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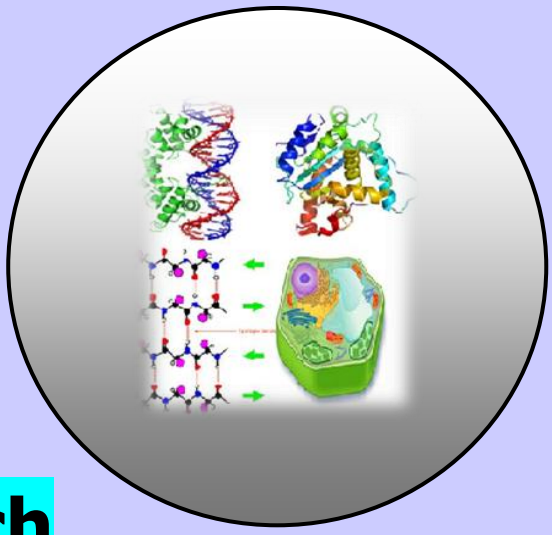

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RESEARCH PAPER

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Isolation of a Novel Oligosaccharide (Dicose) from Sheep Milk

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ABSTRACT

Milk is an important fluid in glycobiology which contains number of carbohydrate chains either in free form or as glyco-conjugates. Milk is one of the important source of biologically active oligosaccharides which have shown various biological activities such as antitumor, anti-inflammatory, anti-cancer, antimicrobial activities. Sheep milk contents have their affect in cardiovascular, nervous and immune system. In order to search for more novel biologically active milk oligosaccharides, Sheep milk was collected and processed by Kobata and Ginsburg method followed by gel filtration and column chromatography which resulted in the isolation of novel milk oligosaccharide namely Dicose. The structure of isolated novel oligosaccharide was elucidated with the help of ^{1}H, ^{13}C NMR and 2D NMR (COSY, TOCSY and HSQC) techniques and mass spectrometry as-

β-GlcNAc(1→3)β-GlcNAc(1→3)
|
α-Gal(1→3) β-GalNAc(1→4)Glc
|
β-GlcNAc(1→3)

DICOSE

Key words: Oligosaccharides, Dicose, Kobata and Ginsburg method.

INTRODUCTION

Milk is a complete food for mammalian neonates and it has many nutritional and medicinal values (Srivastava et al., 2016). Milk contains a variety of components including proteins, endogenous peptides, lipids, carbohydrates and minerals all of which fulfils the nutritional

needs for growth and development mammalian infants. Milk of various origins has proven themselves as a source for biologically active oligosaccharides. Oligosaccharides have inhibitory effect on certain virulence-related abilities of monocytes, lymphocytes and nuetrophils adhesion to endothelial cells (Singh et al. 2016). The development of carbohydrates and glycoconjugates as therapeutics continues to gain interest when biological relevance of these biopolymers were further revealed and understood (Singh et al. 2016). The elephant milk oligosaccharide has 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-specfic and specific immunological resistance (Deepak et al. 1999). Goat milk oligosaccharides play important role 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 trinitrobenzene sulphonic(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 Escherechia coli strains of the calf (Johansson et al. 2005). Mare 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 was established from recent researches that 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 nutrient, 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. Sheep milk is a rich source of amino acid, calcium, fucose which is beneficial for skin disease and cosmetic purpose. Sheep milk provides energy as well as develops immunological system, brain, neurological system and provides strength against infection (Tafaro et al. 2007). Sheep milk has some other biological effects which are due to oligosaccharides (Herrera et al. 2012) present there in. Sheep milk is rich source of fucosylated oligosaccharides which have definite biological effects like α 1, 2-linked fucosylated oligosaccharides, probably in conjugation with other families of oligosaccharide constitute a powerful innate immune system of human milk (Sharon and Ofek et al. 2000). In search for some novel biologically active oligosaccharides sheep milk was collected and their oligosaccharides were isolated and purified by following method.

MATERIAL AND METHOD

General procedure

General procedure was same as described in our earlier articles (Ranjan et al. 2015).

Isolation of milk (Sheep milk) oligosaccharide by Kobata and Ginsburg method

In continuation to our previous studies the process adapted for isolation of Sheep milk oligosaccharide were same as described earlier (Ranjan et al. 2015).

Acetylation of Sheep milk oligosaccharide mixture

Lyophilized oligosaccharides of pooled fractions (12 gm) were acetylated by treatment with pyridine (12ml) and acetic anhydride (12ml) at 60° C for 24 hr. Further the, reaction mixture was evaporated under reduced pressure and viscous residue was taken in $CHCl_3$ and washed in sequence with 2 N HCl, ice cold 2N $NaHCO_3$ and finally with H_2O. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding the acetylated mixture (15.5g). Non-polar acetyl derivative of oligosaccharides were resolved nicely on TLC using $CHCl_3$: MeOH as eluent. Detection of the spots was done by spraying with 50% H_2SO_4 and heating.

Purification of Acetylated milk oligosaccharide on Silica Gel Column

Purification of acetylated oligosaccharide mixture was carried over silica gel column chromatography: silica ratio of 1:100 using various proportion of Hexane: $CHCl_3$, $CHCl_3$, $CHCl_3$:MeOH mixture were used which was resolved into twelve fractions namely I(259mg), II(92mg), III(164mg), IV(2.05gm), V(1.95gm), VI(2.82gm), VII(120mg), VIII(286mg), IX(726mg), X(187mg), XI(342mg) and XII(55mg) respectively. These fractions were containing mixture of two to three compounds. Repeated column chromatography of fraction VI led to the isolation of one chromatographically pure compound C (62mg).

Deacetylation of Dicose

Deacetylation of acetylated oligosaccharide Dicose (62mg) was carried out in 2ml acetone and 2ml NH_3 for 24 hrs in a stoppered hydrolysis flask. After 24 hrs ammonia was removed under reduced pressure, equal volume of $CHCl_3$ and water were added and the compound was recovered in the aqueous phase and the water layer was finally freeze dried giving the deacetylated oligosaccharide Dicose (36mg).

Description of Isolated Compound Dicose

^{1}H NMR of Dicose: δ in D_2O

5.62 [d, 1H, J=4 Hz, αGlc (S-1) H-1], 5.15 [d, 1H, J=4.0Hz, α-Gal (S-6)], 4.55 [d, 1H, J=8.0Hz ,β-GalNAc (S-2)], 4.46 [d, 1H, J=8 Hz, β-GlcNAc(S-4)H-1], 4.41[d, 1H, J=8Hz, β-GlcNAc (S-3)H-1], 4.35 [d, 1H, J=8.0Hz, β-Glc (S-1) H-1], 4.33[d, J=8Hz β-GlcNAc (S-5) H-1] , 3.18[t,J=8.0Hz, β-Glc(S-1)H-2] , 2.04[s,3H, $NHCOCH_3$ β-GalNAc (S-2)], 1.98[s, 3H, $NHCOCH_3$, β-GlcNAc (S-3)], 1.96[s, 3H, $NHCOCH_3$ β-GlcNAc(S-4)], 1.80[s, 3H, $NHCOCH_3$ β-GlcNAc(S-5)]

^{13}C NMR of Dicose Acetate: δ in $CDCl_3$

102[1C β-GlcNAc(S-5) C-1], 101.0 [1C, β-GlcNAc (S-3),C-1], 95.36 [1C, β-GalNAc (S-2), C-1], 95.35 [1C, β-GlcNAc(S-4) C-1], 90.336 [1C, α-Gal (S-6), C-1], 90.208[2C, β-Glc (S-1), α-Glc(S-1)C-1]].

^{1}H NMR of Dicose Acetate: δ in $CDCl_3$

δ6.19 [d, 1H, J=4 Hz, αGlc (S-1) H-1], δ 5.36 [d, 1H, J=7.6Hz, β-Glc (S-1) H-1], 5.208 [d, 1H, J=4.0Hz, α-Gal (S-6) H-1], 4.77[d, J=8Hz β-GalNAc (S-2),H-1], 4.71[d, J=8Hz, β-GlcNAc (S-4), H-1], 4.56 [d, β-GlcNAc (S-3)H-1], 4.48 [d, 1H, J=8 Hz, β-GlcNAc(S-5)H-1].

ES Mass of Dicose

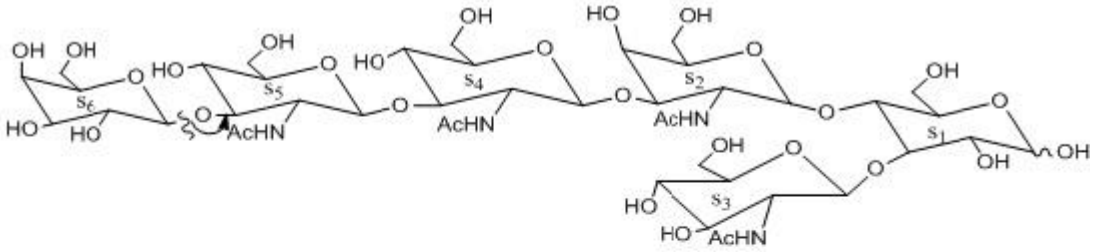
1216[M+Na+K], 1193[M+K], 1154 [M]+, 1124, 1094, 1070, 992, 977, 974, 951,956, 926, 916, 908, 878, 858, 789, 731, 713, 696, 666, 568, 553, 539, 536, 505, 476, 440, 365, 331, 316, 162.

RESULT AND DISCUSSION

The compound 'C' Dicose, $C_{44}H_{74}O_{31}N_4$ gave positive Phenol sulphuric acid test, Fiegl test (Fiegl 1975) and Morgan-Elson test, showing the presence of normal and amino sugars in the compound. The ^{1}H NMR of Dicose in D_2O at 400 MHz showed seven anomeric proton signal for seven anomeric protons in Dicose at δ 5.62 (1H), δ 5.15 (1H), δ 4.55 (1H),δ 4.46 (1H), δ 4.41 (1H), δ 4.35 (1H), δ 4.33 (1H) suggesting it to be hexasaccharide in its reducing form. The hexasaccharide nature of Dicose was also confirmed by the presence of seven crosspeaks in the anomeric region of HSQC spectrum of Dicose Acetate at 400 MHz. The values of cross peaks were present at δ 4.48x102, δ 4.56x101.08, δ 4.71x95.35, δ 4.77x95.36, δ5.20x90.33, δ 5.36x90.20, δ 6.19x90.20. The glycosidic region of HSQC spectrum of Dicose Acetate contains five crosspeaks for glycosidically linked carbons and protons i.e. in the region δ70to δ80 x δ3.5to δ4.1, confirming the nature of Dicose as Hexasaccharide. The 1H NMR of Dicose acetate at 400 MHz contains seven anomeric proton signal at value δ 6.19(1H), δ 5.36(1H), δ 5.20(1H), δ 4.77(1H), δ 4.71(1H), δ 4.56(1H) and δ 4.48. The presence of α and β anomeric protons in the 1H NMR of Dicose and Dicose Acetate confirmed the reducing nature of Dicose. Further presence of four singlets of three protons each in the region δ 1.80-2.04 in the 1H NMR of Dicose confirmed the presence of four-NAcetyl group in Dicose. The reducing nature of Dicose was also confirmed by its Methyl glycosidation followed by its acid hydrolysis which led to the isolation of α and β methyl glucosides , along with Galactose, GlcNHAc and GalNHAc suggesting the presence of Glucose at the reducing end and presence of Galactose, GlcNHAc and GalNHAc as its constituents. The six monosaccharides present in Dicose have been designated as S-1, S-2, S-3, S-4, S-5 and S-6 for convenience starting from the reducing end. To confirm the monosaccharide constituents in Dicose it was hydrolysed under strong acidic conditions by Killiani hydrolysis (Killiani et al, 1930) which gave four types of monosaccharide units i.e. Glc, Gal, GlcNHAc and GalNHAc supporting the fact that Dicose consists of these four type of monosaccharide units. ^{1}H NMR of Dicose in D_2O at 400 MHz contains two anomeric proton signals as doublets at δ5.62 (J=4Hz) and δ4.35 (J=8Hz) confirming the presence of Glucose at the reducing end, which was also confirmed by the ^{1}H NMR data of acetylated Dicose in $CDCl_3$ at 400 MHz which contained two anomeric proton signal at δ 6.19(J=4Hz) and δ 5.36(J=8Hz). The anomeric proton of Glc at δ5.36 in the ^{1}H NMR of Dicose acetate showed five cross peaks with respect to anomeric proton at δ3.71, δ4.08, δ4.46, δ4.78 and δ5.0 in the TOCSY spectrum of Dicose Acetate, showing that the two cross peaks present at δ3.71 and δ4.08 showed that the two protons of reducing glucose S-1were involved in glycosidic linkage by the next monosaccharide unit. Further the cross peak at δ3.71 and δ4.08 were confirmed as H-3 and H-4 of βGlc (S-1) by the COSY spectrum of Dicose Acetate. The ^{1}H NMR of Dicose in D_2O at 400 MHz contained a triplet at δ3.18 indicated , both the equatorially oriented hydroxyl groups present at C- 3 and C- 4 of reducing β Glc were substituted and were involved in glycosidation and also suggested that there was a presence of Lactose type of structure in it. Another anomeric proton present at δ4.55 as a doublet along with a singlet for N-Acetyl group at δ2.04 in the ^{1}H NMR of Dicose in D_2O was due to the presence of GalNHAc in the hexasaccharide as the next sugar. The large coupling constant of anomeric proton at δ4.55 (J=8Hz) confirmed the β glycosidic linkage between GalNHAc(S-2) and Glc(S-1). Since the SRG of Lactose type of linkage was supported by the triplet present at δ3.18 suggested the 1→4 β glycosidic linkage between GalNHAc(S-2) and Glc(S-1).

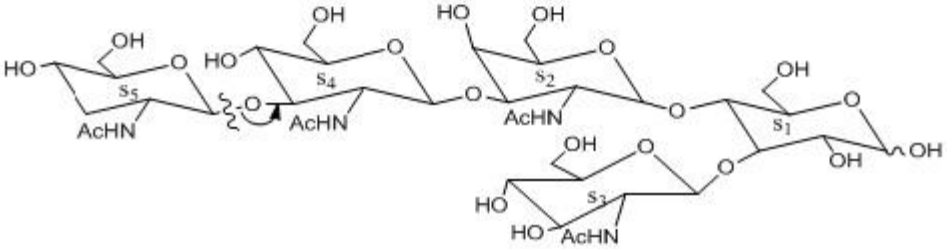
Further the next anomeric proton which was present at δ4.41along with a signal of N-Acetyl group at δ1.98 was due to presence of GlcNHAc moiety in the hexasaccharide Dicose . As it was mentioned earlier that two of the hydroxyl groups (H-3 and H-4) of Glc (S-1)were available for glycosidic linkages and since H-4 of Glc (S-1) was already linked with GalNHAc (S-2) at H-4 of Glc(S-1) the GlcNHAc(S-3) must be attached to H-3 of reducing Glc(S-1).The large coupling constant of anomeric proton of GlcNHAc (S-3) at δ4.41(J=8Hz) confirmed the β-glycosidic linkage between GlcNHAc(S-3) and Glc(S-1) hence confirming β1→3 glycosidic linkage between GlcNHAc (S-3) and Glc(S-1). All these assignments were confirmed by COSY and TOCSY spectrum of Dicose Acetate. The anomeric proton of GlcNHAc (S-3) at δ4.56 in the ^{1}H NMR of Dicose Acetate showed none of its methine proton in the glycosidic region in the TOCSY spectrum of Dicose Acetate, confirming the presence of GlcNHAc (S-3) at the non reducing end .Further the anomeric proton of GalNHAc (S-2) which was present as a doublet at δ4.77 in the ^{1}H NMR of Dicose Acetate showed 4 crosspeaks at δ3.75, δ4.15, δ5.2 and δ5.5 which could be assigned for methine protons of GalNHAc(S-2). The COSY spectrum of Dicose acetate substantiated the signal of GalNHAc H-2 methine proton at δ4.15 and H-3 proton at δ3.75 confirming that H-3 of (S-2)GalNHAc was available for glycosidic linkage by the next monosaccharide . The left over H-4 and H-5 were confirmed by the cross peaks at δ5.2 and δ 5.5 confirming the free hydroxyl of GalNHAc(S-2). The next anomeric proton which was present as a doublet at δ4.46 in the 400MHz ^{1}H NMR of Dicose in D_2O along with a peak at δ1.96 for N-Acetyl group suggested presence of another GlcNHAc in the Dicose. Since H-3 proton of GalNHAc (S-2) was available for glycosidic linkage, it was suggested that GlcNHAc (S-4) may be linked to GalNHAc (S-2). The large coupling constant of anomeric proton of GlcNHAc(S-4) (J=8Hz) suggested the β glycosydic linkage between GlcNHAc (S-4) and GalNHAc (S-2), hence confirming 1→3 β glycosydic linkage between S-4 and S-2. The presence of GlcNHAc S-4 was supported by the anomeric proton signal at δ 4.71 in the ^{1}H NMR of Dicose Acetate at 400MHz in $CDCl_3$. This anomeric proton signal of GlcNHAc showed two consecutive complementary signals in glycosidic linkage at δ3.6 and δ3.75 in the glycosidic region in the TOCSY spectrum of Dicose Acetate. These signals were assigned as H-2 and H-3 of GlcNHAc S-4 which was interpreted by COSY spectrum of Dicose Acetate, suggesting that the signal present at δ3.75 was due to the presence of methine proton attached to H-2 of S-4 containing N-Acetyl group in the other signal which was present at δ 3.6 was due to H-3 methine proton which was available for glycosidic linkage in GlcNHAc (S-4) by the next monosaccharide unit. The next anomeric proton which was present at at δ 4.33 as a doublet along with a signal of N-Acetyl group at δ1.80 in the ^{1}H NMR of Dicose in D_2O was due to the presence of another GlcNHAc unit present in Dicose. Since it was ascertained the H-3 of GlcNHac (S-4) was available for glycosidic linkage it was suggested that the GlcNHAc (S-5) must be attached to H-3 of GlcNHAc(S-4). The large coupling constant of anomeric proton present at δ4.33 (J=8Hz) suggested that GlcNHAc (S-5) was glycosidcaly linked by β 1→3 glycosidic linkage to GlcNHAc (S-4). The presence of GlcNHAc as the fifth sugar in the hexasaccharide was further confirmed by the presence of a doublet in the anomeric ^{1}H region at δ4.48 in the ^{1}H NMR of Dicose Acetate in $CDCl_3$ at 400MHz . This anomeric proton showed four cross peaks at δ 3.72, δ4.1, δ4.95 and δ 5.33. Out of these signals the signal at δ 4.1 was assigned for H-2 methine proton of GlcNHAc containing N-Acetyl group by the COSY spectrum of Dicose Acetate.

While the cross peak present at δ 3.72 was assigned toH-3 of GlcNHAc (S-5) showing that the H-3 of GlcNHAc (S-5) was available for glycosidic linkage by the next monosaccharide. The next anomeric proton which was present at δ5.11 was due to presence of α-Gal present in the hexasaccharide Dicose. Since the position 3 (H-3) of GlcNHAc (S-5) was available for glycosidic linkage , the Gal (S-6) must be linked to H-3 of GlcNHAc(S-5). The small coupling constant of anomeric proton δ5.115 (J=4Hz) confirmed the α-glycosidic linkage between S-6 and S-5, hence confirming α 1→3 glycosidic linkage between Gal (S-6) and GlcNHAc(S-5).



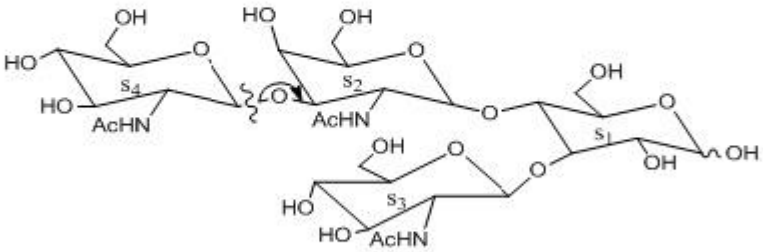
m/z 1154

-S-6



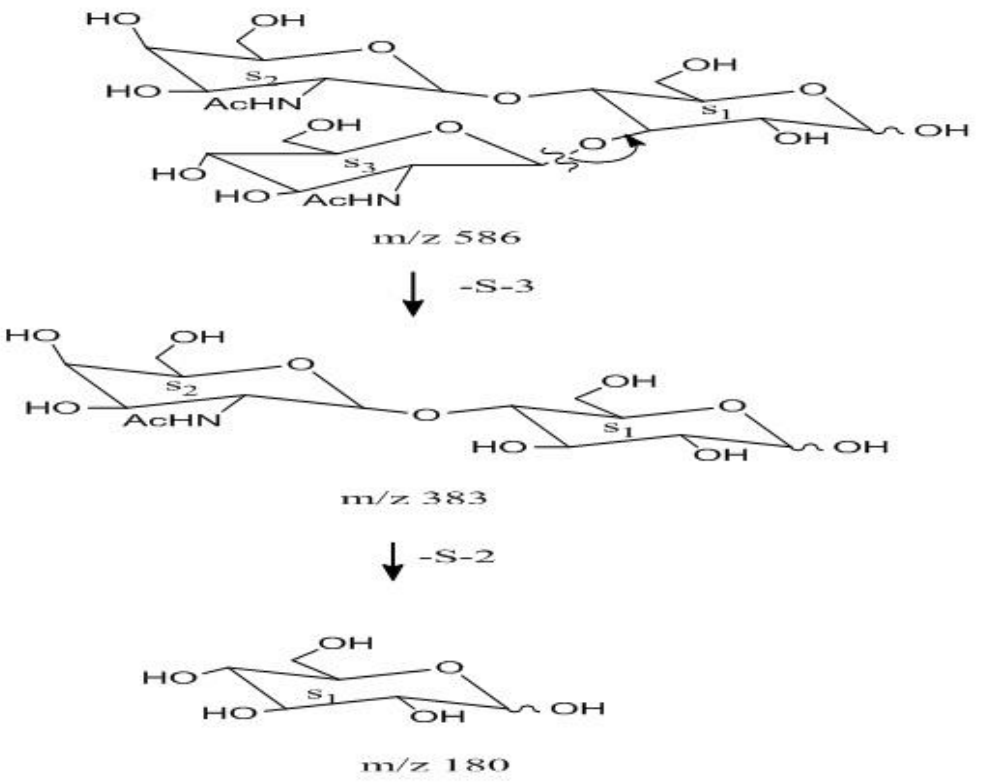
m/z 992

-S-5



m/z 789

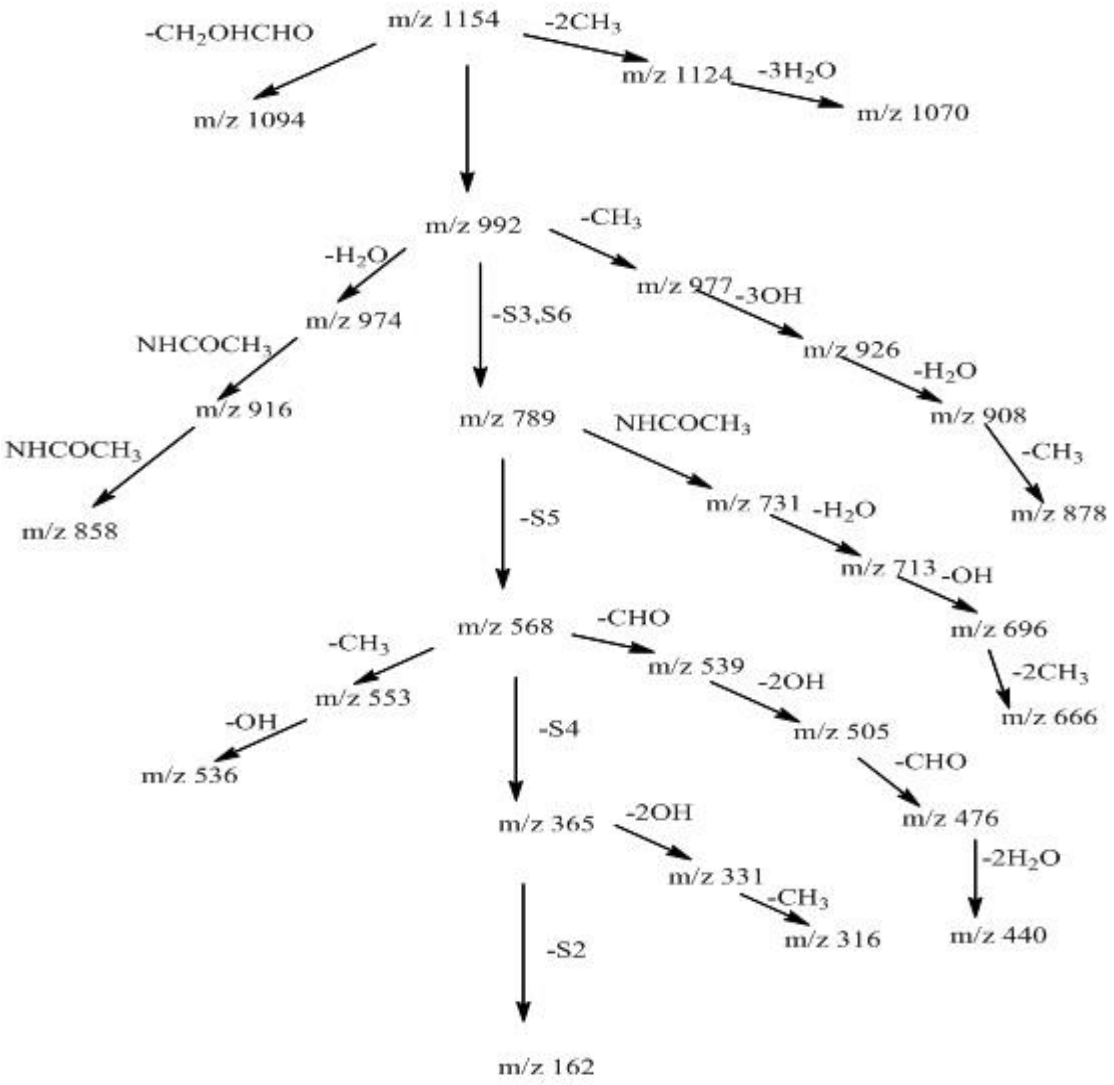
-S-4



MASS FRAGMENTATION OF COMPOUND DICOSE

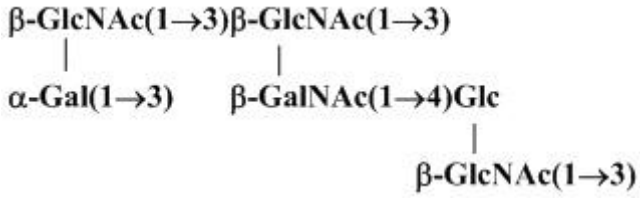
The Electronspray mass spectrometry data of Dicose not only confirmed the derived structure but also supported the sequence of monosaccharide in Dicose. The highest mass ion peaks were recorded at m/z 1216 which were due to [M+Na+K]. It also contains the molecular ion peak at m/z 1154 confirming the molecular weight of Dicose as 1154 and was in agreement with its molecular formula. Further the mass fragments were formed by repeated H transfer in the oligosaccharide and was accompanied by the elimination of terminal sugar less water. The hexasaccharide m/z 1154 (I) fragmented to give mass ion at m/z 951(II) [1154-S_3], this fragment arised due to the loss of terminal GlcNHAc (S_3) moiety from hexasaccharide indicating the presence of GlcNHAc (S_3) at the non -reducing end. It further fragmented to give mass ion peak at m/z 789 (III) [751-S_6] which was due to loss of Gal (S_6) moiety from pentasaccharide.

This fragment of 789 further fragmented to give mass ion peak at m/z 568 (IV) [789-S_5] which was due to loss of GlcNAc (S_5) moiety from the tetrasaccharide, which further fragmented to give mass ion peak at m/z 365 [568-S_4] which was due to loss of GlcNHAc (S_4) moiety from trisaccharide. This fragment of disacharide at 365 further fragmented to give mass ion peak at m/z 162 (IV) [365-S_2], which was due to loss of GalNAc (S_2) moiety from disaccharide. These five mass ion peak II, III, IV, V, VI, appeared due to the consequent loss of S_3 S_6 S_5 S_4 and S_2 from original molecule. The mass spectrum also contain the mass ion peak at m/z 789, 587, 585 and 588 correspond to the mass ion fragment A, B, C, D which confirm the position of S_1, S_2, S_3 ,S_4, S_5, S_6. The mass spectrum also showed the other usual losses of OH and CH_2OH groups.



MASS FRAGMENTATION OF COMPOUND DICOSE

Based on result obtained from chemical degradation/acid hydrolysis, chemical transformation, Electrospray mass spectrometry and ^{1}H, ^{13}C NMR and HOMOCOSY, TOCSY and HSQC 2D NMR technique of acetylated Dicose and Dicose, the structure and sequence of isolated novel oligosaccharide molecule Dicose was deduced as-



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