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Synthesis of Reverse Building blocks of Milk Oligosaccharides

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Abstract: In recent times the importance of oligosaccharides isolated from various milk has emerged as an important source for biologically active compounds which are used as immuno-stimulant and anticancer agents. Various naturally occurring milk oligosaccharides have been synthesized in which the lactose was present at reducing end and glucose, galactose, GlcNAc, GalNAc and fucose were the other monosaccharides present in the oligosaccharides. Since the position and configuration of glycosidic linkage plays a definite role in the biological activity, in the present study we have synthesized the **reverse building blocks** of milk oligosaccharides, meaning in the trisaccharides which we are synthesizing here contain lactose at non-reducing end and they work clubbed with glucose and fucose at the reducing end. In this process lactose was taken as a donor as its trichloro acetamidate and simultaneously the acceptors of glucose and fucose were also been synthesized and glycosidation of these acceptors and donor were made successfully by TMSOTf method, which results in the formation of four trisaccharides namely-***Methyl-2-O.-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl(1→4)-2,3,6-tri-O-acetyl-β-D-glucopyranosyl)-3,4-O-isopropylidene-α-L-fucopyranoside*** [10], ***Methyl-3-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-O-acetyl-β-D-glucopyranosyl)-2,4-di-O-acetyl-α-L-fuco- pyranoside*** [12]**,** ***Methyl-4-O-(2,3,4,6–tetra–O-acetyl-β-D-Galactopyranosyl(1→4)-(2,3,6-tri–O-acetyl-β-D-glucopyranosyl)-2,3-di-O-acetyl-6-O-benzyl-α–D-glucopyranoside*** [24]**,** ***Methyl-4-O-(2,3,4,6–tetra–O-acetyl-β-D-galctopyranosyl(1→4)-(2,3,6-tri–O-Acetyl-β-D-glucopyranosyl)-2,3,4-tri-O-acetyl-α–D-glucopyranoside*** [25]**.** The structures of synthesized trisaccharide were confirmed by NMR spectroscopy.

Keywords: Milk oligosaccharides and reverse building blocks.

INTRODUCTION

Oligosaccharides of natural origin are important class of bioactive products and used for biological, synthetic, medicinal and technological purposes. These complex oligosaccharides have now shown to behave as antigenic determinants, antitumor agents, immuno-stimulant, anticancer agents and anti-inflammatory agents [1-5]. A number of higher plants, fungi, algae, lichen, milk and bacteria serve as rich sources of biologically active

oligosaccharides. The cell-surface carbohydrates serve as attachment sites for infectious bacteria, viruses and toxins resulting in pathogenesis [6] so the synthesis of structurally defined glycoconjugates provides the opportunity to probe and intervene in critical biological process. The synthesis of the partial structure of these oligosaccharides provide a demanding challenge as these structure act as tumor-associated and cancer-associated antigens, which occur as free oligosaccharides in human milk. The tumor-associated carbohydrate structure can only be obtained in small amounts from tumor cells, although they are generally absent or present in undetectable levels in normal cells [7, 8]. Therefore currently human milk oligosaccharides were used for studying the biosynthesis of antigen-I, and antigen-i and Lewis blood group related oligosaccharide [9, 10]. Significantly oligosaccharides isolated from buffalo milk [11] have been found immunostimulant & this isolated oligosaccharide was synthesized by Chinese workers [12]. The oligosaccharides isolated from donkey's milk [13] have also shown high degree of immunostimulant activity & proposed to be very helpful in cure of AIDS patient. It found that demonstrate in vitro stimulation of splenic lymphocytes of BALB/c mice and Immunostimulatory actions of oligosaccharide content present in mares' milk [14]. Since isolation of these oligosaccharides from their various natural sources provides a very meager quantity of the oligosaccharide. It is therefore of paramount importance to synthesize them in larger quantity using various stereo- and regio-selective synthetic strategies.

It has been seen that the milk oligosaccharides are comprised of various core-units and every core-units has lactose at its reducing end, besides that the milk oligosaccharides contain glucose, galactose, GlcNAc, GalNAc, fucose and sialic acid at its non-reducing end. The presence of these mono-saccharides and the position of glycosidic linkages play a definite role in their biological activity. Various building blocks of milk oligosaccharides have been synthesized by different workers in which the lactose was present at the reducing end. In this present paper we have synthesized the **reverse building blocks** of milk oligosaccharide meaning thereby in the trisaccharide which we are reporting here, contain lactose at its non-reducing end.

The synthesis of oligosaccharides requires the preparation of two polyfunctional partners. One partner acts as acceptor and other acts as donor. A major problem in the synthesis of carbohydrate is the presence of several reactive hydroxyl groups in each sugar residue. So to achieve unambiguous synthesis, it is necessary to protect those hydroxyl groups of the sugar that are not to be involved in the reaction. These protecting groups are also important as they influence the reactivity requirements, so various regio and stereoslective protecting groups and strategies have to be taken into consideration. A great deal of care should be taken while selecting the protecting groups for these hydroxyl groups.

MATERIALS AND METHODS

All solvents used were of reagent grade and were purified and dried according to standard procedures. Methanol was refluxed over magnessium turnings and resublimed iodine and stored over $4A^{o}$ molecular seives. Amberlite IR-120 (H^{+}) resins were washed with methanol and dried over P_2O_5 under vacuum in a dessicator before use. Organic solvents were dried over anhydrous sodium sulphate and all the compounds were dried in a high vaccum over P_2O_5 before use. DMF, pyridine and acetic anhydride were distilled over a direct flame before use. THF was dried over sodium and distilled over LAH. Chloroform was distilled over anhydrous $CaCl_2$. Dichloromethane was dried and distilled over P_2O_5 and stored over $4A^{0}$ molecular seives. Acetonitrile was

dried and distilled over CaH_2. K_2CO_3 was dried by heating to approximately 400°C in a muffle furnace and was then cooled to room temperature in a dessicator under vacuum over P_2O_5. The 4A° molecular sieves were activated and cooled to room temperature as K_2CO_3. All the glycosidation reactions were carried out under an atmosphere of N_2. All the evaporations were carried out in an electrically heated Boetius micro melting point apparatus. Column chromatographies were carried over silica gel (60-120 mesh) using organic solvents like hexane, ethyl acetate, chloroform and methanol. ^{1}H NMR spectrum were recorded in $CDCl_3$ at Broker AM 300 FT NMR Perkin-Elmer R-32 or CFT-20 FT-NMR spectrometer using Me_4Si as an internal standard at ambient Temperature. Optical rotations were determined on a Perkin-Elmer 241 Polarimeters, Polarimeter Autopol 3 Jasco DIP 180 digital polarimeters at ambient temperature.

Acetyl-2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl(1→4)-2,3,6-tri-O-acetyl-D-glucopyranoside (2) (scheme-1)

Conventional acetylation of D-lactose **1** (2 gm, 5.8 mmol) with 1: 1 Pyr : Ac_2O (40 ml:40 ml) gave a fully acetylated product **2** (2.1gm). Column chromatography of the residue gave the octa acetate as a crystalline compound.

^{1}H NMR(300MHz): δ5.5(d, 1H,H-1',J=3.6 Hz,S-1), δ5.49 (t,1H,H-3',J=9.2 Hz, S-1), δ5.16 (2t,2H,H-3'', H-4'',S-2), δ5.14 (dd,2H, H-2', S-1, H-2'', S-2), δ4.97(d, 1H, H-1', J=8.1 Hz,S-1), δ4.50 (d,1H, H-1'', J=7.5 Hz, S-2), δ4.31(dd, 1H, H-6'',S-2), δ4.11 (dd, 1H, H-6'', S-2), δ3.90 (t,1H,H-4',S-1), δ3.75 (m, 1H, H-5'', S-2), δ3.74 (m, 1H, H-5', S-1), δ3.67 (m, 1H,H-6', S-1), δ3.49(dd, 1H, H-6',S-1), δ 2.17, δ 2.16, δ2.15, δ 2.13,δ2.12,δ2.09, δ2.03, δ2.00 (8s, 24 H, 8-OAc)

Anal. Calcd. for $C_{28}H_{38}O_{19}$: C, 49.56 % ; H, 5.66%

Found: C, 49.54 %; H, 5.65%

2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl(1→4)-2,3,6-tri-O-acetyl-glucopyranose(3) (scheme-1)

Glacial acetic acid (1.260 ml) was added to ethylenediamine (1.260 ml) in THF (63.68 ml). lactoseoctacetate **2** (1.92gm, 2.8mmol) in THF was added to this solution. The reaction mixture was stirred for 20 hr at room temperature. Column chromatography of the residue gave the hepta acetate **3** as a crystalline compound.

^{1}H NMR(300MHz):δ 5.49 (t,1H,H-3', J=9.6Hz, S-1) , δ5.15 (2t, 2H, H-3'', H-4'', S-2), δ5.04 (dd, 2H,H-2', S-1, H-2'', S-2), δ 4.82 (d,1H,H-1',J=3.6 Hz, S-1), δ4.73 (d, 1H,H-1',J=8.1Hz, S-1), δ4.50 (d,1H,H-1'', J=7.5 Hz, S-2), δ4.3 (dd,1H, H-6', S-1), δ4.08 (dd, 1H, H-6'', S-2), δ3.90 (t, 1H, H-4', S-1), δ3.77 (m, 1H,H-6'',S-2), δ3.74 (m, 1H,H-5'', S-2), δ3.72 (m, 1H,H-5',S-1), δ3.47 (dd, 1H, H-6', S-1), δ2.15 , δ2.13, δ2.12, δ2.09, δ2.03, δ2.00 , δ1.99 (7s,21H, 7OAc).

Anal. Calcd. for $C_{26}H_{36}O_{18}$: C, 48.90% ; H, 5.64%

Found: C, 48.88 % ; H, 5.63%

Methyl α-L-fucopyranoside (6) (Scheme-2)

A suspension of L-fucose (1.5 gm, 9.08mmol) dried over P_2O_5 under vacuum and amberlite IR-120 (H^+) resin (0.300 gm) in absolute MeOH (15 mI) was refluxed with stirring at 70°C for 18 hr. The solution was filtered and the filtrate was concentrated under reduced pressure. The residue was chromatographed on a column of silica gel to give **6** (1.3gm) as a viscous syrup. [α]D-192.7°(.c, 0.9, H_2O)

Anal. Calcd. for $C_7H_{l4}O_5$: C, 47.19% ; H, 7.86%

Found: C, 47.17 % ; H, 7.85%

Methyl-3,4-O-isopropylidene-α-L-fucopyranoside (7) (Scheme-2)

Methyl α -L-fucopyranoside (1.3gm, 7.3 mmol) in acetone (260.34 rnI) containing 2,2 DMP (18.73ml) and PTSA (26.01 mg) was stirred for 3 hrs at room temperature. The acid was neutralized with $NaHCO_3$ and the

solution was evaporated to yield syrup. Column chromatography of the residue yielded **7** (1.22gm) as an amorphous solid. m.p. 89-92°C, [α]D-159.5^0 (.c, 0.4, $CHCl_3$) .

^{1}H NMR(300MHz): δ4. 71 (d, IH, H-l, J=3.6Hz), δ 4. 046(dd, 1H, H-4), δ 4.021 (m, 1 H, H-5), δ 3.86 (dd, 1H, H-2), δ 3.83 (dd, 1 H, H-3, J=4.2), δ 3.44 (s, 3H, OCH_3), δ1.56 (s,3H,CH_3) , δ 1.35 (s,3H,CH_3), δ 1.33 (d, 3H, $^6CH_3$, J= 6. 6Hz)

Anal. Calcd. for $C_9H_{18}O_5$: C, 52.42% ; H, 8.73%

Found: C, 52.41 % ; H, 8.72%

Methyl-2-O-acetyl-3,4-O-isopropylidene-α-L-fucopyranoside(8) (Scheme-2)

Conventional acetylation of **7** (1gm, 4.59mmol) with 1: 1 Pyr: $A_{C2}O$ (15 ml: 15ml) followed by column chromatography of the residue gave **8** (1.08gm) as a crystalline compound. m.p. 99-100°C, [α]D-230.2°(.c, 0.6 $CHCl_3$)

^{1}H NMR(300MHz): δ 4.7 (dd, 1H, H-2), δ4. 73 (d, IH, H-l, J=3.8Hz), δ 4. 1(dd, 1H, H-4), δ 4.08 (m, 1 H, H-5), δ 3.91 (dd, 1 H, H-3, J=4.4,), δ 3.47 (s, 3H, OCH_3), δ1.52(s,3H,CH_3) , δ 2.01 (s,3H,OAc), δ 1.33 (s,3H,CH_3), δ 1.31 (d, 3H, $^6CH_3$, J= 6. 7Hz)

Anal. Calcd. for $C_{11}H_{20}O_6$: C, 53.22% ; H, 8.06%

Found: C, 53.20 %; H, 8.04%

Methyl-2-O-acetyl-α-L-fucopyranoside (9) (Scheme-2)

A solution of **8** (900 mg, 3.45mmol) in 4: 1 acetic acid: water (20ml) was warmed to 80^{0}C and kept at this temperature for 30 min. The reaction mixture was then concentrated under reduced pressure. The residue was chromatographed to give **9** (860 mg) as an amorphous solid. m.p. 81-83^{o}C,[α]D -195.4 (c,O.4, $CHCl_3$).

^{1}H NMR(300MHz): δ4.77 (dd, 1H,H-2), δ 4.7 (d,1H,H-1,J=3.1Hz), δ4.02 (m, 1H,H-5), δ3.4 (s,3H,OCH_3), δ2.0(s,3H,OAc),δ1.33 (d ,3H,$^6CH_3$,J=6.0Hz).

Anal. Calcd. for $C_9H_{l6}O_6$: C, 49.09% ; H, 7.27%

Found : C, 49.05 % ; H, 7.26%

Methyl -2-O.-(2,3,4,6- tetra-O-acetyl-β-D-galactopyranosyl (1→4)- 2,3,6-tri-O-acetyl- β - D-glucopyranosyl)-3,4-O-isopropylidene-α-L-fucopyranoside (10) (Scheme-3)

To a solution of **3** (560mg, 0.659mmol) in dry CH_2Cl_3 was added anhydrous K_2CO_3 (391.30 mg) and trichloroacetonitrile (2.67ml). The suspension was stirred at room temperature for 48 hr under N_2. The reaction mixture was filtered through celite, washed with CH_2Cl_2 (35.89ml) and the filtrate was concentrated under reduced pressure and the oily residue of trichloroacetimidate donor **4** was used immediately for glycosidation **7** (500mg, 1.30 mmol) and **4** (500 mg, 1.005 mmol) were dissolved in dry CH_2Cl_2.TMS-OTf (0.05ml, 3.54meq) dissolved in CH_2Cl_2 was added and the reaction mixture was stirred at 0^{0}C for 1 hr. The reaction mixture was filtered through celite and the residue washed with CH_2Cl_2. Combined filtrate and washings were concentrated and purified by column chromatography using Hex-EtOAc which gave **10** (155mg) as a viscous.

^{1}H NMR(300MHz):δ5.66 (d,1H,H-1'', J=8Hz,S-2), δ5.22 (t,1H, H-3'',S-2), δ5.19 (t, 2H, H-3''', H-4''', S-3), δ5.13(dd,2H,H-2'', S-2'H-2'''S-3), δ5.02 (dd,1H, H-2', S-1), δ4.92 (t, 1H, H-4'', S-2), δ4.71 (d, 1H,H-1', J=3.9 Hz, S-1), δ4.47 (d, 1H, H-1''', J=8.4 Hz, S-3), δ4.20 (1H,H-6'',S-2), δ4.09 (dd, 1H, H-6''', S-3), δ4.08 (1H,H-6',S-1), δ4.07 (dd, 1H, H-4',S-1), 4.04 (m, 1H, H-5', S-1), δ3.88 (dd, 1H,H-3', S-1), δ3.81 (m, 1H,H-5'', S-2), δ3.76 (m, 1H, H-5''', S-3), δ3.42 (s, 3H,OMe,S-1), δ2.17 , δ2.12, δ2.09, δ2.05, δ2.02, δ2.00, δ1.9 (7s, 21H ,7OAc,S-2 ,S-3).

Anal. Calcd. for $C_{35}H_{52}O_{22}$ • C, 50.97 % ; H, 6.31%

Found. C, 50.966 % ; H, 6.308%

Methyl 3-O-(2,3,4,6- tetra-O-acetyl- β - D-galactopyranosyl-(1 →4)- 2,3,6- tri-O-acetyI-(β - D-glucopyranosyl)-2-O-acetyl-α-

L-fucopyranoside (11) (Scheme-3)

To a solution of **3** (560mg, 0.659 mmol) in dry CH_2Cl_2 was added anhydrous K_2CO_3 391.30mg) and trichloroacetonitrile(2.67ml). The suspension was stirred at room temperature for 48hr under atmosphere of N_2. The reaction mixture was filtered through celite, washed with CH_2Cl_2 (35.89ml) and the filtrate was concentrated under reduced pressure. The oily residue of trichloroacetimidate donor **4** without further purification was used immediately for glycosidation.

A solution of **9** (490 mg, 2.293 mmol) and **4** (490 mg, 1.005 mmol) were dissolved in dry CH_2Cl_2, TMS-OTf (0.38ml) was added and the reaction mixture was stirred at 0°C for 1 hr under an atmosphere of N_2. The reaction mixture was filtered through celite and the residue was washed with CH_2Cl_2. Combined filtrate and washings were concentrated and purified by column chromatography using Hex-EtOAc which gave **11** (188 mg) as a viscous product.

^{1}H NMR(300MHz):δ5.67 (t,1H,H-1'', J=8 Hz, S-2), δ5.45 (t,1H, H-3'', S-2), δ5.22 (t,2H,H-3''',H-4''',S-3), δ5.17 (dd, 1H,H-2', S-1), δ5.05 (dd, 1H,H-2'',S-3), δ4.90 (d, 1H, H-1', J=3.9 Hz, S-1), δ4.90 (t,1H ,H-4'', S-2), δ4.04 (dd, 1H,H-6''', S-3), δ4.53 (d, 1H, H-1''', J=8.4 Hz, S-3), δ4.26 (dd, 1H,H-3', S-1), δ4.22 (dd,1H,H-6'', S-2), δ 3.910 (m, 1H ,H-5''', S-3), δ3.90 (dd, 1H,H-4',S-1), δ3.79 (m, 1H,H-5', S-1), δ3.64 (m, 1H,H-5'',S-2), δ3.56 (dd, 1H ,H-6'',S-2), δ3.49 (s,3H, OMe,S-1), δ2.16, δ2.14, δ2.13, δ2.10, δ2.08, δ2.05, δ2.03, δ1.99 (8s,24H, 8OAc,S-1,S-2& S-3), δ1.15 (d, 3H,$^6CH_3$).

Anal. Calcd. for $C_{35}H_{50}O_{23}$ • C, 50.12 % ; H, 5.97%

Found. C, 50.11% ; H, 5.968%

Methyl 3-O-(2,3,4,6- tetra-O-acetyl-β-D-galactopyranosyl-(1→4)- 2,3,6-tri-O-acetyl-β- D-glucopyranosyl)-2,4-di-O-acetyl-α- L-fucopyranoside (12) (Scheme-3)

Compound **11** (188 mg, 0.277 mmol) was dissolved in 24.6ml of pyridine and to it was added 24.6 ml of acetic anhydride. The reaction mixture was stirred at 60°C for 1 hr. The mixture was then co-evaporated with toluene and the resulting compound (164.2 mg) was purified by column chromatography.

^{1}H NMR(300MHz):δ5.67 (d,1H,H-1'',J=8.1Hz,S-2), δ5.3 (d,1H,H-3'',S-2), δ5.32 (dd,1H,H-3', S-1), δ5.22(t,2H,H-3''', H-4''', S-3), δ5.17 (dd,1H,H-2',S-1), δ5.05 (dd, 1H, H-2'', S-2), δ4.94 (dd, 1H,H-4',S-1), δ4.90 (d,1H, H-1',J=3.9Hz, S-1), δ4.901(1H,H-4''S-2), δ4.53 (d,1H,H-1''',J=8.4Hz, S-3), δ4.22 (dd, 1H, H-6'', S-2), δ4.04 (dd,1H,H-6''',S-3), δ3.79 (1H,H-5''',S-3), δ3.791 (m,1H,H-5',S-1), δ3.64 (m, 1H,H-5'',S-2), δ3.53 (dd, 1H, H-6'',S-2), δ3.49 (s,3H,OMe,S-1), δ2.15, δ2.14, δ2.13, δ2.11, δ2.10, δ2.08, δ2.05, δ2.03, δ1.99 (9s,27H, 9OAc,S-1,S-2& S-3), δ1.15(d,3H, $^6CH_3$)

Anal. Calcd. for $C_{37}H_{52}O_{24}$ • C, 50.45% ; H, 5.91%

Found • C, 50.44 % ; H, 5.907%

Methyl -D-glucopyranoside (13) (scheme -4)

A suspension of D-glucose (2 gm, 11mmol) Dried over P_2O_5 under vaccum and amberlite IR-120 (H^+) resin (0.500mg) in absolute methanol (16.6ml) refluxed at 70°C for 18 hr. The solution was filtered and then concentrated under reduced pressure. The residue was chromatographed on silica gel to give **13** (1.9 gm) as a viscous syrup: $[\alpha]_D$+ 137.5° (c, 0.5,H_2O).

Anal. Caled. for $C_7H_{l4}O_6$: C, 43.29% ; H, 7.216%

Found: C, 43.22%; H, 7.213%

Methyl -2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside (16) (scheme -4)

Acetic anhydride (2.5ml) was added to **13** (1.9 gm, 9.5mmol) in pyridine (2.5 ml) at room temperature and the solution stirred overnight. The mixture was concentrated with

co-evaporation with toluene under reduced pressure. Column chromatography of the residue gave **14** which on repeated column chromatography afforded **15** (1.3 gm) and **16** (1.8 gm). Compound **15** and **16**were confirmed by their ^{1}H NMR spectrum.

Methyl -α-D-glucopyranoside (17) (scheme -4)

Sodium (24 mg) was dissolved in dry methanol (22mg) and the solution was added to compound **16** (1.8 gm, 4.95 mmol) and 85 ml of methanol was added. After 7 hr at room temperature de-O-acetylation was completed. The solution was concentrated and chromatographed using chloroform and methanol to afford **17** (722 mg) as a crystalline product.

^{1}H NMR(300MHz):δ5.5 (t,1H,J=9Hz, H-3), δ5.12t,1H, J=9Hz, H-4), δ4.95 (d,1H,J=3.2Hz), δ4.9 (dd, J=3.1Hz,H-2), δ4.27 (dd, 1H,J=3.12Hz and J=8.9 Hz, H-6), δ 4.1 (dd,1H,J=2.1Hz, H-6'), δ4.05-3.90 (m,1H,H-5), δ3.45 (s, 3H,MeO).

Anal. Caled. for $C_7H_{l4}O_6$: C, 43.29% ; H, 7.216%

Found: C, 43.27; H, 7.214%

Methyl 4,6-O-benzylidene- α-D-glucopyranoside.(18)(scheme 4)

A suspension of Methyl - **α** -D-glucopyranoside (400mg, 2mmol), PTSA (1.0mg) and BDMA(0.44ml) in N,N dimethyl formamide (5.1ml)was rotated under pressure for 2 hr at 45° C. The acid was neutralized with $NaHCO_3$ and filtered. The solution was evaporated to yield syrup. Column chromatography of the residue yielded compound **18** (472mg) as a crystalline compound: m.p.150-1510C, $[\alpha]_D$+82.0° (c, 0.5,EtOH).

^{1}H NMR(300MHz): δ7.38 (m,5H,C_6H_5), δ5.5 (s,1H,-CHC_6H_5), δ4.81 (d,1H,H-1,J=3.2 Hz), δ4.17 (m, 1H,H-6), δ4.11 (2H,H-3,H-6), δ3.80 (m,1H,H-5), δ3.48 (t,1H,H-4), δ3.42 (dd,1H,H-2), δ3.35 (s,3H,OMe).

Anal. Calcd. for $C_{14}H_{18}O_6$: C, 59.57% ; H, 6.38%

Found : C, 59.54% ; H, 6.366%

Methyl- 2–di-O-acetyl-4,6-O-benzilydene-α-D-glucopyranoside(19) (scheme-4)

Conventional acetylation of compound **18** (400mg) with 1:1 Pyr::Ac_2O (3ml:3ml) at room temperature was performed and the solution was stirred overnight. The mixture was concentrated under reduced pressure. Column chromatography of the residue gave **19** (480 mg) as a crystalline compound m.p.145-147^{0}C, $[\alpha]_D$+90.5^0 (c, 1, $CHCl_3$).

^{1}H NMR(300MHz):δ7.37 (m,5H,C_6H_5), δ5.58 (s,1H,-CHC_6H_5), δ4.81 (d,1H,H-1, J=3.2HZ), δ4.49 (1H,H-4), δ4.4(dd,1H,H-2), δ4.28 (m, 1H,H-6), δ4.26 (2H,H-3,H-6), δ3.75 (m,1H, H-5), δ3.35 (m,3H,OMe), δ2.01(6H,-OAc).

Anal. Calcd. For $C_{18}H_{22}O_8$: C, 59.016% ; H, 6.010%

Found: C, 59.011%; H, 6.008%

Methyl 2, 3 –di-O- acetyl-,6-O-benzyl- α-D-glucopyranoside(20) (scheme-4)

A suspension of compound **19** (152 mg, .049mmol), $NaCNBH_3$ (132mg), and crushed molecular sieves in N,N-dimethyl formamide (3.1 ml) was stirred at room temperature under an atmosphere of N_2 for 15 min. CF_3COOH (0.325 ml) in DMF (2.1 ml) was added and the suspension was stirred under atmosphere of N_2 at room temperature for 19 hr. More TFA (.305ml) was added to the suspension which was then stirred for another 24 hr under atmosphere of N_2 at room temperature. The reaction mixture was cooled to 0^{0}C, diluted with EtOAc and CH_3OH and stirred for 5 min. This was then filtered and the residue washed with EtOAc. The combined filtrate and washing were washed with cold H_2O, cold aq. $NaHCO_3$ and H_2O and evaporated to dryness. The residue was chromatographed on silica gel to give **20** (118mg).

^{1}H NMR(300MHz): δ7.48 (5H,C_6H_5), δ4.81 (d,1H, H-1,J=3.3Hz), δ4.4 (dd,1H, H-2), δ4.26 (1H,H-6), δ4.24 (1H,H-6), δ 4.16(2H,J=12Hz,-$CH_2C_6H_5$), δ4.14 (1H, H-3), δ3.79 (1H,H-5), δ3.48 (1H, H-4), δ3.35 (3H, OMe), δ2.00 (6H,OAc).
Anal. Calcd. For $C_{18}H_{24}O_8$: C, 58.69% ; H, 6.52%
Found: C, 58.66%; H, 6.51%

Methyl -6-O trityl - α-D-glucopyranoside (21) (scheme-4)
Methyl α-D-glucopyranoside **17** (200 mg, 1.03mmol) was dissolved in pyridine and stirred for 45 min. at 0^0C. Trityl chloride (3.48 gm) was added and the suspension was stirred at 60^0C for 18 hr. $CHCl_3$ was added to the reaction mixture and then filtered to remove the excess of TrCl. Evaporation yielded a crude amorphous solid which was dissolved in chloroform and water was added to it . The organic layer was extracted and this process was repeated twice. Na_2SO_4 was added to the organic layer and filtered and then evaporated .Purification was afforded by column chromatography which gave compound **21** (155 mg) as an amorphous solid.

^{1}H NMR(300MHz): δ7.17 (15H,C_6H_5), δ4.17 (1H,J=3.3 Hz, H-1), δ3.94 (1H, H-6), δ3.88(2H,H-3 & H-6), δ3.47 (1H,H-5), δ3.4 (1H,H-2), δ3.39 (t,1H,H-4), δ3.30 (s,3H,OMe).
Anal. Calcd. For $C_{26}H_{28}O_6$: C, 71.51% ; H, 6.42%
Found: C, 71.50%; H, 6.41%

Methyl 2,3, 4 tri Acetyl-6-O trityl - α-D-glucopyranoside(22) (scheme-4)
Acetic anhydride (7.46 ml) was added to Methyl -6-O trityl - α-D-glucopyranoside (**21**) (155 mg) in pyridine (7.46 ml) the reaction was stirred over night at room temperature . The mixture was concentrated under reduced pressure with co-evaporation with toluene. The compound was purified by column chromatography which yielded **22** (170 mg) as crystalline compound.

^{1}H NMR(300MHz): δ7.17 (15H, C_6H_5), δ4.401 (1H,H-3), δ4.401H,H-2), δ4.38 (1H,H-4), δ4.17 (d,1H,J=3.3,H-1), δ4.08 (1H,H-6), δ3.94 (1H,H-6), δ3.46 (1H,H-5), δ3.30(3H,OMe).
Anal. Calcd. For $C_{32}H_{34}O_9$: C, 68.32% ; H, 6.04%
Found: C, 68.31%; H, 6.03%

Methyl-2,3,4-tri–O-Acetyl-α-D-glucopyranoside(23) (scheme-4)
A solution of **22** (170 ml .372mmol) in 4:1 acetic acid: water (22.83 ml) was warmed at 80^0C for 30 min. The reaction mixture was concentrated under reduced pressure. The residue was chromatographed to give **23** (135 mg) as an amorphous solid.

^{1}H NMR(300MHz): δ5.31(1H,H-3), δ5.00(1H,H-4), δ4.85 (d, 1H,H-1,J=3Hz), δ4.40 (1H,H-2), δ4.31(1H,H-6), δ4.09 (1H, H-6), δ3.95 (1H,H-5), δ3.35 (3H,OMe), δ3.03 (6H,OAc), δ2.09(3H,OAc).
Anal. Calcd. For $C_{13}H_{20}O_9$: C, 48.75% ; H, 6.25%
Found: C, 48.72%; H, 6.24%

Methyl-4-O-(2,3,4,6–tetra–O-acetyl-β-D-Galctopyranosyl(1→4)-(2,3,6-tri–O-Acetyl-β -D-glucopyranosyl)-2,3-di-O-acetyl-6-O-benzyl-α–D-glucopyranoside (24) (scheme-5)
A solution of **20** (100 mg, 307 mmol) lactosyl acetaimidate 9 (100 mg, 0.130 mmol) and molecular sieves in dry CH_2Cl_2 (24.59 ml) was stirred at 0^0C with TMS –OTf (0.017 ml, 1.02 meq) under an atmosphere of nitrogen. Column chromatography of the residue using Hexane: Ethyl acetate gave **24** as a viscous product (42 mg, 34%).

^{1}H NMR(300MHz): δ7.38 (5H, C_6H_5,S-1), δ5.72 (1H,H-1'',S-2), δ5.45 (t,1H,H-3'',S-2), δ5.26 (1H,H-3',S-1), δ5.19(1H,H-3''',S-3), δ5.15 (1H,H-4''',S-3), δ5.10 (dd,1H,H-2''',S-3), δ5.08(dd,1H,H-2'',S-3), δ5.05 (t,1H,H-4,S-1), δ4.96 (1H,H-4''',S-2), δ4.85 (d,1H,J=3Hz,S-1),

δ4.53 (1H,H-1''',J=7.8Hz,S-3), δ4.31 (dd,1H,H-6), δ4.27(d,1H,H-6,S-3), δ4.40(1H,H-2), δ4.25(dd,1H,H-6,S-1), δ4.20(dd, 1H, H-6,S-1), δ4.12 (2H,CH_2C_6H5), δ3.961 (1H,H-5',S-1), δ3.960 (1H,H-6'',S-2), δ3.84 (1H,H-5'',S-2), δ3.79 (1H,H-5'',S-3), δ3.59 (dd,1H,H-6,S-2), δ3.58 (s,3H, OMe,S-1), δ2.10, δ2.08, δ2.07, δ2.04, δ2.03, δ2.02 (s,OAc,S-1,S-2,S-3).

Anal. Calcd. For $C_{44}H_{58}O_{25}$: C, 53.549% ; H, 5.88%

Found: C, 53.5423%; H, 5.87%

Methyl-4-O-(2,3,4,6–tetra–O-acetyl-β-D-Galctopyranosyl(1→4)-(2,3,6-tri–O-Acetyl-β -D-glucopyranosyl)-2,3,4-tri-O-acetyl-α–D-glucopyranoside (25) (scheme-5)

A solution of **23** (135 mg, 0.314 mmol) 9 (135 mg, 0.177 mmol) and molecular sieves in dry CH_2Cl_2 was brought to 0^0C and TMS-OTf (0.023 ml) was added to it. The reaction mixture was stirred at 0^0C for 1 hr under nitrogen .Column chromatography of the residue using Hex: EtOAc gave **25** as a viscous product (82mg).

^{1}H NMR(300MHz): δ5.67 (d,1H,H-1'',J=8.4Hz,S-2), δ5.41(1H,H-3'',S-2), δ5.30(H-3',H-4',S-1), δ5.22 (t,2H,H-3''',H-4''',S-3), δ5.19 (dd,1H,H-2''',H-3''',S-3), δ5.05 (1H,H-2''), δ5.02(dd,1H,H-6,S-1), δ4.83(d,1H,J=3Hz,S-1), δ4.52(d,1H,H-1''',J=7.5Hz,S-3), δ4.50(dd,1H,H-6,S-1), δ4.40(1H,H-2), δ4.22 (1H,H-6,S-3), δ3.94 (2H,H-5,S-1 &H-6'',S-2), δ3.79 (1H,H-5'',S-2), δ3.65(1H,H-5''',S-3), δ3.58 (dd,1H,H-6,S-2), δ3.34 (3H,OMe,S-1), δ2.09, δ2.08, δ2.03, δ2.02, δ2.01, δ2.00, δ1.99 (s,OAc,S-1,S-2,S-3).

Anal. Calcd. For $C_{39}H_{54}O_{26}$: C, 49.893% ; H, 5.75%

Found: C, 49.890%; H, 5.74%

RESULT AND DISCUSSION

After looking into the structure of milk oligosaccharides, it has been seen that most of milk oligosaccharides are made up of basic core units i.e. Lacto-N-tetraose, Lacto-N-neotetraose, Lacto-N-fucopentaose-1, Lacto-N-fucopentaose-2 etc. In all the core units the lactose is the backbone of these core units. The other monosaccharides which are present in the milk oligosaccharides are Gal, GlcNAc, GalNAc, Fucose and sialic acid etc. Keeping in mind the importance of milk oligosaccharides in the present paper it is proposed to synthesize allied and modified trisaccharides involving lactose and monosaccharides present in milk oligosaccharide.

The efficient chemical synthesis of complex oligosaccharides require highly convergent strategies in which well designed glycosyl donor and acceptors were assembled involving a minimum of steps of reactions. This paper deals with the synthesis of some allied and modified trisaccharides where Lactose was used as a donor and monosaccharide units Glucose and Fucose were used as acceptor. Special emphasis has been given to the synthesis of the glycosyl donors, which can act as an electrophile upon activation with suitable activating reagents, the anomeric protecting groups or the hemiacetals has to be converted into a better leaving group. The synthesis of the oligosaccharides was brought about by condensation of the electrophile with the nucleophile.

The first step of synthesis was the preparation of derivatives of lactose which act as active glycosyl donor. For this the disaccharide lactose 1 (β-D-galactopyranosyl-(1→4)-D-glucose) was acetylated using acetic anhydride and pyridine in (1:1) to afford 2 (Acetyl-2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl(1→4)-2,3,6-tri-O-acetyl-D-glucopyranoside) as a mixture of α and β anomers which was characterized by presence of eight singlets for 8 –OAc group in ^{1}H NMR spectrum. In the next step the anomeric deacetylation was achieved by

treating the octaacetate with equimolar mixture of ethylene diamine and acetic acid (1:1) in the presence of THF to give 3 (2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl(1→4)-2,3,6-tri-O-acetyl-glucopyranose) which was mixture of α and β anomers. Further compound 3 was converted into imidate donor by treating it with TCA in the presence of anhydrous K_2CO_3 which afforded active donor 4.

In the next step of synthesis, derivatives of fucose were prepared which act as glycosyl acceptor. For this the introduction of methyl group at anomeric position was first attempted by treatment of L-fucose with absolute methanol and amberlite IR 120 (H^+) resin to afford α and β anomers of fucose **[15]** (5,6) which were further separated through column chromatography. In this step the α-anomer 6 (Methyl-α-L-fucopyranoside) was obtained in higher yield due to anomeric effect of oxygen atom. Further the subsequent protection of 3,4-position of 6 as isopropylidine ring**[16]** was achieved by treatment of 6 with 2,2-DMP in the presence of an acid catalyst PTSA affording 7 (Methyl-3,4-O-isopropylidene-α-L-fucopyranoside) which was characterized by its ^{1}H NMR spectrum which showed two singlets of the two CH_3 groups of isopropylidine ring at δ1.357 and δ1.563 for three protons each. Now compound 7 was acetylated with acetic anhydride and pyridine to get 8 (Methyl-2-O-acetyl-3,4-O-isopropylidene-α-L-fucopyranoside). In the next step 8 was treated with a mixture of AcOH: H_2O in 4:1 for removal of isopropylidine ring to afford 9 (Methyl-2-O-acetyl-α-L-fucopyranoside) which was confirmed by its ^{1}H NMR spectrum which showed a downfield shifted double doublet for H-2 and a singlet for three protons of acetate group at δ2.00 and absence of singlets of two CH_3 group of the isopropylidine ring. This was an active acceptor for glycosidation.

Further derivatives of Glucose were prepared by various protecting and deprotecting methodologies. In the first step, Glucose anomeric group was protected by methyl group through Fischer's method **[15]**. In this method Glucose was refluxed with absolute methanol and amberlite IR 120 (H^+) resin to afford α and β anomers of glucose (Methyl -D-glucopyranoside) (13).In the next step conventional acetylation of 13 with acetic anhydride and pyridine (1:1) was done which afforded 14(anomeric mixture). The separation of α and β anomers of methyl tetra-O- acetyl-(α, β)-D-glucopyranosides 15 and 16 (Methyl-2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside) were achieved only after extensive column chromatography. α-Glycosidic linkage was confirmed by its ^{1}H NMR spectrum which showed presence of a doublet at δ4.9 with small coupling of 3 Hz for H-1 along with four acetoxy group singlets at δ 2.1, δ2.08, δ2.06, δ2.03 and a singlet of three protons at δ 3.4 confirming the presence of anomeric methoxy group. Now compound 16 was deacetylated with sodium methoxide in methanol which afforded compound 17(Methyl-α-D-glucopyranoside) mp 160^{0}C, which was confirmed by its ^{1}H NMR spectrum which showed absence of singlets for acetyl group. The subsequent protection of 4, 6-position in compound 17 as 1, 3-dioxane ring**[11]** was afforded by treating it with benzaldehyde dimethyl acetal and a mild acid, p-toluene sulphonic acid as a catalyst, yielding 4,6-acetal 18 (Methyl-4,6-O-benzylidene-α-D-glucopyranoside). The most stable arrangement was the six membered (1, 3-dioxane) ring in which chair conformation had the phenyl substituent in an equatorial position. This arrangement was more stable than the five membered (1, 3-dioxolane) ring in which the ring substituents necessarily experience eclipsing interaction. Besides the other appropriate signals the 1, 3-dioxane derivative 18 exhibited singlet at δ5.5 for benzylidine methine proton and the five aromatic protons multiplet appeared at δ7.38 in ^{1}H NMR

spectrum. Further acetylation of 18 with Ac_2O in pyridine (1:1) gave Methyl 2, 3 –di-O- acetyl-4, 6-O-benzylidene- α-D-glucopyranoside (19) which was characterized by the presence of singlet of six protons at δ2.01 for O-acetyl group in its ^{1}H NMR spectrum. In the next step, the selective debenzylidination of compound 19 (Methyl-2–di-O-acetyl-4,6-O-benzilydene-α-D-glucopyranoside) was achieved by treating it with CF_3COOH and $NaCNBH_3$ affording 20 (Methyl-2,3–di-O-acetyl-,6-O-benzyl-α-D-glucopyranoside) in which O-6 was protected by benzyl group and O-4 became free for glycosidation providing a glycosyl acceptor which was confirmed by its ^{1}H NMR spectrum which showed benzylic methylene protons as doublet at δ4.16 (J=12 Hz). ^{1}H NMR spectrum of compound 19 and 20 has the same ratio of aromatic protons suggesting that benzylidene acetal group of 19 was not completely removed during selective debenzylidination but it had undergone reductive cleavage giving free hydroxyl groups at 4-position. The selective protection of the 6-OH position of compound 17with a trityl group **[17]** was achieved by treating it with trityl chloride and pyridine to afford compound 21 (Methyl-6-O-trityl-α-D-glucopyranoside) which was charaterized by its ^{1}H NMR spectrum which showed multiplet at δ 7.17 for 15 aromatic protons. The subsequent protection of 2, 3 and 4 positions of 21 was done by acetylation with acetic anhydride and pyridine in 1:1 to afford 22 (Methyl-2,3,4-triacetyl-6-O-trityl-α-D-glucopyranoside). Now trityl group was removed by acid hydrolysis of compound 22 yielding 23 (Methyl-2,3,4-tri–O-Acetyl-α-D-glucopyranoside) which was confirmed by its ^{1}H NMR spectrum which showed absence of aromatic proton signals and presence of three acetoxy group signals. This was an active acceptor for glycosidation.

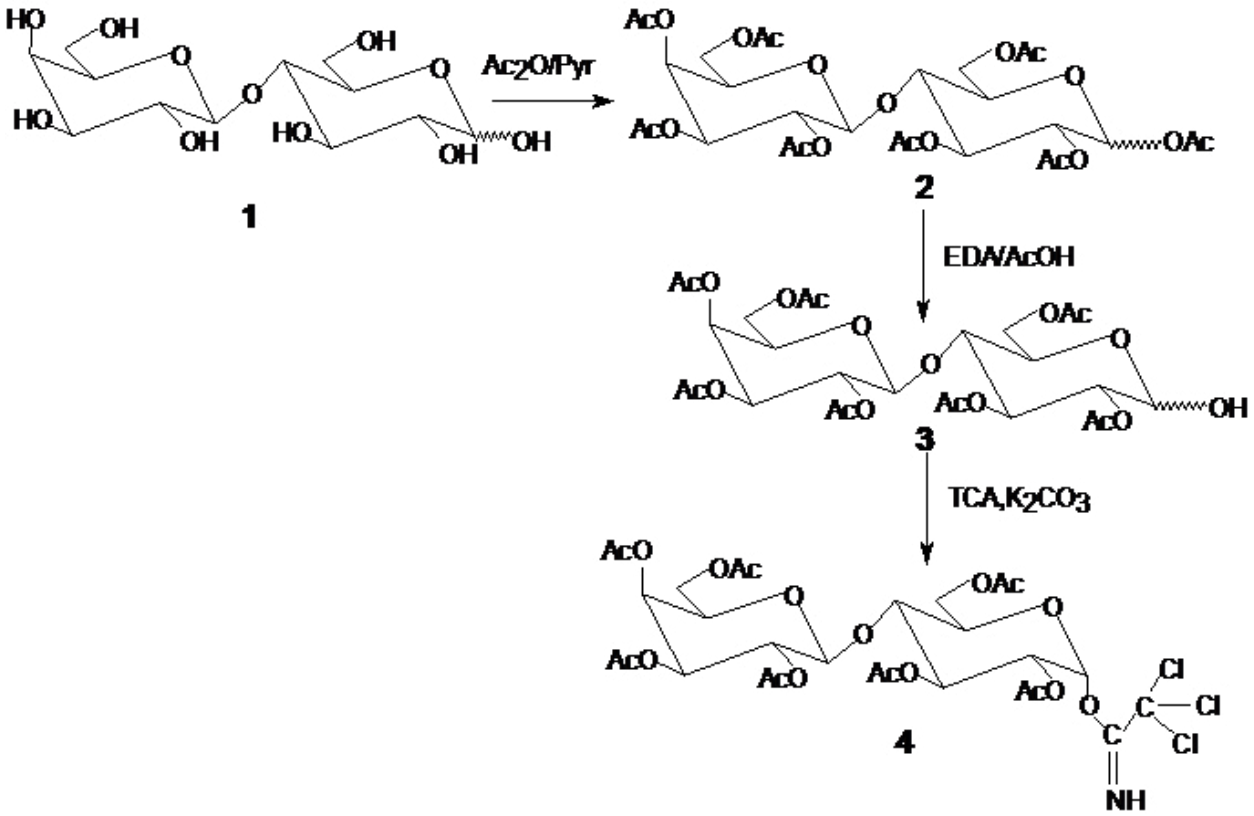
In the final step of synthesis of trisaccharide, lactose was linked to fucose at different position. For this the trichloroacetimidate lactosyl donor was activated in the presence of lewis acid catalyst TMS-OTf **[18]**, which permitted the synthesis of trisaccharide by condensing it with **7** to afford 10 (scheme-3), regio and stereo-specifically in 55% yield. The anomeric configuration of newly formed glycosidic linkage in compound 10 was confirmed by by its ^{1}H NMR spectrum which showed anomeric proton resonance of S-2 at δ5.66 with a large coupling constant J=8Hz.In the same way, the trisaccharide 11 was synthesized by condensing 9 with 4 in the presence of TMS-OTf as an promoter to afford 1, 2-trans-β linked trisaccharide 11. The anomeric configuration of newly formed glycosidic linkage in the trisaccharide was confirmed by its ^{1}H NMR spectrum which showed anomeric proton resonance of S-2 at δ5.67 with a large coupling constant J=8Hz and two more doublets for S-1and S-3 anomeric protons resonance at δ4.90 (J=3.4Hz) and δ4.53 (J=8.4Hz) also supported the structure of the trisaccharide. The formation of the glycosidic bond at (1→3) position in 11 was confirmed by acetylating 11 with acetic anhydride and pyridine which afforded 12. The ^{1}H NMR spectrum of this derivative 12 showed one additional singlets for the three protons of the O-acetyl group along with downfield shifting of H-4 methine proton from δ3.90 to δ4.94. The (1→3) linked trisaccharide 11 was formed in preference to (1→4) because of less crowded environment at equatorially oriented hydroxyl groups at C-3 position in 9. While the axial –OH group at C-4 position exert more steric crowding.

In the next scheme-5, glycosylation of acceptor 20 with lactosyl acetimidate 4 in trimethylsilyltrifluromethansulphonate (TMSOTf)**[19]** as a promoter afforded the desired trisaccharide β-D-Galp(1→4)-β-D-Glcp(1→4)-α-DGlcp 24 (Methyl-4-O-(2,3,4,6–tetra–O-acetyl-β-D-Galctopyranosyl(1→4)-(2,3,6-tri–O-Acetyl-β-D-glucopyranosyl)-2,3-di-O-acetyl-6-O-benzyl-α–D-glucopyranoside). The

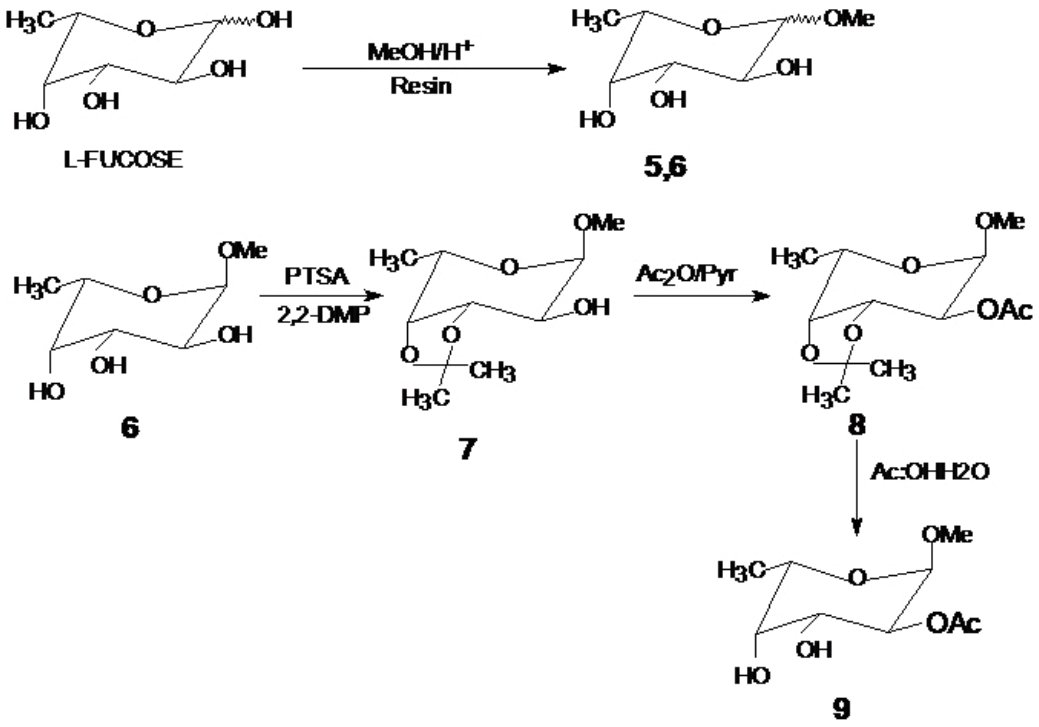
nature of glycosidic linkage in 24 was assigned by its ^{1}H NMR spectrum which showed downfield shifted anomeric proton of S-2 at δ5.72 with large coupling constant along with a downfield shifted H-4 resonance from δ3.48 to δ5.05. This confirmed the formation of β (1→4) glycosidic linkage in compound 24. Further another trisaccharide 25 (Methyl-4-O-(2,3,4,6–tetra–O-acetyl-β-D-Galctopyranosyl(1→4)-(2,3,6-tri–O-Acetyl-β-D-glucopyranosyl)-2,3,4-tri-O-acetyl-α–D-glucopyranoside) was synthesized by glycosylation of acceptor 23 with lactosyl acetimidate 4 in equimolar quantity by using TMS-OTf as a promoter. The anomeric configuration and position of the newly formed glycosidic linkage for compound 25 was determined by its ^{1}H NMR spectrum **[20]** which showed anomeric proton resonance of S-2 appeared at δ5.67with a large coupling constant of 8.4 Hz suggesting the formation of β-glycosidic linkage in compound 25. Further the downfield shifted H-6 resonance of S-1 confirmed the formation of β (1→6) glycosidic linkage in compound 25.

CONCLUSION

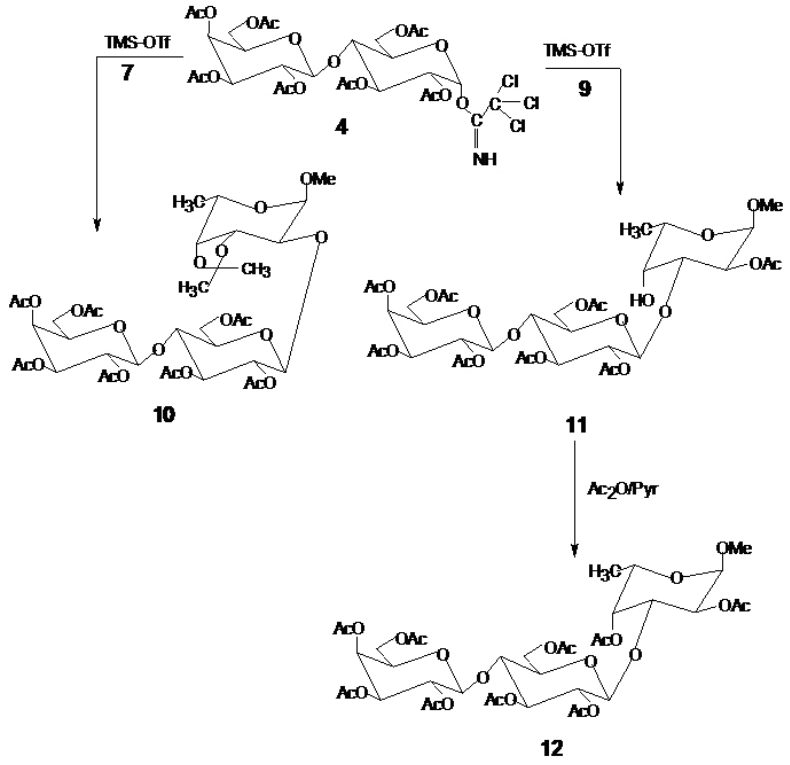
SCHEME-1



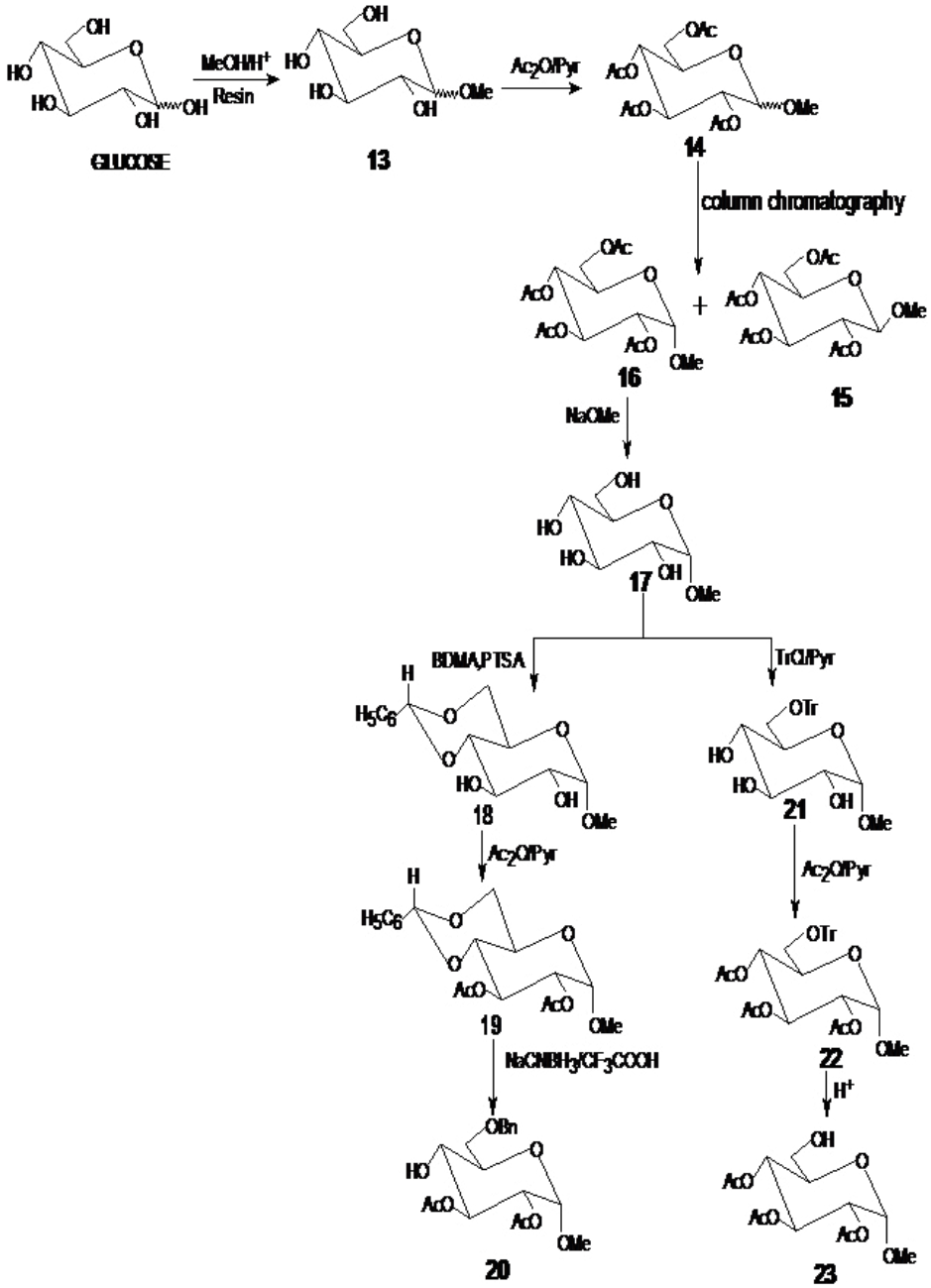
SCHEME-2



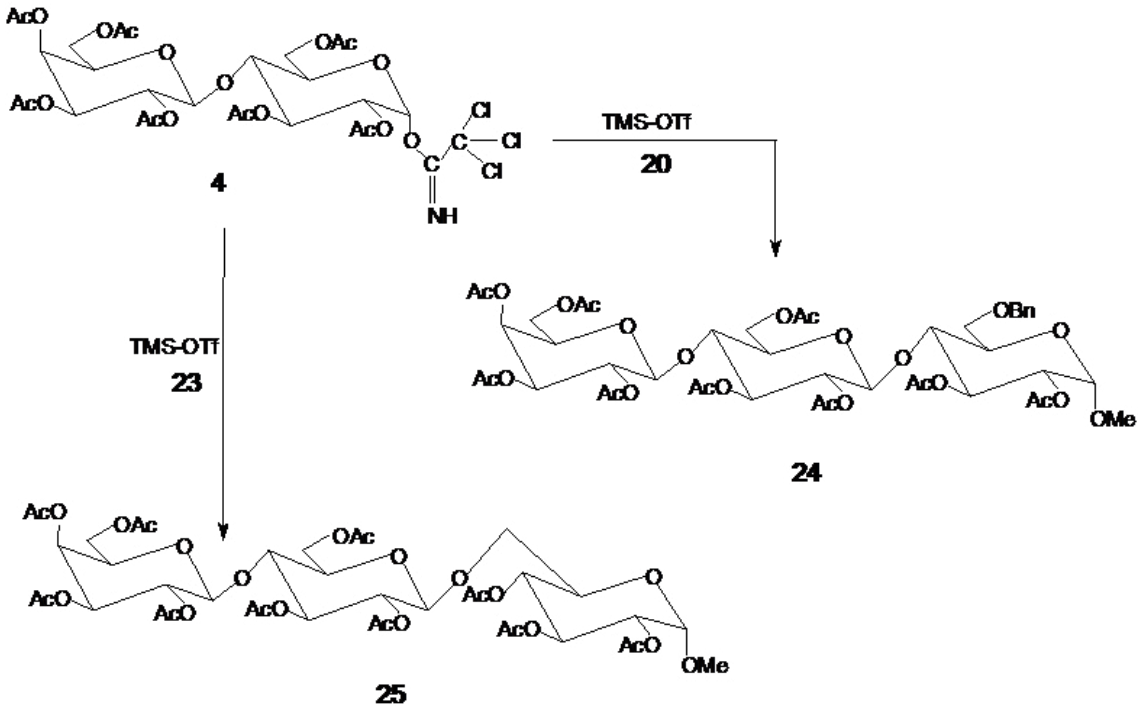
SCHEME-3



SCHEME-4


GLUCOSE
MeOH/H+
Resin
13
Ac2O/Pyr
14
column chromatography
16
15
NaOMe
17
BDMA,PTSA
TrCl/Pyr
18
21
Ac2O/Pyr
19
22
NaCNBH3/CF3COOH
H+
20
23

SCHEME-5



In this work we have synthesized the lactose as a donor as its trichloro acetamidate and simultaneously the acceptors of glucose and fucose were also been synthesized and glycosidation of these acceptors and donor has been made successfully by TMSOTf method, which results in the formation of **four trisaccharide,** by the reverse building blocks of milk oligosaccharides method, which contain lactose at its non-reducing end and glucose and fucose at reducing end.

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