligosaccharide by Modified Method of Kobata and Gingsburg (Kobata and Gingsburg, 1999)

10 liter Camel milk was collected from a domestic Camel from rural area of Ujjain city in Madhya Pradesh.

The milk was fixed by addition of equal amount of ethanol. The preserved milk was taken to laboratory and there it was centrifuged for 30 min at 5000 rpm at 4^0C. The solidified layer was removed by filtration through glass wool column in cold. More ethanol was added to clear filtrate to a final concentration of 68% and the resulting solution was left overnight at 0^0C. The white precipitate formed, mainly of lactose and protein was removed by centrifugation and washed twice with 68% ethanol at 0^0C. The supernatant and washings were combined and filtered through a micro filter and lyophilized affording crude oligosaccharide mixture (210gm).

Sephadex G-25 Gel Filtration of Camel Milk Oligosaccharide Mixture

The repeated gel filtration was performed on 25gm of crude oligosaccharide mixture by Sephadex G-25. Camel milk oligosaccharide mixture was packed in a column (1.6 x 40 cm) (void volume = 25 ml) equilibrated with glass triple distilled water (TDW) and it was left for 10-12 h 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-sulphuric acid test. In this U.V. monitored Sephadex G-25 chromatography of Camel milk oligosaccharide mixture showed four peaks i.e. I, II, III and IV. A substantial amount of proteins, glycoproteins and serum albumin were eluted in the void volume which was confirmed by positive coloration with p-dimethylaminobenzaldehyde reagent (Frehden and Golschmidt,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 Camel milk. These fractions (peak II and III) were pooled and lyophilized affording 15.2 gm of oligosaccharide mixture.

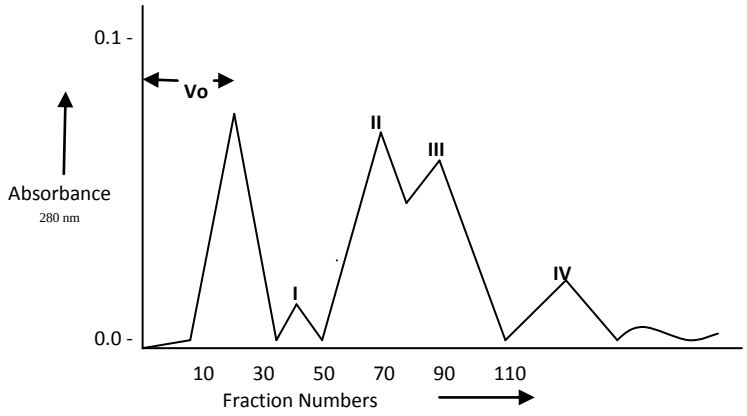

Figure 1. Spehadex G-25 chromatography of Camel Milk Oligosaccharides Detected by Phenol Sulphuric Acid Method. Elution was Made with TDW 25 g of Camel Milk Oligosaccharide Mixutre Chromatographed over Sephadex G-25 Chromatography

Confirmation of Homogeneity of Camel Milk Oligosaccharide by RP-HPLC

Oligosaccharide mixtures were quantitatively 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 plotters. The cyano column used for this purpose was a binary gradient system.

The eluents were detected at 240 nm. Thirteen peaks were noticed in the sample at the varied retention times from 5.00min. to 28.8 min. for convenience the peaks were numbered in their increasing order of retention time i.e. 5.00 min. (R_1), 8.5 min. (R_2), 11.3 min. (R_3), 12.1 min. (R_4), 13.2 min. (R_5), 13.3 min. (R_6), 17.00 min. (R_7), 18.5 min. (R_8), 19.3 min. (R_9), 22.8 min. (R_{10}), 24.5 min. (R_{11}), 25.5 min. (R_{12}) and 28.8 min. (R_{13}).

Fig

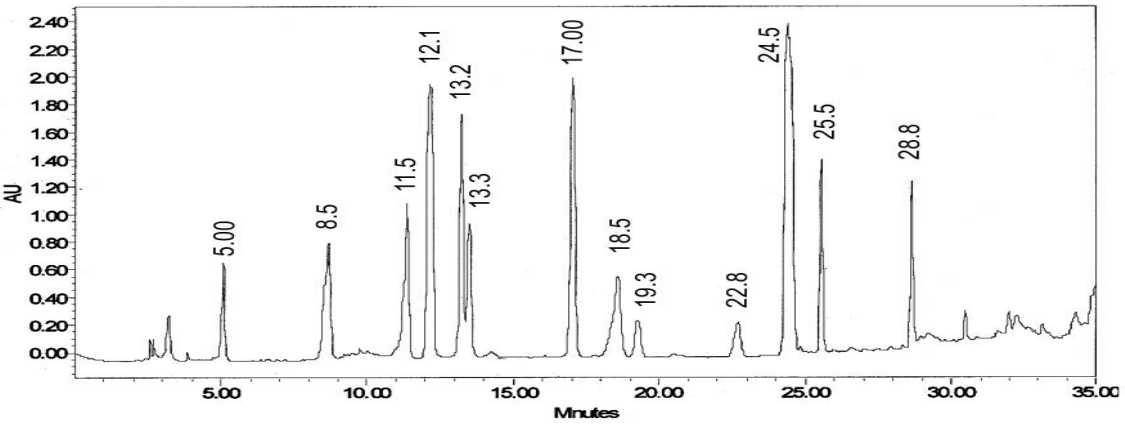

Figure 2. HPLC Chromatogram of Camel milk oligosaccharides.

S.No.	Retention time (RT)	% area of each peak
1	5.00	4.2
2	8.5	5.3
3	11.3	6.5
4	12.1	7.00
5	13.2	12
6	13.3	6
7	17.00	11
8	18.5	5
9	19.3	4
10	22.8	4
11	24.5	15
12	25.5	7
13	28.5	9

Figure 3. Table giving retention time and % area of each compound.

ACETYLATION OF OLIGOSACCHARIDE MIXTURE

11g of crude oligosaccharide mixture was acetylated with pyridine (11 ml) and acetic anhydride (11 ml) at 60^0C and solution was stirred overnight. The mixture was evaporated under reduced pressure and the viscous residue was taken in $CHCl_3$ (250ml) and washed in with ice cold water. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding the acetylated mixture (13g). The acetylation converted the free sugars into their non-polar acetyl derivatives which were resolved nicely on TLC, giving eight spots on TLC i.e. a, b, c, d, e, f, g and h respectively.

To obtain oligosaccharides in their purified form the acetylaed oligosachharides mixture was subjected to column chromatography over silica gel using hexane: $CHCl_3$ and $CHCl_3$:MeOH as eluents in various proportion which resulted into 8 fractions namely I(1.45gm), II(1.10gm), III(2.15gm), IV(1.2gm), V(2.30gm), VI(2.10gm), VII(1.40 gm) and VIII(800mg) respectively. These fractions were containing mixture of 3 to 4 compounds shown on TLC. These fractions 1-6 were re-chromatographed over silica gel at the compound: silica gel in the ratio 1:100 and varied proportions of $CHCl_3$:MeOH that resulted into the isolation of four novel oligosachharides b (320mg), c(204mg), e(349mg) and f (241mg) respectively.

METHODS OF DEACETYLATION OF ISOLATED COMPOUNDS

The acetylated compounds (b, c, e and f) were obtained from column chromatography. The mixture of acetylated oligosaccharides was dissolved in acetone and ammonia and left overnight in a Stoppard hydrolysis flask. Ammonia was removed under reduced pressure and the compound was washed with $CHCl_3$ and was finally freeze-dried giving the natural oligasaccharides AMCM-1(for b), AMCM-2(for c), AMCM-3(for e) and AMCM-4(for f) respectively (analytical notation for deacetylated oligosaccharides are AMCM) having the percentage value of 5.45%, 4.09%, 3.63%, 7.27% respectively.

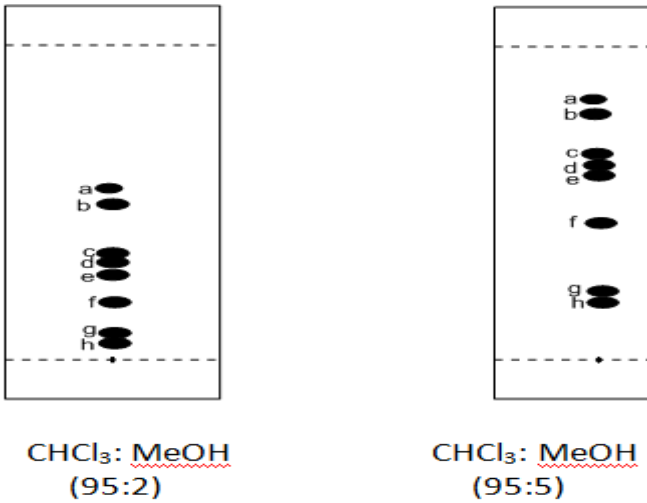

Fig. 4 TLC of acetylated oligosaccharides mixture at different polarity.

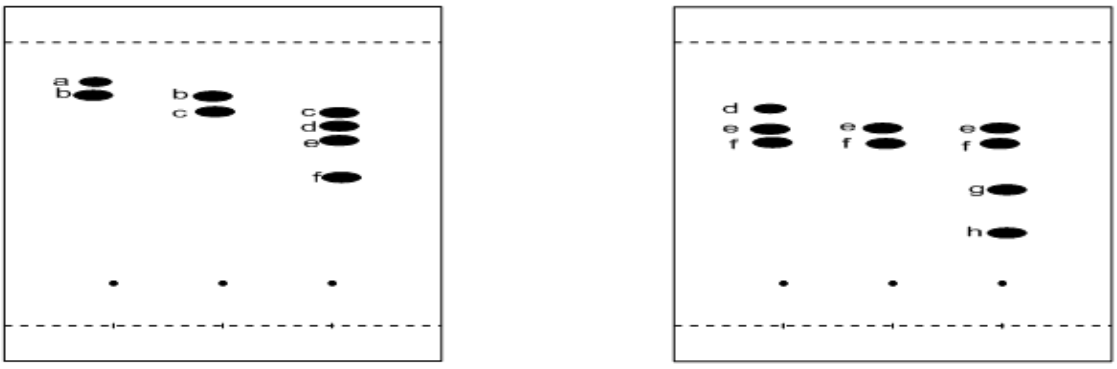

Fig. 5 TLC of acetylated oligosaccharide fractions from 1 to 8.

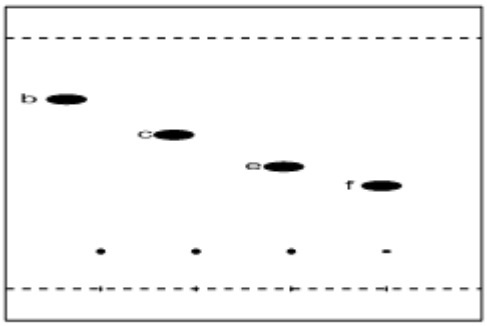

Figure 6. TLC of purified acetylated isolated compounds b, c, e and f.

RESULT AND DISCUSSION

Recently a number of oligosaccharides have been isolated from milk of different 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 is already discussed in a previous paper (Ashok etal 2015).

The structures of these basic core oligosaccharides were assigned by chemical degradation and spectroscopic techniques by previous workers and further, the structures were determined by comparing the (^{1}H NMR) chemical shifts of anomeric signals and ring protons of unknown milk oligosaccharides with the chemical shifts of the core units (LNT and LNnT etc) and structure reporter group (Dabrowski et al, 1983 and Wengang et al, 2005) have been detected so far. In the present scenario numbers of chromatographic techniques like HPLC, HPTLC, Pressure chromatography etc acquainted for the isolation and purification but 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 camel milk oligosaccharide we have combined the High performance liquid chromatography, gel filtration, thin layer chromatography and Column chromatography for obtaining novel camel milk oligosaccharides. The process followed by us includes various stages. In the 1st stage we obtained the crude Camel milk oligosaccharide by processing the milk by Modified Kobata and Ginsburg method which resulted into the isolation of the mixture of protein, glycoprotein, oligosaccharides and lactose. With a view to isolate camel milk oligosaccharide this crude mixture was subjected to sephadex G-25 chromatography which is a process of molecular sieving, Gel filtration separates the compounds present in the mixture according to their molecular weight. In this chromatography sequence of the compound is based on the molecular weight, compounds with higher molecular wt. comes first followed by the compound with lesser mol. wt. i.e. in the present case the glycoprotein come first followed by proteins, oligosaccharides, lactose and finally the monosaccharide. For this purpose a total amount of (25 gm) 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 1.2gm, 7.5 gm, 3.6gm and 0.72 gm respectively(fig. 1).The pooled fractions II and III comprising of oligosaccharides which were 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 13 peaks R1 to R13 (fig.2) with their respective retention times confirming the presence of 13 oligosaccharides in the camel milk (fig.3).

These oligosaccharides were very polar in nature so it was not possible to isolate these oligossacharides in good amount for their structure elucidation from routine chromatographic techniques therefore this oligosaccharide mixture was subjected to acetylation by AC2O/pyridine at 60^0C for obtaining them in their acetylated form. These acetylated oligosaccharides were less polar in nature therefore they were easily separated by classical chromatographic techniques i.e. Thin layer chromatography and column chromatography. The acetylated oligosaccharides mixture obtained after acetylation (13 gm) was examined and analysed over TLC showing eight spots a to h (fig 4) they were resolved nicely 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 isolaton of four novel camel milk oligosaccharides in their acetylated form b,c, e and f(fig 6) which was further deacetylated to obtain compounds AMCM-1, AMCM-2, AMCM-3 and AMCM-4 as natural oligosaccharides of camel milk. The spectroscopic data of isolated compounds are given as follows:

1. COMPOUND A (AMCM-1)

^{1}H NMR of AMCM-1: δ in D_2O at 400 MHz

δ 5.55 [d, 1H, J=4.0 Hz, α-Glc (S-1) H-1], 5.18 [d, 1H, J=8.0Hz, β-Glc (S-1) H-1], 4.58 [d, 1H, J=8.0Hz, β-GlcNAc (S-3), 4.42 [d, 2H, J=8.0 Hz, β-Gal (S-2), β-Gal (S-5) H-1], 4.35 [d, 1H, J=8.0Hz, β-GalNAc (S-4) H-1], 3.20 [t, 1H, J=8.0 Hz, β-Glc(S-1), H-2], 1.95 [s, 3H, $NHCOCH_3$, β-GlcNAc (S-3)], 1.85 [s, 3H, $NHCOCH_3$, β-GalNAc (S-4)]

^{1}H NMR of AMCM-1: δ in $CDCl_3$ at 400 MHz

δ 6.20[d, 1H,α-Glc (S-1) H-1], 5.63 [d, 2H, β-Glc (S-1), β-GlcNAc (S-3) H-1], 4.46 [d, 1H, β-Gal (S-5), H-1)], 4.44 [d, 1H, β-Gal (S-2), C-1], 4.42 [d, 1H, β-GalNAc (S-4) H-1], 4.18[m, 1H, β-Gal (S-2), H-4], 3.80 [m, 1H, β-Glc (S-1), H-4], 3.75 [m, 1H, β-Gal (S-2), H-2],

^{13}C NMR of AMCM-1: δ in $CDCl_3$ at 400 MHz

89.00 [1C, α-Glc (S-1) C-1], 91.38 [2C, β-Glc (S-1, β-GlcNAc (S-3) C-1], 100.73 [2C, β-Gal (S-2), β-Gal (S-5) C-1], 100.98 [1C, β-GalNAc (S-4) C-1]

ES Mass

m/z 972 $[M+Na+K]^+$, 949 $[M+K]^+$, 910 $[M]^+$, 892, 861, 850, 819, 748, 702, 659, 545, 509, 483, 382, 342, 284, 277, 180.

2. COMPOUND B (AMCM-2)

^{1}H NMR of AMCM-2: δ in D_2O at 400 MHz

δ 5.55 [d, 1H, J=4.0 Hz, α-Glc (S-1) H-1], 5.15 [d, 1H, J=8.0Hz, β-Glc (S-1) H-1], 4.58 [d, 1H, J=8.0Hz, β-Glc (S-5), 4.45 [d, 2H, J=8.0 Hz, β-Gal (S-2), β-Gal (S-4) H-1], 4.35 [d, 2H, J=8.0 Hz, β-GlcNAc (S-3), β-GalNAc (S-6) H-1], 3.20 [t,1H, J=8.0Hz, β-Glc (S-1) H-2],1.95 [s, 3H, β-GlcNAc (S-3), $NHCOCH_3$] and δ1.90 [s, 3H, β-GalNAc (S-6), $NHCOCH_3$].

^{1}H NMR of AMCM-2: δ in $CDCl_3$ at 400MHz

δ 6.15[d,1H, J==4.0Hz, α-Glc(S-1) H-1], 5.35 [d, 2H, J=8.0Hz, β-Glc (S-1), β-GlcNAc (S-3), H-1], 4.75[d, 1H, J=8.0Hz, β-Glc, (S-5), H-1], 4.50 [d, 2H, J=8.0Hz, β-Gal (S-2), β-Gal (S-4) H-1], 4.45 [d, 1H, J=8.0Hz, β-GalNAc(S-6), H-1], 4.10 [m, 1H, β-Gal (S-2), H-3], 3.90[m, 1H, β-Gal (S-4), H-3], 3.80[m, 1H, β-Glc (S-1), H-4].

^{13}C NMR of AMCM-2 : δ in $CDCl_3$ at 400MHz

δ 89.00 [1C, α-Glc (S-1) C-1], 90.05 [2C, β-Glc (S-1), β-GlcNAc (S-3) C-1], 95.19 [1C β-Glc (S-5) C-1], 100.91 [2C, β-Gal (S-2), β-Gal (S-4),C-1], 101.01 [1C β-GalNAc (S-6) C-1].

ES Mass

m/z 1111 $[M+K]^+$, 1095 $[M+Na]^+$, 1072 $[M]^+$, 1025, 1012, 965, 929, 919, 869, 838, 820, 804, 780, 747, 707, 676, 659, 586, 565, 545, 529, 516, 499, 422, 342, 324, 295, 180.

3. COMPOUND C (AMCM-3)

^{1}H NMR of AMCM-3: δ in D_2O at 400 MHz

δ 5.45 [d, 1H, J=4.0Hz, α-GalNAc (S-3) H-1], 5.35 [d, 1H, J=4.0 Hz, α-Gal (S-4) H-1], 4.32 [d, 1H, J=8.0Hz, β-Glc (S-1), 4.28 [d, 1H, J=8.0 Hz, β-Gal (S-2) H-1], 3.15 [t, 1H, J=8.0 Hz, β-Glc(S-1), H-2] δ1.80 [s, 3H, α-GalNAc (S-3), $NHCO\underline{CH_3}$]

^{1}H NMR of AMCM-3: δ in $CDCl_3$ at 400 MHz

δ 5.72 [d,1H, J=8.0Hz α-GalNAc (S-3), H-1)], 5.71 [d, 1H, J=8.0Hz, α-Gal (S-4) H-1], 4.40 [d, 2H, J=8.0Hz, β-Glc (S-1), β-Gal (S-2), H-1], 4.08 [m, 1H, β-Gal (S-2), H-3), 3.90 [m, 2H, β-Glc (S-1), α-GalNAc (S-3), H-4], 3.53 [s, 1H, -OCH3 (S-1) H-1].

^{13}C NMR of AMCM-3: δ in $CDCl_3$ at 400 MHz

δ 92.098 [1C, α-GalNAc (S-3), C-1], 92.558 [1C, α-Gal (S-4), C-1], 102.461 [2C, β-Gal (S-2), β-Glc (S-1) C-1], 57.340 [1C, $-OCH_3$, C-1]

ES mass

m/z 760 $[M+K]^+$, 744 $[M+Na]^+$, 721$[M]^+$, 703, 663, 559, 499, 468, 465, 356, 296, 286, 193.

4. COMPOUND D (AMCM-4)

^{1}H NMR of AMCM-4: δ in D_2O at 400 MHz

δ 5.61 [d, 1H, J=3.0 Hz, α-Glc (S-1) H-1], 5.20 [d, 1H, J=7.0Hz, β-Glc (S-1) H-1], 4.40 [d, 1H, J=7.0 Hz, β-Gal (S-2)], β-Glc (S-4) H-1] , 4.35 [d, 1H, J=7.0Hz, β-GlcNAc (S-3), H-1], 4.32 [d, 1H, J=7.0Hz, β-Glc(S-4) H-1], 4.28 [d, 1H, J=7.0 Hz, β-Gal (S-5)], 3.25 [t, 1H, J=8.0 Hz, β-Glc(S-1), H-2], δ1.90 [s, 3H, βGlcNAc (S-3) $NHCO\underline{CH_3}$].

^{1}H NMR of AMCM-4: δ in $CDCl_3$ at 400 MHz

δ 6.20 [d, 1H, α-Glc (S-1) H-1], 5.61 [d, 2H, β-Glc (S-1), β-GlcNAc (S-3), H-1], 4.45 [d, 1H, β-Glc (S-4) C-1], 4.44 [d, 1H, β-Gal(S-2), H-1)], 4.40 [β -Gal (S-5) H-1], 4.10 [m, 1H, β-Gal(S-2), H-3], 3.80 [m, 1H, β-Gal(S-2), H-2], 3.75 [m, 1H, β-Glc(S-1), H-4],

^{13}C NMR of AMCM-4: δ in $CDCl_3$ at 400 MHz

δ 88.80[1C, α-Glc (S-1) C-1], 91.37 [2C, β-Glc (S-1), β-GlcNAc (S-3) C-1], 100.77 [2C, β-Gal (S-2), β-Glc (S-4), C-1], 101.02 [1C, β-Gal (S-5) C-1].

ES mass

m/z 931 $[M+Na+K]^+$, 892 $[M+Na]^+$, 869[M]+, 811, 777, 733, 707, 676, 649, 582, 545, 510, 465, 342, 306, 289, 180.

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