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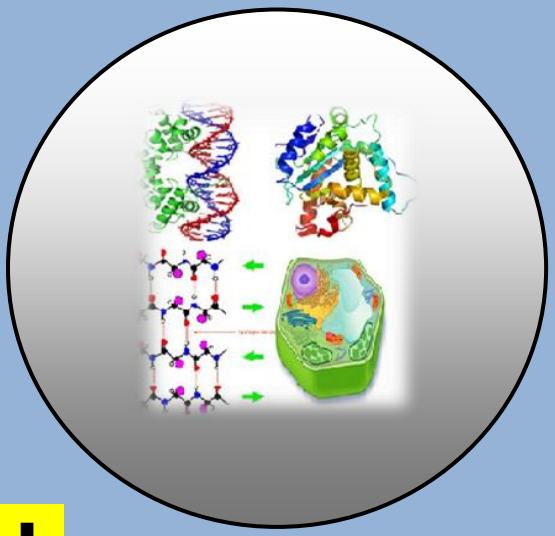

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RESEARCH PAPER

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Structure Elucidation of a Novel Pentasaccharide Meeniosefrom *Babalus bubalis* Colostrum

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ABSTRACT

Oligosaccharides present in the milk of different origin have shown varied biological activities. Oligosaccharide isolated from Buffalo milk has shown encouraging immune stimulant activities. With a view to isolate more biologically active oligosaccharides from colostrum of buffalo (Babalus bubalis). Colostrum was collected and processed by modified method of Kobata and Ginsberg, involving deproteination, centrifugation and lyophilization followed by acetylation and silica gel column chromatography of derivatized oligosaccharides. The structure of isolated penta saccharide was elucidated by chemical transformation, chemical degradation, ^{1}H NMR, ^{13}C NMR, 2D NMR (COSY, TOCSY and HSQC) and mass spectrometry. The geometry of isolated compound Meeniose was optimized at B3LYP method and 6-311+G (d, p) basis set. In the light of results obtained from above experiments, the structure of novel pentasaccharides meeniose was confirmed as β-GalNAc (1→2)- β-Gal(1→4)- β-GlcNAc (1→3) -β-GalNAc (1→4)- Glc.
KeyWords: Carbohydrate, Babalus bubalis, Colostrum, Spectroscopy, Pentasaccharide and Meeniose.

INTRODUCTION

Milk is a natural source for the physiological and biological development of any neonate provided to him by his mother in any mammal. It is a biological fluid of unique complexity and richness (Casado et al., 2009). It contains all necessary nutrients for the growth and development of the newborn. Besides the normal milk, the milk of early days i.e. 1-5 days which is called colostrum have important role in the development of infant. Both milk and colostrum are rich resource of oligosaccharides. Oligosaccharides have established themselves as an effective class of organic bio molecules impacting various physiological and pathological processes such as molecular recognition, signal transaction, differentiation and developmental events and exhibit varied biological activities such as antitumor

(Schwonzen et al., 1992), immune stimulant (Abe et al., 1983), anticancer (Fang et al.,1985), & immunological activities (Srivastava et al.,1989). Oligosaccharides isolated from various milk sources are categorized under classes i.e. sialylated and non sialylated. Both these class of oligosaccharide have been tested for their varied biological activities (Ferdouse et al., 2014). Milk oligosaccharides are important parts of these functional ingredients (Peterson et al., 2013 and Barboza, et al., 2012). Numbers of biologically active oligosaccharides have been isolated from human, buffalo, donkey, cow, mare, sheep and goat milk (Claeys et al., 2014). The milk oligosaccharides recognize cancer associated antigens, used as antimicrobial agents; tumor associated antigens and has physiological significance in infants (Fujimura et al., 2010). Donkey and Buffalo milk oligosaccharides have shown promising immune stimulant activity. (Deepak et al., 1998 and Saxenaet al., 1999). Keeping in mind the immune stimulant activity of buffalo milk and importance of colostrum it was thought to isolate some novel, biologically active oligosaccharide from colostrum of buffalo (*Babalus bubalis*).Present research work includes isolation of novel oligosaccharide from colostrum of buffalo and elucidation of its three dimensional structure by chemical degradation, chemical transformation and spectroscopic techniques like ^{1}H NMR, ^{13}C NMR, 2D NMR (COSY, TOCSY and HSQC) and mass spectrometry, Further, quantum chemical calculation has been performed to determine the lower geometry structure and stability of the isolated and characterized compounds.

MATERIAL AND METHODS

Collection and storage of buffalo colostrum-Twelve (12) liter of buffalo colostrum (1 to5 day) was collected from a domestic buffalo (*Babalus bubalis*) from the Kharika village near Telibagh of district Lucknow, Uttar Pradesh. The colostrum was fixed by addition of equal amount of ethanol (12 liter).

Isolation of colostrum oligosaccharides by modified method of Kobata and Ginsburg-Isolation of colostrum oligosaccharides was done by modified Kobata and Ginsburg (1970) method. The preserved colostrum was taken to laboratory and there it was cooled at 4°C and centrifuged for 30 min at 5000 rpm at 4°C. The solidified lipid layer was removed by filtrating 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 over night at 0°C. The white precipitate formed, mainly of lactose and protein was removed by centrifugation and washed twice with 68% ethanol at 0°C. The supernatant and washing were combined and filtered through a micro filter and lyophilized affording crude oligosaccharides mixture.

Acetylation of oligosaccharide mixture-The oligosaccharides are very polar in nature hence there isolation is not an easy task. Therefore, oligosaccharide mixture (8 gm) was acetylated by acetic anhydride and pyridine for getting the oligosaccharides as its acetyl derivative (9.60 gm). Theacetylated oligosaccharide mixtures were purified by column chromatography.

The structure elucidation of isolated and purified novel oligosaccharide was performed by chemical transformation, chemical degradation and spectroscopic technique ^{1}H NMR, ^{13}C NMR, 2D NMR (COSY, TOCSY and HSQC) and mass spectrometry,. Further, quantum chemical calculation was performed to determine the lower geometry structure and stability of the isolated and characterized compound.

Deacetylation of compound

Compound (44 mg) was obtained from column chromatography of acetylated oligosaccharide mixture. 30 mg of compound was dissolved in acetone and NH_3 was added in it and was left overnight in a stopper hydrolysis flask. After 24 h ammonia was removed under reduced pressure and the compound was washed with $CHCl_3$ (to remove acetamide) and the water layer was finally freeze dried giving the deacetylated oligosaccharide (25 mg).

General Procedure for structure elucidation

The ^{1}H and ^{13}C NMR spectra of oligosaccharides were recorded in D_2O and the spectra of acetylated oligosaccharides were recorded in $CDCl_3$ at 25°C on a Bruker AM 300 and 400 FT NMR spectrometer. The electro spray mass spectra were recorded on a MICROMASS QUATTRO II triple quadruple mass spectrometer. The sample (dissolved in suitable solvents such as methanol/acetonitrile/water) was introduced into the ESI source through a syringe pump at the rate 5µl per min. The ESI capillary was set at 3.5 KV and the cone voltage was 40 V. The spectra were collected in 6s scans and the printouts are recorded spectra of 6-8 scans. The C, H and N analysis were recorded on elemental analyzer CARLO-ELBA 1108. The sugars were visualized on TLC with 30% aqueous H_2SO_4 reagent and on paper chromatography (PC) sugars were visualized with acetyl acetone and p-dimethyl amino benzaldehyde reagents. The absorbent for TLC was silica gel G (SRL) and CC silica gel (SRL, 60-120 mesh). PC was performed on Whatman No.1 filter paper using solvent system ethyl acetate-pyridine (2:1) saturated with H_2O. To check the homogeneity of the compounds reverse phase HPLC system was used equipped with Perkin Elmer 250 solvent delivering system, 235 diode array detector and G.P. 100 printer plotter. Authentic samples of glucosamine, galactosamine, galactose, glucose, fucose and sialic acid were purchased from Aldrich Chemicals.

Methyl glycosidation/Acid hydrolysis of compound

Compound (5 mg) was refluxed with absolute MeOH at 70°C for 18 h in the presence of cation exchange IR-l20 (H) resin. The reaction mixture was filtered while hot and filtrate was concentrated. In the solution of 1, 4-dioxane (1 ml), and 0.1N H_2S0_4 (1 ml) was added and the solution was warmed for 30 minutes at 50°C. The hydrolysis was complete after 24 h. The hydrolysate was neutralized with freshly prepared $BaCO_3$ filtered and concentrated under reduced pressure to afford α-and β-methylglucosides along with the Glc, GalNAc and GlcNAc. Their identification was confirmed by comparison with authentic samples (TLC, PC).

Kiliani hydrolysis of compound

Compound (5 mg) was dissolved in 2 ml Kiliani mixture (AcOH-H_2O-HCl, 7:11:2) and heated at 100°C for 1 h followed by evaporation under reduced pressure. It was dissolved in 2 ml of H_2O and extracted twice with 3 ml $CHCl_3$. The aqueous residual solution was made neutral by addition of 1-2 drops of 2N NaOH and was evaporated under reduced pressure to afford Glucose, Galactose, GalNAc and GlcNAc on comparison with authentic samples of Glucose, Galactose, GalNAc and GlcNAc. (Killiani, 1930).

RESULT AND DISCUSSION

Compound Meeniose, $C_{36}H_{61}O_{28}N_3$, gave positive Phenol-sulphuric acid test (Dubois et.al., 1956), Fiegltest (Fiegl, 1975) and Morgon-Elson test (Gey,et.al., 1996) showed the presence of normal and amino sugar moieties.

The HSQC spectrum of acetylated Meeniose showed the presence of six cross peaks of anomeric protons and carbons in the respective region at δ 6.19 x 88.82, δ 5.62 x 91.40, δ 5.62 x 91.40, δ 5.03x 101.04, δ 4.44 x 100.79, δ 4.43 x 100.79 suggesting the presence of six anomeric protons and carbons in it. The presence of six anomeric protons were further confirmed by the presence of six anomeric proton doublets at δ6.19 (1H), δ5.62 (2H), δ5.03 (1H), δ4.44 (1H), δ4.43 (1H) in the ^{1}H NMR spectrum of acetylated Meeniose in $CDCl_3$ at 400 MHz. The presence of six anomeric carbons also confirmed by the presence of six anomeric carbon signals at δ101.04 (1C), δ100.79 (2C), δ91.40 (2C) and δ88.82 (1C) in the ^{13}CNMR spectrum of acetylated Meeniose in $CDCl_3$Meeniose at 400 MHz. The presence of down field shifted α and β anomericprotons and also α and β anomeric carbons in ^{13}CNMR of Meeniose acetate suggested that compound Meeniose may be a pentasaccharide in its reducing form. The reducing nature of compound Meeniose was further confirmed by methylglycosylation of compound Meeniose by MeOH/H^+ followed by its acid hydrolysis which led to the isolation of α and β-methyl glucosides, suggested the presence of glucose at the reducing end. Forconveniencethe five monosaccharide present in compound Meeniose have been designated as S-1, S-2, S-3, S-4 and S-5 respectively starting from glucose (S-1) at the reducing end. The monosaccharide constituents of Meeniose were confirmed by its Killianihydrolysis (Killiani et al., 1930), under strong acidic conditions, followed by paper chromatography (PC) and thin layer chromatography (TLC). In its hydrolysis four spots were found on TLC and PC which were found identical with Glucose, Galactose, GlcNAc and GalNAc by co-chromatography with the authentic samples, thus confirming that the pentasaccharide contained four types of monosaccharide units i.e., Glc, Gal, GalNAc and GlcNAc in it. The chemical shifts values of anomeric protons and carbon observed in ^{1}H NMR and ^{13}C NMR spectrum of Meeniose were also in agreement with the reported values of ^{1}H and ^{13}C anomeric chemical shifts of Glc, Gal, GalNAc and GlcNAc confirming the presence of these monosaccharides in the compound Meeniose. The presence of two anomeric proton signals at δ6.19 (J=3.6 Hz) and δ 5.62 (J=8.0 Hz) in the ^{1}H NMR spectrum of Meeniose acetate in $CDCl_3$ at 400 MHz for α and βanomers of reducing monosaccharides (S-1) which was later identified as glucose by comparing the chemical shift data of ^{1}HNMR and ^{13}C NMR and also from the results obtained by methylglycosylation by MeOH/H^+ followed by its acid hydrolysis. The anomeric protons signal present at 5.62 in the^{1}HNMR of Meeniose acetate contains cross peaks at δ5.62 x3.80, δ5.62 x 5.0, δ5.62 x 5.20, in its TOCSY spectrum .Out of which one cross peak arised at δ5.62 x 3.80 suggested the position of proton of linkage into reducing S-1 which was later identified as H-4 of glucose S-1 by COSY spectrum of meeniose acetate. Suggesting that the H-4of glucose S-1 was available for glycosidation by the next monosaccharide S-2 confirming the 1→4 linkage between S-2 → S-1 .The 1→4 linkage between (S-2) and Glc (S-1) was also supported by the position of H-4 of S-1at δ3.80 acetylated Meeniose at 400 MHz in $CDCl_3$. Further the presence of another anomeric proton doublet at δ4.43 (J=8.0 Hz) along with a singlet of amide methyl (-$NHCOCH_3$) at δ2.10 was due to presence of β-GalNAc (S-2) moiety. The coupling constant of anomericsignal (S-2) with J value 8.0 Hz confirmed the β-configuration of the S-2 moiety. This anomeric proton signal at δ4.43 contain four cross peaks at δ4.43 x3.74, 4.43 x4.07, 4.43 x4.90, 4.43 x5.30 in its TOCSY spectrum, out of which one cross peak arised at δ 4.43 x4.07 corresponds to H-2 position of β-GalNAc (S-2).

And another peaks arised at δ 4.43 x 3.74 corresponds to linkage region of β-GalNAc(S-2)which is later defined as H-3 of β-GalNAc (S-2) by COSY spectrum of Meeniose acetate which was available for (1→3) glycosidic linkages by the next monosaccharide i.e.(S-3) unit. The next anomeric proton signal which appeared as doublet at δ 5.62 (S-3) (J=8.0 Hz) along with a singlet of amide methyl (-$NHCOCH_3$) at 1.92 was due to presence of GlcNAc (S-3).Suggesting that the H-3 of β-GalNAc (S-2) was available for glycosidation by the next monosaccharide S-3, confirming the 1→3 linkage between S-3 → S-2. The 1→3 linkage between GlcNAc (S-3) and β-GalNAc (S-2) was also supported by the position of H-3 of S-2 at δ3.74 acetylated Meeniose at 400 MHz in $CDCl_3$.Further another anomeric proton signal present at δ 5.62 (J=8.0) was assigned to β- GlcNAc (S-3). Contains four cross peaks δ5.62 x3.80, δ5.62 x 5.00, δ5.62 x 5.20, δ5.62x5.63 in its TOCSY Spectrum. Out of which the one cross peaks δ 5.62x3.80 corresponding to linkage region of GlcNAc (S-3) which was later defined as H-4 of β- GlcNAc (S-3) by COSY spectrum of Meeniose. Later this signal of δ5.62 x3.80 was ascertained as H-4 of β-GlcNAc (S-3) by COSY and TOCSY spectrum of Meeniose acetate showing that H-4 of β-GlcNAc (S-3) was available for glycosidic linkage by the next monosaccharide unit (S-4). Further another anomeric proton signal present at δ 5.03 (J=8.0) was assigned to β-Gal (S-4).Confirming the 1→4 linkage between S-4 → S-3. The 1→4 linkage between β-Gal (S-4) and β-GlcNAc (S-3) was also supported by the position of H-4 of S-3 at δ3.80 acetylated Meeniose at 400 MHz in $CDCl_3$.Further anotheranomeric proton signal present at δ 5.03 (J=8.0) was assigned to β-Gal (S-4). This anomeric proton contains two cross peaks at δ5.03 x3.74, δ5.03 x4.50 in its TOCSY Spectrum of Meenioseacetate. Out of which the one cross peaks δ 5.03 x3.74 corresponded to linkage region showing that H-2 of β-Gal (S-4) .The H-2 of S-4 available of glycosidic linkage by the next monoaccharide unit S-5. Another anomericprotn signal δ 4.44 (J=7.6 Hz) along with a singlet amide methyl (-$NHCOCH_3$) at δ 1.96 was defined for the presence of β-GalNAc (S-5), confirming the 1→2 linkage between S-5→S-4. The 1→2 linkage between GalNAc (S-5) and β-Gal (S-4) was also supported by the position of H-2 of S-4 at δ3.74 acetylatedMeeniose at 400 MHz in $CDCl_3$.The coupling constant of anomeric signal (S-4) δ5.03 with J value 8.0 Hz confirmed the β-configuration of the S-4 moiety which linked with β-GlcNAc (S-3). Another anomericproton signal δ 4.44 (J=7.6 Hz) along with a singlet amide methyl (-$NHCOCH_3$) at δ 1.96 was defined for the presence of β-GalNAc (S-5) this anomeric proton contain only one cross peak at δ4.44 x 4.08 in the TOCSY spectrum of Meeniose acetate. This cross peak was later defined for H-2 of S-5 i.e. H-2 of GalNAc (S-5) by the COSY spectrum of meeniose acetate, This also suggested that it does not contain any methine protons in glycosidic linkage region i.e., δ 3-4 ppm confirming that none of -OH group of β-GalNAc (S-5) were involved in glycosidic linkages hence, confirmed that β-GalNAc(S-5)were present at non-reducing end and none of their -OH group were available for glycosidic linkages, which was confirmed by the TOCSY spectrum of acetylated Meeniose . All the ^{1}HNMR assignments for ring proton of monosaccharide units of Meeniose were confirmed by COSY and TOCSY experiments. The positions of glycosidation in the oligosaccharide were confirmed by position of anomeric signals, comparing the signals in ^{1}H and ^{13}C NMR of acetylated Meeniose. The glycosidic linkages in Meeniose were also confirmed by the cross peaks for glycosidically linked carbons and protons in the HSQC spectrum of acetylated Meeniose. The values of these cross peaks was appeared as Glc(S-1) H-4xC-4 at δ 3.80 x 75 showed (1→4) linkage between S-2 and S-1, β-GalNac(S-2) H-3 x C-3 at δ 4.07 x 70 showed (1→3)

linkage between S-3 and S-2, β-GlcNAc (S-3) H-4xC-4 at δ 3.80x73 showed (1→4) linkage between S-4 and S-3, β-Gal(S-4) H-2 x C-2 at δ 3.74X 73 showed (1→2) linkage between S-5 and S-4. All signals obtained in ^{1}H and ^{13}C NMR of compound Meeniose was in conformity by 2D ^{1}H-^{1}H COSY, TOCSY and HSQC experiments. Thus, based on the pattern of chemical shifts of ^{1}H, ^{13}C, COSY, TOCSY and HSQC NMR experiments it was interpreted that the compound Meeniose was a pentasaccharide as shown in Figure 3.

The electro spraymass spectra of Meeniose is shown in Figure 1 and confirmed the derived structure but also supported the sequence of monosaccharide in Meeniose.

Figure 1.ES Mass fragmentation of Meeniose.

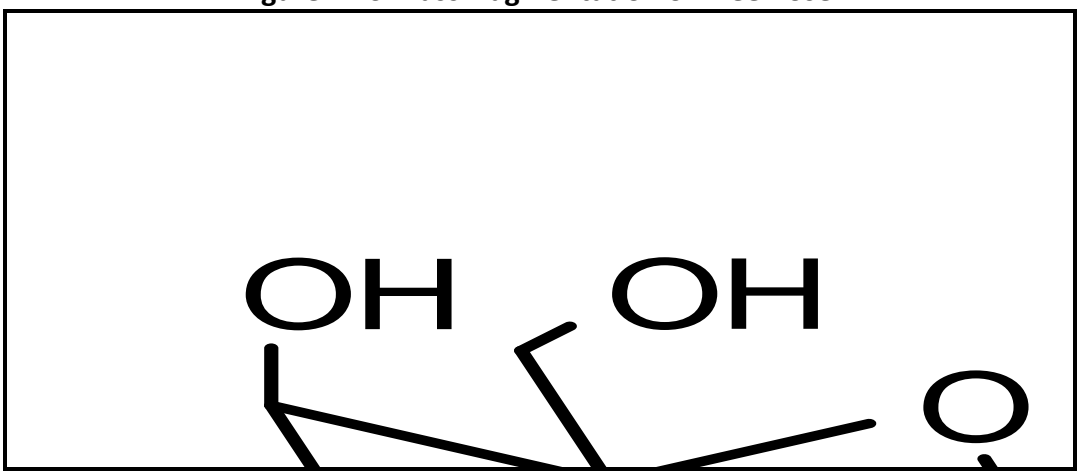
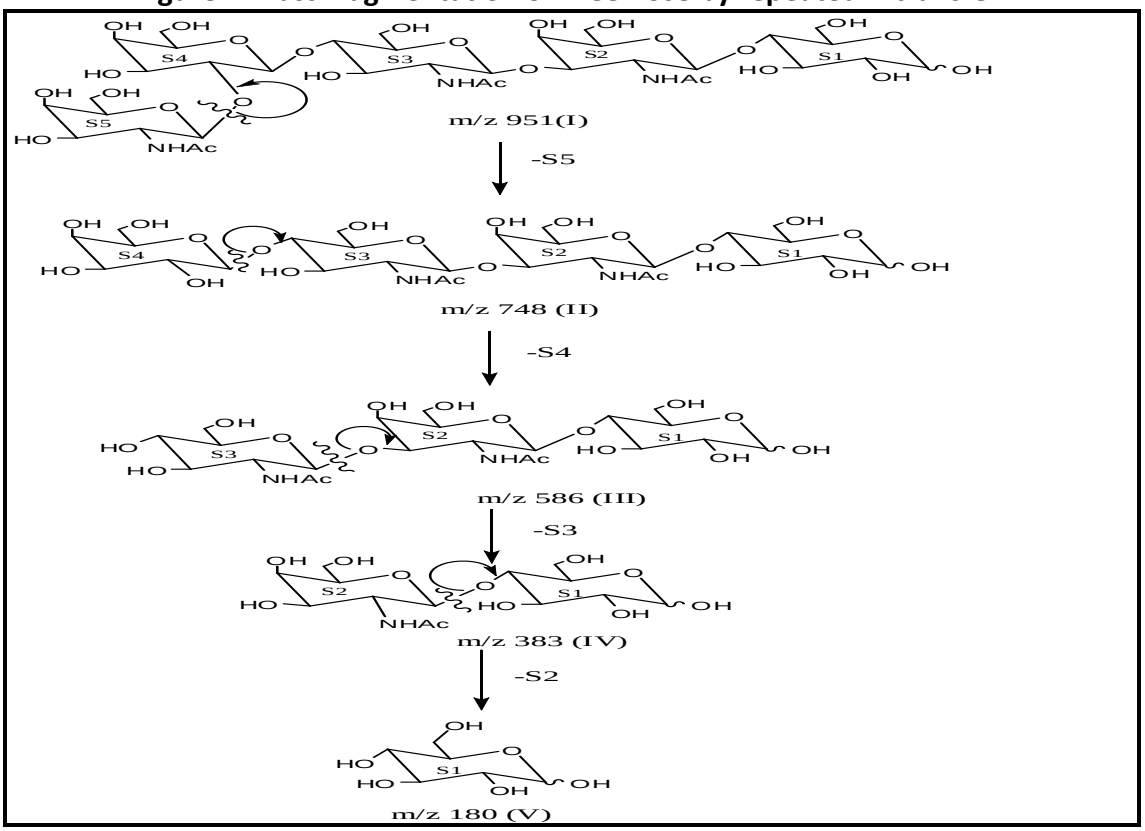


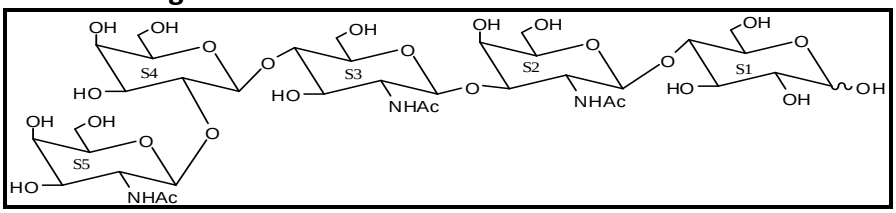
Figure 2.Mass fragmentation of Meeniose by repeated H transfer.



The highest mass ion peaks were recorded at m/z 1013 assigned to $[M+Na+K]^+$ and m/z 974 assigned to $[M+K]^+$, it also contain the molecular ion peak at m/z 951 confirming the molecular weight as 951 which was in agreement with its molecular formula $C_{36}H_{61}O_{26}N_3$. 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 pentasaccharide m/z 951 (I) fragmented to give mass ion at m/z 748(II) [951-S_5] which was a tetrasaccharide (II) ,this fragment was arised due to the loss of 203 in GalNAc (S_5) moiety from pentasaccharide (I). The complete fragmentations of compounds is shown in Figure 2

It further fragmented to give mass ion peak at m/z 586(III) which was a trisaccharide moiety (III) [748-S_4] and was due to loss of 162 in Gal (S-4) moiety from tetrasaccharide. This fragment of 586 further fragmented to give mass ion peak at m/z 383 (IV) [586-S_3] which was a disaccharide (IV), was due to loss of 203 as GlcNAc (S_3) moiety from the tetrasaccharide. This disaccharide (IV) unit further fragmented to give mass ion peak at m/z 180 (V) [383-S_2], which was due to loss of 203 in GalNAc (S_2) moiety from disaccharide. These four mass ion peak II, III, IV, V were appeared due to the consequent loss of S_5, S_4, S_3 and S_2 from original molecule. The mass spectrum also contain the mass ion peak at are m/z 586, 424 correspond to the mass ion fragment A,B,(Figure 1) which confirm the position of S_1 S_2, S_3 ,S_4, S_5. The m/z of fragmented moieties of compound Meeniose as -$NHCOCH_3$ (58), -CH_2OH (31), -OH(17), -CHO(29), -H_2O(18), -CH_2OHCHO(60). The other fragmentation pathway in ES Mass spectrum of compound Meeniose at m/z 951 shows the mass ion peaks at 933 [951-H_2O], 893 [910-$NHCOCH_3$], 862 [893-CH_2OH], 700 [748-OH, CHO], 657 [748-CH_2OHCHO, CHO], 490 [586-$2H_2O$, CH_2OHCHO],528[586-$NHCOCH_3$], 318 [383-CH_2OH, 2OH], 258[318-CH_2OHCHO], 296[383-$NHCOCH_3$,CHO], 180[383-S-2]. Based on result obtained from chemical degradation/acid hydrolysis, chemical transformation, electro spray mass spectrometry and ^{1}HNMR,^{13}C NMR and 2D NMR technique of acetylated Meeniose shown in Figure 3.

Figure 3.The elucidate structure of Meeniose.



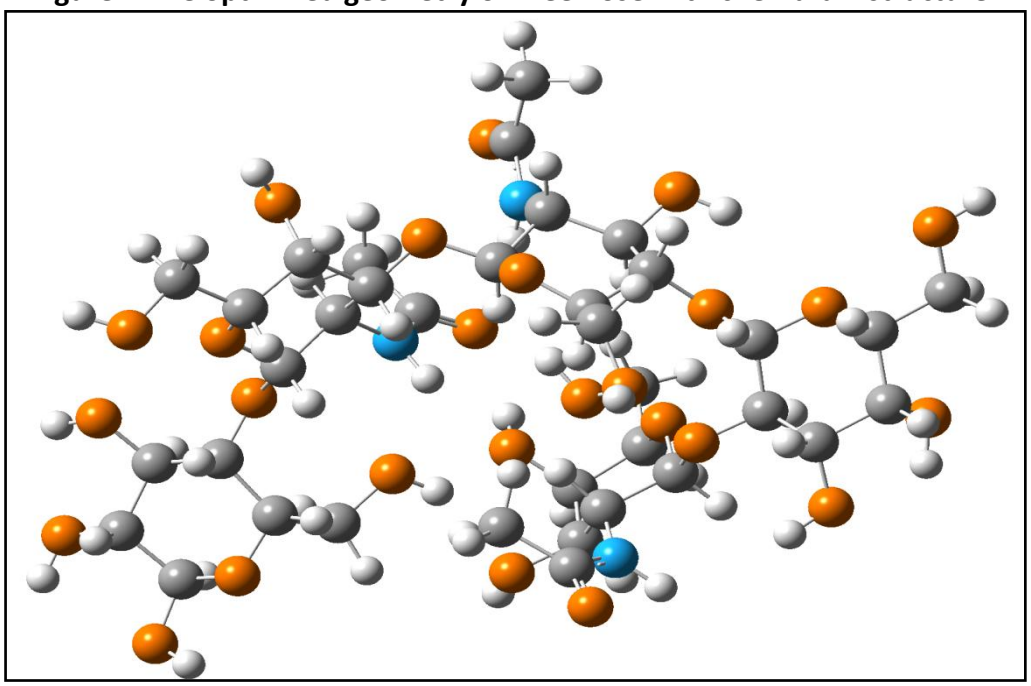
β-GalNAc(1→2)- β-Gal(1→4)- β-GlcNAc(1→3) -β-GalNAc(1→4)- Glc

Computational study and stability of Molecular geometry of the isolated compound

The quantum chemical calculation has been performed on B3LYP functional and 6-311+G (d, p) basis set. Geometry of compound Meeniose has been first optimized and the presence of positive wave numbers values for all the optimized geometry indicates stability of the compounds. All computations were performed using the Gaussian 09 program package (Frisch, 2009). As we know that molecular geometry for determining the structure-activity relationship, conformational analysis plays a very important role. The geometries of compound Meeniosehas been optimized at B3LYP method and 6-311+G (d,p) basis set. The optimized molecular geometry of compound Meeniose has been given in Figure 4.

The molecular geometries can be determined by the quantum mechanical behavior of the electrons and computed by *ab-initio* quantum chemistry methods to high accuracy. Molecular geometry represents the three-dimensional arrangement of the atoms that determines several properties of a substance including its reactivity, polarity, phase of matter, color, magnetism, and biological activity. The optimized geometries of compounds show positive wave numbers values indicated the stability of the compound Meeniose. The total energy level of compound is 54 kcal/mol.

Figure 4-The optimized geometry of Meeniose with chemdraw structure.



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