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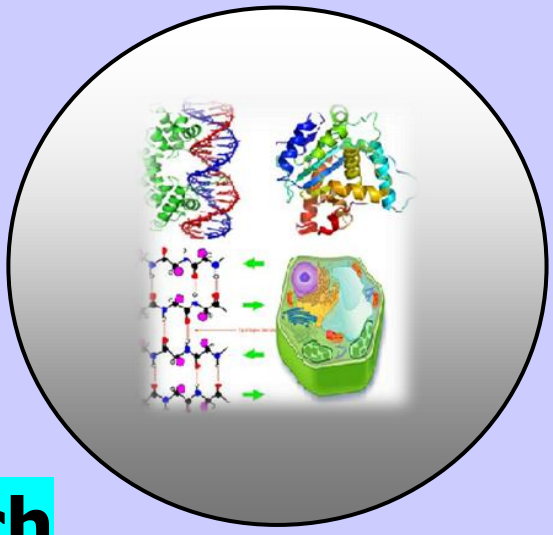

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Muzeeb Khan
http:// www.sasjournals.com
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jbiolchemres@gmail.com

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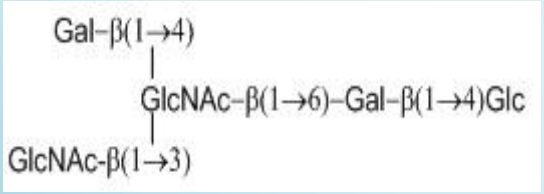
Structure Elucidation of Novel Oligosaccharide from Shyama Dhenu Milk and their DFT Studies

Muzeeb Khan, Sheelu Sharma, Deepali Narain, Anil Mishra, Anakshi Khare and Desh Deepak
Department of Chemistry, University of Lucknow, Lucknow 226007, U.P., India

ABSTRACT

Oligosaccharides present in milk are exhibiting an assortment of biological activities, which are responsible for development of immune system of new born babies as well as adults. Oligosaccharides which have been isolated from different bovine milk exhibit high potent biological activities like antiviral, anticancer, antitumor, antioxidant, antibacterial, antifungal, antimicrobial, antihepatitis, anti-inflammatory, anticoagulant, immunostimulant, antituberculosis. For this cow milk was chosen in search of novel milk oligosaccharides which have importance in medicinal field. The milk oligosaccharide was isolated by given method of Kobata and Ginsburg followed by gel filtration HPLC and CC resulted into the isolation of a novel milk oligosaccharide Indose. The stereoscopic structure of the purified compound was elucidated with the help of chemical degradation, chemical transformation and stereoscopic techniques like 1H, 13C and 2D NMR and mass spectrometry. The optimized geometry of compound B (Indose) at B3LYP method and 6-311+G basis set on Gaussian 09 program; show that the compound B was stable compound.



INDOSE

Keyword: Antimicrobial, Bovine milk, Oligosaccharide, Indose, Immunostimulant 2D NMR and mass spectrometry.

INTRODUCTION

Milk is complete food source for developing mammals. Milk have glycoprotein's which play key role in protection of infants by reducing the number of pathogen infections and promoting the development of the intestinal epithelium (G.V. Coppa, et. al. 2006, A.M. Zivkavic, et. al. 2010). Milk oligosaccharides relatively resistant to digestion (2000) and contribute to the anti-infective and prebiotic activities (C. Kunz, et. al. 2000, G.V. Coppa, et. al. 2004). Oligosaccharides present in the milk of different animals e.g. cow, buffalo, donkey, elephant, mare, yak, human, etc contains high concentration of bioactive oligosaccharides (G. Boehm, et. al. 2007, U. K. Sundekilde, et. al. 2012). These oligosaccharide exhibits wide-ranging biological activities like anti-tumour, anti-cancer, anti-viral, anti-inflammatory, anticoagulant, antioxidant and immunostimulant activity (M. Schwonzen, et. al. 1992, K. Abe et al. 1983, R. Srivastava, 1989). Cow milk oligosaccharides reduce the adhesion of enterotoxic Eschererchia coli strains of the calf (P. Johansson, et. al. 2005). Goat milk oligosaccharides have ability to intestinal protection and repair after damage caused by DSS (Dextron sodium sulphate) induced colitis and their implication in human intestinal inflammation (F. Lara-Villoslada et al. 2006) and also have anti-inflammatory effects in rats with trinitrobenzenesulfonic (T) acid induced colitis and useful in the management of inflammatory bowel disease (J. Hakkarainen et. al. 2005). Buffalo milk oligosaccharides have capability to stimulate non-immunological resistance of the host opposed to parasitic infections (R. Saksena, et. al.1999). Donkey milk oligosaccharides have ability to stimulate specific and non-specific immunological resistance (Desh Deepak et al, 1998) and helpful in cure of AIDS patients and in prevention of atherosclerosis (A. Tafaro, et al., 2007). Mare's milk has shown anti oxidant, lipid lowering and post heparin lipolytic activity (Srivastava A. et. al. 2012). Elephant milk oligosaccharides have high ratio of sialyl oligosaccharide; this help to the formation of brain components, such as gangliosides of the suckling calves (G. Osthoff et. al. 2007). The Dog milk oligosaccharide has dominant N-acetylneuraminlactose sulphate (W. A. Bubb, et. al.1999) which plays a key role in the nutrition of the rat pups. Human breast milk plays an important role in gut colonization and modulation of the infant's guts (S. Divya et. al. 2013). The cow milk is important for human life which is written in ancient literature. Indian ancient Physician Dhanvantri confirmed that it protects the human from heart diseases and leucoderma. The Ayurveda has described the medicinal importance of black cow milk and Rigveda says that Cow milk is Amrita, protects human being from diseases, its milk have the curative and prophylactic effects. Several studies supported the constructive effects of supplementation of cow milk in diarrhea in human with immune-deficiency syndrome, NSAID-induced gastrointestinal disturbances.

For this purpose Shyama Dhenu (black cow) milk was collected in bulk and processed by method of Kobata and Ginsburg (A. Kobata et.al. 1970). In continuation to our previous work on isolation of shyama Dhenu (Gunjan et al. 2016, Lata et al. 2016) another novel milk oligosaccharide was isolated from the cow's milk and then its structure was elucidated with the help of chemical degradation, chemical transformation and spectroscopic method like ^{1}H NMR, ^{13}C NMR and 2DNMR (COSY TOCSY, HSQC) technique as well as mass spectrometry.

Theoretical study

The quantum chemical calculations have been analyzed on basis set of B3LYP functional and 6-311+G (d, p).

Geometries of compound B have been first optimized and the presence of positive wave numbers values for all the optimized geometry indicates stability of the compounds. All the isolated compounds were described by computational data using the Gaussian 09 program package (M. J. Frisch et al. 2009).

MATERIAL AND METHODS

General procedure

General procedures were same as described in our previous articles (Gunjan et. al. 2016).

Isolation of Shyama dhenu milk oligosaccharide by Kobata and Ginsburg method

In continuation to our previous studies the Shyama dhenu milk was processed for isolation of its oligosaccharide contents as described in our earlier communications (Gunjan et al. 2016) Obtaining 150g of oligosaccharide mixture.

Acetylation of Shyama dhenu milk oligosaccharide mixture

12g of crude oligosaccharide mixture was acetylated with pyridine (12 ml) and acetic anhydride (12 ml) at 60^0C and solution was stirred overnight. Further the mixture was evaporated under reduced pressure and the viscous residue was taken in $CHCl_3$ (250ml) and washed with water (25 ml). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding the acetylated mixture (15.5g). The acetylation converted the free sugars into their nonpolar acetyl derivatives which were resolved nicely on TLC, giving eight spots on TLC i.e. A, B, C, D, E and F of which two compounds were finally separated by column chromatography over silica gel (60-120 mesh) using hexane: $CHCl_3$ and MeOH: $CHCl_3$ as eluents.

Purification of Acetylated milk oligosaccharide on Silica Gel Column

The acetylated oligosaccharide mixture (10 g) was purified by column chromatography. the silica was used in the ratio of 1:100 using various proportions of Hexane $CHCl_3$, $CHCl_3$, $CHCl_3$:MeOH mixture 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 B (59mg).

Deacetylation of Compound B (Indose)

Compound **B** (Indose) (59mg) was dissolved in acetone (2 mL) and NH_3 (4 mL) and left overnight, hydrolysis flask and ammonia was removed under reduced pressure, washed with $CHCl_3$ and were finally freeze dried giving the deacetylated oligosaccharide b(Indose) (34 mg).

Description of Isolated Compound b (Indose)

Compound B (Indose) (59 mg) obtained from fraction 20-22 of column chromatography-4, on deacetylation with NH_3/ acetone it afforded compound D (34 mg) as a viscous mass. For elemental analysis, this compound was dried over P_2O_5 at 100°C and 0.1 mm pressure for 8 hr.

$C_{34}H_{58}N_2O_{26}$		% C	% H	% N
	Calcd	44.83	6.42	3.08
	Found	44.83	6.42	3.07

It gave positive Phenol-sulphuric acid test, Feigl test, Morgon-Elson test.

^{1}H NMR: δ in D_2O (ppm)

δ5.09(d, 2H J= 3.6Hz), δ4.54(d, 2H J=8.1 Hz), δ4.39(d, 1H J= 7.8Hz), δ4.33(d, 2H J=7.8Hz), δ3.80(d, 1H J=2.7 Hz), δ3.22(t, 1H J=8.7 Hz), δ2.09(s, 3H $NHCOCH_3$), δ1.97(s, 3H $NHCOCH_3$).

^{13}C NMR: δ in D_2O (ppm)

δ 173.4, δ 104.6, δ 102.7, δ 95.5, δ 91.6, δ 78.2, δ 78.1, δ75.1, δ 74.6, δ 74.2, δ 73.6, δ 72.3, δ 71.1, δ 70.9, δ 70.8, δ 69.9, δ 69.1, δ 68.3, δ 60.8, δ 60.3, δ 59.9, δ 59.7, δ 56.5, δ 56.4, δ 51.9.

FAB-MS

1563 $[M+Na]^+$, 904, 881, 830, 801, 779, 759, 741, 717, 701, 700, 659, 636, 577, 559, 499, 457, 390, 229, 211, 169, 109.

RESULT AND DISCUSSION

Structure elucidation of 'Indose' isolated from Shyama dhenu milk oligosaccharide

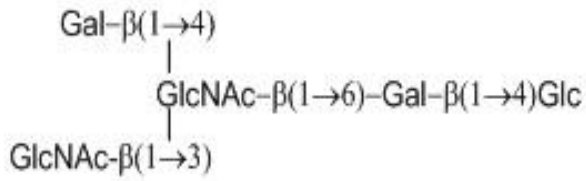
Compound b (Indose) $C_{34}H_{58}O_{26}N_2$ gave positive Phenol sulphuric acid test (Dubois M et al. 1956), Fiegl test (Fiegl F. et al. 1975) and Morgan-Elson test (Partridge S.M. et al. 1948), showing the presence of normal and amino sugars in the compound. The ^{1}H NMR spectrum of compound B at 300 MHz exhibited four signals for protons in the anomeric region at δ 4.33 (2H), 4.39(1H) δ 4.54(2H) and 5.09 (1H) indicating that the compound B may be a pentasaccharides in its reducing form. It was further supported by the appearance of four signals for six anomeric carbons at δ 104.6(1C), 102.7 (3C), 95.5(1C) and 91.6 (1C) in the ^{13}C NMR spectrum of B. The five monosaccharides present in compound B have been designated as S_1, S_2, S_3, S_4 and S_5 for convenience starting from the reducing end. The acid hydrolysis of compound B gave three spots on the paper chromatography, which were identified as Glc, Gal and GlcNAc by co-chromatography with authentic samples. These data led to the suggestion that it may be a tetrasaccharide in its reducing form. Methylglycosidation of B by MeOH/H^+ followed by its acid hydrolysis led to the isolation of α and β–methyl glucoside, GlcNAc and Gal which suggested the presence of glucose at the reducing end in the oligosaccharide and Gal and GlcNAc at the non-reducing end. The reducing and free nature of glucose was further supported by the presence of α and β anomeric proton signals as doublets at δ 5.09 (J= 3.6Hz) and δ 4.54 (J= 8.1 Hz) respectively. Further another anomeric proton doublet at δ 4.33 (J=7.8 Hz), 4.39 (J=7.8 Hz) and two singlets of three protons at δ 1.91 and 1.88 which were assigned to NHAc group, thereby confirming the presence of two GlcNAc moieties in the compound B. B also contains a lactosyl moiety at its reducing terminal i.e. Gal linked to glucose with β(1→4) linkage, which was also confirmed by the presence of anomeric protons as doublets at δ 4.33 (J=7.8 Hz and δ 4.54 (J= 8.1 Hz) for Gal and Glc residues respectively and characteristic signal of H-2 of β-Glc that B like in compound A. The only deference from the compound A is the presence of an addition anomeric proton at δ 4.54 (J=8.1 Hz) which may be due to the presence of an unsubstituted β-Gal residue (G. Groongerg et al, 1992).

In the H^1-H^1 COSY spectrum of acetylated compound B, the anomeric proton doublet at δ 4.33 (2H) (J=7.8 Hz) showed connectivity with H-2 at δ 4.06, H-2 showed connectivity with H-3 at δ 3.62 and H-3 showed connectivity with H-4 at δ3.71. This implies that one of the β-GlcNAc (S_3) was glycosidically linked at its C-3 as well as C-4 positions to S_4 and S_5.

At C-3 it is linked with the other β-GlcNAc (S_4) having its anomeric proton resonating as doublet at δ 4.39 (2H) (J=7.8 Hz) and at C-4 with a β-Gal moiety with its anomeric proton resonating as doublet at δ 4.54 (2H) (J=8.1 Hz). The chemical shifts of the anomeric carbons of B at δ 91.6 (1C α-Glc), 95.5(1C, β-Glc), 102.7 (3C, 2β-GlcNAc & β-Gal), and δ 104.6(1C, β-Gal) as observed in the ^{13}C NMR spectrum are in accordance with the anomeric carbons values of Glc, Gal and GlcNAc (C.A. Bush, et al, 1985). The comparison of chemical shifts of ring carbons of this tetrasaccharide with the reported values also supports the derived structure. Based on the pattern of chemical shift of ^{1}H, ^{13}C and HOMOCOSY, TOCSY NMR experiments of acetylated B and the ^{1}H, ^{13}C chemical shifts of deacetylated compound B, it was interpreted that the compound B was a pentasacchaaaride having following structure.

The FAB mass spectrum of acetylated compound B further helped in substantiating the sequence of monosaccharide units in it. It showed the highest mass ion peak at m/z 1563 $[M+Na]^+$ as psuedomolecular ion peak for the expected molecular ion at m/z 1540 and derived molecular formula ($C_{64}H_{88}N_2O_{41}$). The fragment ion at m/z 331 and m/z 287 showed the presence of terminal hexosyl and hex-NAc moieties present in the pentasaccharide, which were produced by cleavage glycosidic bonds between S_3 and S_4 and S_3 and S_5 at the non reducing terminal of pentasaccharide i. e. [M-S_4] and [M-S_5] thus indicating the presence of branching at the non reducing end. The formation of fragment ion at m/z 881 indicating the presence of trisaccharide consisting of β-GlcNAc(S_3)-βGal(S_2)-βGlc(S_1) which was the formed as a result of loss of terminal Gal and GlcNAc moieties. The presence of lactosyl (S_2-S_1) moiety was further confirmed by the presence of mass ion fragment at m/z 619 produced by the cleavage at N-acetylglucosamine residues starting from the non reducing terminal of the trisaccharide m/z 881. The lactosyl disaccharide at m/z 619 further fragmented to give fragment ion at m/z 289 and m/z 331 which also confirmed the presence of Glc at the reducing terminal of the pentasaccharide. The FAB mass spectrum of compound B also contained other mass on peak at m/z 904 [881-Na], 830 [904-CH_3CO-OCH_3], 801 [904-CH_3COOH-CH_3CO], 779 [881-CH_3COOH-CH_2=C=O], 759 [801-CH_2=C=O], 741 [801-CH_3COOH], 717 [759-CH_2=C=O], 701 [759-$NHCOCH_3$], 700 [759- CH_3COO], 659 [701-CH_2=C=O], 577 [619-CH_2=C=O], 559 [619-CH_3COOH], 499 [619-2CH_3COOH], 457 [559-CH_3COOH-CH_2=C=O], 390 [577-CH_3COOH-3CH_2=C=O], 229 [331-CH_3COOH-CH_2=C=O], 211 [331-CH_3COOH], 169 [229-CH_3COOH], 109 [169-CH_3COOH]. This fragmentation also supported the formation of the various fragment ions that served as anchoring units of the pentasaccharides. The fragmentation pattern also confirmed by presence of Gal (S_2) (1→4) Glc (S_1) at the reducing end and branching at GlcNAc (S_3) of the pentasaccharide.

On the basis of results obtained from physico-chemical techniques and chemical transformation, the structure of the pentasaccharide was determined as



Indose

Stability of Molecular geometries of the isolated compounds

The geometry optimization of indose has been done using B3LYP method at 6-311G basis set employing density functional theory (DFT). The theoretical calculations have been performed using Guassian 09W package. The optimized geometry is visualized using Gauss View 5.0.9 utility software. All the rings are present in the most stable chair form. The indose molecule possesses C1 symmetry. The molecule is found to be highly polar in nature with the total dipole moment of 12.5629 Debye. The molecule has total energy of -3394.6860 a. u. The distribution of Mulliken charges shows that oxygen atom O (-0.677) has maximum negative charge and atom C (-0.557) has maximum positive charge. The highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) for indose is shown in Fig-1 and fig-2. The energy gap of the molecule is also shown. The molecular electrostatic potential map also shows the electron cloud distribution in the overall molecule. The red colored area shows the most electronegative region.

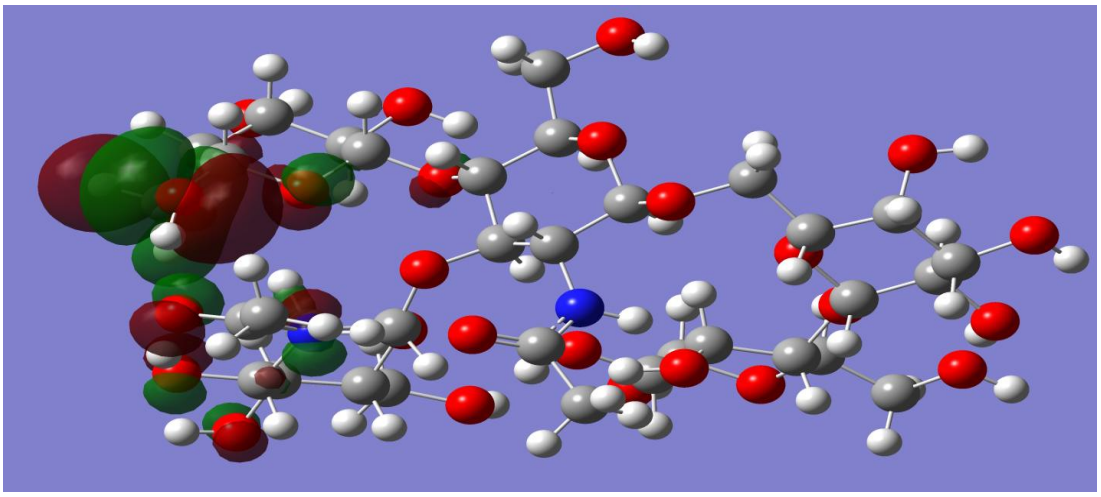


Fig 1. HOMO of Indose.

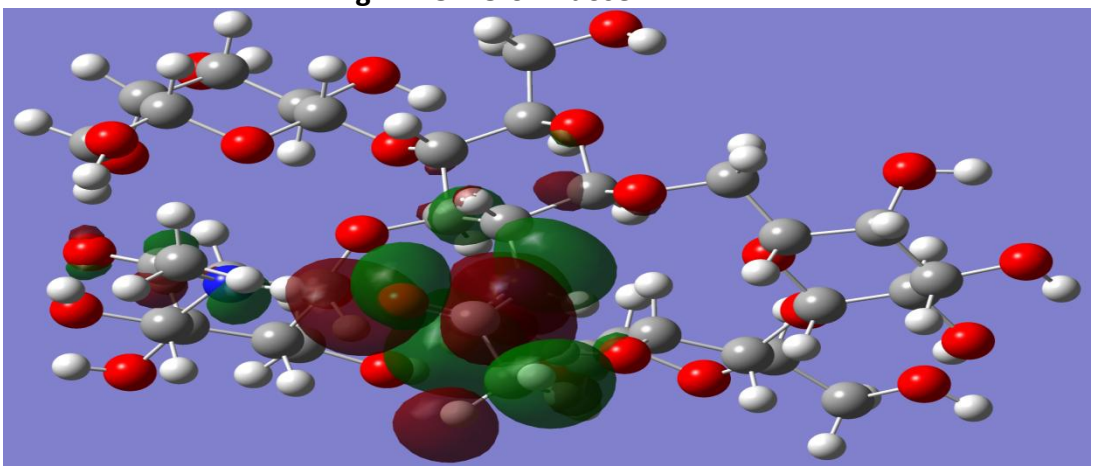


Fig 2. LUMO of Indose.

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Corresponding author: Dr. Desh Deepak, Department of Chemistry, University of Lucknow, Lucknow 226007, U.P., India.
Email: deshdeepakraju@rediffmail.com muzeebkhan0786@gmail.com