CHAPTER-1

INTRODUCTION

Carbohydrates are the most abundant class of organic compounds found in all living organisms. The carbohydrate are major source of metabolic energy both for plants and animals that's depends on plant for food. The formula of many carbohydrates can be written as carbon hydrates, ($C_n(H_2O)_n$) hence their name. Aside from the sugars and starches that meet metabolic energy or vital nutritional role in organisms, carbohydrates also serve as a structural material (cellulose), a component of the energy transport compound ATP, recognition sites on cell surfaces, and one of three essential components of DNA and RNA. Carbohydrates are the most abundant and the most diverse polymers in nature. Due to their highly specific interactions with physiological receptors, they participate in many crucial biological processes. All these processes are potential targets for therapeutic intervention and carbohydrates based drugs are rapidly being engaged by the modern biotechnology and pharmaceutical industry. Some of the recognized ccarbohydrate roles include conferring unique properties on proteins and lipids, functioning as biological semaphores and in plants, serving as a nutritional energy reserve and as a means of skeletal support. Carbohydrate makes an ideal therapeutic platform because they are known to be involved in diverse biological processes.

Carbohydrates also play an important role in various biochemical processes which includes embryogenesis, fertilization, hormonal activities, cellular adhesion, cell-cell recognition, glycosylation of protein, xenotransplantation, cell proliferation and blood group specificity and immune response. Carbohydrates based modified therapeutics exhibits varied biological activity such as anti-inflammatory (Srivastava and Kulshrestra 1989), antitumor (Lee et.al., 1987), antithrombotic (Witzak and Nieforth, 1977), immunostimulant (Piere 1982), anticancer (Mitchell and Wilks 1922), anticomplementary (Yamada and Haruki, 1986), antiviral (Yang and Chuang 2012), antimicrobial (Lee et. al., 2012), lipid-lowering (Halas and Nochta 2012; Tao.et.al., 2011; Castillo et.al., 2008 and Schaatsma et. al., 1998), regulation of mineral absorption, cardioprotective and immunological activitie (Yarema and Bertozzi 1998). Carbohydrate based vaccines are used for typhus, pneumonia, meningitis and vaccines for anthrax and malaria are under development. Carbohydrate associated drugs that are under development such as heparin sulphate binders, lectins, acarbose, aminoglycosides, tamiflu, and technologies using carbohydrate and lectin microarrays offer

improved diagnostic and drug development possibilities. Some of these antigens were conjugated to a carrier such as keyhole limpet haemocyanin (KLH), a more powerful immune response was induced. Immunological adjuvants such as QS–21A a plant derived complex saponin from the tree *Quillaja saponaria molina* have also been used in combination with carbohydrate antigens and potentiators to improve immune responses. Carbohydrate antigen–based vaccines are now in development or in clinical trials and some early results look promising e.g., melanoma patients exhibited a consistent antibody response against ganglioside GD2 using a GD2 lactone-keyhole limpet hemocyanin conjugate vaccine plus immunological adjuvant QS-21. Advances in glycochemisty and glycobiology are not only producing tumor antigens and adjuvants but are also leading to the development of novel carbohydrates antigens. Ley is a blood group determinant, has been identified as important epitope for elicting antibodies against colon cancer, liver cancer, sand prostate, breast and ovarian tumors (Kudryashov et.al., 1998). Globo-H-KLH, a poly-hexasaccharide vaccine used in clinical trials for the treatment of breast, ovarian, and prostate cancer (Gilewski et.al., 2001) NPr PSA (a poly sialic acid vaccine) mimics a different epitope on the surface of group *B. meningococci* and *E. coli K1*. Recently this epitope has been located through the use of an NPr-PSA specific monoclonal antibody (13 D9), in the capsular layers of both group *B. meningococci* and *E. coli K1* (Jennings, et. al., 1987a, 1989b). NPr-PSA protein has been used successfully without deleterious consequences as experimental human vaccine in a number of animal species (Jennings, et.al., 1986 and Fusco, et.al., 1997). NPr-PSA-TT vaccine was even found to be safe and immunogenic in human (Danve, et.al., 1996). Other important vaccines are Ley(c) peptide MBH-KLH conjugate for elicting both IgM and IgG responses (Kudryashov, et. al., 2001) and ST-MMCCH-KLH for the treatment of OSM and STn-positive cells.

Aminoglycosides are used to treat various infections by gram negative bacteria, as they inhibit protein synthesis by binding to bacterial ribosomes. Acarbose is a glycosidase inhibitor that is used to treat type-2 diabetes by regulating carbohydrate digestion, intestinal absorption and carbohydrate metabolism. It has been shown to cause a significant decrease in plasma glucose in patients with non-insulin dependent diabetes. Sugar containing structures are demonstrated to play fundamental roles in the regulation of biological activity and consequently, in the cause and treatment of many diseases as well as in drug action. Doxorubicin, Fornivirsen and Zalcitabine are the sugar containing compounds (Mimaki, et. al., 2003) for the treatment of cancer chemotherapy and AIDS-related Kaposi's sarcoma.

Ribavirin, containing D-Ribofuranosyl ring, act as anti-viral which is active against DNA and RNA viruses (Mimaki, et. al., 2003). Roximycin, containing N-Oxime side chain in the lactone ring is used as an antibiotic (Mimaki, et. al., 2003). Similarly, there are some other drugs which contain carbohydrate or their derivatives as important components e.g., Azithromycin, is a commonly used antibiotic for bacterial infections (Noedl, et. al., 2006) and is also effective against asthma and malaria (Cook and Reich, 1999). Adriamycin is a drug containing carbohydrate used in treatment of various solid tumors. Bleomycin is a drug (Classen, and Long 1999) produced by bacterium *Streptomyces verticillus*, is a glycosylated linear non-ribosomal peptide antibiotic. Bleomycin A2 and B2 is used as anti-cancer agent. These drugs are used in the treatment of lymphomas (especially Hodgkin's disease), squamous cell carcinomas and testicular cancer as well as pleurodesis. Nojirimycin of α-glucosidases is an iminosugar shown to be a potent inhibitor & β-glucosidases, acts as a carbohydrate catabolic enzymes (Inone, et. al., 1966). Miglitol & Voglibiose are synthetic carbohydrate (Kajimoto and Node, 2009) mimics used for treatment of type-2 diabetes, by decreasing the carbohydrate digestion rate & reduce post prandial hyperglycaemia (PPHG). Ralenza is an iminosugar containing drug (Kajimoto and Node 2009), which is used as an inhibitor against influenza viral neuraminidase. It is used as 'flu drug' which plays important role in combating the recent flu pandemic & epidemics. Miglustat **(**Kajimoto and Node, 2009) is an azasugar used as an anticancer drug. It acts as inhibitor against catabolic glycosidases associated with cancer progress. Lobatoside E (Kajimoto, and Node, 2009) & Candicanoside A are triterpene saponins exhibit potent anticancer activity. CE-108 (Saleem et. al., 2010) is a cyclotoxic tetraene macrolide showing antifungal activity. Biselyngbyaside is a 18-membered macrolide glycoside has been reported to exhibit uncommon broad-spectrum anti-tumour activity in human tumour cell line panel (Teruya, et. al., 2009). Hassallidin B is a glycosylated cyclic peptides has an extra rhamnose attached to the 3-hydroxyl group of the acyl chain & was shown to have increased water solubility without decreasing its potent antifungal activity (Neuhof, et. al., 2005). Anthracycline, an anti-tumor antibiotic are amongst the most potent and widely used anticancer agent (Ning and Zhang, 2003). The anthracycline structure is based on a tetracyclic aglycone which is attached to at least one sugar residue. Algucerase, used in treatment of Type-1 Gaucher's disease, is a modified form of glucocerebrosidase where the non-reducing ends of the oligosaccharide chains have been terminated with mannose residue (Lee et. al., 2005 and Stroke, 1994). Isepamicin, the 1-N-(S-alpha-hydroxy-beta-aminopropionyl) derivative of gentamicin B, is

a new aminoglycoside antibiotic with activity against both gram negative (–) and gram positive (+) bacteria producing Type I 6'–acetyltransferases (Tod and Padonic, 2000). The Macrolides are important group of compounds which have been succeeded due to their excellent activity against Gram-positive bacteria. Many of them are used clinically which includes erythromycin, oleandomycin, spiramycin, josamycin and midecamycin (Asano, 2009). Auranofin is an organogold compound, where thioglucose is linked to gold exhibits anti-arthritic, anti-inflammatory and immunomodulating properties **(**Kim et. al., 2004 and Venardos et. al. 2004). It is also known as aktil or crisofin. Doxorubicin is a conjugated of an antracycline adriamycinone and a sugar daunosamiine, used in treatment of cancer chemotherapy and AIDS – related Kaposi's sarcoma (Arcamon, et al., 1999) and also known as Adriamycin or 14-hydroxydaunomycin (Kusmer and Guyton, 2006). Fondapaarinux is synthetic pentasaccharide with O-methyl group at the reducing end of the molecule is used as an anticoagulant (Katzung, 2007). Fomivirsen, treats against cytomegalovirus retinitis (CMV) in immunocompromised patients including those with AIDS, also used as an anti-viral drugs antimicrobial stubs and orphan drugs. Sodium ferric gluconate complex (Edwards, 2003), Iron dextran and Iron sucrose are some of the potential carbohydrate associated iron drugs used for maintaining iron storage in human body (Kusmer and Guyton, 2006). Heparin which is mixture of various oligosaccharides is a widely used injectable anticoagulant, is a highly sulphated glycosaminoglycan. It is currently used in prophylaxis and treatment of thrombosis. Pentosan polysulphate is a first oral medication for intestinal cystitis or painful bladder syndrome contains sugar residue (Coombe and Kett, 2005) and also known as elmiron. Ribavirin or ribasphere (Alvarez et. al., 2006 and Bani-Sadr et. al., 2005) or virazole or viramidine act as an anti-viral which is active against DNA and RNA viruses, contains D-Ribofuranosyl ring. Streptomycin was the first antibiotic remedies (Hurt et. al., 2003 and Kingston, 2004) for tuberculosis which contains an aminoglycosides. Zalcitabine, is a nucleoside analog reverse transcriptase inhibitor (NARTI) also called dideoxycytidine contains a carbohydrate residue, is used in treatment of immunodeficiency virus (HIV) (Moyle et. al., 1998). Oligosaccharides which are present in glycoproteins represent the determinant, which is responsible for the specific biological action and determines the antigenic properties of the cells (Gottschalk, 1972, Horowitz and Pigman, 1978). Antigenic specificity is due to the structural variability of the outer chain comprising of one, two, three or additional glycosyl units as it is only the small part of carbohydrate or hapten which response to immune system (Aspinall, et. al., 1991 and

Brennan, 1989). The oligosaccharide containing moiety of saponin halotoxin which is isolated from sea cucumber *Stichopus japonicus* exhibits interesting antifungal properties (Taguchi, 1985). Fungi belonging to basidiomycetes have been reported to contain useful anti-tumor oligosaccharides e.g. Lantinan and Schizophyllan are used in cancer immunotherapy (Gardiner and Glucan, 2001 and Venardos, et. al., 2004). The β-Glucan, a branched glucose polymer found in mushroom, yeast, bran act as a potent regulator of the immune system and have shown anti tumor activity. It inhibits cancer cell growth and metastasis and prevents bacterial infection (Roberfroid and Delzenne, 1998)

1.1 MILK OLIGOSACCHARIDES - It has been seen that in all of glyco-compounds i.e. glycosides; glycoconjugates etc. carbohydrate are present as mono, dimer or oligomers, which play a decisive role in the biological function of active constituents. Besides this in some of the cases the glycan is present as free oligomer of monosaccharide known as oligosaccharides. Oligosaccharides are amongst the most biologically diverse and important carbohydrate in biological system. Oligosaccharides are found as natural constituents in fruits, vegetables, milk, blood, bacteria and fungus etc. and have various physiological functions such as improvement of mineral absorption, and improvement of both plasma cholesterol and blood glucose level (Rawle, et. al., 2000, Kanemitsu and Kanie, 1999, Imberty and Perez, 2000). The enormous structural variability possible in oligosaccharide structures is the probable reason for nature using them for the purpose of molecular recognition, transformation of oligosaccharide into glycoconjugates then yield a specific diagnostic material, a non-toxic and highly specific vaccine or therapeutic product. Milk oligosaccharides are an important source of complex carbohydrates as supplements for the food and the pharmaceutical industries. More than 250 milk oligosaccharides have been isolated from milk of cow, buffalo, donkey, horse, sheep, goat, bear etc. Human milk oligosaccharides are known to protect breast fed infants from a host of bacterial infection. A broad range of oligosaccharides and their derivatives act as an effective drug against most of acute and chronic diseases. Oligosaccharides play an essential role in many molecular processes impacting eukaryotic biology and diseases and exhibit varied biological activity such as immunostimulant, hypoglycemic, antitumor, antiviral, anticancer, anticoagulant, anticomplementary, immunological and antiinflammatory activities (Ehresmann, et. al., 1979, Yamada and Haruki, 1986, Piere, 1982). Certain cell surface oligosaccharides act as potent antigen and used in tumor vaccines has inspired new approaches to the management of tissue rejection, subsequent to xenotransplantation. Glycosphingolipids is the Globo,

Ganglio and lacto-series have been investigated as components of potential tumor vaccines (Horwacik, 2004). Milk oligosaccharides inhibit the adhesion of pathogenic microorganisms to the intestinal and urinary tract by acting as receptor analogues, thus preventing gastric and urinary infections. More than 250 oligosaccharides have been isolated from various mammalian (Urashima, et. al., 2001) milk of different origin e.g. buffalo, equine, caprine, ezobrown, bear, Japanese black bear, elephant, donkey, rat, dog, echidna, platypus, kangaroo, cow, sheep, goat, mare, camel and human etc. Human milk and colostrum contain more than 80 different oligosaccharides (e.g. fucosyl and sialyl–lactose and lacto-N-tetraose etc.) constituting over 20% of total milk carbohydrate. The oligosaccharides isolated from various milk sources are categorized in two classes i.e. sialylated oligosaccharides and non-sialylated oligosaccharides which have been tested for their varied biological activities. Sialylated oligosaccharides appear to be an essential receptor component for many animal virus families such as newcastle disease virus (paramyxovirus), cardiovirus (picornavirus) murine and primale polyomavirus (papovirus), rheovirus and enterotoxigenic and uropathogenic *E.Coli*, influenza A, B and C viruses (orthomyxoviruses), rotavirus binds in both *in vivo* and *vitro* (Silfverdal-Sa, 2002, Kunj and Rudolff, 1993). Human milk oligosaccharide binds to a wide range of lectins on the surface of epithelial cells living the mouth, oesophagus and stomach and throughout the gastrointestinal system in the new born baby. This in turn prevents opportunistic infection whilst the baby's immune system is developing. Oligosaccharides lectin binding has also been used to target therapeutic agents to diseased cells which express high densities of specific lectins on their surface e.g. GalNAc clusters have been used to potentially allow treatment of hepatitis A. Many milk oligosaccharides contains the basic lactose and lacto-N-tetraose sequence (Gal-GalNAc-GalGlc) or one of its derivatives which has been shown to be a particularly potent bifidus factor. It has been seen that human milk oligosaccharides structures like LNneoT and LNFPII suppress the production of IL10 cytokines which is a potent immunosuppressive cytokine found in breast milk produced by mammary cells (Velupillai and Harn, 1994). The fucose containing oligosaccharides Fuc α-1, 2 Gal (Chaturvedi, et. al., 2001) also enhance immune system of neonates. Fucose suppresses the skin reaction of allergic contact dermatitis and sialic acid inhibits bronchial allergic reaction in animals (Mario, et. al.,). Human milk oligosaccharides are currently used for studying the biosynthesis of I, Lewis blood group related antigens. In fact, some of the oligosaccharides

i.e., SialylLe hexasaccharide have recently been characterized as tumor associated antigen or as differentiation antigens (Martesson, et. al.,1988).

1.1.1 Biological importance of milk oligosaccharides- At one time it was thought that human milk oligosaccharides have no specific function and that they arise neither by specific synthesis nor by degradation of glycolipids. Instead, they could be the incidental result of the high concentration of lactose found in milk, the oligosaccharide serving as a glycosyltransferases found in milk. This view is no longer held by leading authorities in this field. We have seen, moreover, that in several species of mammals, lactose is only a minor component and other oligosaccharides are dominant. It has been recently suggested that central nervous system is not fully developed at the time of birth; the galactose and sialic acid present in milk oligosaccharides may be required for optimal development of the infant brain. It is worth noting that the osmotic pressure exerted by a given mass of that exerted by an equal mass of lactose. This could be significant as it permits milk to contain a greater concentration of saccharides and electrolytes without it becoming hyperosmotic. One can speculate, therefore, that altricial newborns may have greater requirement for saccharides and/ or electrolytes such as sodium and potassium, than precocial newborns (Kunz, et. al., 1982 and Messer, et. al., 1987). Some milk oligosaccharides may have specific functions for example the sulphated oligosaccharides present in rat milk may be of nutritional significance. Although sulphate is not an essential nutrient in mature mammals, the activity of the rate-limiting postnatally in rats, suggesting that sulfate may be an essential nutrient in the neonate. In an experiment using N-acetylneuraminyl lactose sulfate was found to be hydrolyzed in the gut of rat, neonates and the sulphur absorbed as inorganic sulfate (Nakamura, et. al., 1982). The presence of this compound may permit the simultaneous delivery of two essential nutrients, sulfate and calcium in early life, avoiding the precipitation of insoluble calcium sulphate in the milk (Cumar et. al., 1965). The phosphorylated oligosaccharides found in bovine and equine colostrum may similarly permit the simultaneous delivery of phosphate and calcium, avoiding the precipitation of calcium phosphate (Newburg, 2000 and Urashima, et. al., 2000). By way of contrast, it has been suggested that, in human infants, the function of milk oligosaccharides is primarily protective rather than nutritional (Urashima et. al., 1991). Oligosaccharides may inhibit the adhesion of pathogenic microorganisms to the intestinal and urinary tract by acting as receptor analogues, thus preventing gastric and urinary infections. In addition milk oligosaccharides may function as prebiotics, promoting the growth of microorganisms, such

as *Bifidobacterium bifidus,* within the lower gastrointestinal tract and inhibiting the proliferation of pathogenic organisms. One can speculate that these oligosaccharides could have similar functions in non-human species for example the Gal(α1→3)Gal(β1→4)GlcNAc sequence in rabbit glycolipids was identified as a receptor for the binding of colostridium difficile toxin A (Urashima, et. al., 1989). It is possible that the trisaccharide Gal(α1→3)Gal(β1→4)Glc which is found in bovine, ovine, caprine colostrum and bear, coati and elephant milk, is an inhibitor of the binding of this toxin to the intestinal mucosa in the suckling young of these species. (Urashima, et. al., 1994a, 1999b, Kunz, et. al., 1999, Kyogashimam, et. al., 1989, Veh, et. al., 1981, Viverge, et. al., 1997). Glycolipids containing N-glycolylneuraminic acid (Fig. 1.1) in the form of the trisaccharide sequence Neu5Gc (α2→3)Gal(β1→4)Glc were recognized to be receptors for the binding of *Eischerichia coli* K99, an organism which can cause life- threatening diarrhea in piglets (Nakamura, et. al., 1998). The free trisaccharide which is found in bovine and ovine colostrum may therefore be an inhibitor of the binding of pathogenic organisms to the intestinal mucosa of newborn calves and lambs. Milk carbohydrates, the disaccharide lactose mostly represents the dominant from especially in eutherian mammals, but less so in monotremes and marsupials, where free oligosaccharides predominate. Free oligosaccharide represents an interesting fraction of milk carbohydrates, varying by species and time of lactation, and exhibiting a diversity of structures and quantity. Generally, they are extensions of lactose by one or several monosaccharides such as galactose, N-acetylglucosamine, N-acetyl-galactosamine, sialic acid (N-acetyl-neuraminic acid, N-glycoyl-neuraminic acid), and fucose (Rostami, et. al., 2014). Mammalian milk or colostrum contain a trace to 10% of carbohydrate in which the disaccharide lactose (Gal (β1-4) Glc) usually dominates along with lower concentrations of many varieties of oligosaccharides that have a lactose unit at their reducing ends (Anraku, et. al., 2012). Milk oligosaccharides and glycoproteins have demonstrated protective qualities against enteric pathogen infections in infants by enhancing the binding of IgA with pathogens, having an antibacterial activity against pathogens and competing with pathogen binding sites (Wickramasinghe, et. al., 2011). Oligosaccharides play an important role in the prevention of adhesion of pathogenic bacteria to the epithelial surface and have recently also been found to be metabolized by *B. infantis* (Zivkovict and Barile, 2011) and recent suggest that the neutral fraction of HMO containing fucose could play an important role in the development of the typical breast-fed infant micro-biota. Bovine colostrum collected immediately post partum contains only

around 1g/L of oligosaccharides, and this concentration rapidly decreases after 48h (Urashima, et. al., 2013). Bovine milk oligosaccharides (BMOs) have progressed recently, especially in regard to structural characterization, with the development of methods termed glycomics (Urashima, et. al., 2013). Milk from domestic animals contained a much larger variety of complex oligosaccharides than previously assumed, and thirteen of these structures have been identified previously in (Albrecht, et. al., 2014). Human milk oligosaccharides (HMOs) participate in several protective and physiological roles, including immunoregulation and inhibition of pathogen adhesion in the gastrointestinal tract of infants. Bovine colostrum is currently being used in a variety of health promoting supplements worldwide (Danielle, et. al., 2013). The elephant milk oligosaccharide fraction contained a high ratio of sialyl oligosaccharide; this may be significant with respect to the formation of brain components, such as gangliosides of the suckling calves (Osthoff, et. al., 2007). N-acetylneuraminlactose sulphate plays an important role in the nutrition of the rat pups, which is the dominant oligosaccharide in the dog milk (William, et. al., 1999). Buffalo and donkey milk oligosaccharides have ability to stimulate non-specific immunological resistance of the host against parasitic infections (Saxena, et. al., 1999 and Deepak et. al., 1998). Goat milk oligosaccharides play an important role in intestinal protection and repair after a damage caused by DSS (Dextron sodium sulphate) induced colitis and their implication in human intestinal inflammation (Federico, et. al., 2006). Goat milk oligosaccharides have anti-inflammatory effects in rats with trinitrobenzene sulfonic acid induced colitis and may be useful in the management of inflammatory bowel disease (Hakkarainen, et. al., 2005). The cow's milk oligosaccharides reduce the adhesion of enterotoxic *E. coli* strains of the calf (Johansson, et. al., 2005). The anti-infective effect of human milk has been partly attributed to the high amount of free oligosaccharides as well as glycoconjugates because these structures might prevent intestinal attachment of infections agents by acting as receptor analogues. As HMOs are resistant against digestion, they can be detected in the faeces as well as in the urine of breast fed infants (Deepak, et. al., 1999). Human milk protects the infant against infection, and oligosaccharides play an important role in other factors (Chonan and Watanuki, 1996). In human infants, the calcium absorption from human milk is significantly higher than from any formula. There are many factors in human milk that might influence calcium absorption (Kitagawa, et. al., 1989a, 1988b). HMOs antigen such as sialyl Lewis-X and Globo-H are suitable targets for both active and passive anticancer immunotherapies because they have been carefully characterized as being

over expressed on the surface of malignant cells. The 3'-sialyl- lactose and 3'-sialyl-3-fucosyl-lactose present in human milk serves as antiinflammatory components by inhibition of monocyte, lymphocyte and neutrophill adhesion to endothelial cells. Lacto-N-Fucopentaose III (LNFPIII) is a human milk sugar containing the biologically active Lewis-X (LeX) trisaccharide. LNFPIII / LeX are also expressed by immunosuppressive helminthes parasites, by bacteria and on a number of tumor or cancer cells. LNFPIII activates macrophages *in vitro* as indicated by up regulation of Gr-1 expression on F 4/80 (+) cells and F 4/80 (+) cells is able to activate natural killer cells, inducing up regulation of CD69 expression and gamma interferon production (Matesson, et. al., 1988, Yamashita, et. al., 1982, Wieruszeski, et. al., 1985, Rudloff, et. al., 2002 and Cervantes, et. al., 1995). LNFPIII-stimulated macrophages secrete prostaglandin Einterleukin-10 (1L-10) and tumor necrosis factor alpha. An oligosaccharide fraction isolated from goat milk reduces intestinal inflammation in a rat model of dextran sodium sulfate-induced colitis and contribute to the recovery of damaged colonic mucosa (Meyer, et. al., 2001). The human milk is involved in the generation of anti-inflammatory mediators that suppress Th-1-type and inflammatory responses (Toshio and Hiroshi, 1998). Sialic acid is an essential component of breast milk glyco-conjugates and is important for the normal development of the brain. It also acts as a skin cosmetic, which prevent skin roughening, fine wrinkles and stains. Sialylated milk oligosaccharides have possibility to treat various types of neoplastic diseases. The typical tumor associated carbohydrate antigen LeA and sialyl LeX are found in human milk (Martesoon, et. al., 1988, Katsuko, et. al., 1976, Davies et. al., 1989). Deoshexonic and arachidonic acid constitute a relatively small fraction in human breast milk participates in immune development. Some studies have suggested that breast feeding may protected type I diabetes and type II diabetes, it also decrease the risk of developing multiple sclerosis and rheumatoid arthritis (immune system inflammatory disease) (Vande Laar, et. al., 2005, Lefkowitz, 2000, Mario, et. al., 2002). Fucose suppresses the skin reaction of allergic contact dermatitis and sialic acid inhibits bronchial allergic reaction in animals (Eglinton, et. al., 1994). The majority of lymphocytes in milk are T cells and the higher proportion of CD8+ (expressing L-selectin, & 946:7 integrin, mucosal addressin cell adhesion molecule-1) and & 948;+ lymphocytes. Breast milk CD4+ cells are also present in an activated state (expressing activation markers CD40L, sCD30, IL-2 receptor, human mucosa lymphocyte antigen-1, or late activation protein-1) and express CD45RO+, a surface protein associated with immunological memory (Portelli and Gordon, 1998 and Osthoff, et. al., 2007).

1.1.2 Factors affecting the biological activities of milk oligosaccharides

1. Human milk oligosaccharides containing α 1, 2-linked fucose inhibits the stable toxin- producing *E. coli* in *vitro,* and its toxin induced diarrhoea *in vitro* and *in vivo.*
2. Sialylated human milk oligosaccharides also inhibit binding of pathogenic strains of *E. coli* and ulcer- causing human pathogen *H. pylori*.
3. Specific fucosyl oligosaccharides of human milk have been observed to inhibit specific pathogens.
4. The ability of rotavirus to infect MA-104 cells in cultures is inhibited by human milk, and this inhibition is due to a mucin associated 46 KDa milk glycoprotein named lactadherin. Lactadherin from human milk also inhibits rotavirus (EDIM strain) gastroenteritis in mice.
5. Glycoconjugates found in human milk also inhibit binding by *Camphylobacter jejuni in vitro* and *in vivo* and also inhibit binding by calciviruses *in vitro*.
6. Presence of sialic acid in human milk serves as anti-inflammatory components and reduces platelet-neutrophill complex formation leading to a decrease in neutrophill B2 integrin expression.
7. Galactose and sialic acid present in milk oligosaccharide are required for optimal development of the infant's brain.
8. Milk oligosaccharides are non-digested due to presence of β-glycosidic linkage. So this B-Glycosidic linkage plays an important role for its prebiotic activity.
9. Oligosaccharide mimics containing galactose and fucose specifically label tumor cell surfaces and inhibit cell adhesion to fibronectin.
10. Supplementation of milk formula with galacto-oligosaccharides improves intestinal microflora and fermentation in term infants.
11. N and O-linked oligosaccharide causes the release of histamine and other mediators of the allergenic response which then lead to the development of allergenic symptoms.
12. The structures of a series of human milk oligosaccharides which were retained by an immobilized column of anti-CA19-9 antibody.

1.1.3 Effect of constituent monosaccharides and linkages on biological activity of milk oligosaccharides

1. Human milk oligosaccharides containing α 1, 2-linked fucose inhibits the stable toxin-producing *E. coli in vitro* and its toxin induced secretory diarrhea *in vitro* and *in vivo* and (Brennan, 1989 and Svensoonl, et. al., 1992). Glycoconjugates found in human milk also

inhibit binding by *Campylobacter jejuni in vitro* and *in vivo* and also inhibit binding by calciviruses *in vitro*. Some specific focosyl oligosaccharides of human milk have been observed to inhibit specific pathogens. Thus, it can be concluded that the family of α 1, 2-linked fucosylated oligosaccharides, probably in conjugation with other families of oligosaccharide, constitute a powerful innate system of human milk and (Sharon and Ofek, 2000).

2. Due to the presence of sialic acid in human milk they serve as anti-inflammatory components and reduce platelet-neutrophill complex formation leading to a decrease in neutophill B2 integrin expression, while neutral milk oligosaccharide fraction has no effect. Sialylated human milk oligosaccharides also inhibit binding of pathogenic strains of *E. coli* and ulcer causing human pathogen *H. pylori*. On the other hand neutral human milk oligosaccharide may protect the intestinal tract of neonates from *Vibrio cholera* (Kunz, et. al., 2000 and Mehra, et. al., 2006)

3. Prebiotic is non-digestible food ingredients that beneficially affect the host by selectively affecting the growth and activity of bacteria in colon that can improve the host health. Milk oligosaccharides are non-digested due to the presence of β-glycosidic linkage. So this β-glycosidic linkage plays an important role for prebiotic activity (Chonan and Watanuki, 1996 and Kitagawa, et. al., 1989) of milk oligosaccharides.

4. Infection by rotavirus is responsible for much of the diarrhea in infants around the world. The ability of rotavirus to infect MA-104 cells in culture is inhibited by human milk and this inhibition is due to mucin-associated 46kDa milk glycoprotein named lactadherin (Newburg, et. al, 2005). Furthermore after sialic acid is removed from lactadherin, its ability to inhibit rotavirus is essentially lost, which suggests that the glycon portion of the molecule is responsible for inhibition and specific terminal sialic acid is required for inhibition.

5. N and O-linked oligosaccharides cause the release of histamine and other mediators of the allergic response which then lead to the development of allergenic symptoms. Oligosaccharides mimics containing galactose and fucose specifically label tumor cell surface and inhibit cell adhesion to fibronectin (Tisch and Stefan, 2001)

6. Supplementation of milk formula with galacto-oligosaccharides improves intestinal microflora and fermentation in infants. Galactose and sialic acid present in milk oligosaccharide are required for optimal development of the infant's brain (Ben, et. al., 2004)

1.2 ISOLATION OF MILK OLIGOSACCHARIDE - The physiological significance of the milk oligosaccharides in biological systems is widely appreciated, but their isolation from milk is a difficult task because of their very high polar nature, low concentration, low resolution on chromatography and their microheterogeneity. In the earlier days, the structural study of oligosaccharides depends on the paper chromatography, sequential exoglucosidase glycosidation, quantitative methylation analysis etc. But in present days due to modern chromatographic techniques (various forms of HPLC like ion-exchange, size-exclusion, high performance, reverse phase etc.) and spectroscopic techniques (PMR, CMR, 2D NMR, EI mass, ES mass and FAB mass), the isolation as well as structure elucidation of complex oligosaccharides and determination of their anomericity, ring sizes and absolute configuration is very interesting task.

1.2.1 Process for isolation of milk oligosaccharides - A number of milk oligosaccharides have been isolated and characterized by the various workers in the last few decades by processing the milk of different origins but still new oligosaccharides are being isolated from the milk of different species, due to qualitative variation which arise due to genetic factors, which reflected in their biosynthesis. The most pioneer workers among all the workers who have isolated a number of milk oligosaccharides from the milk of different origins by developing the various processing techniques were Victor Ginsburg, Akira Kobata, Hiroshi Kitagawa, Richard Khun, Heinz Egge, David F. Smith, Messer and Mossop and T. Urashima. (Urashima, et. al., 1990, Smith, et. al., 1997, Egge, et. al., Weiruszeski, et. al., 2006 and Kobata and Ginsburg methods 1970a, 1970b)

1.2.2 Kobata & Ginsburg method - In the Kobata and Ginsberg method the samples of milk were obtained and stored at -20^{o}C until used. Milk was centrifuged for 15 minutes at 5000 rpm at 20^{o}C. The solidified lipid layer was removed by filtration through glass wool. Ethanol was added to clear filtrate to final concentration of 68 % and the resulting solution was left overnight at 0°C. The white precipitate formed mainly Lactose and protein, was removed by centrifugation, and washed twice with 68% ethanol at 0^{o}C. The supernatant and washings were combined, concentrated under reduced pressure, and passed through a column of fine grade G-25 sephadex that had been washed with triple distilled water overnight. The column was eluted with triple distilled water and effluents were collected in fractions and aliquots of each fraction were analyzed for oligosaccharides (Kobata and Ginsburg, 1970a, 1970b).

1.2.3 Modified Kobata & Ginsburg method - The colostrum were fixed by addition of equal amount of ethanol. The preserved milk was taken to laboratory and there it was centrifuge for 30 min at 5000 rpm at 4ºC. The solidified lipid layer was removed by filtration through glass wool column in cold. More ethanol was added to clear filtrate to a final concentration of 68% and the resulting solution was left over night at 0ºC. The white precipitate formed, mainly of lactose and protein was removed by centrifugation and washed twice with 68% ethanol at 0ºC. The supernatant and washing were combined and filtered through a micro filter and lyophilized affording crude oligosaccharides mixture (Kobata and Ginsburg, 1970a, 1970b, Deepak, et. al., 1998)

1.3 PURIFICATION OF MILK OLIGOSACCHARIDES - Oligosaccharides obtained from colostrum contained heterogeneous mixture of oligosaccharides. The isolated O-linked colostrum oligosaccharides are highly heterogeneous in structure. Further the colostrum oligosaccharides have been purified by chromatography or electrophoresis which is a prerequisite to many structural elucidation methods. Chromatography plays a major role in purification and analysis of milk oligosaccharides. Chromatographic separations can be carried out by using a variety of supports, including immobilized silica on glass plates (thin layer chromatography), volatile gases (gas chromatography), paper (paper chromatography), and liquids which may incorporate hydrophilic, insoluble molecules (liquid chromatography). Apart from ion exchange chromatography, affinity chromatography and gel permeation chromatography (GPC), high-performance liquid chromatography (HPLC) is the most frequently used techniques. Stationary phases, normal phases, reversed phases and graphite carbon columns are also used for separation of oligosaccharides. Refractive index, UV and fluorescence radioactivity are used as detection principles, with evaporative light scattering detection as a technique with great potential. High pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is a powerful method, which is widely used for carbohydrate analysis. Special continuous preparative chromatographically techniques, such as simulated moving chromatography and annular chromatography are also used in separation of colostrum oligosaccharides. Colostrum oligosaccharides are polar in nature. So separation of compound crude oligosaccharide mixture was acetylated with pyridine and acetic anhydride at 60°C and solution was stirred overnight. The acetylation converted the free sugars into their nonpolar acetylated derivatives which were resolved nicely on TLC. The details of various chromatographic techniques used in separation of oligosaccharides are described as below-

1.3.1 Thin layer chromatography - Thin layer Chromatography is also a very pioneer chromatography in which the stationary phase is applied to a glass or plastic (usually polyester) sheet in a layer about 250 μm thick. It is another useful technique used in the isolation and purification of the natural products. The adsorbent used for the TLC separation of sugars are magnesium silicate, alumina, Keiselghur, silica gel or a mixture of Keiselghur, silica gel and amino propyl bonded silica. The resolution of mixture of compounds depend on the choice of suitable solvent system. Starting from the non-polar single solvent system to highly polar three solvent systems are available to the thin layer chromatography.

1.3.2. Paper chromatography - Paper Chromatography techniques for the isolation and qualitative determination of oligosaccharides. Paper chromatography uses a strip of paper as the stationary phase and is based on liquid-liquid partition process. Capillary action is used to pull the solvents through the paper and separate the solutes. In this, chemical interaction with the paper makes compound travel at different rates. Two-way paper chromatography, also called two-dimensional chromatography, involves using two solvents and rotating the paper 90°C in between. This is useful for separating complex mixtures of similar compounds. For milk oligosaccharides descending paper chromatography is performed with the following solvents on Whatman filter paper (Wieruszeski, et. al., 1985, Kobata and Ginsburg, 1969)**.**

1. Lower layer of phenol-formic acid-2-propanol-H_2O (80:5:100)
2. Upper layer of pyridine-ethyl acetate-H_2O (6:4:3)
3. Phenol-H_2O-conc. NH_4OH (150:40:1)
4. Upper layer or ethyl acetate-pyridine-H_2O (2:1:2)
5. Ethyl acetate-pyridine-acetic acid-H_2O (5:5:1:3)
6. 2-propanol-H_2O (4:1)
7. 1-butanol-pyridine-H_2O (6:4:3)
8. Upper layer of ethyl acetate-acetic acid-H_2O (3:1:3)

After the separation, any strongly colored spots are visible on the chromatogram. Colourless materials can be visualized by heating the paper in an oven so that substances (but not the paper) char and leave black spots or can also be sprayed with a solution of sulphuric acid for better charring. Oligosaccharides were located with $AgNO_3$ reagent, aniline oxalate reagent or periodate-benzidine reagent, Oligosaccharides containing N-acetyl amino sugars with Morgan-Elson reagent while oligosaccharides containing sialic acid were visualized with Thio-barbituric (TBA) reagent.

1.3.3. High performance liquid chromatography - In recent years, HPLC method to purify complex mixtures of glycoprotein/oligosaccharides. All the traditional systems like gel filtration, ion exchange chromatography etc. have been replaced by this attractive alternative. This has also provided a more expedient and refined separation technique of complex oligosaccharide mixture. The wide applicability, speed, and sensitivity of this technique have made it most popular chromatographic technique. HPLC is commonly used at the last step in the purification process. The gradual development of HPLC over the last three decades has enabled higher resolution through both the column efficiency and the ever-expanding range of new sorption materials. For HPLC purification, a judicious selection of operating parameters is required for achieving the desired purity and yield. The following sequence is followed for better resolution and yield (Chaplin, et. al., 1986 and Lee, 1996)

1.3.3.1. Better choice of HPLC system - The separation of different compounds depends on different physical and chemical properties of the solvent. In certain cases, TLC analysis of the sample is used as a first indication of the correct operating conditions, silica gel plates for normal phase column, and sialylated silica gel plates for reversed phase columns.

1.3.3.2. Optimization of analytical columns of small quantities - A preliminary analytical search is necessary for the right choice of conditions, which saves time, sample, and solvent, required in a HPLC system. A good analytical HPLC separation is usually a prerequisite for a successful preparative operation. Relative intention (selectively, α) is a very important parameter in determining possible sample size and it is necessary to maximize this value.

1.3.3.3. Scaling of preparative HPLC apparatus - In many preparative HPLC examples, the column is actually overloaded, nonlinear adsorption isotherms are obtained, and peaks are not symmetrical. Scaling-up a successful analytical separation may cause problem associated with the solubility of the sample. This is especially true for reversed phase HPLC, if the compound under investigation does not dissolved in aqueous solvents. Diluting the samples may help but if the volume injected is too great, separation efficiency decreases. If on the other hand the sample is too concentrated, precipitation on the column may occur. One possible solution is to mix the crushed samples with solvent and dry pack into a sample column or into a chromatography column itself. Beside all the above discussed factors choice of specialized column also plays a decisive role in isolation of mixture of compounds. Oligosaccharides isolated from biological sources are often obtained in reduced and/or de-N-acetylated form. Analysis of reduced oligosaccharides has an

additional advantage as reduction destroy the anomerization at the reducing end thus simplifying chromatography and subsequent structural analysis.

1.3.3.4. Normal phase HPLC - The mode of liquid chromatography in which a polar stationary phase is used in combination with a less polar mobile phase has been known as "normal phase" or "straight phase" liquid chromatography. This method is sensitive to chain length, showing limited selectivity among oligosaccharides of similar size but different stereochemistry. The stationary phase used in normal phase HPLC including silica gel, sorbent in which a polar phase is chemically bonded to silica gel, and polymers bearing polar functional groups. Normal phase HPLC using amino bonded silica gel has been the two most common methods to fractionate oligosaccharides utilizing either ultraviolet (UV) or refractive index (RI) for detection. Detection by UV or RI is also relatively insensitive unless either fluorescent or UV absorbing derivatives can be prepared.

1.3.3.5 Reversed phase HPLC - In contrast to the hydrophilic interactions in normal phase HPLC, the hydrophobic interactions in Reversed-Phase HPLC have been the most popular means of HPLC for a long time. However, appreciation for this separation mode in the analysis of oligosaccharides came relatively late. In reversed phase chromatography packings are characterized by hydrocarbon chains bonded to surface of the silica matrix. The essential criterion responsible for separation is the interaction of the packing with polar materials. Using aqueous solutions or solvent of medium polarity the more polar species elute first and as the polarity decreases the more tightly bounded less polar species are eluted. The retention of underivatized sugars in reversed-phase liquid chromatography (RP-LC) roughly increases with molecular mass. It was observed that the retention times for branched oligosaccharides were shorter than those of the linear counterparts.

The charge on the sialic acid residue causes problems in reverse phase HPLC, which can be solved by adding tertiary amines to the eluant. The formation of an ion-pair will make a sialylated oligosaccharides behave as a neutral compound and interact with the hydrophobic phase. Of several amines tested the best results was obtained with 5 mM triethylamine adjusted to pH 5.0 with acetic acid. For larger oligosaccharides, the retention times could be reduced by the addition of methanol. For example in case of disialylated oligosaccharides addition of 2 % methanol to 5 mM triethylamine, adjusted to pH 5.0 with acetic acid and 1-3 % methanol for nonfucosylated disialylated oligosaccharides.

1.3.4. High performance anion-exchange chromatography - The principle of ion exchange chromatography is based on the separation of molecules on the basis of their molecular

charge. The separation proceeds because ions of opposite charge are retained to different extent. The use of ion-exchange resin is preferred to their increased selectivity, lower operational back pressure, increased reproducibility and the ability to use eluants, which do not decrease the solubility of higher oligosaccharides as in the case with mixture of alcohols in water. The resolution is influenced by the pH of the eluant which affects the selectivity and by the ionic strength of the buffer which mainly affects the retention. Hardy and Townsend were the first to explore systematically the separation power of HPCE-PAD in the analysis of carbohydrate. They succeeded in separating natural oligosaccharides according to their molecular weight, sugar composition and the linkage of the monosaccharide residue. (Thurl, et. al., 1991, Viverge, et. al., 1985)

1.3.5. Size exclusion chromatography - Monosaccharide and low molecular-weight oligosaccharides have relatively small molecular dimensions and therefore macroporous chromatographic supports currently used for the SEC of higher molecular weight polymers are only seldom used for the (GPC) Gel Permeation Chromatography of oligosaccharide. This technique involves the transport of a liquid mobile phase trough a column containing the separation medium, a porous material. A gel is a heterogeneous phase system in which a continuous solid phase, the gel matrix. The pores have a specifically controlled range of sizes and the matrix is chosen for its chemical and physical stability and inertness (lack of adsorptive properties) gels may be formed from polymers by cross linking to form a three dimensional for example Bio Gel is formed by cross linking polyacrylamide and sephadex by cross linking dextrans. The pores in the gel matrix, which are filled with the liquid phase, are usually comparable in size to the molecules of interest for separation (Kitagawa, et. al., 1989a, 1993b)

1.3.6. Gas chromatography - Gas chromatography in combination with mass spectroscopy is a sensitive analytical technique for monosaccharide as well as certain di- and tri-saccharide composition analysis, allowing detection of sub-nanomole amounts of carbohydrates. The method provides information of both identity and quantity of the component monosacchaides. The detection is by means of Flame Ionization Detector (FAD), which responds to all carbohydrate related molecules over an extremely wider linear range. GLC separation is dependent upon the differential extractive distillation of the component in the mixture; therefore the volatile derivatives of the carbohydrates are prepared. The earlier strategies involved the use of Gas chromatography (GC) and GC/MS for quantification of monosaccharides and linkage determination. Liberation of components

sugars from glycoconjugates by use of aqueous acid catalysis has been considered to be the main cause of loss in carbohydrate analysis. To increase the volatility and hydrophobicity of sugar molecules, permethylation was utilized in the gas phase analysis (Kunkel, et. al., 1998 and Urashima, et. al., 1997)

1.4 STRUCTURAL CHARACTERIZATION OF MILK OLIGOSACCHARIDES

In the world of natural product chemistry, the determination of three dimensional structures is often very difficult, sometimes even for a monosaccharide; because many carbohydrates differ only in their stereochemistry, which produces the similar spectral data. Thus, the structure elucidation of oligosaccharide is not an easy task because of the close similarity in the structures of constituent sugar residues and due to the existence of multiple substitution points. Tradationally the structure elucidation of oligosaccharides were based on sequential digestions with exoglycosidases, regiospecific chemical degradation, and methylation / acetylation analysis, but with the recent advances in physico-chemical techniques like LC-NMR-MS, GC-MS, FAB, TOF, ESI-MS, MALDI-MS, High-field proton, carbon and various 2-dimensional NMR experiments (COSY, HSQC, HMBC, TOCSY, NOESY, HOHAHA) it has become possible to define the all-structural aspects of oligosaccharides. In structure elucidation of oligosaccharide, NMR and Mass spectroscopy plays an important role and are extremely powerful tool for the structure elucidation of oligosaccharides. NMR and Mass spectrometry methods still represent the good standard for structure analysis of oligosaccharides and help in determination of the configuration, linkage positions, and anomericity of monosaccharide residues of milk oligosaccharides. For structural characterization of milk oligosaccharides simple as well as complex methods was developed. Many milk oligosaccharides contain the units of ABH antigens, α-Gal, Lewis a, b, x or y, and/or α (2-3)/(2-6) sialyl residues linked to core structures such as lacto-N-tetraose, lacto-N-neotetraose, lacto-N-hexaose, lacto-N-neohexaose, para lacto-N-hexaose, lacto-N-octaose, etc. As some similarities among milk oligosaccharide structures were found, analysis of structure reporter groups as well as anomer resonances in their ^{1}H NMR spectra, which had been developed the oligosaccharides structure elucidation has became very helpful for oligosaccharides analysis with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) or fast atom bombardment mass spectrometry (FAB-MS) and ES-MS plays important role in structure elucidation of oligosaccharides. Milk oligosaccharides can also be characterized by ^{1}H-^{13}C correlated spectroscopy (^{1}H-^{13}C COSY), heteronuclear single quantum coherence experiment

(HSQC), heteronuclear multiple bond correlation experiments (HMBC), $^{1}H–^{1}H$ Homonuclear Hartmann–Hahn experiments ($^{1}H–^{1}H$ HOHAHA), etc., characterises the milk oligosaccharides.

1.4.1 Sequencing techniques of oligosaccharides - Once a milk oligosaccharide is isolated, the process of determining its monomer sequence is not an easy effort. In oligosaccharide sequencing, one must also determine how each sugar is linked. There are number of techniques used to identify and sequence the monosaccharides in an oligosaccharide.

1.4.1.1 Acid hydrolysis - The acid hydrolysis of pure oligosaccharide molecule helps in confirmation and identification of constituent monosaccharides. In carbohydrate chemistry, different types of acid hydrolysis are used according to the structure, linkage and nature of oligosaccharide molecule. In Kiliani acid hydrolysis (AcOH-H_2O-HCl, 7:11:2) strong acid condition is used, it is commonly used for the hydrolysis of oligosaccharides comprised of normal sugars. Mannich and Siewart hydrolysis (conc. HCl/Acetone) is used for the acid hydrolysis of oligosaccharides made up of normal and 2 deoxy sugars, it also helps in knowing the sequence of monosaccharides in oligosaccharides. The Trifluro-Acetic acid could also used, instead of HCl or H_2SO_4 because apart from expected destruction of sialic acid, all other sugar molecule can be determined. Rangaswami and Reichstein, method of hydrolysis is used for the sequences of the sugar in oligosaccharides involving 2-deoxy hexoses. It has been observed that in very mild acid hydrolysis condition, the sugars are hydrolysed in sequence from the terminal end and the liberated sugars can be identified by PC or TLC. Some times hydrolysis carried out in the presence of Ethanol (AcOH, H_2SO_4 or HCl in EtOH) or Methanol (H_2SO_4 or HCl in MeOH). After Acid Hydrolysis, for confirmation of each monosaccharides molecule are compared with commercially available authentic sample ([α] D, TLC and PC) followed by their chemical transformation (Mechref, 2002 and Deepak, et. al., 1986)

1.4.1.2 Methylation - Methylation is a useful transformation technique used in carbohydrate analysis, which provides information that, which hydroxyl groups are engaged in glycosidic linkage/substituted. Methylation with CH_3I and NaOH followed by its acid hydrolysis gives useful information about glycosidic linkages. Investigated oligosaccharides in H_2O at temperatures below $0^{o}C$ by supercooling or addition of acetone to prevent freezing. This method can be used to identify positions in the monosaccharide residues of oligosaccharides which are glycosidically linked. The aliphatic protons at carbons with OH attached will show couplings to the OH group at low temperature and can be identified by

comparison of spectra obtained in D_2O and H_2O using, e.g., 1D TOCSY or by the line broadening. The remaining aliphatic protons, often with sharper signals, will then correspond to positions of the glycosidic linkages or substituted positions. This method requires only small amounts of material compared to the amounts required for full NMR structural analysis. If this indirect method fails to identify the glycosidic positions due to overlap, the positions bearing OH can be identified in a 2D COSY by the correlation between OH protons and aliphatic protons. Similar experiments can be carried out in DMSO, where the exchange of OH-protons is slow even at room temperature **(**Killiani, et. al, 1930).

1.4.1.3 Peracetylation - This is another method based on a similar idea peracetylation of free hydroxyl groups with ^{13}C-carbonyl-labeled acetic anhydride either fully enriched or only at the carbonyl carbon. This method can be used to separate free hydroxyl positions from positions involved, e.g., in glycosidic linkages. The protons at acetyl protected positions will show a three bond ^{13}C-^{1}H coupling constant and can be readily identified. This coupling constant has been shown to be n the range from 2.5 to 4.7 Hz and with no interference from the four bond heteronuclear coupling constant, which are small and buried within the line width. The assignment of an acetylated position can be performed by a comparison of a COSY (gradient COSY) and carbon-decoupled COSY spectra. In complex structures, where the protons cannot be identified directly, the advantage of using a ^{13}C label is the increase in sensitivity in HMBC or heteronuclear COSY spectra. Additionally, the protection with acetate increases the chemical shift range where the aliphatic protons are observed and thereby allows for assignment of more complex structures. (Townsend, et. al., 1988)

1.4.1.4 Digestion with exoglycosidases - The specificity of the exoglycosidases in oligosaccharide sequencing cannot be ignored. Judicious use of the enzymes of known specificity can give insight into the glycosylation of a purified oligosaccharide. Sequential exoglycosidase digestion could combine with the chromatographic (gel permeation or size exclusion) or electrophoretic techniques for exploring the "sequence" of an oligosaccharide. Some of the important glycosidases used in milk oligosaccharides are jack bean β-galactosidase [cleave β Gal(1→6) or (1→4) linked], diplococcus β-galactosidase cleave β-D-Galp-(1→4)-D-GlcNAc not (1→3) or (1→6)], jack bean N-acetyl-β-D-hexosaminidase (cleave all β-Gal and GlcNAc linkages), the bovine testes β-galactosidase (cleave all three types of Gal linkage), the bovine epididymis α-L-Fucosidase (cleaves all the α-L-

fucopyranosyllinkages), bacillusfulminas α-fucosidase (cleaves fuc (1→2)Gal) and almond emulsin a-fucosidase (cleave α-L-fuc linkages of the β-D-Galp(1→4)-[α-L-fucp-(1→3)]-D-GlcNAc and (β-D-Galp(1→3)-[α-L-fucp-(1→4)]-D-GlcNAc) etc. (Ersser, et. al., 1979 and Endo, et. al., 1986).

1.4.1.5 Methyl glycosidation/Acid hydrolysis of compound - Compound was ref1uxed 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 methylglycoside of, 1, 4-dioxane, and 0.1N H_2S0_4 was added and the solution was warmed for 30 minutes at 50ºC. 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).

1.5 NMR OF MILK OLIGOSACCARIDES - In the field of Glyco-chemistry and particularly in structure elucidation of oligosaccharides, application of NMR spectroscopy is enormous. For primary structural analysis of a monosaccharide, oligosaccharide, or polysaccharide by NMR spectroscopy, different research groups performed different approaches for structure elucidation of carbohydrate. The 'structural-reporter-group' concept, which is based on signals outside the bulk region (3-4 ppm) in the ^{1}H NMR spectra of carbohydrates. This approach is used to identify individual sugar or sequences of residues. NMR-based structure elucidation is combined with data obtained from chemical degradation and mass spectrometry. Carbohydrates normally have at least two NMR-active nuclei, ^{13}C and ^{1}H, but also less frequently used nuclei like ^{2}H, ^{3}H, ^{11}B, ^{15}N, ^{17}O, ^{19}F and ^{31}P can be used for studies of natural or synthetic oligosaccharides. The dispersion of resonances in the carbon spectra is favourable, but the amount of material needed to acquire such spectra is relatively high due to the low natural abundance of ^{13}C but in proton NMR the dispersion of NMR signals are a limitation. The signals are divided into two parts *i.e*., anomeric region and the ring proton region. This problem of complexity was overruled by various 2-D experiments like HOMOCOSY, HETROCOSY, TOCSY and NOESY experiments. For elucidating the structure of oligosaccharides following procedure may be adopted.

1. Number of sugar residues - The number of anomeric proton chemical shift and the integration of the anomeric resonances gives an idea about the number of different monosaccharide residues present in the oligosaccharides. The anomeric proton resonances

are found in the shift range 4.2-5.5 ppm in ^{1}H NMR. The remaining ring proton resonances are found in the range 3-4.2 ppm in the unprotected oligosaccharides. Similarly, the range of anomeric carbon varies from 90 – 110 ppm (Utille, et. al., 1981).

2. Constituents monosaccharides - Homonuclear TOCSY and DQF-COSY spectra are useful in the identification of individual monosaccharides residues. In TOCSY spectra of oligosaccharides acquired with a fairly long mixing time (>100 ms), it is often possible to measure the size of the coupling constants and the correlation to reveal the oligosaccharides residue. Both ^{1}H and ^{13}C chemical shifts for most of the monosaccharaides can be found in the literature, and based on such values an assignment of the individual sugars can be made (Jens, et. al., 2002).

3. Anomeric configuration - In oligosaccharide molecule normally the α-anomer resonates downfield compared to the β-anomer in D-pyranoses in $^{4}C_{1}$ conformation. If H1 and the H2 are both are in an axial configuration in pyranose structure, a large coupling constant (8-10 Hz) is observed, whereas if they are equatorial-axial configuration, this is smaller (*J* 1, 2~4 Hz), and for axial-equatorial or equatorial-equatorial oriented protons, even smaller coupling constants are observed (<2 Hz). The ^{13}C chemical shift reveals the anomeric configuration in a similar manner to the proton chemical shifts.

4. Linkages and sequence- Both the ^{1}H and the ^{13}C chemical shift may give an indication for the linkage of complete oligosaccharide moiety. The effect of glycosylation shift depends on the linkage type, and the changes in the chemical shift are in general larger at the glycosylation site than at neighbouring positions (Uhrin, 1997 and Sheng, et. al, 1988).

5. Position of appended groups-The non-carbohydrate groups like methyl, acetyl, sulphate, or a phosphate group could be pointed by the proton and carbon NMR chemical shifts. Presence of these groups affects the proton and carbon resonance where the group is located. Normally downfield shifts of 1 ppm are observed for protons and higher δ values for ^{13}C. This places these resonances in a less crowded area of the spectra and helps the identification of novel residues (Vinogradov, et. al., 1998). Different NMR experiments used in structure elucidation of milk oligosaccharides are as follows-

1.5.1 ONE DIMENSIONAL NMR SPECTROSCOPY

1.5.1.1 Proton NMR (^{1}H NMR) - High resolution proton spectroscopy of carbohydrate gives the complete information about the finer stereo-chemical details such as configuration of the glycosidic linkages and ring conformation. Despite the fact that most resonances are clustered between ~δ 3.4 and ~δ 4.0, ^{1}H spectra of carbohydrates do contain some well-resolved signals,

including those of anomeric protons (δ 4.4- δ 5.5), acetyl (~δ 2.0-δ 2.1) and methyl (~δ 1.2) groups, and other protons that are influenced by specific functionality, including amino groups, phosphorylation, sulphation, glycosylation, and acetylation, or the lack of functionality as in deoxysugars. Apart from this diagnostic chemical shift data, comparison of the integrated intensities of anomeric protons can reveal the number of monosaccharide residues. The chemical shift of a particular anomeric proton and its splitting pattern provides the configuration of sugar linkage and conformation of that monosaccharide unit. Since the chemical shift of the anomeric proton of various sugars and various methine protons of different sugars are confined to the region δ 4.2-5.5 and δ 3.2-4.5, respectively, hence it requires expert interpretation of spectra for monosaccharide identification. In the pyranosides, the six-member ring generally forms a chair conformation ($^4C_1$) providing axial and equatorial protons. Therefore, the coupling patterns are characteristic of the stereochemistry of monosaccharide units usually the anomeric resonances of α-glycosides resonate at a downfield position by 0.3 to 0.5 ppm than its corresponding β-glycosides. The anomeric doublets appear in the region from 4.8-5.3 ppm, with coupling constant (3J1,2) in range 2-4Hz (H_{eq}-H_{ax}; dihedral angle 60^o) are those for α-anomeric proton, where as β-anomeric proton appear as doublets between 4.4-4.8 ppm with coupling constant (3J1,2) in range 8-10 Hz157 (H_{ax}-H_{ax}; dihedral angle 180^o) in monosaccharides with glucose and galactose stereochemistry. The analysis of reducing oligosaccharides showed that the anomeric configuration of the reducing end sugar also exerts its influence on the spectral parameters of residues in its spatial neighborhood, being sometimes even the non-reducing end sugar. To resolve the spectral complexities of oligosaccharides (Vligenthart et. al, 1983), introduced the "structural reporter group" concept, which was based on signals outside the bulk region (δ 3-4) in the ^{1}H NMR spectra of the carbohydrates. This approach is used to identify individual sugars or sequence of residues. These structural reporter groups include anomeric proton, equatorial protons, deoxy protons and that distinct functional group such as amide group, *i.e.* anomeric protons at 4.3-5.5 ppm, methyl doublets of 6-deoxy sugars at 1.1-1.3 ppm, methyl singlets of acetamido groups at 2.0-2.2 ppm and various others with distinctive chemical shift. These in conjunction with vicinal 1H-1H coupling constants (3J H, H) can be correlated with known structures to yield relevant information in terms of monosaccharides units and their relative abundance. The structure of different linkages can be defined in terms of NMR parameters of their structural reporter groups. In case of milk oligosaccharides the anomeric proton resonances are found in the chemical shift range 4.4-5.5 ppm and the remaining ring proton resonance are found in the

range 3.0-4.2 ppm in unprotected oligosaccharides. But in case of acetylated oligosaccharides, the acetyl groups induce a strong downfield shift of proton linked to hydroxylated carbons. The signals of methine protons shifts further downfield (4.8-5.5 ppm) than those of methylene protons (~4.0-4.8 ppm). The resonances of protons linked to the non-acetloxylated carbons at the site of glycosidic linkage and at the ring C-5 occupy the interval between 3.5 and 3.9 ppm. The H-2 signal of 2-acetyl amine-2 deoxy glucose occurs in the highest field (δ 2.9-3.5)(Van, 1996, Agarwal,1992 and Kobata and Ginsburg, 1972).

Some of the common spectral feature of the ^{1}H NMR structural reporter groups of milk oligosaccharides can be summarized as follows-

1. The ^{1}H NMR spectra show the typical feature of reducing oligosaccharides ending in lactose. The reducing Glc residue is characterized by the H-1 signals of or it's α and β anomers at δ 5.221 (J1, 2 3.7 Hz) and δ 4.688 (J1, 2 8.0 Hz) respectively with ratio of 7:10.
2. The 4 substituted reducing Glc shows anomeric signals from both the α- and the β-anomeric at δ 5.22 and 4.66 ppm, with H-2 of the β-from in the range of δ 3.2-3.3 ppm.
3. The 3, 4- disubstituted reducing Glc shows anomeric signals from both the α- and the β-anomeric at δ 5.22 and δ 4.66 ppm, with H-2 of the β-from at a typical downfield shift above δ3.33 ppm.
4. The 3, 6-disubstituted β-linked Gal shows signal for H-1 at 4.4 ppm and H-4 at a typical downfield shift around δ 4.13-4.15 ppm due to substitution in the 3-position by a β-linked GlcNAc residue.
5. The H-4 of (1→6) linked β-Gal is around δ 3.8-3.9 ppm and H-4 of (1→3) linked β-Gal is around δ 3.9-4.2 ppm.
6. β-linked GlcNAc residues with anomeric signals appear at δ 4.6-4.7 ppm and CH_3 signals in the range of δ 2.02-2.08 ppm. H-1 of the (1→6) linked GlcNAc appears at lower chemical shift value (δ 4.6 ppm.) than the (1→3) linked GlcNAc residue (δ 4.7 ppm). A splitting of the anomeric doublets is due to the anomerization of the reducing terminal.
7. The H-2 of β-GlcNAc at δ 3.6-3.8 ppm and β-GalNAc at δ 3.8-4.2ppm.
8. Presence of anomeic signals with a integration of two proton at δ 4.44-4.6 ppm suggest a LNT structure in which one β-Gal is attached to Glc by (1→4) linkage while another β-Gal unit is attached to β-GlcNAc or β-Glc by (1→3) linkage i.e. β-Gal(1→3)β-

GlcNAc (1→3/6) β-Gal (1→4) Glc or β-Gal (1→3) β-Glc (1→3/6) β-Gal (1→4) Glc moieties is present.

9. Signals for H-1 of the unsubstituted Gal residues appear around δ 4.44-4.47 ppm.

10. The N-acetyl CH_3 signals are found at δ 2.026 and δ 2.056 ppm for the 1→3 and 1→6 linked GlcNAc residue, respectively.

11. α-linked Gal residues with anomeric signals appears at δ 4.945-5.2 ppm.

12. α-linked Gal residues with anomeric signals at δ 5.02 ppm is (1→4) linked, at δ 5.2 ppm is (1→2) linked, and between two (1→3) linked.

13. α-linked Fuc residues with anomeric signals appears at δ 5.02-5.435 ppm.

14. The presence of fucose subunit could be inferred by the presence of CH_3 doublet at δ 1.1-1.3, H-5 at δ 4.2-4.9 and the anomeric doublet at δ 5.02-5.2 ppm.

15. α-linked Fuc residues with anomeric signals at δ 4.984ppm (1→4) linked, at δ 5.384 ppm (1→2) linked, and between two (1→3) linked.

16. The presence of sialic acid residue could be ascertained by the characteristic resonances of H-3 axial and equatorial protons of sialic acid at δ 1.78 and δ 2.75 respectively.

17. The anomeric configuration of Neu5Ac can usually be inferred from the chemical shift of the Neu5Ac H-3eq and H-4 resonances. Analysis of a series of model substance shows that for α anomers, H-3eq varies between δ 2.6 and δ 3.8 ppm, for β anomers, these ranges are δ 2.1 to δ 2.5 ppm & δ 3.9 to δ 4.2 ppm, respectively.

18. The location of a Neu5Ac residue can be deduced as follows, (a) the signal for H-3a and H-3e of the Neu5Ac residue can be used to discrimination between (2→3) and (2→6)-α-linkage to Gal. (b) for an α-Neu5Ac(2→3)-β-Gal-(1-sequence, the signal for H-3 of the Gal residue is shifted down field of the ring protons by ~0.6 ppm. Also, in a) β-GlcNAc-(1→3)-β-Gal-(1-sequence, the signal for H-4 of the Gal residue appears at ~δ4.15) ppm.

The information signals, assignments for an oligosaccharide can be obtained by comparison with reported compounds, particularly with monosaccharides or with oligosaccharides with fewer monosaccharide residues. Once individual resonances have been assigned to specific sugar residue, and then NOE and relaxation experiments involving these resonances can decisively prove the position of attachment of sugar moiety of milk oligosaccharide (Kobata and Ginsburg, 1972b, William, 2003, Vilengenthart, et. al., 1983, Kamerling, et. al., 1982, Urashima, et.al., 2003a, 2004b, 2005c 2006d, 2007e, Gopal and Gill, 2000 and Horst, 1998).

The spectra are very helpful in providing information about the finer stereochemical details such as configuration of the glycosidic linkage and ring conformation. However, it is also of value for the determination of the number of methoxy groups present, the position of hydroxyl group and nature and position of acyl groups. The characteristic splitting pattern of anomeric protons(s) signals of sugar moieties present in the glycosides reveal not only the configuration of glycosidic linkages but also indicates the size and conformation of monosaccharides. In case of 2-deoxy sugars, the anomeric proton usually appears as a double doublet (Yuan, et. al., 1992) in the region δ 4.0-5.2 and some times as a triplet (Abe and Yamauchi, 1988) obtained by the merger of inner notches of the doublet, while the anomeric proton of normal sugars appear only as a doublet (Deepak, et. al., 1992) in the region δ 4.2-5.5. In 2-deoxy sugars, if the splitting constant of the double doublet is of 7-10 and 1-2 Hz, then it confirms the presence of β-glycosidic linkage in $^{4}C_{1}$ conformation (Allgeier, 1968) where H-1 is axial, whereas a smaller coupling of 3-4 and 1 Hz indicates the nature of glycosidic linkages as α where the sugar is in $^{1}C_{4}$ conformation (Allgeier, 1968) and H-1 is equatorially obtained. The splitting pattern of the anomeric proton of normal sugars depends on the conformation of H-1 as well as that of H-2 are trans to each other the resultant coupling observed in the anomeric proton signal would be large (J = 7-11 Hz) and when they are cis, a small coupling (J = 3-1 Hz) would result. A downfield glycosidation shift of 0-6.1 ppm is observed in the anomeric proton signal of glycosidic ally linked sugar with respect to the free sugar. In the higher field the characteristic signal for secondary methyl groups appear as doublets (J = 6 Hz) in the region δ 1.0-1.5.The signal for methylene protons of 2-deoxy sugar appear separately for equatorial and axial protons as two sets of multiplet in the region δ 2.0-2.5 and 1.5-2.0. The signal of H-3, H-4 and H-5 in hexoses appears as multiplets in the region δ 3.5-4.5.

1.5.1.2 ^{13}C NMR spectroscopy -The introduction of high field proton NMR spectroscopy has brought improved criteria of precision to the structural identification of glycopeptides and the complex oligosaccharides of related structure. This non destructive method can be used simultaneously to verify the purity of an oligosaccharide preparation and to identify the residues, their anomeric configuration and linkage, as well as their position in the oligosaccharide chain. In principle it would appear the ^{13} C NMR spectroscopy with its greater chemical shift dispersion could offer an even more powerful method for oligosaccharide structure identification. Because of the similarity in chemical shift of most of the methine protons of the carbohydrate ring, assignments in proton NMR spectra have often been limited to certain “Structural reporter groups” such as anomeric protons, equatorial protons, de-oxy

protons and those of distinct functional groups such as amide. In contrast, the carbon spectra of carbohydrates give resolved lines for nearby all of the carbon atoms even in oligosaccharides of moderate size, reducing the chance for misidentification of an oligosaccharide. Proton decoupled ^{13}C NMR spectra are well resolved and usually provide an unambiguous identification of a monosaccharide, especially when anomeric chemical shift is compared with a collection of ^{13}C NMR data of monosaccharides. This spectroscopy is also useful for the structure elucidation of oligosaccharides because of greater chemical shift dispersion in lack of complexities arising from spin-spin coupling overlap of resonances. The number of sequence and linkage of a sugar could be assigned by the ^{13}C NMR data and identify of monosaccharide has also been established by the comparison of chemical shift of anomeric carbon with the reported values. The total number of monosaccharide units presents in milk oligosaccharide can be fixed by counting the number of anomeric carbons presents in the ^{13}C NMR of that particular compound. These signals generally appear between the ranges of δ 90-110. The chemical shift of the anomeric gives perfect information regarding the nature of the monosaccharides. It also provides information regarding the nature of glycosidic linkages. The signals due to β-linkages usually appear 2.0-6.0 ppm downfield from their α-counterpart. The resonances of $–CH_3$ of 6-deoxy sugars, methoxy function, $-CH_2OH$ of normal hexoses and ring carbons generally appear in the region δ 16-199, δ 55-62, δ 60-64 and δ 65-85, respectively. The presence of sialic acid residue could also be well determined by ^{13}C NMR spectroscopy. The anomeric signals (C-2) appear at δ 100-101 ppm while signal for –COOH group appears at δ 174 ppm **(**Horst, 1998, Jihui, et. al., 2004, Elin, et. al., 2004, Ticoa, et. al., 2005, Simone, et. al., 2006, Colin, et. al., 2006, Christian, et. al., 2006, Lipkind, et. al., 1990, Jansson, et. al., 1999, and Agarwal, et. al., 1992).

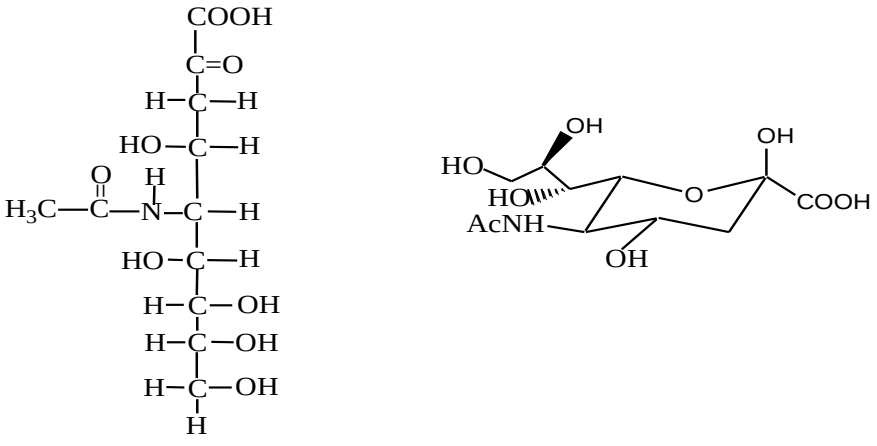

Fig.1.1 N-acetyl neuraminic acid (5-amino3, 5 dideoxy-D-glycero-D-galacto-2-nonulosonic acid)

1.5.2 Two - dimensional NMR spectroscopy - The NMR spectroscopy is today probably the most often used analytical technique which can provide invaluable information about

carbohydrate molecules. 1D NMR methods provide limited information for the determination of the complete structure and stereochemistry of oligosaccharides due to the substantial overlapping of multiplets and other overlapped signals. These difficulties can be overcome by the use of modern high-field NMR experiments. For interpretation of ^{1}H NMR spectra of oligosaccharides which are not identical to those closely related two known compounds, complete assignment of the methine and methylene resonances in the poorly resolved group of signals in the region 3.2-4.0 ppm adds greatly to the structural information. Recognition of NMR signals belonging to closed spin system i.e. to individual sugar residues is always the first stage of structural analysis. The general approach is to assign an isolated resonance often an anomeric proton (4.3-5.4 ppm) or the methyl resonance (1.2-1.4 ppm) in 6-deoxy sugars, then to correlate spins in a step-wise manner around the spin system of the ring. However, spin correlation can be done by one-dimensional difference decoupling if only few assignments are needed. These difficulties could be overtaken by the use of modem two-dimensional NMR experiments because they are more efficient for the simultaneous determination of a large number of spin correlations. 2D NMR spectroscopy provides actual, high quality and well interpretable data of the sugar molecule. There are two fundamental types of 2D NMR spectroscopy: *J*-resolved spectroscopy in which one frequency axis contains spin coupling (*J*) and other chemical shift (δ) information and correlated spectroscopy in which both frequency axes contain chemical shift (δ) information.

1.5.2.1 Correlated spectroscopy (COSY) - The conventional way to identify monosaccharide units involves use of COSY which identifies direct *J*-coupling (e.g. geminal and vicinal). This technique was invented by Belgian Jeenar and is one of the important 2D NMR spectroscopic methods for structure determination. ^{1}H-^{1}H COSY (correlated Spectroscopy) is useful for determining which signals arise from neighbouring protons, especially when the multiplets overlap or there is extensive second order coupling. A COSY spectrum yields through bond correlation via spin-spin coupling. There are two types of correlation spectroscopy *i.e.* HOMOCOSY and HETROCOSY. In homonuclear shift correlation 2D experiments, the correlation is between similar nuclei *i.e.* either ^{1}H-^{1}H or ^{13}C-^{13}C is observed. The normal NMR spectra are plotted on a two frequency axis and the conventional 1D spectrum appears along the diagonal. The clear representation of 2D NMR spectrum is obtained as contour plots of mutual coupling which exists between two nuclei (^{1}H-^{1}H, ^{13}C-^{13}C), cross peak appears at the chemical shift coordinates (X, Y) and (Y,

X). Identification of monosaccharide units is first approached by analyzing the ^{1}H homonuclear shift-correlation spectra. The COSY spectra contain information on spin coupling networks within the constituent's residues of the oligosaccharide through the observation of cross peaks. Assignment of this spectrum by coupling-correlation requires an initial point for the identification of the individual spin system of sugar rings. The most downfield ^{1}H signals (anomeric) are always a convenient starting point for the assignment. With in typical aldohexopyranosyl ring, the coupling network is unidirectional *i.e.* H-1 couples to H-2; H-2 couples to H-1 and H-3, H-3 to H-2 and H-4 and so on. However, the presence of no or small coupling between H-4 and H-5 ($J_{4,5}$ = 2-3 Hz) of galactopyranosyl residue and coupling between H-1 and H-2 in mannopyranosyl residue prevents detection of cross peaks. The COSY experiments and its RELAY extensions give coupling pattern along with shift information, which allow each monosaccharide residue to be identified and designated as α or β and also provide information about sugar identity and substitution pattern. Sugar analysis indicated the presence of 3Gal, 1Glc, 2GlcNAc and 3NeuAc residues, in the sialyl oligosaccharide present in milk. Further, on the basis of the upfield resonances of the easily distinguished the H-1 signals of Gal and GlcNAc. The H-1 signal of Glc-β was correlated with H-2 signal at δ 3.32 ppm. The structure was also deduced by correlating the characteristic chemical shift of H-3 ax and NeuAc residue α-2, 6-linked to GlcNAc III and the low field shifted value of Gal IV H-3 at 4.078 ppm with H-1 and H-2 resonance on the COSY spectrum. There are different types of 2D COSY NMR experiments found to be extremely useful in the identification of each monosaccharide unit of oligosaccharides moiety (Gronberg, et. al., 1992 and Keeler, 2010).

1.5.2.1.1 Relay correlation spectroscopy (RELAY-COSY) - In this 2D technique the correlation of anomeric proton is observed not only with H-2 proton, but also with other intra residue protons (H-3, H-4, H-5 and H-6) in a well resolved region of the 2D spectrum. But the limitation of this technique is H-6 proton.

1.5.2.1.2 Double-quantum filtered correlation spectroscopy (DQF-COSY) - It provides a clear and accurate way of obtaining chemical shift values coupled protons. It not only provides characteristic multiplicity within the cross peak, enabling identification of particular sugar units but also provides semi quantitative information of the coupling constants of protons involved in the cross peak. For better visualization of cross peaks, which are close to diagonal axis can be achieved by the introduction of a double quantum

filter, which generates a COSY spectrum having both cross peaks and a diagonal multiplet anti phase structure (Findlay, et. al., 1972).

1.5.2.1.3 Triple quantum filtered correlation spectroscopy (TQF-COSY) - In this method all the spin system that contain less than three or more mutually coupled spins are eliminated by the use of a triple quantum filtration. This technique is useful in making assignments of mutually couple H-5, H-6 in hexopyranosides system (Piantini, et. al., 1982).

1.5.2.1.4 IN ADEQUATE spectroscopy (IN ADEQUATE) - The two dimensional 'incredible natural abundance double quantum transfer experiment' provides direct information on carbon bounding, Relayed techniques generally combine two coherence transfer steps, one of which is used to modulate the signals and generate the one dimension, while the other remains fixed and in series to pass on the signal to a more interesting destination. For instance, the most common path is to transfer magnetization from one proton to another and then on to a heteronucleus coupled to the second proton. Thus, the original proton can be correlated not only with the heteronucleus to which it is directly attached, but also with another one nearby providing the required information about the molecular skeleton (Davis, et. al., 1991).

1.5.3 Total correlation spectroscopy (TOCSY) - TOCSY is a more recent 2D NMR experiment for identifying extended couplings is TOCSY (Total Correlation Spectroscopy) it is also known as HOHAHA (Homonuclear Hartman Hahn Spectroscopy). In this technique an isotropic mixing is added after the evolution time by applying a sequence of pulse which effectively averages out chemical shifts. This can be thought of as a sequence of pulses, each of which refocuses the chemical shifts. In fact, all coupled spins will have the same precession frequency, so they will be strongly coupled and their transitions will be thoroughly mixed. So, this method mainly used for the structural assignments of complex carbohydrates. The 2D HOHAHA technique is helpful in analysis of the sub spectrum of each monosaccharide unit present in the oligosaccharides. This experiment is especially helpful in sugars where similar chemical shifts of methine protons leads to confusion. TOCSY/HOHAHA can give total correlation of all protons in a chain with each other and it helps in the identification of single residue (Dunkel, et. al, 1992 and Keeler, 2010).

1.5.4 Nuclear overhauser effect spectroscopy (NOESY) - A NOESY spectrum yields through space correlation via the Nuclear Overhauser Effect Spectroscopy. Inter residue NOEs are the principle assignment tool for determination of the sequence of sugar residue

and also in determining their linkage positions. The Nuclear Overhauser effect (NOE) holds a position of great importance in organic structure elucidation as it enables us to define the three dimensional stereochemistry of sugar molecules of interest. ^{1}H-^{1}H NOESY is useful for determining which signals arise from protons, which are close in space (distance smaller than 5Å) but not closely connected by chemical bonds. By measuring cross peak intensity, distance information can be extracted. In practice semi selective excitation of one carbohydrate proton, combined with multistep-relayed coherence transfer and a terminal NOE transfer has been used for the sequential analysis of oligosaccharides. Assignment of the anomeric and some of the other protons resonance may be made with the help of data from decoupling and NOE experiments. In a glycoside (G-O-S1-O-S2), because the proton on C-1 of S-1 is close enough to the proton bonded to C-4 of S-1, it is possible to demonstrate coupling between the two rings from a NOESY experiment. The PK 1,3–diaxial and eq-ax proton pairs in pyranosyl ring produce intra NOESY crosspeak *i.e.* for β-glycopyranosyl residue crosspeaks are observed between H-1and H-3 (and H-5) whereas strong crosspeak observed between H-1 and H-2 in an α-glucopyranosyl configuration. In the structural determination of Lacto-N-hexaose, selective decoupling irradiation at each of the three doublets assigned to the β-Galactose H-1 at 4.424, 4.454 and 4.504 ppm identified the resonances of the corresponding H-2 at 3.546, 3.489 and 3.496 ppm, respectively. Selective decoupling irradiation of the narrow doublet at 4.145 ppm, which is assigned to H-4 of a β-galactose, is C-3, substituted. Decoupling at the gal H2 resonance at 3.546 ppm identifies this same gal H-3 resonance this completing the assignment of H-1, H-2 H-3 and H-4 of the branching galactosyl residue. Confirmation of β-GlcNAc H-1 is found in NOE observed at that doublet on irradiation of β-GlcNAc H-3 at 4.077 ppm. The appearance of β-GlcNAc H-3 as a structural reporter group result from fucosylation at C-4 was observed in case of LNF-II. Irradiation at 4.077 ppm also gives NOE at the doublet at 4.504 ppm allowing its assignment to β-gal H-1. Irradiation of α-Fuc H-1 NOE at β-GlcNAc H-4 (3.749 ppm) due to inter ring NOE and at α-Fuc H-2 (3.683 ppm) due to intra-ring NOE. Selective decoupling at β-GlcNAc H-3 and decoupling at H-1 identifies H-2 completing the assignment of the resonances of β-GlcNAc. An effective way of connecting the monosaccharide residue is by monitoring the nuclear Overhauser effect from signal for an anomeric reporter group to the hydrogen of the substituted position in the adjacent ring **(**Ernst and1987).

1.5.5 Rotating frame overhauser enhancement spectroscopy (ROESY) - In cases where attempts to obtain reliable NOE crosspeaks are unsuccessful, a ROESY spectrum can show all NOE crosspeaks defining interglycosidic linkage. Because NOE is a function of molecular rotation time, which itself depends on the size and shape of the molecule. Viscosity of the medium and temperature are important in this type of experiment. The NOESY technique has the disadvantage that for molecules with a molecular mass in the order of 1000 to 3000 the signal may disappear, since the NOE effect change its sign depending on the molecular correlation time. Due to the well know problems involved with NOE measurements at medium field strength form medium-sized molecules, a 2D NOE in a rotating frame (ROESY) can be of importance **(**Keeler, 2010a, 2010b).

1.5.6 Heteronuclear multiple quantum coherence (HMQC) spectroscopy - The ^{1}H-^{13}C one-bond correlations through a ^{13}C-decoupled 1H detected heteronuclear multiple quantum coherence spectrum at a higher contour level led to the assignment of all ^{13}C resonances of the α anomer. Some times the proton resonances of oligosaccharides are too overlapping to be disentangled by homonuclear correlation alone. In such cases heteronuclear correlation maps may enable the assignment of ^{1}H resonances, because in such a spectrum one observes connectivities between ^{1}H and ^{13}C chemical shifts. This method spreads the ^{1}H NMR spectrum in the ^{13}C dimension, thus greatly improving the resolution and eliminating the effects of strong ^{1}H couplings. Usually ^{1}H-^{13}C cross peaks do not superimpose until the ^{1}H and ^{13}C chemical shifts are identical due to the presence of a very similar chemical environment correlation of H-2 and H-3 resonances at 4.05 and 4.36 ppm with ^{13}C resonances at 53.5 and 51.5 ppm led to the assignment of these amido substituted carbons to C-2 and C-3, respectively. The chemical shift for NeuAc with H-3 at 1.786/2.756 ppm (ax/eq) and a β- linked Gal with H-1 at 4.503 ppm is linked α 2$\rightarrow$3 to a Gal residue. The presence of α-linked Fuc is verified by signals of H-1 at 5.099 ppm, H-5 at 4.825 ppm, and CH_3 at 1.170 ppm. The chemical shifts for C-1 at 101.6 ppm of GlcNAc residue showed that this sequence is connected to the 6-position of the 3, 6 disubstituted Gal residue. The ^{1}H-^{13}C one-bond dipolar coupling values were measured for samples of the human milk oligosaccharide (Kiji, 1990).

1.6 MASS SPECTROMETRY - Mass spectrometry is one of the oldest and broadly applicable analytical tools in the chemical sciences in biological research. Mass spectrometry is an important analytical tool for structure elucidation of complex oligosaccharides/oligoglycosides comprising composition, sequence, branching, and linkage

analysis, including anomericity and finally also the rings sizes and absolute configuration *i.e.*, identity of the subunits. It can give accurate mass of molecular or fragment ions, which provide peripheral structural information of the oligosaccharide molecule. The characterization and analysis of glycoconjugates and oligosaccharides by mass spectrometry has undergone a number of improvements in the last 10 years, especially with the development of methods capable of ionizing and analyzing these compounds in their native states. The purified milk oligosaccharides can be identified with greater certainty by mass spectroscopic technique. Most of the oligosaccharides are composed of five unique monosaccharide units with different incremental masses and knowledge of the molecular weight can be used to determine the potential composition of the oligosaccharide. In general, mass spectrometry provides the possibility of structural elucidation based on characteristic fragmentations of the molecules under investigation.

Most of the human milk oligosaccharides consist of building blocks, *i.e.*, lactose at the reducing end linked to multiple units of *N*-acetyl lactosamines, which differ in size, branching, and linkage, with additional fucose or sialic acid residues linked to the core oligosaccharides. The structural analysis of milk oligosaccharides must be addressed to the following aspects:

Composition analysis.

Determination of branching positions.

Differentiation of the two core-constituents *N*-acetyllactosamine, *i.e.*, Galβ (1-3) GlcNAc (LacNAc, lacto-series or "type I") and Galβ (1-4) GlcNAc (Lac-*neo*-NAc, lacto-neo-series or "type II").

Determination of the position of fucose and / or sialic acid residues.

Linkage of the *N*-acetyllactosamine subunits (and the lactose to the first *N*-acetyllactosamine) is β (1-3).

Linkage of the fucose residues to *N*-acetylglucosamines depends on the linkage of the corresponding *N*-acetyllactosamine subunits: in the lacto-series, the fucose is (α 1-4) linked and in the lacto-neoseries the linkage is (α 1-3).

All carbohydrate researchers trace the Mass spectroscopic data and elucidate an exact and precise mass of the complex milk oligosaccharide. This spectrometric technique offers the possibility of structural investigations of each purified milk oligosaccharides; it has been demonstrated by using of "soft" ionization techniques (FAB-MS, LC-MS, thermospray and electrospray MS) has even expanded the utility of MS for the analysis of large biopolymers.

Fast-atom bombardment (FAB) and electrospray-ionization (ESI), mass spectrometry have both been utilized successfully to this end. Matrix-assisted laser desorption /ionization (MALDI) is the most suitable ionization method for the analysis of carbohydrates collected after HPAEC, because MALDI is 10-100 times more sensitive than FAB for detection of underivatized oligosaccharides and is more tolerant of salts than either FAB or ESI. The molecular ion was fragmented into the fragment units which were formed by the decomposition pathways in which repeated H transfer in the oligosaccharide is accompanied by the elimination of terminal sugars less water, such fragmentation goes on until the monosaccharide is left **(**William, 2003) and given in Fig.1.2.

1.6.1 Fast atom bombardment Mass spectrometry (FAB-MS) - Fast atom bombardment (FAB) ionisation has been shown to be a very useful method in the mass spectrometric analysis of a wide range of natural compounds, such as glycosides, saccharides, antibiotics, and other highly polar compounds. However, although FAB itself can provide relative molecular mass information of glycosides and saccharides, the low abundance of $[M+H]^+$ ions and chemical noise from the background of the FAB matrix greatly limit the molecular mass information. In this method ions are produced in a mass spectrometer from non-volatile or thermally fragile organic molecules by bombarding the compound in the condensed phase with energy-rich neutral particles. This technique is very helpful in the mass spectrometric determination of milk oligosaccharides. Purified milk oligosaccharides may be analyzed directly by FAB-MS but derivatization of the oligosaccharides to enhance their surface activity markedly improves the sensitivity of the method. A first insight into the composition of milk oligosaccharides can be obtained by the determination of the molecular ions either using negative ion desorption of native compounds positive ion desorption with peracetylated or permethylated compounds. The spectra of the peracetylated oligosaccharides show intense pseudo molecular ion M^+, Na^+ and $M+Na^+$ together with the daughter ion formed by the elimination of one or two $CH_2=C=O$ units, *i.e.*, 42 or 84 a.m.u. in comparison to the FAB spectra could be obtained with 1-mercapto-2, 3-propanediol and the addition of sodium acetate to the target. Besides the very intense pseudo molecular ion M^+, Na^+ and $M+K^+$, often constituting the base peak, fragments ions characteristic for the carbohydrate constituent are also present in the reduced and peracetylated neutral oligosaccharides glucose as glucitol, fucose, galactose and N-acetylglucosamine each contributes specific mass increments to the molecular weight

(Heide, et. al, 2004, Barber, et. al., 1981, Martinez-Fereza, et. al., 2006, Niggemann, et. al., 1988, Viverge, et. al., 1997 and Rudolff, et. al., 1996).

1.6.2 Matrix assisted laser desorption / ionisation Mass spectroscopy (MALDI -MASS) - One of the most intriguing of the recent MS technologies is matrix-assisted laser desorption MS (known as LD-MS or MALDI-MS), this spectrometric technique has become an important tool for the analysis of milk oligosaccharides. Matrix-assisted laser desorption/ionization coupled with Fourier-transform mass spectrometry (MALDI-FTMS) offers high resolving power and high sensitivity and makes it especially suited for analization of large biomolecules. With this mass spectrometric method, neutral and anionic milk oligosaccharides are analyzed simply in positive and negative mode, respectively and derivatization of compound is not required in this technique for structure determination.

Matrix-assisted laser desorption -PSD (MALDI-PSD) mass spectrometry is now widely used in the molecular mass determination of unsderivatized oligosaccharides. An analysis of the relative ion intensities in the MALDI-PSD mass spectra oligosaccharides was very useful for distinguishing the linkage isomers and for the characterizing the type of glycosyl linkage. The native oligosaccharides of lacto-N-neo tetraose (Gal β 1-4 GlcNAc β 1-3Gal β1-4 Glc; LNnT) and lacto-N-tetraose (Gal β 1-3 GlcNAc β 1-3Gal β1-4 Glc; LNT) were analyzed by using curved field reflection matrix-assisted laser desorption/ ionization time of flight mass spectrometry (MALDI-TOFMS). Since a curved-field reflection TOFS enables a simultaneous focusing of a wide mass range of metastable fragment ions, the relative ion intensities in the PSD mass spectra can be discussed. The PSD mass spectra of LNnT and LNT were distinguish in the relative ion intensities. In case of LNT, β-elimination could occur in the GlcNAc at the C-3 position, which was bonded by Gal; however, it did not occur in LNnT. The 3-0 elimination caused a difference in the relative ion intensities in the PSD mass spectra of LNnT and LNT. The β 1-3 glycosyl linkage cleaved more easily than the β 1-4 glycosyl linkage in the MALDI-PSD fragmentation. In the PSD-mass spectra of the oligosaccharides, the PSD ions produced by cleavage of the α1-6 had higher intensities than those of the β 1-4 glucosyl linkage, indicating that the α1-6 linkages cleaved much more readily than the β 1-4 glycosyl linkage (Brunz, et. al., 1998).

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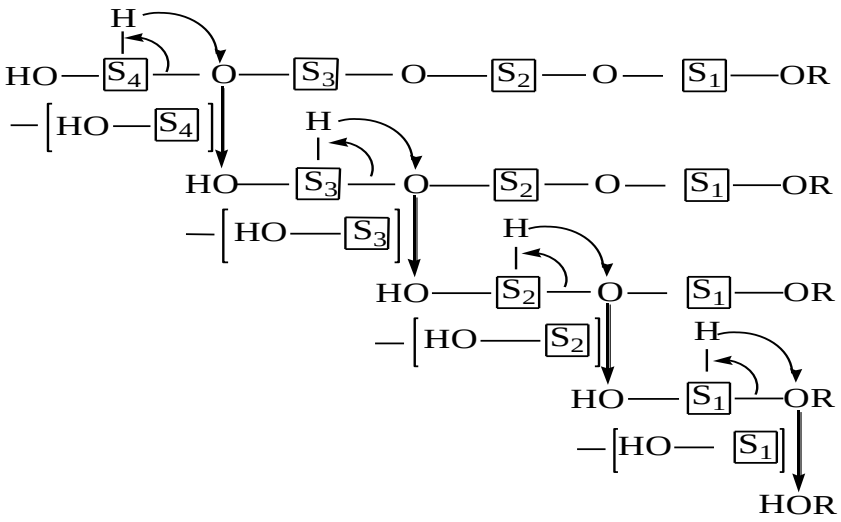


Fig. 1.2 H-Transfer in oligosaccharide and elimination of nonreducing sugar residue

CHAPTER-2

ISOLATION

2.1 GENERAL - Buffalo (*Bubalus bubalis)* has been an integral part of livestock agriculture in Asia for over 5000 years producing, milk, meat, hides and draft power. They are the second most important species for milk production in the world after cows. The importance of the buffaloes is also conferred by a longer longevity, high dry content of milk and a strong organic resistance when compared with cows. There are two general types of water buffaloes, the swamp type (*B. carabanesis*) and the river type (*Bubalus bubalis*). *Bubalus bubalis* is the most appreciated for milking in countries around the world. The world total buffalo population is about 94 million of which 56% are in India Murrah breed of water buffalo (*Bubalus bubalis*) is common domestic variety of buffalo, originally from Punjab and Haryana states of India, but has been used to improve the milk production of dairy buffalo in other countries such as Pakistan, Italy, Bulgaria, Egypt etc. It is a large, extremely strong, dark grey animal, standing nearly 6 ft (180 cm) at the shoulder and weighing up to 2,000 lb (900 kg). It's widely spread horns curve out and back in a semicircle and may reach a length of 6 ft (180 cm). Their diet consists chiefly of grass and wheat hey. It delivers young ones mostly are in number; the young's are suckled for one year for which there mammies and mammary glands are produces milk. It is warm blooded with hair, sweat glands and heterodant teeth.

2.1.1 Systematic (zoological) description of buffalo –

Kingdom	-	Animalia
Phylum	-	Chordata
Class	-	Mammalia
Order	-	Artiodactyla
Family	-	Bovidae
Sub family	-	Bovinae
Tribe	-	Bovini
Genus	-	*Bubalus*
Species	-	*Bubalis*

2.1.2 Buffalo milk - Buffalo milk is a natural product that can be consumed like any other milk. It is nearly complete food and contains all the essential components in balanced form. Buffalo milk is lower in cholesterol and higher in calcium than cow, sheep or goat milk. In addition to the significant cholesterol and calcium benefits, buffalo milk is also a rich source of Iron, phosphorus and also contains sodium, zinc, copper and manganese in small

amounts. It is a rich source of riboflavin, vitamin B_{12}, vitamin A, vitamin C and thiamin. Small amounts of folate, pantothenic acid vitamin B_6 and niacin are also found in buffalo milk. 100 gm of buffalo milk has 97 calories. Buffalo milk also contains high levels of natural antioxidant tocopherol. Its peroxidate activity is normally 2-4 times that of cow's milk. An unfortunate sign of the times is the growing number of people suffers from Cows Milk Allergy (CMA). Fortunately, this is not the case with buffalo milk which is suitable for many who suffers from CMA. Buffalo milk is good for healthy bones, dental health, cardiovascular health, weight gain etc.

2.1.3 Normal composition of buffalo milk - Milk is very complex, naturally possessing many chemical and physical components; all milks contain the same kind of major constituents but in varying amount. Within a given species, genetic factors and environmental condition such as the climate and the storage of lactation influence the compositions. A compositional chart of different constituents of milk of four different species i.e. cow, goat, sheep, buffalo is as follows-

Table 2.1 Composition analysis of major four different milk producing species

MILK COMPOSITION ANALYSIS					
Constituents	**Unit**	**Cow**	**Goat**	**Sheep**	**Buffalo**
Protein	Grm	3.2	3.1	5.4	4.5
Fat	Grm	3.9	3.5	6.0	8.0
Carbohydrate	Grm	4.8	4.4	5.1	4.9
Energy	K cal K J	66 275	60 253	95 396	110 463
Sugars (Lactose)	Grm	4.8	4.4	5.1	4.9
Fatty Acids- Saturated Monounsaturated Polyunsaturated	 grm grm grm	 2.4 1.1 0.1	 2.3 0.8 0.1	 3.8 1.5 0.3	 4.2 1.7 0.2
Cholesterol	Mg	14	10	11	8
Calcium	Iu	120	100	170	195

2.1.4 Colostrum - In general, milk obtained from different types of animal origin is of two types. One is "colostrum" means early milk and another is "mature milk" that is late or normal milk. So, colostrum is a special type of milk produced in eutherian mammals during the 1st few days after birth, is loaded with immune, growth and tissue repair factors. It is a complex biological fluid, which help in the development of immunity in the newborn. It contains significant quantities of complement components that act as natural antimicrobial agent to actively stimulate the maturation of an infant's immune system. The composition of

colostrum and mature milk is quite different. Colostrum contains various nutrients which changed toward normal milk from the day after birth.

2.1.5 Collection and storage of colostrum - Twelve (12) litre buffalo colostrum (1 to 5 day) were collected from a domestic buffalo (murrah) from the Kharika village near Telibagh of district Lucknow, Uttar Pradesh. The milk was fixed by addition of equal amount of ethanol. The preserved milk was taken to laboratory and there it was centrifuged for 15 min at 5000 rpm at -4 ^{o}C. It shows following physicochemical properties –

Colour	-	Shiny pale white
Odour	-	Characteristic
Taste	-	Sweetish
Existence form	-	Thick liquid
P^{H}	-	6.7
Boiling Point	-	101.75°C
Freezing point	-	-0.560°C

Colostrum is a rich source of biologically active oligosaccharides but their isolation from colostrum is difficult task because of their high polar in nature, low concentration, and microheterogeneity.

2.2 ISOLATION OF COLOSTRUM OLIGOSACCHARIDES BY MODIFIED KOBATA AND GINSBURG METHOD - 12 liter buffalo colostrum was collected from domestic buffalo (murrah) from the Kharika village near Telibagh of district Lucknow, Uttar Pradesh. The milk was fixed by addition of equal amount of ethanol, the preserved milk was taken to laboratory and there it was centrifuge for 30 min at 5000 rpm at -4^{o}C. The solidified lipid layer was removed by filtration through glass wool column in cold. More ethanol was added to clear filtrate to a final concentration of 68% and the resulting solution was left over night at 0^{o}C. The white precipitate formed, mainly of lactose and protein was removed by centrifugation and washed twice with 68% ethanol at 0^{o}C. The supernatant and washing were combined and filtered through a microfilter and lyophilized affording crude oligosaccharides mixture.

Buffalo Colostrum (12 lit)

Equal amount of alcohol was added, at 4ºC, filtered through a loosely packed Glass wool column in cold

Lipid Layer

Residue discarded

Supernatant

Was precipitated by addition of 68% ethanol and separated by centrifugation

Supernatant

Was filtered through a microfilter (0.2µ) to remove remaining lactose. It was then lyophilized

Protein and Lactose

Residue discarded

Lyophilized material

(Mixture of oligosaccharides) was then fractionated on a Sephadex G-25 column, eluted with triple distilled water at flow rate 3ml/min. Fractions were analyzed for sugars by phenol-sulphuric Acid reagent

Carbohydrate containing fractions

(Fractions were pooled, lyophilized and analyzed by HPLC)

Analytical HPLC

The carbohydrate fractions were eluted with TDW (containing 0.1% TFA & CH_3CN) at a flow rate 1ml/min., to check homogeneity of the oligosaccharide mixture. The elution was monitored by UV absorbance at 220 nm.

Chemical transformation

Fractions with (+) test for sugars could not be separated over silica gel, hence the oligosaccharide mixture was acetylated with Ac_2O and pyridine converting free sugar into non-polar acetyl derivatives which were resolved nicely on TLC and were separated by column chromatography over silica gel which resulted in the isolation of chromatographically pure compounds

Deacetylation

↓

The chromatographically pure acetylated Milk oligosaccharides were deacetylated by dissolving them in acetone & NH_3 and left overnight. Ammonia was removed under reduced pressure and the compound was washed with $CHCl_3$ and was finally freeze dried giving the deacetylated milk oligosaccharides.

2.3 CONFIRMATION OF HOMOGENEITY OF *Bubalus bubalis* COLOSTRUM OLIGOSACCHARIDES BY REVERSE PHASE HPLC –

Oligosaccharide mixture were quantitatively analysed by reverse phase HPLC. The HPLC system was equipped with Perkin–Elmer 250 solvent delivering system 235 diode array detector and G.P.100 printer plotters.The cyano column used for this purpose was a binary gradient system. The eluents were detected at 240 nm. Twelve peaks were noticed in the sample at varied retention times from 2.5 min. to 34.5 min. For convenience the peaks were numbered in their increasing order of retention time i.e. 2.50 (R_1), 4.00 (R_2), 10.2 (R_3), 12.0 (R_4), 12.6 (R_5), 19.5 (R_6), 26.6 (R_7), 26.8 (R_8), 27.0 (R_9), 27.3 (R_{10}), 33.0 (R_{11}), 34.5(R_{12}).

Table 2.2 HPLC chromatogram of *Bubalus bubalis* colostrum

S.No.	Retention time (Rt)	Percentage area of each peak
1	2.50	12.0
2	4.00	10.0
3	10.2	8.00
4	12.0	3.00
5	12.6	2.50
6	19.5	2.50
7	26.6	3.50
8	26.8	3.00
9	27.0	19.0
10	27.3	8.50
11	33.0	10.0
12	34.5	18.0

HPLC finger print profile was established by MSBC. Elution was carried out at a flow rate of 1.5 ml/min with water: phosphoric acid (100:0.3 v/v) as solvent A and acetonitrile: water: phosphoric acid (79.7:20:0.3 v/v) as solvent B, using a gradient elution in 0-5 min. with 88-85% A, 5-15 min. with 85-70% of A, 15-20 min. with 70-50% A and 20-25 min. with 50-30% of A and isocratic till 35 min. with 30% of mobile phase A. Detection was done at 320 nm using 2996 PDA detector.

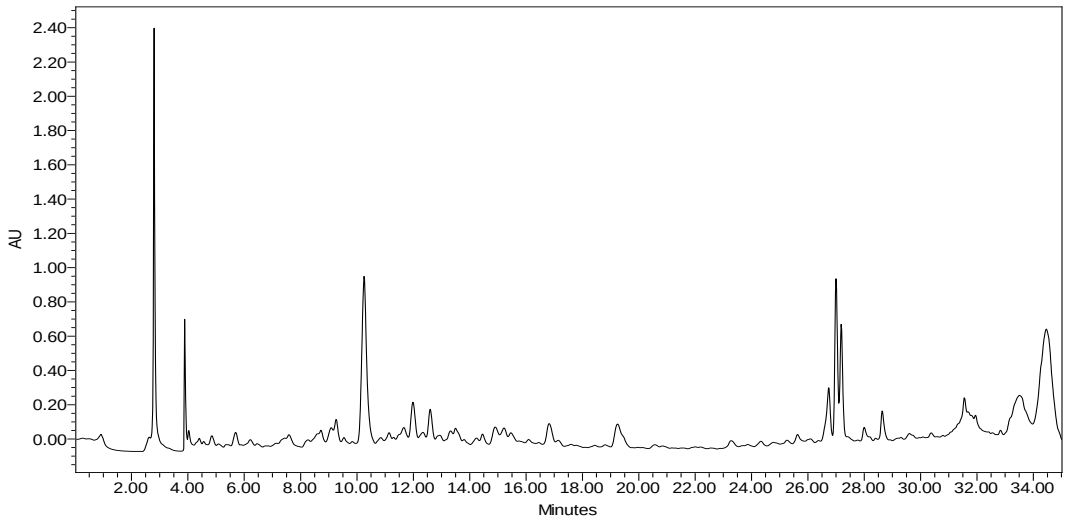
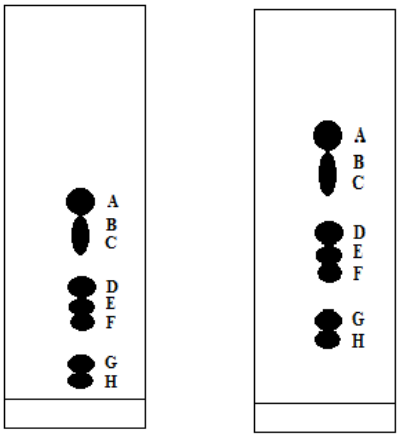


Fig 2.1 HPLC of oligosaccharide mixture *Bubalus bubalis* colostrum

2.4 ACETYLATION OF OLIGOSACCHARIDES - 8.0 gm of crude oligosaccharide mixture was acetylated by adding pyridine (8.0 ml) and acetic anhydride (8.0 ml) at 60^{o}C with constant stirring and kept overnight. The mixture was evaporated under reduced pressure and the viscous residue was taken in $CHCl_3$ (250 ml) and washed with ice cold water. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding the acetylated mixture (9.60 gm). 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, F, G and H, out of which four compounds have been finally separated by column chromatography over silica gel using hexane: chloroform, chloroform: chloroform, and chloroform: methanol as eluents (Fig 2.2). The thin layer chromatogrphy of acetylated buffalo colostrum oligosaccharide is as follows:



$CHCl_3$: MeOH (98:2) $CHCl_3$: MeOH (95:5)

Fig 2.2 Thin layer chromatography of acetylated oligosaccharide mixture

2.5 PURIFICATION OF ACETYLATED OLIGOSACCHARIDE MIXTURE

A- First column chromatography -The purification of acetylated oligosaccharide mixture of about 8.0 gm was carried out over silica gel using different proportions of Hexane: $CHCl_3$, $CHCl_3$ and $CHCl_3$:MeOH as mobile phase or eluent. Fractions were collected after every 200 ml and were checked by thin layer chromatography and the fractions which were found similar were collected together for further investigations. Details are given in Table 2.3

Table 2.3 8.0 gm mixture chromatographed over 400 gm silica

Fraction No.	Solvent	Eluted Residue Amorphous	Spots on TLC	Further Investigation
1-15	Hex : $CHCl_3$ 10 : 90	30 mg	Streaking	-
16-40	$CHCl_3$	218 mg	streaking a with a, b	-
41-60	$CHCl_3$:MeOH 99.8 : 0.2	1.242 g	a, b, c	CC-2, CC-3
61-100	$CHCl_3$:MeOH 99.5: 0.5	1.302 g	b, c	CC-4, CC-5
101-120	$CHCl_3$:MeOH 99 : 1	504 mg	c, d, e	-
121-140	$CHCl_3$:MeOH 98 : 2	432 mg	d, e, f	CC-6, CC-7
141-165	$CHCl_3$:MeOH 98 : 2	650 mg	f, g	CC-8
166-188	$CHCl_3$:MeOH 95 : 5	215mg	Washing	CC-9
189-200	$CHCl_3$:MeOH 90:10	-	-	-

B – Second column chromatography -The fractions of 41-60 (800 mg) from first column chromatography, which contains 'a' and 'b' were chromatographed over 100 gm silica gel. The elution was carried out using solvents Hexane:$CHCl_3$, $CHCl_3$ and $CHCl_3$:MeOH as eluants in different proportions and collecting fractions of 200 ml each. Details are given in Table 2.4

Table 2.4 800 mg mixture chromatographed over 100 gm silica

Fraction No.	Solvent	Eluted Residue Amorphous	Spots on TLC	Further Investigation
1-34	Hex : $CHCl_3$ 10 : 90	14 mg	Streaking	-
35-55	$CHCl_3$	56 mg	a with streaking	-
56-75	$CHCl_3$: MeOH 99.5 : 0.5	236 mg	a, b	-
76-81	$CHCl_3$: MeOH 99 : 1	128.4 mg	a, b	-
82-95	$CHCl_3$: MeOH 98 : 2	150 mg	b with streaking	-
96-105	$CHCl_3$: MeOH 98 : 2	22 mg	b with streaking	-
106-121	$CHCl_3$: MeOH 98 : 2	-	Washing	-

C - Third column chromatography -The fractions of 56-81 (364.4 mg) from second column chromatography, which contains 'a' and 'b' were chromatographed over 40 gm silica gel. The elution was carried out using solvents Hexane:$CHCl_3$, $CHCl_3$ and $CHCl_3$:MeOH as eluants in different proportions and collecting fractions of 150 ml each. Details are given in Table 2.5

Table 2.5 364.4 mg mixture chromatographed over 40 gm silica

Fraction No.	Solvent	Eluted Residue Amorphous	Spots on TLC	Further Investigation
1-10	Hex : $CHCl_3$ 10 : 90	23 mg	Streaking	-
11-27	$CHCl_3$	25 mg	a with streaking	-
28-45	$CHCl_3$: MeOH 99 : 1	47 mg	a	Physico-chemical investigation
46-70	$CHCl_3$: MeOH 98 : 2	12 mg	a, b	-
71-83	$CHCl_3$: MeOH 95 : 5	25.5 mg	b with streaking	-
84-100	$CHCl_3$: MeOH 90 : 10	-	Washing	

D - Fourth column chromatography - The fractions of 61-100 (500 mg) from first column chromatography, which contains 'a', 'b' and 'c' were chromatographed over 50 gm silica gel. The elution was carried out using solvents Hexane:$CHCl_3$, $CHCl_3$ and $CHCl_3$:MeOH as eluants in different proportions and collecting fractions of 150 ml each. Details are given in Table 2.6

Table 2.6 500 mg mixture chromatographed over 50 gm silica

Fraction No.	Solvent	Eluted Residue Amorphous	Spots on TLC	Further Investigation
1-18	Hex : $CHCl_3$ 10 : 90	18 mg	Streaking	-
19-37	$CHCl_3$	23 mg	a with streaking	-
38-50	$CHCl_3$: MeOH 99.5 : 0.5	54 mg	a, b	-
51-78	$CHCl_3$: MeOH 99 : 1	40 mg	b with streaking	-
79-97	$CHCl_3$: MeOH 98 : 2	180 mg	b, c	CC-5
98-107	$CHCl_3$: MeOH 95 : 5	44 mg	b, c	CC-5
108-115	$CHCl_3$: MeOH 90 : 10	-	Washing	-

E – Fifth column chromatography -The fractions of 79-107 (224 mg) from fourth column chromatography, which contains 'b' and 'c' were chromatographed over 30 gm silica gel. The chromatographic process was carried out by using solvents Hexane:$CHCl_3$, $CHCl_3$ and $CHCl_3$:MeOH as eluants in different proportions and collecting fractions of 150 ml each. Details are given in Table 2.7

Table 2.7 224 mg mixture chromatographed over 30 gm silica

Fraction No.	Solvent	Eluted Residue Amorphous	Spots on TLC	Further Investigation
1-15	Hex : $CHCl_3$ 10 : 90	4 mg	Streaking	-
16-22	$CHCl_3$	10 mg	streaking	-
23-40	$CHCl_3$: MeOH 99 : 1	54 mg	b with streaking	-
41-59	$CHCl_3$: MeOH 98 : 2	34.0 mg	b	physico- chemical investigation
60-72	$CHCl_3$: MeOH 95 : 5	46 mg	b, c	-
73-80	$CHCl_3$: MeOH 90 : 10	-	Washing	-

F - Sixth column chromatography -The fractions of 120-140 (432 mg) from first column chromatography, which contains 'c', 'd' and 'e' were chromatographed over 40 gm silica gel. The solvents Hexane: $CHCl_3$, $CHCl_3$ and $CHCl_3$:MeOH were used in different proportions for elution and collecting fractions of 150 ml each. Details are given in Table 2.8

Table 2.8 432 mg mixture chromatographed over 40 gm silica

Fraction No.	Solvent	Eluted Residue Amorphous	Spots on TLC	Further Investigation
1-12	Hex : $CHCl_3$ 10 : 90	12 mg	Streaking	-
12-25	$CHCl_3$	46 mg	c with streaking	-
26-36	$CHCl_3$: MeOH 99 : 1	168 mg	c, d	CC-7
37-50	$CHCl_3$: MeOH 98 : 2	44 mg	c, d	CC-7
51-78	$CHCl_3$: MeOH 95 : 5	45 mg	d, e	-
79-87	$CHCl_3$: MeOH 90 : 10	-	washing	-

G - Seventh column chromatography -The fractions of 26-50 (212 mg) from sixth column chromatography, which contains 'c' and 'd' were chromatographed over 30 gm silica gel. The solvents Hexane: $CHCl_3$, $CHCl_3$ and $CHCl_3$:MeOH were used in different proportions for elution and collected fractions of 150 ml each. Details are given in Table 2.9

Table 2.9 212 mg mixture chromatographed over 30 gm silica

Fraction No.	Solvent	Eluted Residue Amorphous	Spots on TLC	Further Investigation
1-10	Hex : $CHCl_3$ 10 : 90	12 mg	Streaking	-
11-27	$CHCl_3$	14 mg	c with streaking	-
28-40	$CHCl_3$: MeOH 99 : 1	20 mg	c, d	-
41-63	$CHCl_3$: MeOH 98 : 2	78 mg	c	physico-chemical investigation
64-75	$CHCl_3$: MeOH 95 : 5	7.5 mg	d with streaking	-
76-90	$CHCl_3$: MeOH 90 : 10	-	washing	-

H - Eighth column chromatography - The fractions of 141-165 (650 mg) from first column chromatography, which contains 'd', 'e' and 'f' were chromatographed over 70 gm silica gel. The solvents Hexane:CHCl3, CHCl3 and CHCl3:MeOH were used in different proportions for elution and collected fractions of 150 ml each. Details are given in Table 2.10

Table 2.10 - 650 mg mixture chromatographed over 100 gm silica

Fraction No.	Solvent	Eluted Residue Amorphous	Spots on TLC	Further Investigation
1-25	$CHCl_3$	16 mg	d, e with Streaking	-
26-51	$CHCl_3$: MeOH 99.5 : 0.5	44 mg	d	Physico- Chemical Investigation
52-78	$CHCl_3$: MeOH 99.5 : 0.5	180 mg	d, e with streaking	-
79-85	$CHCl_3$: MeOH 99.5 : 0.5	54 mg	e	physico-chemical investigation
86-91	$CHCl_3$: MeOH 99.5 : 0.5	125 mg	e, f with streaking	CC-9
92-115	$CHCl_3$: MeOH 99.5 : 0.5	24 mg	e, f with streaking	CC-9
116-125	$CHCl_3$: MeOH 99 : 1	12 mg	Washing	-

I - Ninth column chromatography -The fractions of 86-115 (149 mg) from eight column chromatography, which conntains 'e' and 'f' were chromatographed over 20 gm silica gel. The solvents Hexane:$CHCl_3$, $CHCl_3$ and $CHCl_3$:MeOH were used in different proportions for elution and collected fractions of 50 ml each. Details are given in Table 2.11

Table 2.11 149 mg mixture chromatographed over 20 gm silica

Fraction No.	Solvent	Eluted Residue Amorphous	Spots on TLC	Further Investigation
1-30	$CHCl_3$	16mg	e,f with Streaking	-
26-51	$CHCl_3$: MeOH 99.5 : 0.5	12mg	e,f with Streaking	-
52-78	$CHCl_3$: MeOH 99.5 : 0.5	58mg	f	physico-chemical investigation
79-85	$CHCl_3$: MeOH 99.5 : 0.5	13mg	f with streaking	-
86-90	$CHCl_3$: MeOH 99 : 1	12mg	Washing	-

2.6 DEACETYLATION OF ISOLATED COMPOUNDS - Acetylated oligosaccharide compound was obtained from column chromatography was dissolved in equal amount acetone and ammonia 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 3 x $CHCl_3$ (to remove acetamide) and water layer was finally freeze dried giving the deacetylated oligosaccharide. The acetylated compounds 'a', 'b', 'c' and 'd' are obtained from column chromatography on deacetylation gave respective native oligosaccharides. Details are given in Table 2.12

Table 2.12 Acetylated & Deacetylated oligosaccharides of buffalo colostrum

Acetylated Compound				Deacetylated Compound		
Alphabetical name	Analytical notation	Quantity (mg)		Alphabetical name	Analytical notation	Quantity (mg)
		Obtained by column	taken for deacetylation			
A	MSBC-4 A	44.0	30.0	A	MSBC-4	29
B	MSBC-3B	78.0	50.0	B	MSBC-3	35
C	MSBC-2C	34.0	34.0	C	MSBC-2	31
D	MSBC-1D	47.0	25.0	D	MSBC-1	16

2.7 DESCRIPTION OF ISOLATED COMPOUNDS

A - Compound A (MSBC-4) Meeniose:

Compound A (44 mg) was obtained from fraction 41-63 of eight column chromatography. On deacetylation of 30 mg of substance with NH_3 / acetone, it afforded compound A (29mg). For experimental analysis, this compound was dried over P_2O_5 at 100^0 C and 0.1 mm pressure for 8 hr.

$C_{36}H_{61}O_{26}N_3$		%C	%H	%N
	Calculated	45.42	6.41	4.41
	Found	45.42	6.46	4.41

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

^{1}H NMR of Meeniose: δ in at 300 MHz

5.69 [d, 1H, J = 3.6 Hz, α-Glc (S-1) H-1], 4.50 [d, 1H, J = 8.0Hz, β-Glc (S-1) H-1], 2.10 [s, 3H, $NHCOCH_3$, β -GalNAc (S-2)], 1.96 [s, 3H, $NHCOCH_3$, β-GalNAc (S-5)], 1.92s, 3H, $NHCOCH_3$, β -GlcNAc (S-3)].

^{1} H NMR of Meeniose: Acetate δ in $CDCl_3$ at 400 MHz

6.19 [d, 1H, J = 3.6 Hz, α-Glc (S-1) H-1], 5.62 [d, 1H, J = 8.0Hz, β-Glc (S-1) H-1], 4.43 [d, 1H, J = 8.0Hz, β-GalNAc (S-2) H-1], 5.62 [d, 1H, J = 8.0Hz, β-GlcNAc (S-3) H-1], 5.03 [d, 1H, J = 8.0Hz, β-Gal (S-4) H-1], 4.44 [d, 1H, J = 7.6Hz, β-GalNAc (S-5) H-1], 3.80 [d, 1H, , β-Glc (S-1) H-4], 3.80 [d, 1H, β-GlcNAc (S-3) H-4], 3.74 [d, 1H, β-GalNAc (S-2) H-3], 3.74 [d, 1H, β-Gal (S-4) H-2], 4.07 [d, 1H, β-GalNAc (S-2) H-2], 4.08 [d, 1H, β-GalNAc (S-5) H-2]

^{13}C of Meeniose acetate: **δ in $CDCl_3$ at 400 MHz**

88.82[1C, α-Glc (S-1) C-1], 91.40[2C, β-Glc (S-1) & β-GlcNAc (S-3) C-1], 100.79[2C, β-GalNAc (S-5) & β-GalNAc (S-2C-1)], 101.04 [1C, β-Gal (S-4) C-1].

ES Mass of compound A Meeniose

1013[M+Na+K], 990 $[M+K]^+$, 951$[M]^+$, 933, 893, 862, 748, 657, 729, 700, 657, 586, 550, 528, 490, 383, 352, 325, 310, 296, 258, 180

B - Compound B (MSBC-3) Murtiose:

Compound B (78 mg) was obtained from fraction 26-51 of seventh column chromatography. On deacetylation of 50 mg of substance with NH_3 / acetone, it afforded

substance B (35mg). For experimental analysis, this compound was dried over P_2O_5 at 100^0 C and 0.1 mm pressure for 8 hr.

$C_{34}H_{58}O_{26}N_2$		%C	%H	%N
	Calculated	44.80	6.30	3.07
	Found	42.83	6.42	3.08

It gave positive Phenol-Sulphuric acid test, Feigl test and Morgon-Elson test

^{1}H NMR MSBC-3 Murtiose: δ in D_2O at 300 MHz

δ5.69 [d,1H, J = 4.0,α- Glc(S-1),H-1], 4.63,[d, 1H, J = 8.0, β Glc(S-1), H-1], 5.20 [d,1H, J = 1.0, α- GalNAc (S-5),H-1], 4.49 [d,1H, J=8.0, β-Gal (S-3), H-1], 4.42,[d,1H, J=8.0, β-Gal (S-2),H-1], 1.96[S, 3H, α-GalNAc (S-4)NHCOCH$_3$], 1.90[S,3H, α-GalNAc (S-5)NHCOCH$_3$].

^{1}H NMR of Murtiose: Acetate δ in $CDCl_3$ at 400 MHz

6.08 [d, 1H, J = 4.0 Hz, α-Glc (S-1) H-1], 5.62 [d, 1H, J = 8.0Hz, β-Glc (S-1) H-1], 5.26 [d, 1H, J = 1.0 Hz, α-GalNAc (S-5) H-1], 4.70 [d, 1H, J = 2.0 Hz, α-GlcNAc (S-4) H-1], 4.45[d, 1H, J = 8.0 Hz, β-Gal (S-3) H-1], 4.37 [d, 1H, J = 8.0Hz, β-Gal (S-2) H-1], 4.05 [d, 1H, β-Gal(S-2) H-2], 3.74 [d, 1H, β-Gal (S-2) H-3], 3.72 [d, 1H, β-Glc (S-1) H-4], 3.70 [d, 1H, α-GalNAc (S-4) H-3].

^{13}C of Murtiose acetate: δ in $CDCl_3$ at 400 MHz

89.61[1C,α-GalNAc (S-5) C-1], 90.93[1C, α-Glc (S-1) C-1], 91.29[1C, β-Glc (S-1) C-1], 94.69 [1C, αGalNAc (S-4) C-1], 100.63[1C, β-Gal(S-2)], 100.70[1C, β-Gal (S-3) C-1].

ES Mass of compound B Murtiose

972 [M+Na+K], 949[M+K] $^+$, 910[M] $^+$, 875, 851, 820, 785, 748, 671, 717, 545,487, 507, 485, 342, 325, 284, 281,180.

C - Compound C (MSBC-2) Vediose:

Compound C (34mg) was obtained from fraction 41-59 of fifth column chromatography. On deacetylation of 34 mg of substance with NH_3 / acetone, it afforded compound C (31mg), For experimental analysis, this compound was dried over P_2O_5 at 100^0 C and 0.1 mm pressure for 8 hr.

$C_{52}H_{88}O_{41}N_2$		%C	%H	%N
	Calculated	44.69	6.30	2.00
	Found	44.70	6.35	2.00

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

^{1}H NMR Vediose: δ in D_2O at 300 MHz

δ 5.20 [d, 1H, J = 3.2, α- Glc(S-1), H-1], 4.62[d, 1H, J = 7.8, β Glc(S-1), H-1], 5.70 [d,1H, J = 2.0 Hz, α Glc(S-3), H-1], 5.54 [d, 1H, J = 3.0, α-Glc (S-6), H-1], 4.52 [d, 1H, J = 7.4, β-Gal (S-7), H-1], 4.51 [d, 1H, J = 7.8, β-Gal (S-8), H-1], 4.43 [d, 1H, J = 7.8, β-GalNAc (S-4),H-1], 1.96 [S,3H, β-GalNAc (S-4)$NHCOCH_3$], 1.92 [S,3H, α-GalNAc (S-5)$NHCOCH_3$].

^{1} H NMR of Vediose acetate: δ in $CDCl_3$ at 400 MHz

6.22 [d, 1H, J = 3.2 Hz, α-Glc (S-1) H-1], 5.65 [d, 1H, J = 8.4 Hz, β-Glc (S-1) H-1], 5.57 [d, 1H, J = 2.0 Hz, α-Glc (S-3) H-1], 5.34 [d, 1H, J = 3.0 Hz, α-Glc (S-6) H-1], 5.27[d, 1H, J = 1.6 Hz, α- GalNAc (S-5) H-1], 4.67 [d, 1H, J = 6.0Hz, β-Gal (S-2) H-1], 4.60 [d, 1H, J = 7.2, β-Gal (S-7) H-1], 4.54 [d, 1H, J = 8.4, β-Gal (S-8) H-1], 4.46 [d, 1H, J = 7.8, β-GalNAc (S-4) H-1], 3.52 [m, 1H, β-Glc (S-1) H-4], 3.73 [m, 2H, β-Glc (S-1) H-3& β-Gal (S-2) H-3], 3.70 [m, 2H, α-Glc (S-3) H-3& β-GalNAc (S-4) H-3], 3.53 [m, 1H, α-Glc (S-3) H-2, 3.40 [m, 1H, β-Gal (S-2)H-2]

^{13}C of Vediose acetate: **δ in $CDCl_3$ at 400 MHz**

89.00[1C,α-Glc (S-1) C-1], 91.08[1C, β -Glc (S-1) C-1], 93.06[1C, α-Glc (S-3) C-1], 90.00 [1C, α-Glc (S-6) C-1], 92.01[1C, α-GalNAc(S-5) C-1], 96.01[1C, β-Gal (S-2) C-1], 102.02 [3C, β-Gal(S-7, S-8, S-4), C-1]

ES Mass of compound C Vediose

1458[M+Na+K], 1435$[M+K]^+$, 1396$[M]^+$, 1297, 1277, 1234 ,1217, 1205, 1072, 1041, 1013, 910, 861, 835, 852, 707, 545, 504, 486, 428, 473, 342, 310, 283, 291, 180

D - Compound D (MSBC-1) Bebiose

Compound D (47mg) was obtained from fraction 28-45 of third column chromatography. On deacetylation of 25 mg of substance with NH_3 / acetone, it afforded substance D (16mg). For experimental analysis, this compound was dried over P_2O_5 at 100^0 C and 0.1 mm pressure for 8 hr.

$C_{40}H_{68}O_{31}N_2$		%C	%H	%N
	Calculated	44.77	6.34	2. 60
	Found	44.78	6.39	2.21

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

^{1}H NMR Bebiose: δ in D_2O at 300 MHz

δ5.69 [d, 1H, J = 4.0, α- Glc(S-1), H-1], 5.20[d, 1H, β Glc(S-1), H-1], 4.52[d,1H, J = 7.8, β-Gal(S-2), H-1], 4.42 [d, 1H, J = 6.3, β-Gal(S-6), H-1], 1.97 [S, 3H, α-GlcNAc (S-5)$NHCOCH_3$], 1.96 [S, 3H, β-GalNAc (S-6)$NHCOCH_3$].

^{1}H NMR of Bebiose: acetate δ in $CDCl_3$ at 400 MHz

6.17 [d, 1H, J = 4.0 Hz, α-Glc (S-1) H-1], 5.69 [d, 1H, J = 5.2Hz, β-Glc (S-1) H-1], 5.40 [d, 1H, J = 2.8Hz, α -GlcNAc (S-5) H-1], 4.72 [d, 1H, J = 8.0Hz, β-Gal (S-4) H-1], 4.59 [d, 1H, J = 8.0Hz, β-Gal (S-3) H-1], 4.52 [d, 1H, J = 8.4, β-Gal (S-2) H-1], 4.44 [d, 1H, J = 6.3, β-GalNAc (S-6) H-1], 3.81 [d, 1H, β-Glc(S-1) H-4], 3.80 [d, 2H, β-Gal (S-4) H-2& β- Gal (S-2) H-3], 3.64 [d, 1H, β-Gal (S-4)H-3]

^{13}C of Bebiose acetate: **δ in $CDCl_3$ at 400 MHz**

91.00[1C,α-Glc (S-1) C-1], 91.08[1C, β -Glc (S-1) C-1], 90.00 [1C, α-GlcNAc (S-5) C-1], 96.00[1C,β-Gal(S-4)C-1],102.00[1C,β-Gal(S-3),C-1],102.00[1C,β-Gal(S-2)C-1], 102.00[1C, β-GalNAc (S-6)C-1].

ES Mass of compound D Bebiose

1134[M+Na+K], 1095[M+Na], 1072[M^+] ,1054, 1013, 869, 833, 811, 666, 648, 606, 504, 473, 467, 342, 324, 180

Table 2.13 Description of isolated oligosaccharides from *Bubalus bubalis* colostrum

	A	**B**	**C**	**D**
Analytical notation	MSBC-4	MSBC-3	MSBC-2	MSBC-1
Name of compound	Meeniose	Murtiose	Vediose	Bebiose
Physical state	Syrupy	Syrupy	Syrupy	Syrupy
Mol. Formula	$C_{36}H_{61}O_{26}N_3$	$C_{34}H_{58}O_{26}N_2$	$C_{52}H_{88}O_{41}N_2$	$C_{40}H_{68}O_{31}N_2$
ES mass (m/z)	951	910	1396	1072
Phenol Sulphuric test*[1]	+ve	+ve	+ve	+ve
Morgon-Elson test*[2]	+ve	+ve	+ve	+ve
Thiobarbituric acid test*[3]	-ve	-ve	-ve	-ve
Bromocresol green test*[4]	-ve	-ve	-ve	-ve

*[1] Test of normal sugar.

*[2] test of amino sugar

*[3]Test of sialic acid

*[4]Test of carboxylic acid.

CHAPTER-3

RESULTS AND DISCUSSION

3.1 STRUCTURAL ASPECTS OF MILK OLIGOSACCHARIDE

On the basis of their structure, milk oligosaccharides are divided into two classes, neutral and acidic. Neutral oligosaccharide does not contain any charged carbohydrate residues while acidic oligosaccharide contains one or more residues of sialic acid which are negatively charged. The biological activity of oligosaccharide is closely related to their configuration and conformation. Most of milk oligosaccharides are made up of basic core units which are comprised of D-Glucose, D-Galactose, D-GlcNAc, D-GalNAc, fucose and sialic acid. The structures of basic core units of milk oligosaccharide are given as under

Lactose :-

Gal-(β1→4)-Glc

Lacto-N-tetraose (LNT):-

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

Lacto-N-neotetraose (LNnT):-

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

Lacto-N-hexaose (LNH):-

Gal- β (1→4)-GlcNAc- (β 1→6) ＼
Gal- (β 1→4)-Glc
Gal- (β 1→3)-GlcNAc- (β 1→3) ／

Lacto-N-neohexaose (LNneoH):-

Gal- (β 1→4)-GlcNAc- (β 1→6) ＼
Gal- (β 1→4)-Glc
Gal- (β 1→4)-GlcNAc- (β 1→3) ／

Para-Lacto-N-neohexaose (paraLNH):-

Gal-(β1→4)-GlcNAc-(β1→3)-Gal-(β1→4)-GlcNAc-(β1→3)-Gal-(β1→4)-Glc

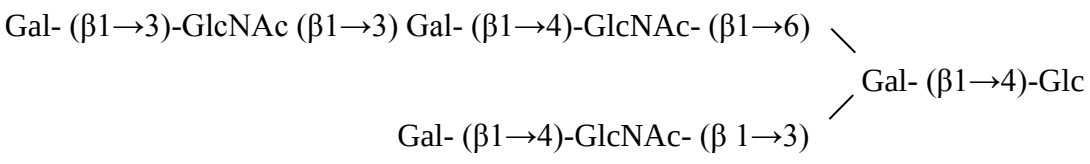
Para-Lacto-N-neohexaose (paraLNneoH):-

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

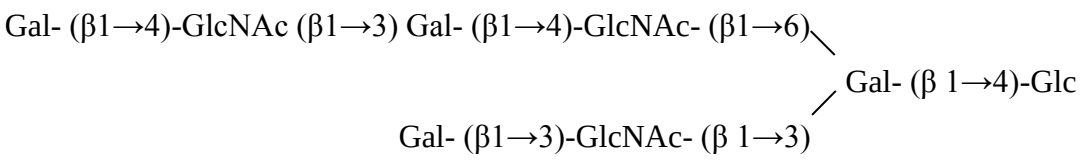
Lacto-N-octaose:-

Gal-(β1→4)-GlcNAc (β1→3) Gal-(β1→4)-GlcNAc- (β1→6) ＼
Gal- (β 1→4)-Glc
Gal- (β1→4)-GlcNAc- (β 1→3) ／

Lacto-N-neooctaose:-



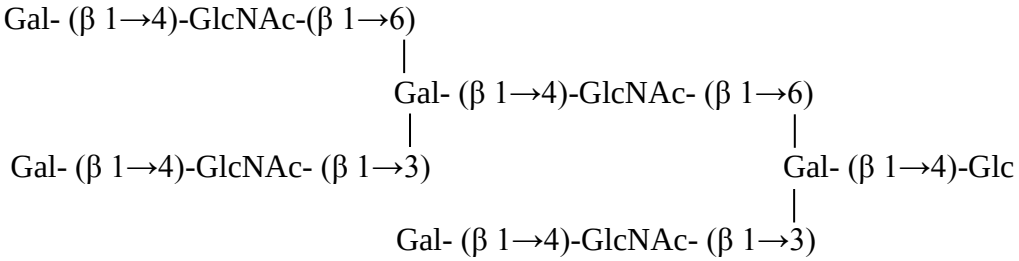
Iso-Lacto-N-octaose:-



Para-Lacto-N-octaose:-

Gal-(β1→3)-GlcNAc-(β1→3)-Gal-(β1→4)-GlcNAc-(β1→3)-Gal-(β1→4)-GlcNAc-(β 1→3)- Gal-(β 1→4)-Glc

Lacto-N-decaose:-



With few exceptions, all core units contained lactose at their reducing end. The scientists have elucidated the structure of milk oligosaccharides by chemical degradation and spectroscopic techniques (NMR and ES-MS). Keeping above mentioned basic core units in mind, the structures of various milk oligosaccharides were established by comparing the chemical shift data (^{1}H and ^{13}C NMR) of anomeric signals and other important signals of unknown milk oligosaccharides with the chemical shifts of known milk oligosaccharides. In the present study analogies between chemical shift of certain 'structural reporter group resonances' were used to make proton resonance assignments as well as structural assignments of the oligosaccharides. All chemical shifts of anomeric proton signals of milk oligosaccharides were further confirmed by 2D (^{1}H-^{1}H COSY, TOCSY and HSQC) NMR experiments, which were earlier assigned with the help of ^{1}H and ^{13}C NMR data. Other techniques like deacetylation, methylation, hydrolysis, chemical degradation and mass spectrometry were also used in the structural elucidation of the oligosaccharides.

3.1.1 Structural elucidation of compound - A (Meeniose)

Compound A, $C_{36}H_{61}O_{26}N_3$, gave positive Phenol-sulphuric acid test (Dubois et.al., 1956), Fiegl test (Fiegl, F., 1975) and Morgon-Elson test (Gey, et. al., 1996) showing the presence of normal and amino sugar moieties in the compound Meeniose. 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 5.03 x 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 (Fig. 3.1). The presence of six anomeric protons were further confirmed by the presence of six anomeric proton doublets at δ 6.19 (1H), δ 5.62 (2H), δ 5.03 (1H), δ 4.44 (1H), δ 4.43 (1H) in the ^{1}H NMR spectrum of acetylated Meeniose in $CDCl_3$ at 400 MHz (Fig. 3.2). The presence of six anomeric carbons also confirmed by the presence of six anomeric carbon signals at δ 101.04 (1C), δ 100.79 (2C), δ 91.40 (2C) and δ 88.82 (1C) in the ^{13}C NMR spectrum of acetylated Meeniose in $CDCl_3$ at 400 MHz (Fig. 3.3). The presence of downfield chemical shift of α and β anomeric protons and carbons in ^{13}C NMR of Meeniose acetate, respectively, suggested that compound Meeniose may be a pentasaccharide in its reducing form. The reducing nature of compound Meeniose was further confirmed by methylglycosylation 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. For convenience the five monosaccharides 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 monosachharide constituents of Meeniose were confirmed by its Killiani hydrolysis (Killiani, 1930) under strong acidic conditions, followed by paper chromatography (PC) and thin layer chromatography (TLC). In its hydrolysis four spots were found on PC and TLC which were found identical with glucose (Glc), galactose (Gal), 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 and ^{13}C NMR spectra of Meeniose were also in agreement with the reported values of ^{1}H and ^{13}C anomeric chemical shifts of Glc, Gal, N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GlcNAc) confirming the presence of these monosaccharides unit in the compound Meeniose. The presence of two anomeric proton signals at δ 6.19 (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 β anomers of reducing monosaccharides (S-1) which was later identified as glucose by comparing the chemical shift data of ^1H and ^{13}C NMR spectrum 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 ^1H NMR of Meeniose acetate contains cross peaks at δ 5.62 x 3.80, δ 5.62 x 5.0, δ 5.62 x 5.20, in its TOCSY Spectrum (Fig. 3.4). Out of which one cross peak arised at δ 5.62 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 (Fig. 3.5), suggesting that the H-4 of 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-1 at δ 3.80 acetylated Meeniose at 400 MHz in $CDCl_3$. Further the presence of another anomeric proton doublet at δ 4.43 (J = 8.0 Hz) along with a singlet of amide methyl ($-NHCOCH_3$) at δ 2.10 was due to presence of β-GalNAc (S-2) moiety. The coupling constant of anomeric signal (S-2) with J value 8.0 Hz confirmed the β-configuration of the S-2 moiety. This anomeric proton signal at δ 4.43 contain four cross peaks at δ 4.43 x 3.74, 4.43 x 4.07, 4.43 x 4.90, 4.43 x 5.30 in its TOCSY spectrum, out of which one cross peak arised at δ 4.43 x 4.07 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 δ 3.74 acetylated Meeniose at 400 MHz in $CDCl_3$. Further another anomeric proton signal present at δ 5.62 (J = 8.0 Hz) was assigned to β- GlcNAc (S-3), contains four cross peaks δ 5.62 x 3.80, δ 5.62 x 5.00, δ 5.62 x 5.20, δ 5.62 x 5.63 in its TOCSY spectrum. Out of which the one cross peaks δ 5.62 x 3.80 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 δ 5.62 x 3.80 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 Hz) 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 δ 3.80 acetylated Meeniose at 400 MHz in $CDCl_3$. Further another anomeric proton signal present at δ 5.03 (J = 8.0 Hz) was assigned to β-Gal (S-4). This anomeric proton contains two cross peaks at δ 5.03 x 3.74, δ 5.03 x 4.50 in its TOCSY spectrum of Meeniose acetate. Out of which the one cross peaks δ 5.03 x 3.74 corresponds 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 anomeric proton 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 δ 3.74 acetylated Meeniose at 400 MHz in $CDCl_3$. The coupling constant of anomeric signal (S-4) δ 5.03 with J value 8.0 Hz confirmed the β-configuration of the S-4 moiety which linked with β-GlcNAc (S-3). Another anomeric proton 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 δ 4.44 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 which 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}H NMR assignments for ring proton of monosaccharide units of Meeniose were confirmed by COSY and TOCSY spectra. 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 were appeared as Glc (S-1) H-4 x C-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-4 x

C-4 at δ 3.80 x 73 showed (1→4) linkage between S-4 and S-3, β-Gal (S-4) H-2 x C-2 at δ 3.74 x 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 spectra. Thus based on the pattern of chemical shifts of ^{1}H, ^{13}C, COSY, TOCSY and HSQC NMR spectra it was interpreted that the compound 'A' Meeniose was a pentasaccharide having the following structure as-

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

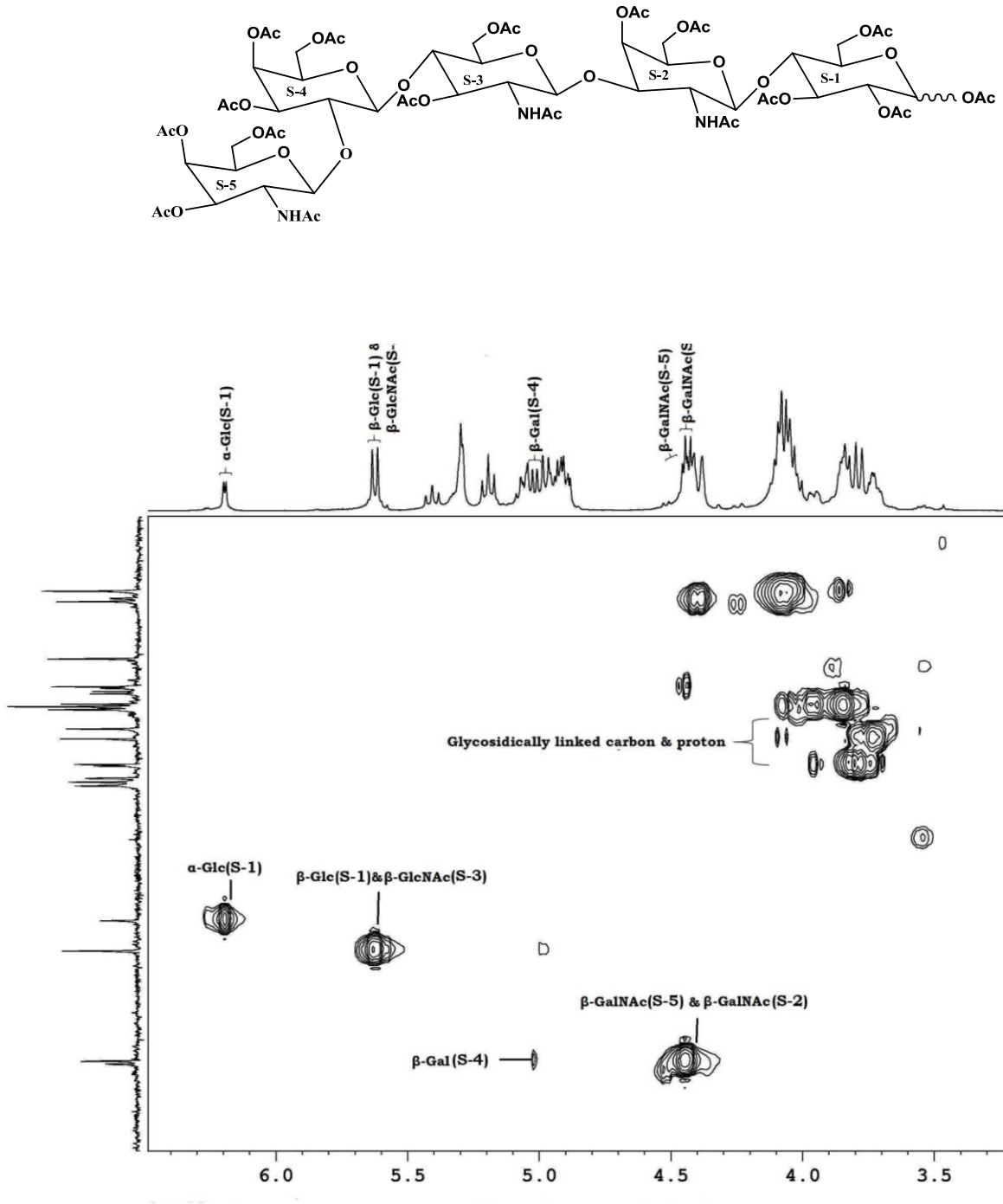


Fig. 3.1 ^{1}H-^{13}C HSQC spectrum of Meeniose acetate in $CDCl_3$ at 400 MHz

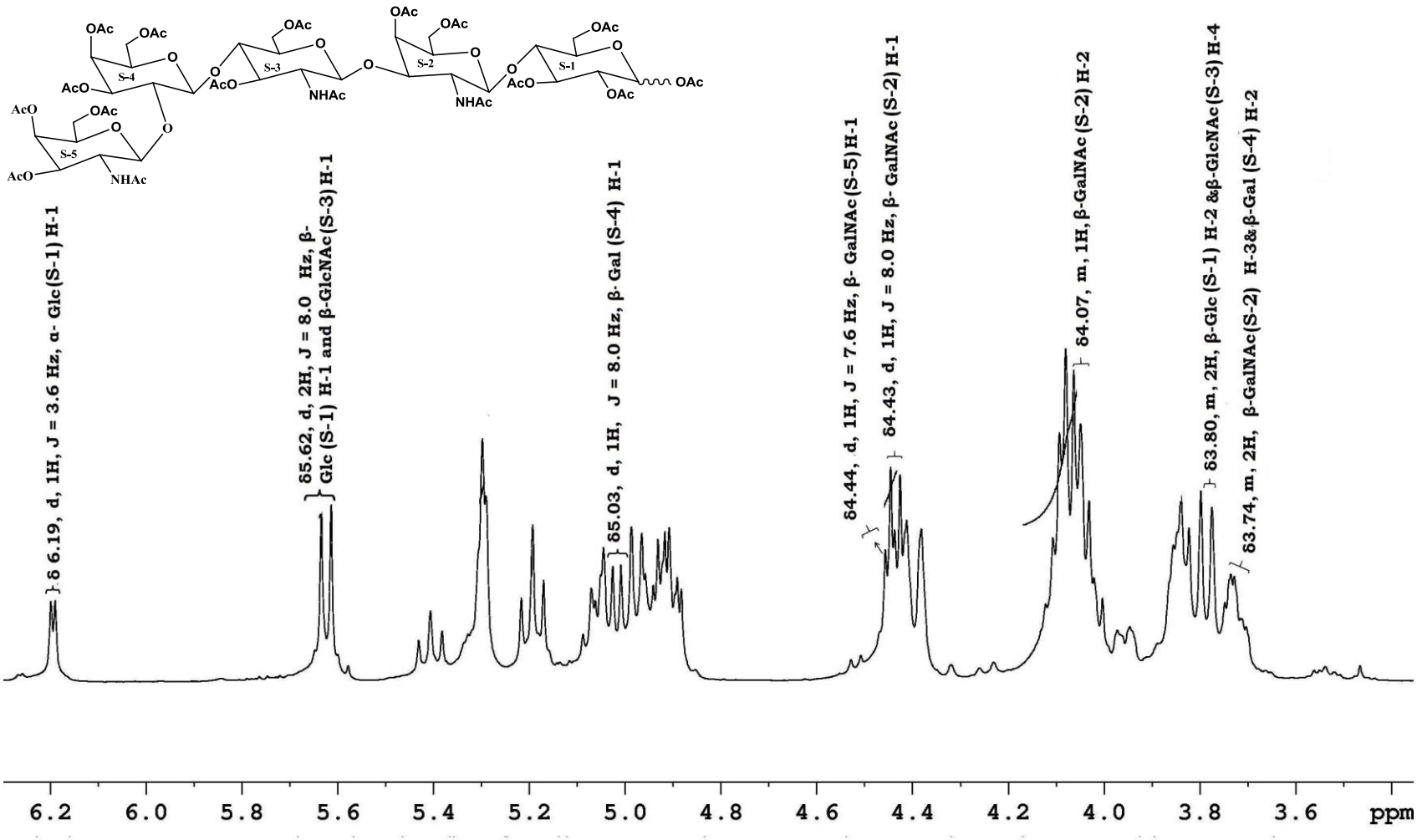


Fig. 3.2 ^{1}H NMR spectrum of Meeniose acetate in $CDCl_3$ at 400 MHz

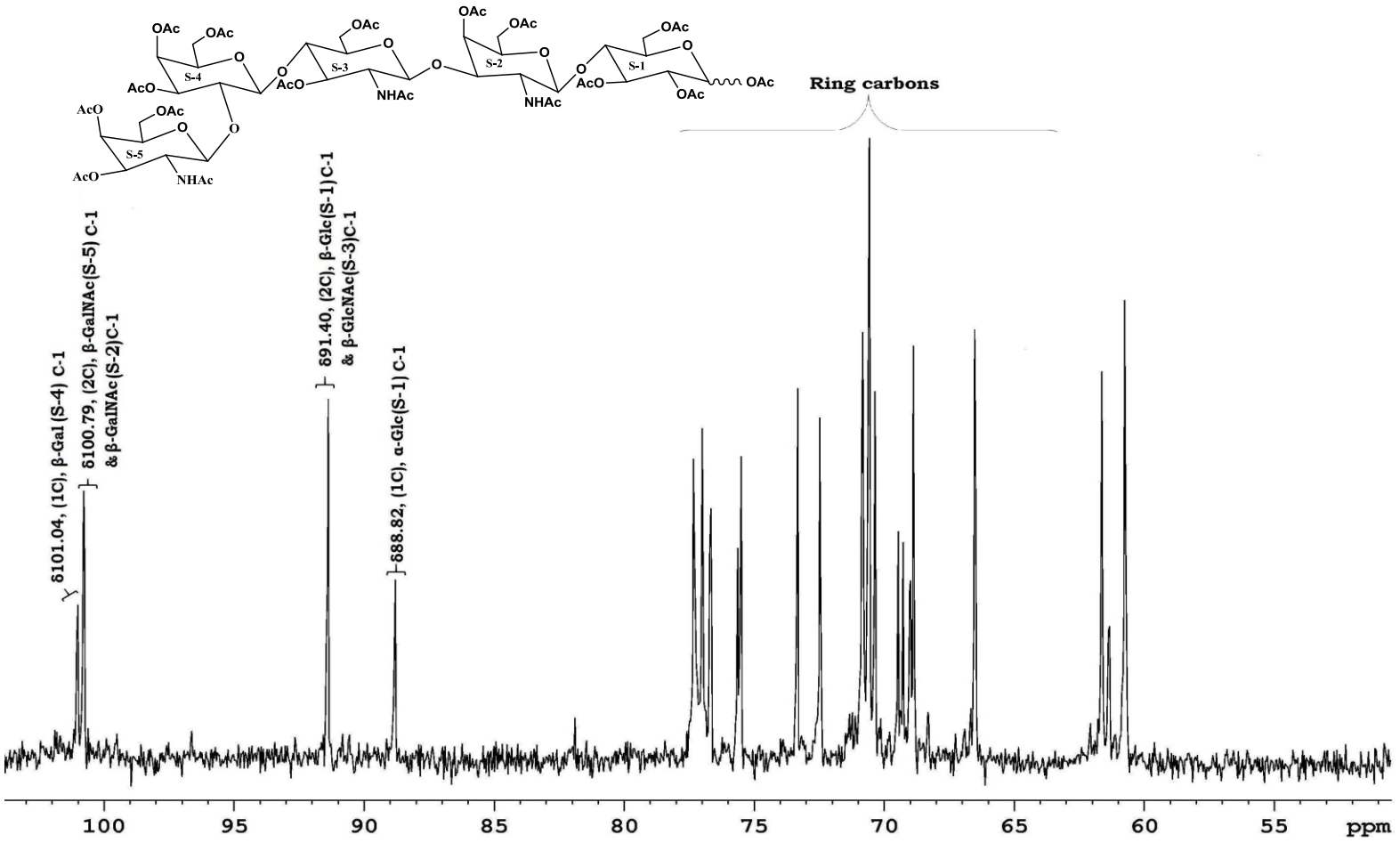


Fig. 3.3 ^{13}C NMR spectrum of Meeniose acetate in $CDCl_3$ at 400 MHz

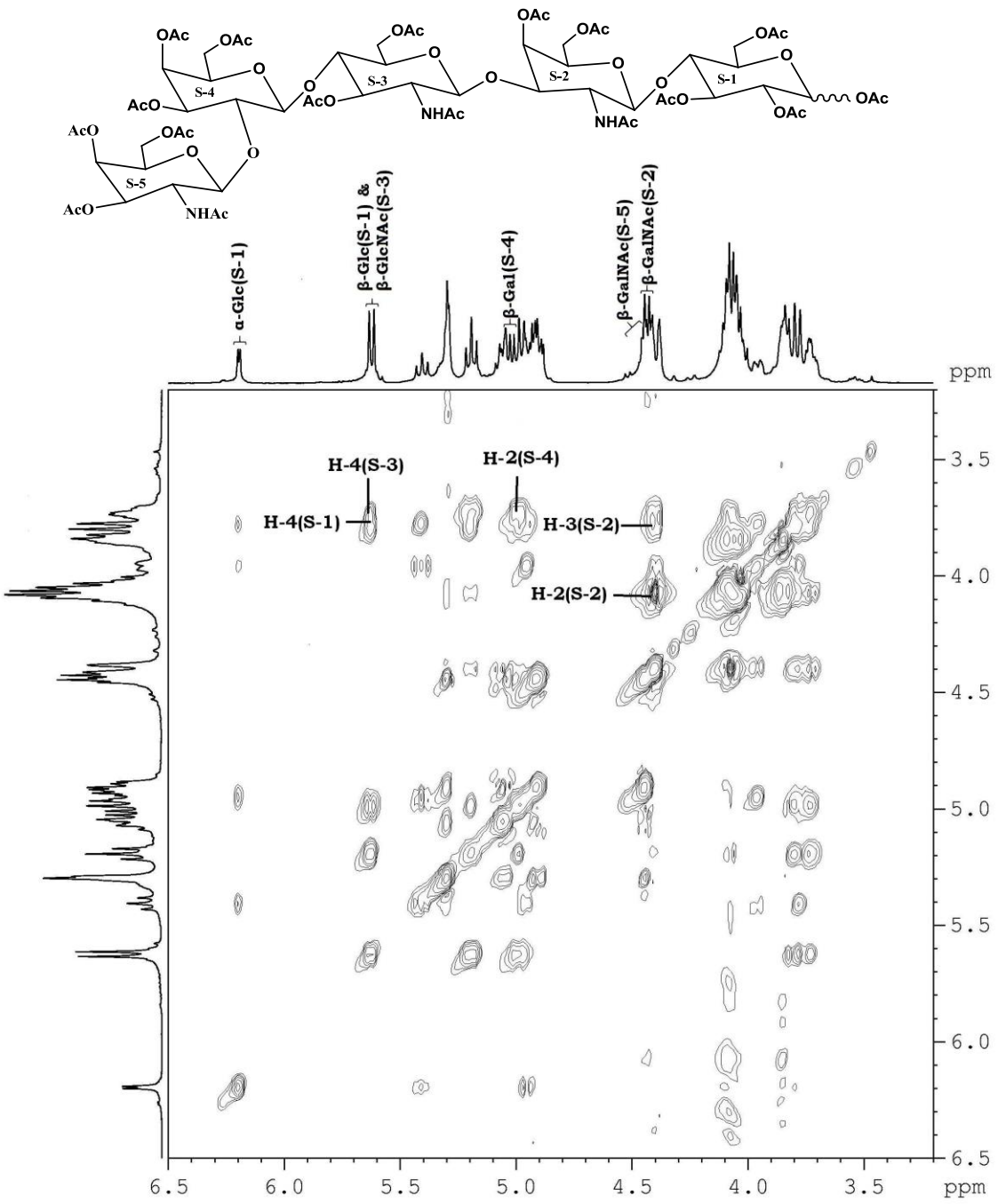


Fig. 3.4 TOCSY spectrum of Meeniose acetate in $CDCl_3$ at 400 MHz

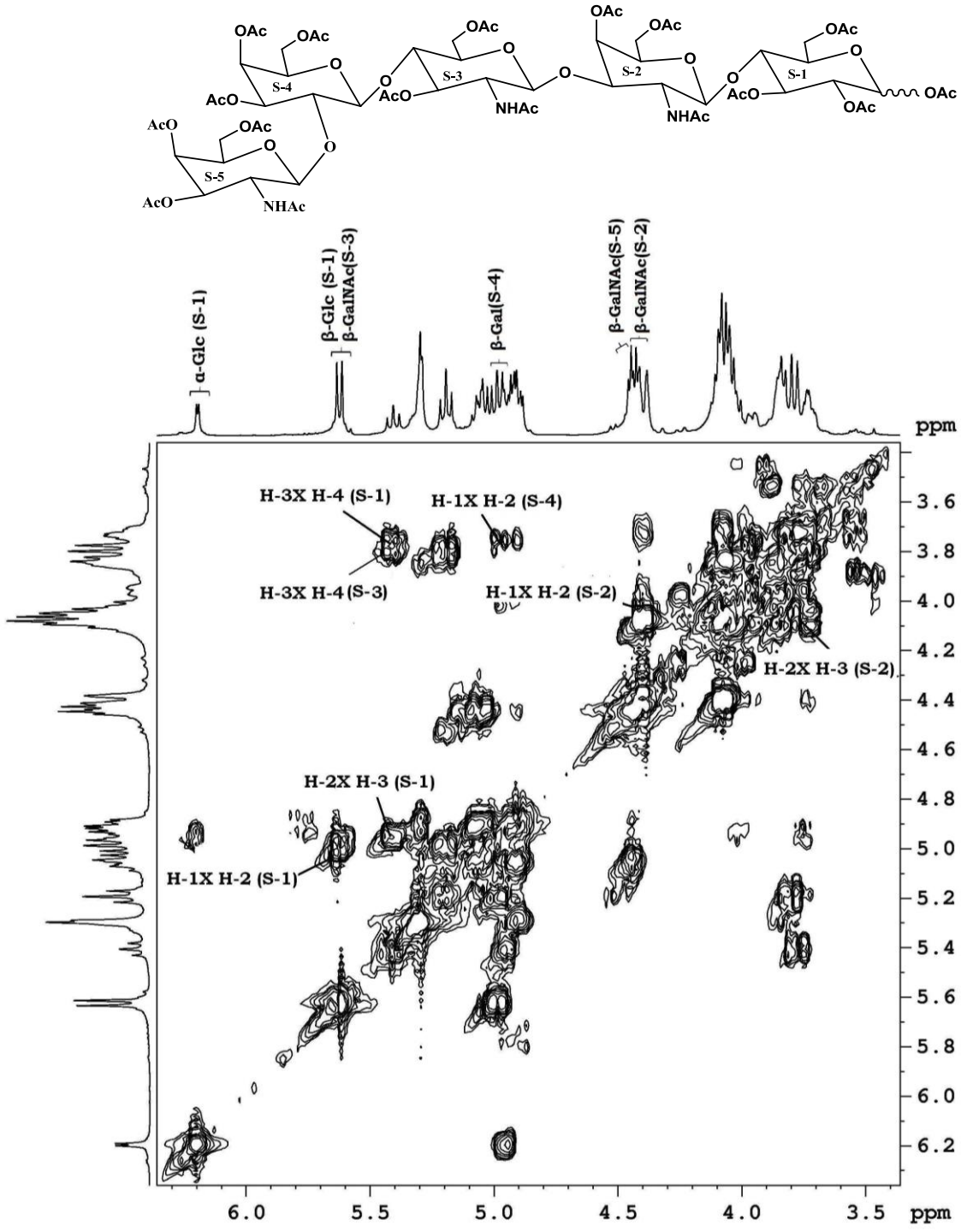


Fig. 3.5 COSY spectrum of Meeniose acetate in $CDCl_3$ at 400 MHz

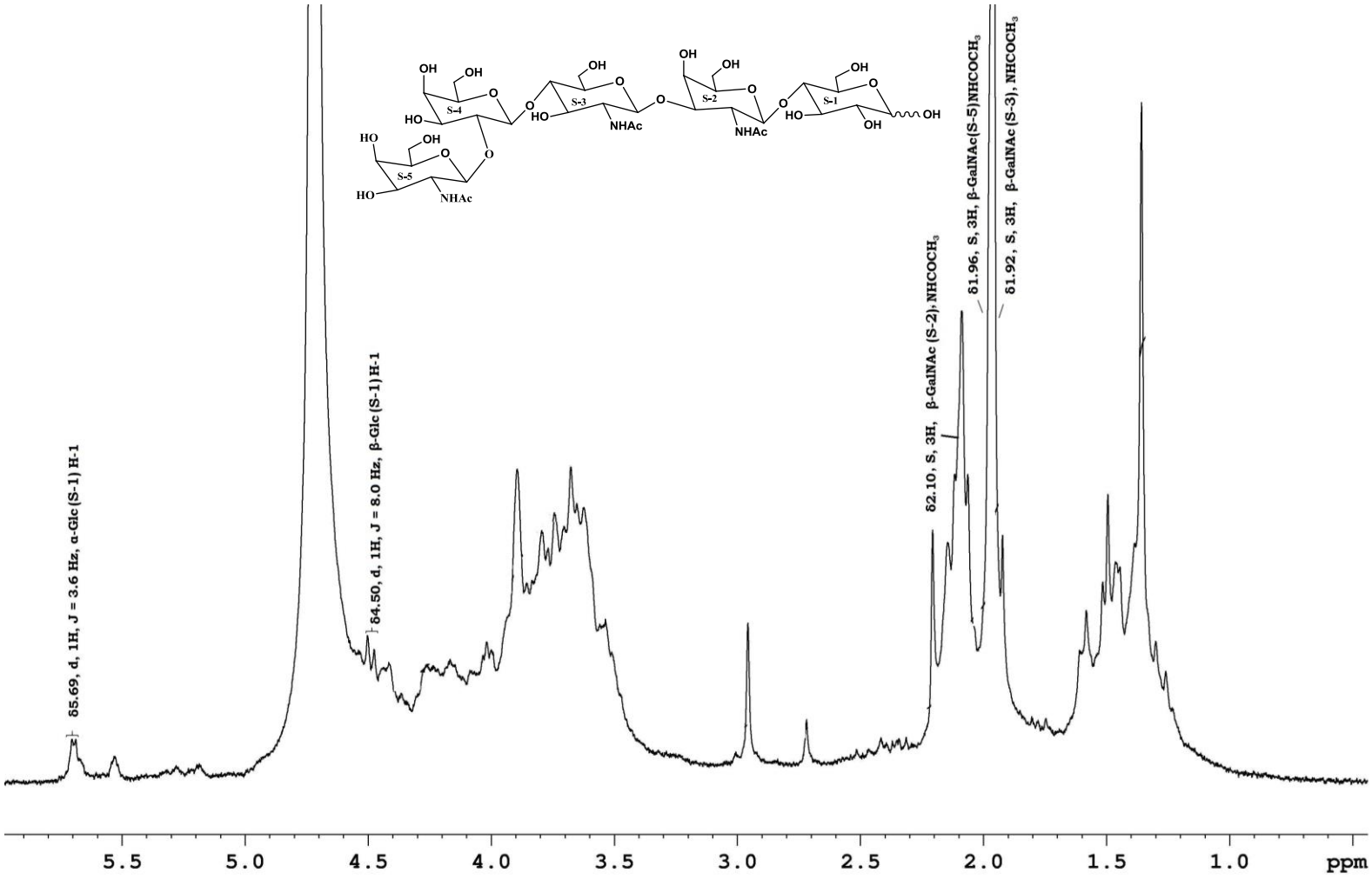
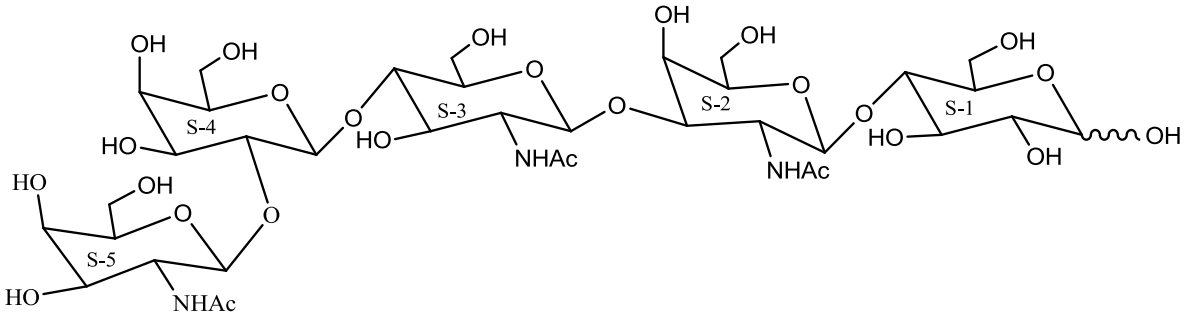


Fig. 3.6 ^{1}H NMR spectrum of Meeniose in D_2O at 300 MHz

The electrospray Mass spectrometry data of Meeniose not only confirmed the derived structure but also supported the sequence of monosaccharide in Meeniose (Fig. 3.8). The highest mass ion peaks were recorded at m/z 1013 assigned to $[M+Na+K]^+$ and m/z 990 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). It further fragmented to give mass ion peak at m/z 586(III) which was a trisaccharide moiety (III) [748-S-4] and 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-1], which was due to loss of 203 in GalNAc (S-1) moiety from disaccharide (Fig. 3.9). These four mass ion peak II, III, IV and V were appeared due to the consequent loss of S-5, S-4, S-3 and S-1 from original molecule. The mass spectrum also contain the mass ion peak at m/z 586, 424 correspond to the mass ion fragment A and B, which confirm the position of S-1, S-2, S-3, S-4, S-5 (Fig.3.10). The m/z of fragmented moieties of compound A 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 A 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] (Fig. 3.11).

Based on above results obtained from chemical degradation / acid hydrolysis, chemical transformation, electrospray mass spectrometry ^{1}H, ^{13}C NMR and 2D NMR COSY, TOCSY and HSQC technique, the structure of isolated novel oligosaccharide Meeniose was deduced (Fig. 3.7) as-



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

Fig. 3.7 Structure of Meeniose

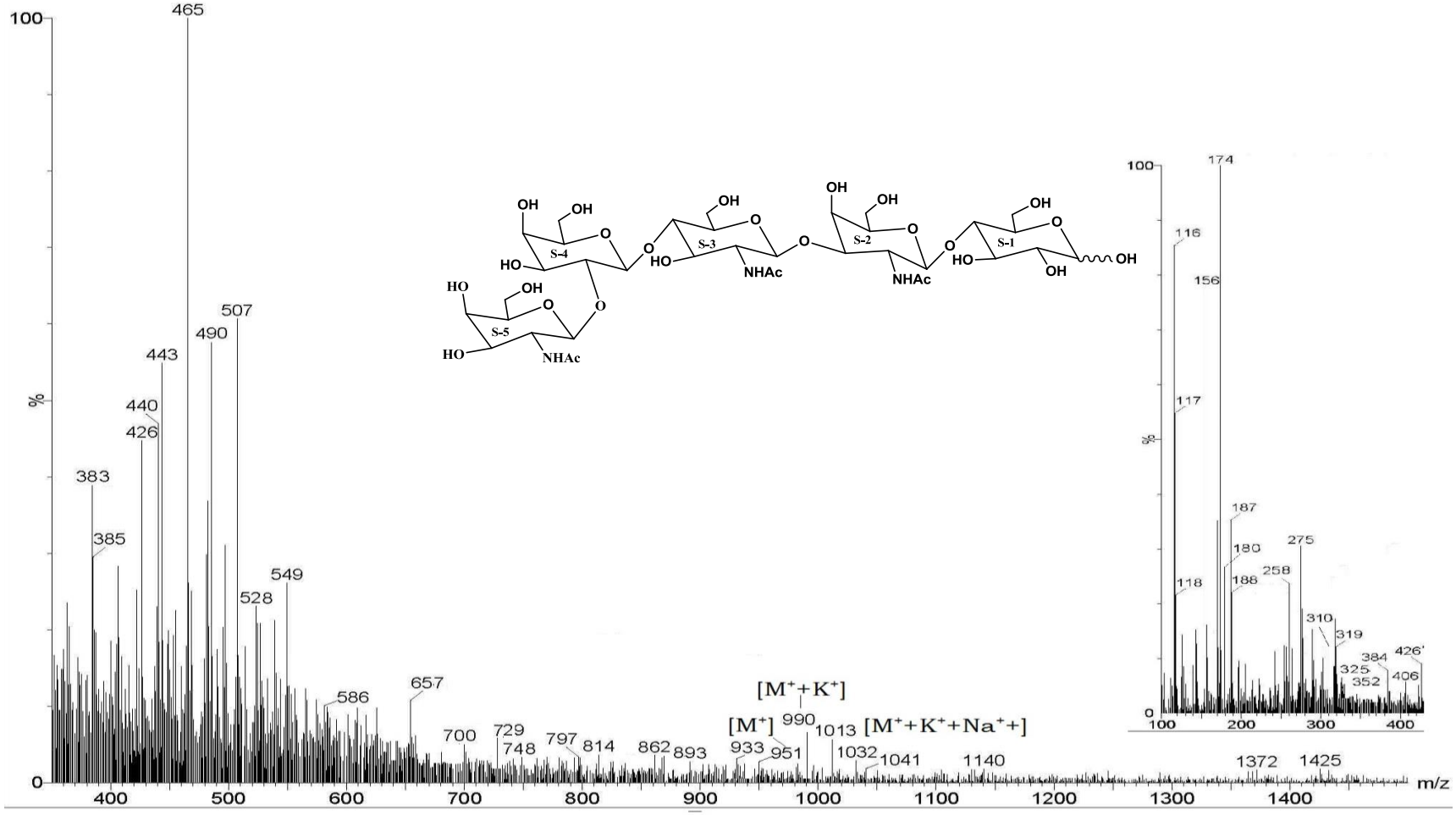


Fig. 3.8 ES Mass spectrum of compound Meeniose

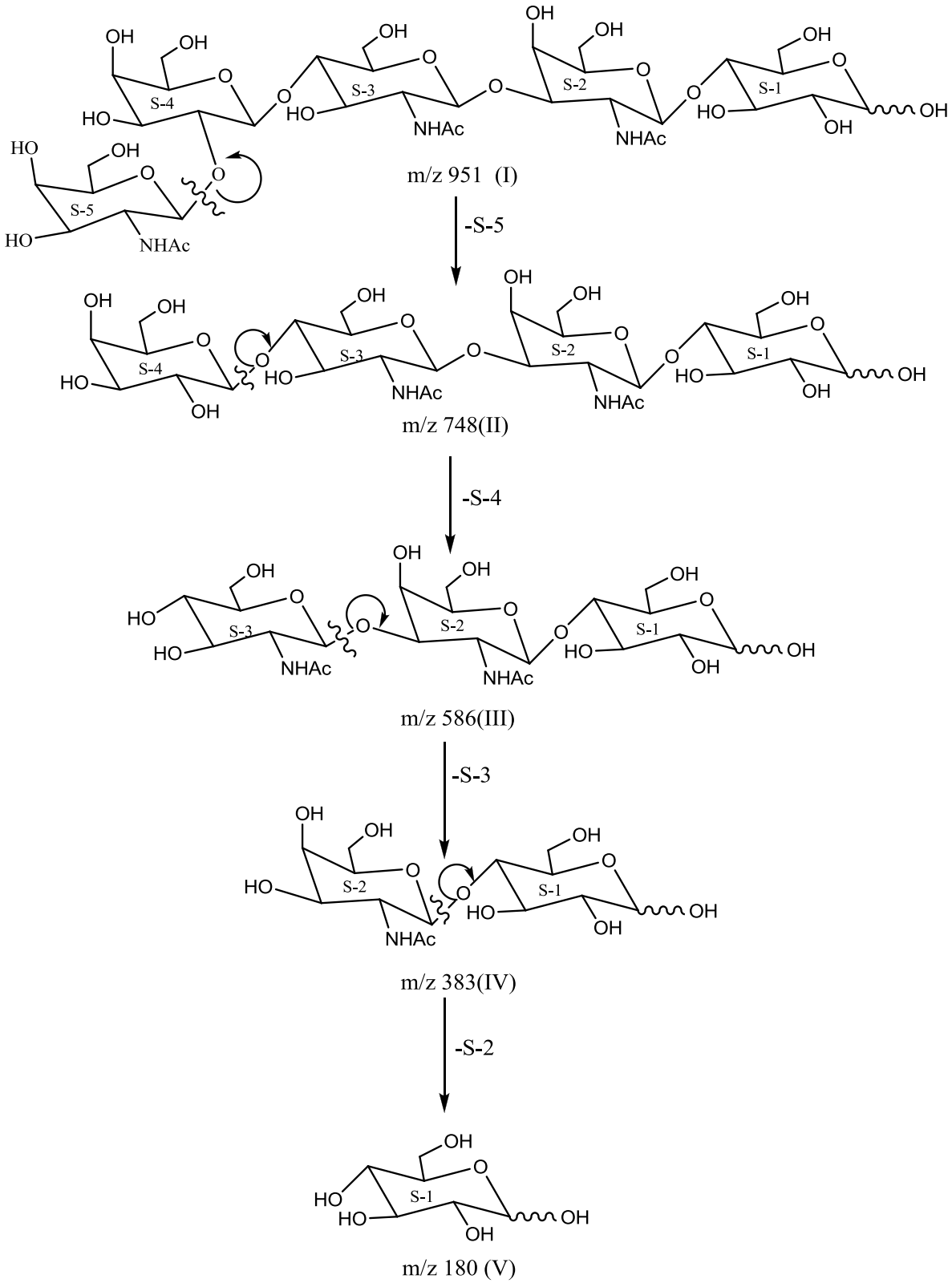


Fig. 3.9 Mass fragmentations of compound Meeniose

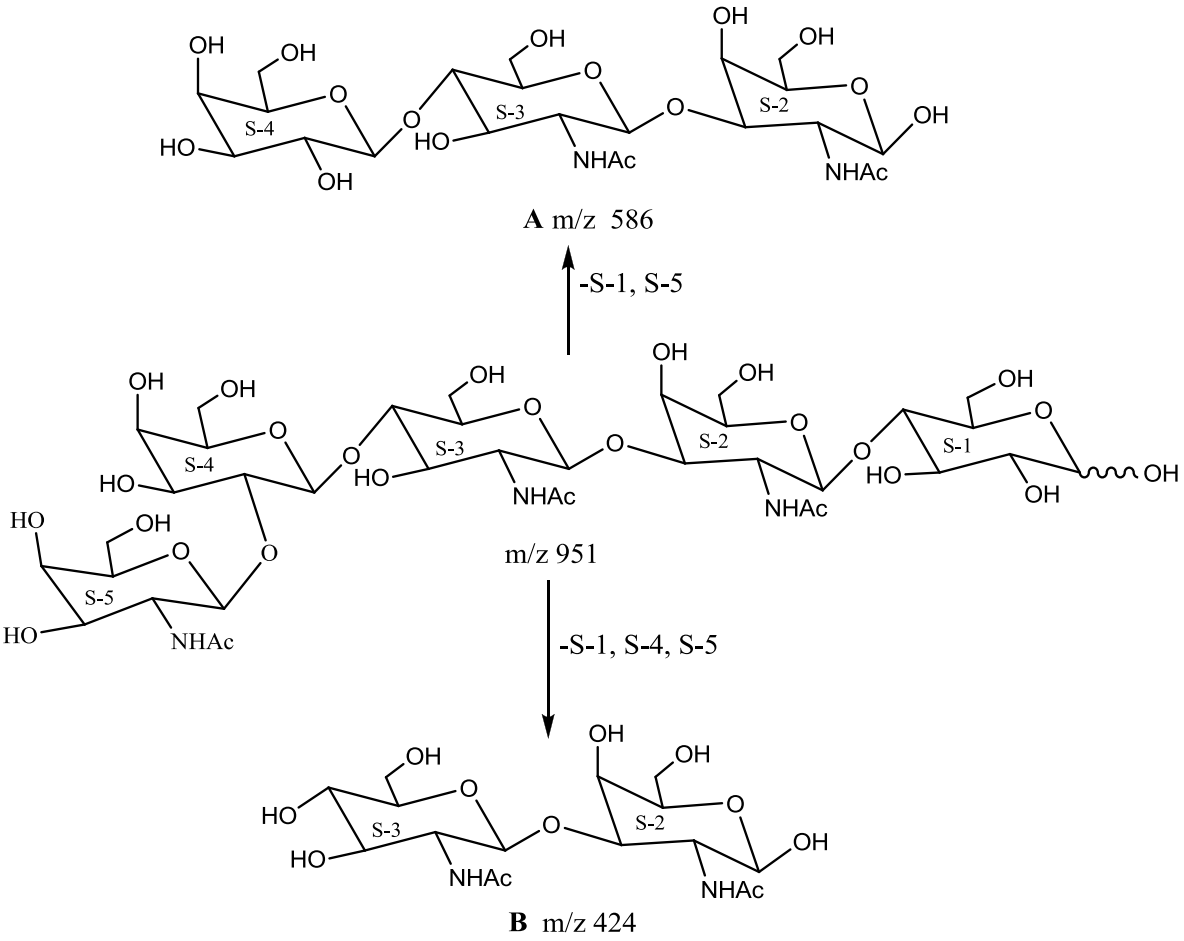


Fig. 3.10 ES-MS fragments of compound Meeniose.

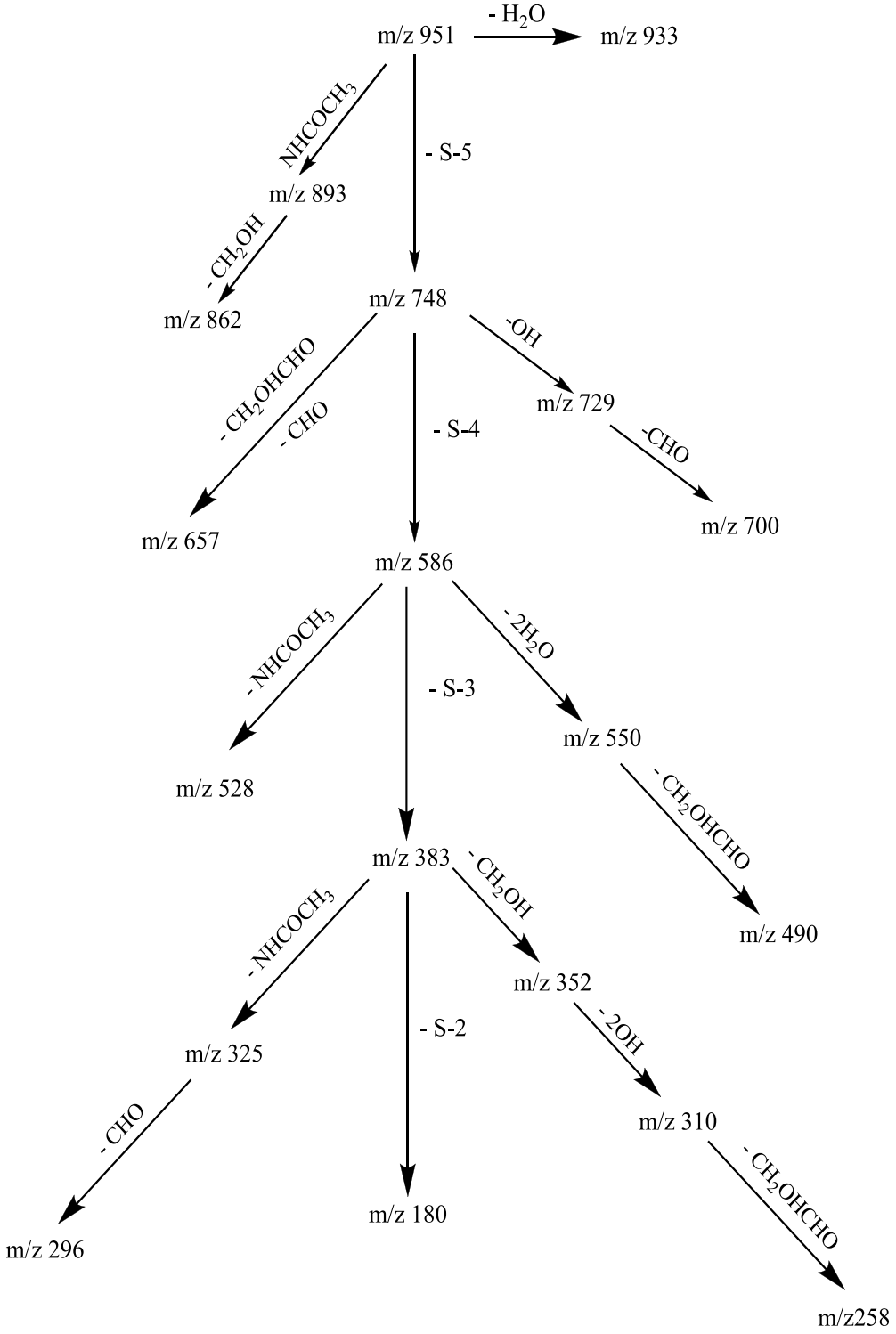


Fig. 3.11 Line diagram of ES-MS fragmentations of compound Meeniose

3.1.2 Structural elucidation of compound – B (Murtiose)

Compound B, $C_{34}H_{58}O_{26}N_2$, gave positive Phenol-sulphuric acid test (Dubois et.al., 1956), Fiegl test (Fiegl, F., 1975) and Morgon-Elson test (Gey, et. al., 1996) showing the presence of normal and amino sugar moieties in the compound Murtiose. The HSQC spectrum of acetylated Murtiose showed the presence of six cross peaks of anomeric protons and carbons in their respective region at δ 6.08 x 90.93, δ 5.62 x 91.29, δ 5.26 x 89.61, δ 4.70 x 94.69, δ 4.45 x 100.70, δ 4.37 x 100.63 suggesting the presence of six anomeric protons and carbons in it (Fig. 3.12). The presence of six anomeric protons were further confirmed by the presence of six anomeric doublets at δ 6.08(1H), δ 5.62 (1H), δ 5.26 (1H), δ 4.70 (1H), δ 4.45 (1H), δ 4.37(1H) in the ^{1}H NMR spectrum of acetylated Murtiose in $CDCl_3$ at 400 MHz (Fig. 3.13). The presence of six anomeric carbons were also confirmed by six anomeric carbon signals at δ 89.61 (1C), δ 90.93 (1C), δ 91.29 (1C), δ 94.69(1C), δ 100.63(1C) and δ 100.70 (1C) in the ^{13}C NMR spectrum of acetylated Murtiose in $CDCl_3$ at 400 MHz (Fig. 3.14). The ^{1}H NMR spectrum of Murtiose in D_2O at 300 MHz showed anomeric proton signals as doublets at δ 5.69 (1H), δ 5.20(1H), δ 4.63 (1H), δ 4.49 and δ 4.42 (1H). Since all these NMR spectrum of Murtiose and Murtiose acetate contained downfield shifted α and β anomeric proton and carbon signals suggested that the compound Murtiose may be a pentasaccharide in its reducing form. The reducing nature of compound Murtiose was further confirmed by methylglycosylation of compound Murtiose by MeOH/H^+ followed by its acid hydrolysis which led to the isolation of α and β-methyl glucosides, suggesting the presence of glucose at the reducing end. For convenience, the five monosaccharides present in compound Murtiose have been designated as S-1, S-2, S-3, S-4 and S-5, respectively, starting from glucose (S-1) the reducing end.

The monosaccharide constituents in compound Murtiose were confirmed by its Killiani hydrolysis (Killiani, 1930) under strong acidic conditions, followed by PC and TLC. In its hydrolysis four spots were detected on PC and TLC which were found identical with glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) by co-chromatography with 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 and ^{13}C NMR spectrum of Murtiose 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 Murtiose. The presence of two anomeric proton signals at δ 5.69 (J = 4.0 Hz) and δ 4.63 (J = 8.0 Hz) in the ^{1}H NMR spectrum of Murtiose in D_2O at 300 MHz were assigned for α and β anomers of glucose (S-1) confirming the presence of Glc (S-1) at the reducing end. In the ^{1}H NMR of Murtiose in D_2O another anomeric proton doublet at δ 4.42 (J = 8.0 Hz) showed the presence of β-Gal (S-2) residue as the next monosaccharide. The ^{1}H NMR of Murtiose also contain a triplet δ 3.27 (Stucture Reporter Groups) (Dua, and Bush, 1983) suggested the presence of lactose type of structure at reducing end confirming the 1→4 linkage between β-Gal (S-2) and β-Glc (S-1), confirming the presence of lactosyl moiety (Dua, and Bush, 1983) at the reducing end. Since, the HSQC spectrum of Murtiose acetate shows six anomeric proton and carbon signal. The anomeric signal for α and β anomers were detected at δ 6.08 and δ 5.62, respectively, which were assigned for reducing glucose. In the TOCSY spectrum of Murtiose acetate the anomeric signal of β-Glc(S-1) at δ 5.62 gave cross peak at 5.62 x 3.72, 5.62 x 5.00, 5.62 x5.20, out of which one cross peak at 5.62 x δ 3.72 suggested that only one position in glucose (S-1) was available for glycosidic linkage by the next monosaccharide unit (Fig. 3.15), which was later assigned as H-4 of β-Glc(S-1) by the COSY spectrum of Murtiose acetate (Fig. 3.16). This shows that β-Glc(S-1) was 1→4 linked with the next monosaccharide unit. Further another anomeric proton signal at δ 4.37 in the ^{1}H NMR of Murtiose acetate assigned for β-Gal(S-2), in the TOCSY spectrum of Murtiose acetate showed four cross peaks at 4.37 x 3.74, 4.37 x 4.05, 4.37 x 4.58 , 4.37 x 5.10, out of which two cross peaks at δ 4.37 x δ 3.74 and δ 4.37 x 4.05 in the ^{1}H NMR of Murtiose acetate in $CDCl_3$ showed that the two position in S-2 were available for glycosidic linkages, confirming that the two hydroxyl groups of β-Gal (S-2) were involved in glycosidic linkages by other monosaccharide moieties. These signals were identified for H-2 and H-3 of β-Gal (S-2) by the COSY spectrum of Murtiose acetate suggesting that H-2 and H-3 of β-Gal (S-2) were available for glycosidic linkages by the next monosaccharide units. The coupling

constant of anomeric signal β-Gal (S-2) with J value of 8.0 Hz confirmed the β-configuration of the β-Gal (S-2) moiety and hence β 1→4 glycosidic linkage between S-2 and S-1 were confirmed. The next anomeric proton signal appeared as doublet at δ 4.45(J = 8.0 Hz) in the ^{1}H NMR spectrum of Murtiose in $CDCl_3$ at 400 MHz was due to the presence of β Gal (S-3) moiety. The anomeric proton signal at δ 4.45 in the ^{1}H NMR spectrum of Murtiose acetate showed its complementary ^{13}C anomeric signal at δ 100.70 in the HSQC spectrum, later this ^{13}C anomeric signal at δ 100.70 showed its cross peak at 100.70 x 3.74 in the HMBC spectrum of Murtiose acetate showing the glycosidic linkages between S-3 and S-2 (Fig. 3.17). The signal of δ 3.74 was assigned as H-3 of S-2 by COSY spectrum confirming the 1→3 glycosidic linkage between S-3→S-2. The coupling constant of anomeric signal of (S-3) with J value 8.0 Hz confirmed β-configuration of the β-Gal(S-3) moiety. Therefore the glycosidic linkage between S-3 and S-2 was confirmed as β 1→3. Since, the anomeric proton signal at δ 4.45 in ^{1}H NMR of Murtiose acetate does not have any cross peak in the TOCSY spectrum of Murtiose acetate in the linkage region and none of methine proton of β-Gal (S-3) was found in the linkage region which confirms that β-Gal (S-3) linked at the non reducing end. Another anomeric proton signal which appeared as a doublet at δ 4.70 (J = 2.0 Hz), along with a singlet of amide methyl (-$NHCOCH_3$) at δ 1.96 in $CDCl_3$ at 400 MHz, was assigned for the presence of α-GalNAc (S-4) moiety. Since it was ascertained by COSY and TOCSY spectrum of Murtiose acetate that the positions 2 and 3 of β-Gal (S-2) were available for glycosidic linkages and position 3 of β-Gal (S-2) was already linked with β-Gal (S-3). Hence the leftover H-2 position of S-2 must be linked by α-GalNAc (S-4). The position of linkage between GalNAc (S-4) and β-Gal (S-2) was further confirmed by the appearance of H-2 signal of β-Gal δ 4.05 (S-2) in the ^{1}H NMR spectrum of Murtiose acetate which was also confirmed by COSY and TOCSY spectrum at 400 MHz in $CDCl_3$. The small coupling constant of α-GalNAc (S-4) (J = 2.0 Hz) confirmed the α-glycosidic linkage between GalNAc (S-4) and β-Gal (S-2). Further another anomeric proton signal which appeared as a doublet at δ 4.70 (J = 2.0 Hz) in the ^{1}H NMR of Murtiose acetate at 400 MHz gave signal in the TOCSY spectrum of Murtiose acetate, the anomeric proton of α-GalNAc (S-4) at δ 4.70 showed cross peak at 4.70 x 3.70, 4.70 x 4.07, 4.70 x 5.10, out of

which one cross peak 4.70 x 3.70 suggested that one position of α-GalNAc (S-4) was available for glycosidic linkage by the next monosaccharide. Later this signal of δ 4.70 x 3.70 was ascertained as H-3 of α-GalNAc (S-4) by COSY spectrum of Murtiose acetate showing that H-3 of α-GalNAc (S-4) was glycosidically linked by H-1 of next monosaccharide unit while the other cross peak of 4.70 x 4.07 was assigned for H-2 of GalNAc (S-4). Further, the presence of another anomeric proton as doublet at δ 5.26 (J = 1.0 Hz) along with a singlet of amide methyl (-$NHCOCH_3$) at δ 1.90 in $CDCl_3$, was identified due to the presence of α-GalNAc (S-5) as the next monosaccharide unit. As ascertained by the COSY and TOCSY spectrum of Murtiose acetate the position of H-3 of S-4 was available for glycosidic linkage. The α-GalNAc (S-5) must be attached to H-3 of S-4. Hence 1→3 linkage between α-GalNAc (S-5) and α–GalNAc (S-4) was confirmed. The small coupling constant of α-GalNAc (S-5) of (J = 1.0 Hz) confirmed the α-glycosidic linkage between α-GalNAc (S-5) and α-GalNAc (S-4). Since the anomeric proton of α-GalNAc (S-5) at δ 5.26 does not contain any cross peak in the linkage region in TOCSY spectrum of Murtiose acetate does not contain any methine protons in glycosidic linkage region i.e., δ 3-4 ppm, confirmed that none of -OH group of α-GalNHAc (S-5) was involved in glycosidic linkages. Hence, confirming that α-GalNAc (S-5) were present at non-reducing end and none of their -OH group were available for glycosidic linkages.

All the ^{1}H NMR assignments for ring proton of monosaccharide units of Murtiose were confirmed by COSY and TOCSY spectra. The positions of glycosidation in the oligosaccharide were confirmed by position of anomeric signals, and comparing the signals in ^{1}H and ^{13}C NMR of acetylated Murtiose. The glycosidic linkages in Murtiose were also confirmed by the cross peaks for glycosidically linked carbons with their protons in the HSQC spectrum of acetylated Murtiose. The values of these cross peaks appeared as Glc (S-1) H-4 x C-4 at δ 3.72 x 76 showed (1→4) linkage between S-2 and S-1, β-Gal (S-2) H-3 x C-3 at δ 3.74 x 73 showed (1→3) linkage between S-3 and S-2, α-GalNAc (S-4) H-3 x C-3 at δ 3.70 x 70 showed (1→3) linkage between S-5 and S-4, β-Gal (S-2) H-2 x C-2 at δ 4.05X 71 showed (1→2) linkage between S-4 and S-2.

All signals obtained in ^{1}H and ^{13}C NMR of compound Murtiose was in conformity by 2D COSY, TOCSY and HSQC spectrum. Thus based on the pattern of chemical shifts of ^{1}H, ^{13}C, COSY, TOCSY and HSQC NMR spectra it was interpreted that the compound 'B' Murtiose as a pentasaccharide having the following structure as-

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

Further the ^{1}H NMR of Murtiose in D_2O at 300 MHz was also in conformity with the derived structure contain anomeric proton signal at δ 5.69 (1H), δ 5.20(1H), δ 4.63 (1H), δ 4.49 (1H) and δ 4.42 (1H), i.e. the anomeric proton signal of glucose S-1 gave signal for α and β anomers at δ 5.69 (J = 3.2 Hz) and 4.63 (J = 8.0 Hz), respectively, while the anomeric proton signal of S-2 was arised at δ 4.42 (J = 7.2 Hz) along with a triplet at δ 3.27 which is structure reporter group for the presence of lactose structure at the reducing end (Dua, and Bush, 1983). Further it contain two more anomeric proton signal at δ 5.20 (J = 2.0 Hz) and δ 4.49 (J = 8.0 Hz) along with signal of methyl of NAc groups δ 1.965 and δ 1.904 suggesting the presence of two N-acetylated monosaccharides which were already detected as α-GalNAc (S-5) by 2D NMR of Murtiose acetate. Moreover, the anomeric proton doublet detected at δ 4.49 (J = 8.0 Hz) was assigned to β-Gal (S-3). The anomeric proton signal for S-4 was not detected but its complementary signal of 1.904 for NAc group was present. The position of inter glycosidic linkage for (S-1-S-2) i.e. H-4 of S-1 at δ 3.72, (S-4-S-1) i.e. H-2 of S-2 at δ 4.05, (S-3-S-1) i.e. H-3 of S-2 at δ 3.74, (S-5-S-4) i.e. H-3 of S-4 at δ3.70, thus confirming the structure of Murtiose as-

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

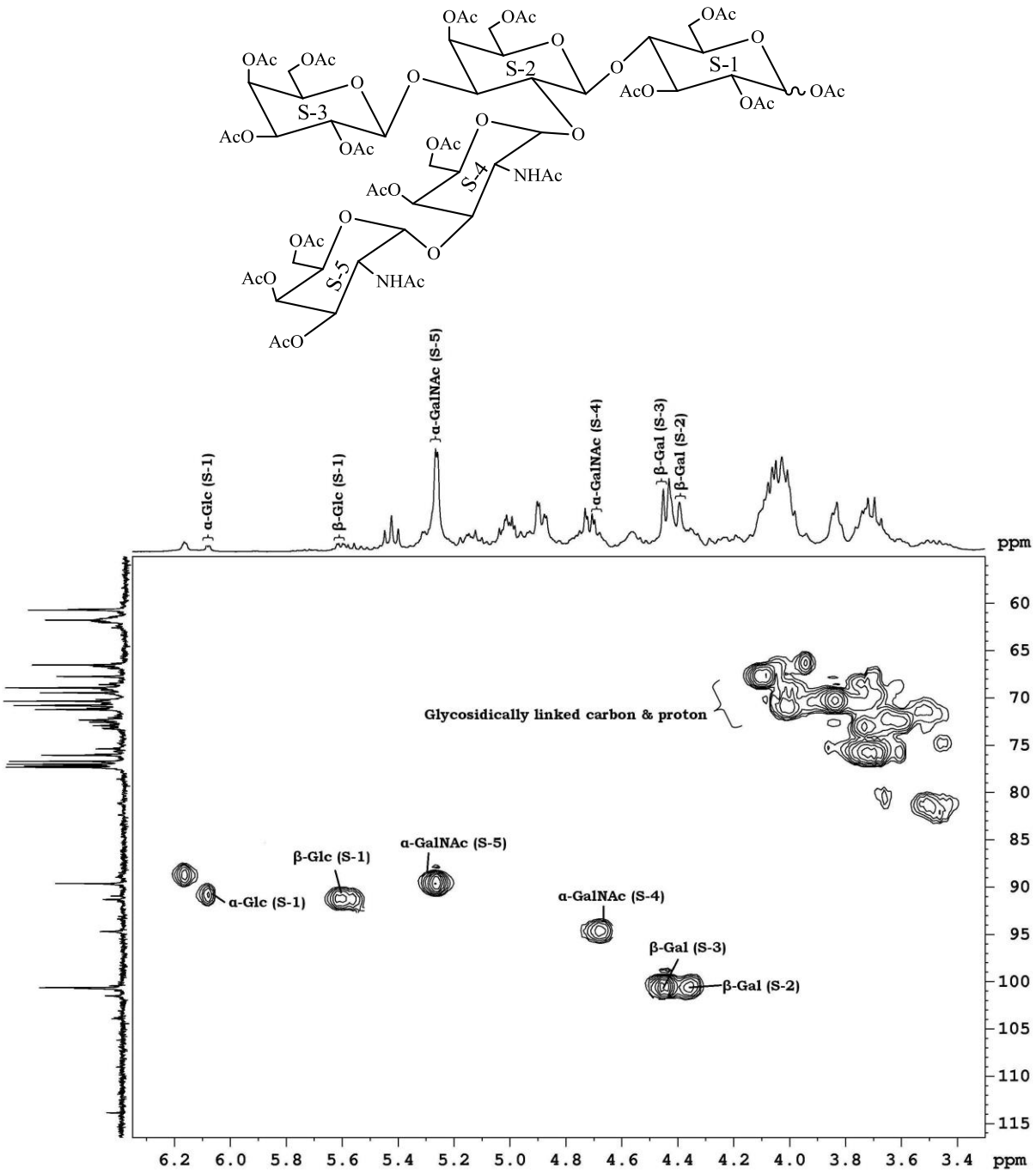


Fig. 3.12 ^{1}H-^{13}C HSQC spectrum of Murtiose acetate in $CDCl_3$ at 400 MHz

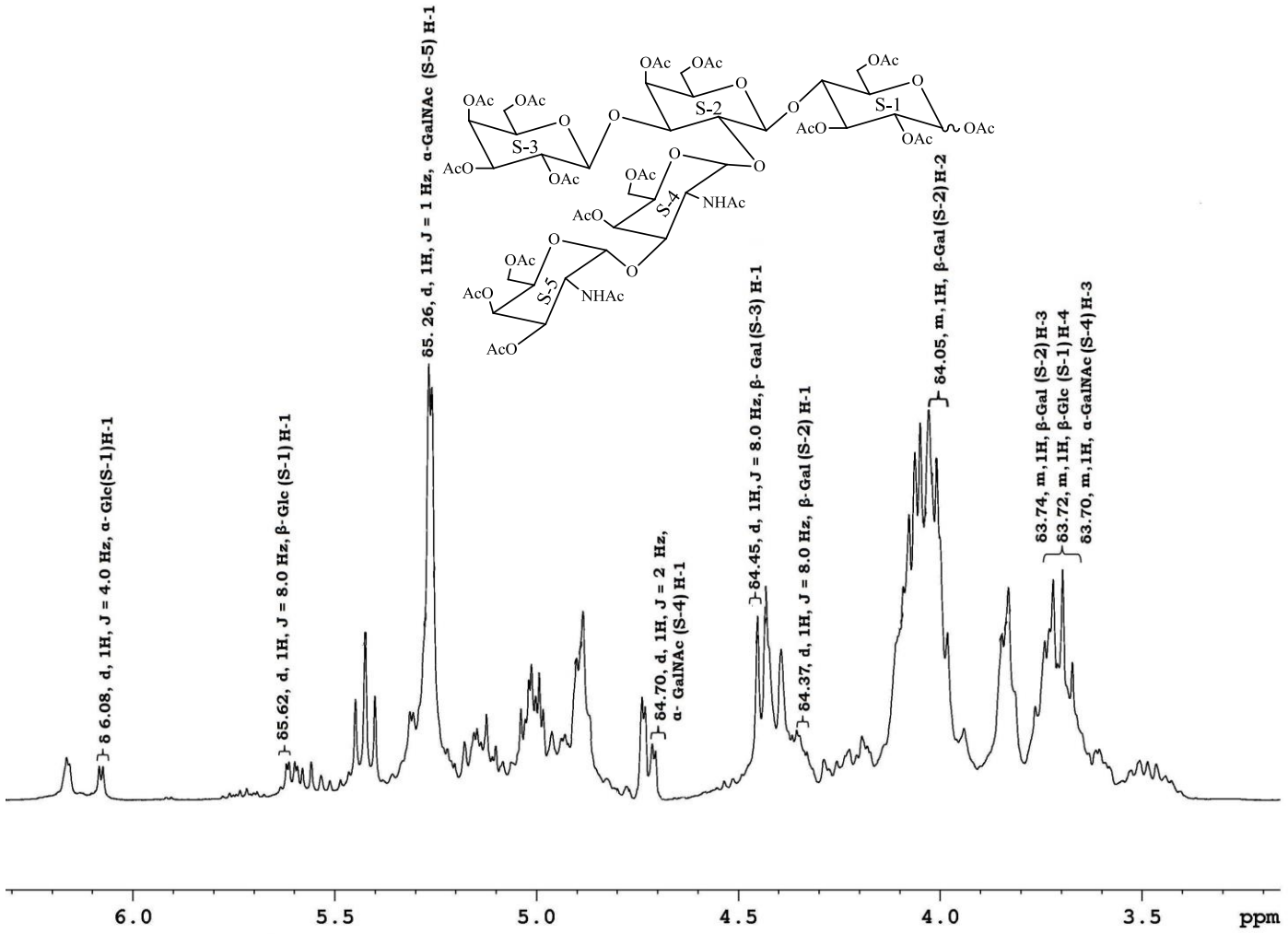


Fig. 3.13 ^{1}H NMR spectrum of Murtiose acetate in $CDCl_3$ at 400 MHz

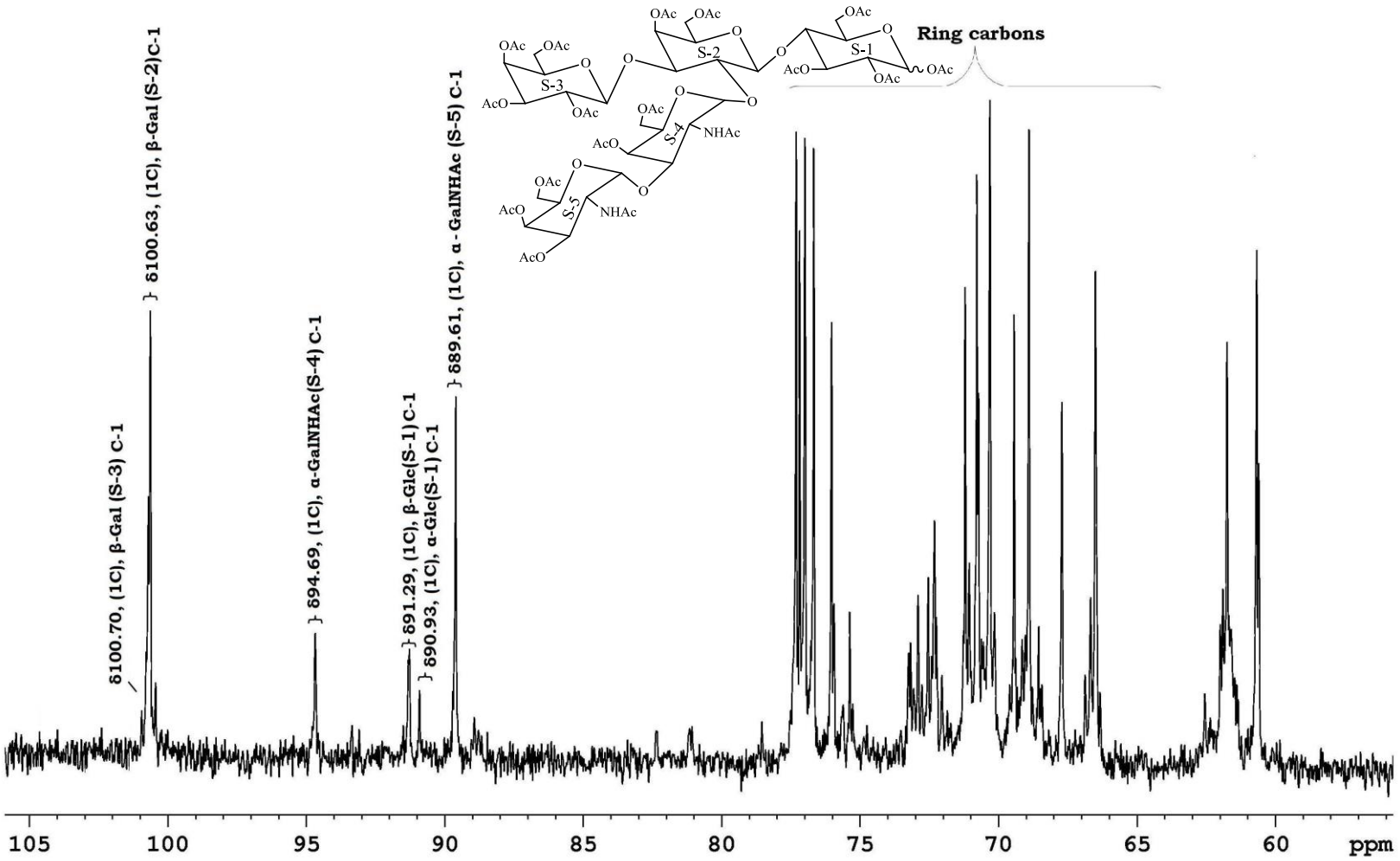


Fig. 3.14 ^{13}C NMR spectrum of Murtiose acetate in $CDCl_3$ at 400 MHz

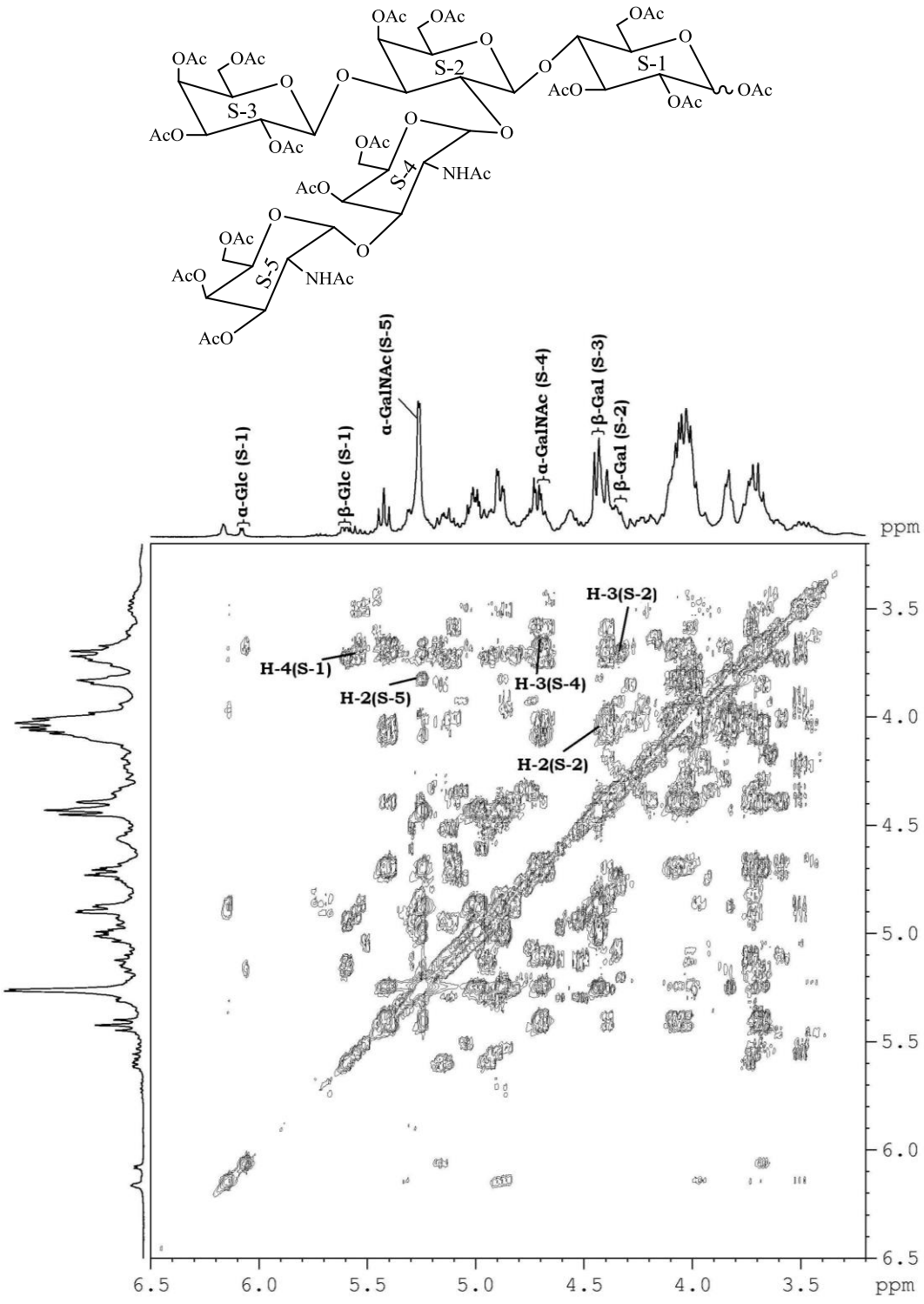


Fig. 3.15 TOCSY spectrum of Murtiose acetate in $CDCl_3$ at 400 MHz

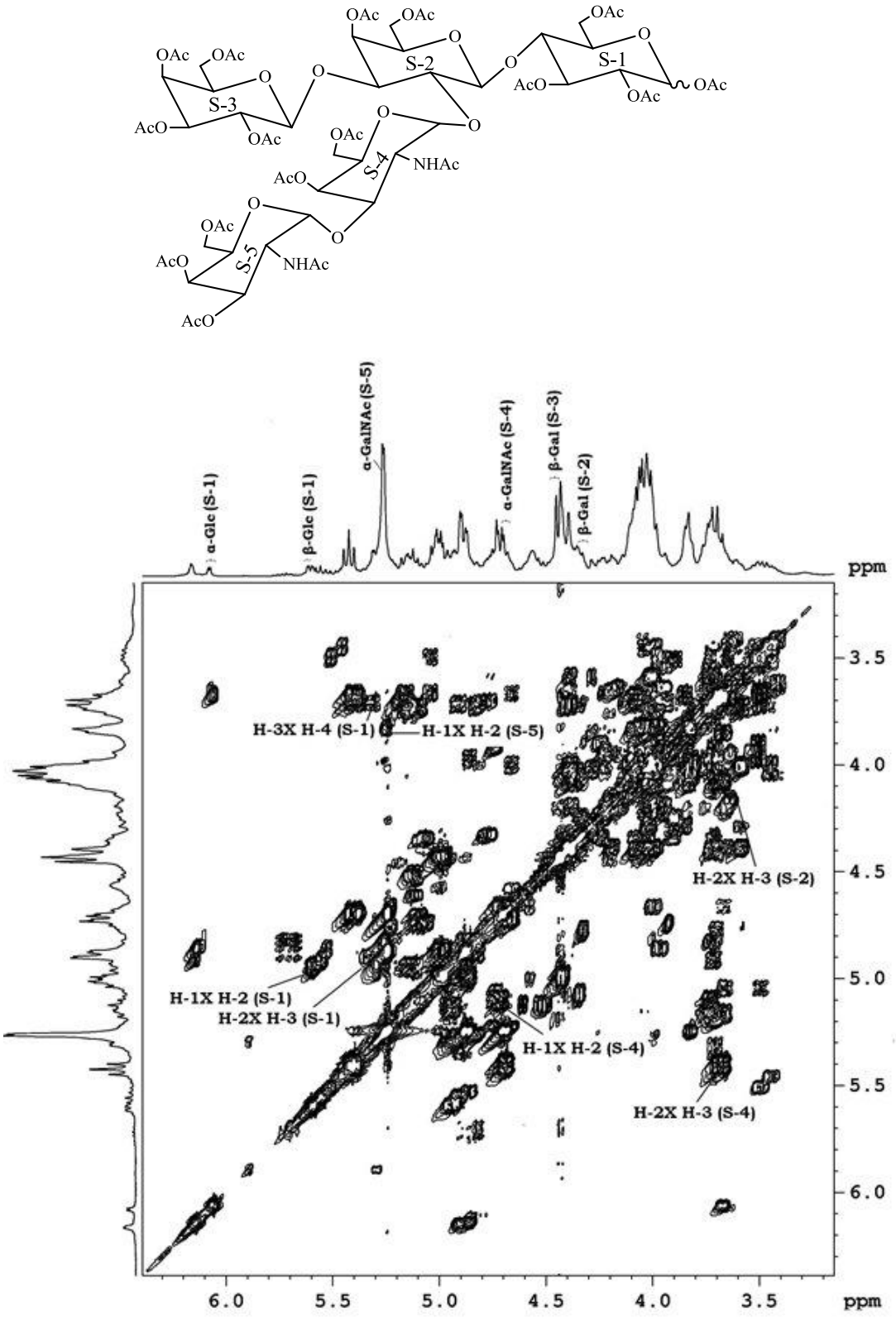


Fig. 3.16 COSY spectrum of Murtiose acetate in $CDCl_3$ at 400 MHz

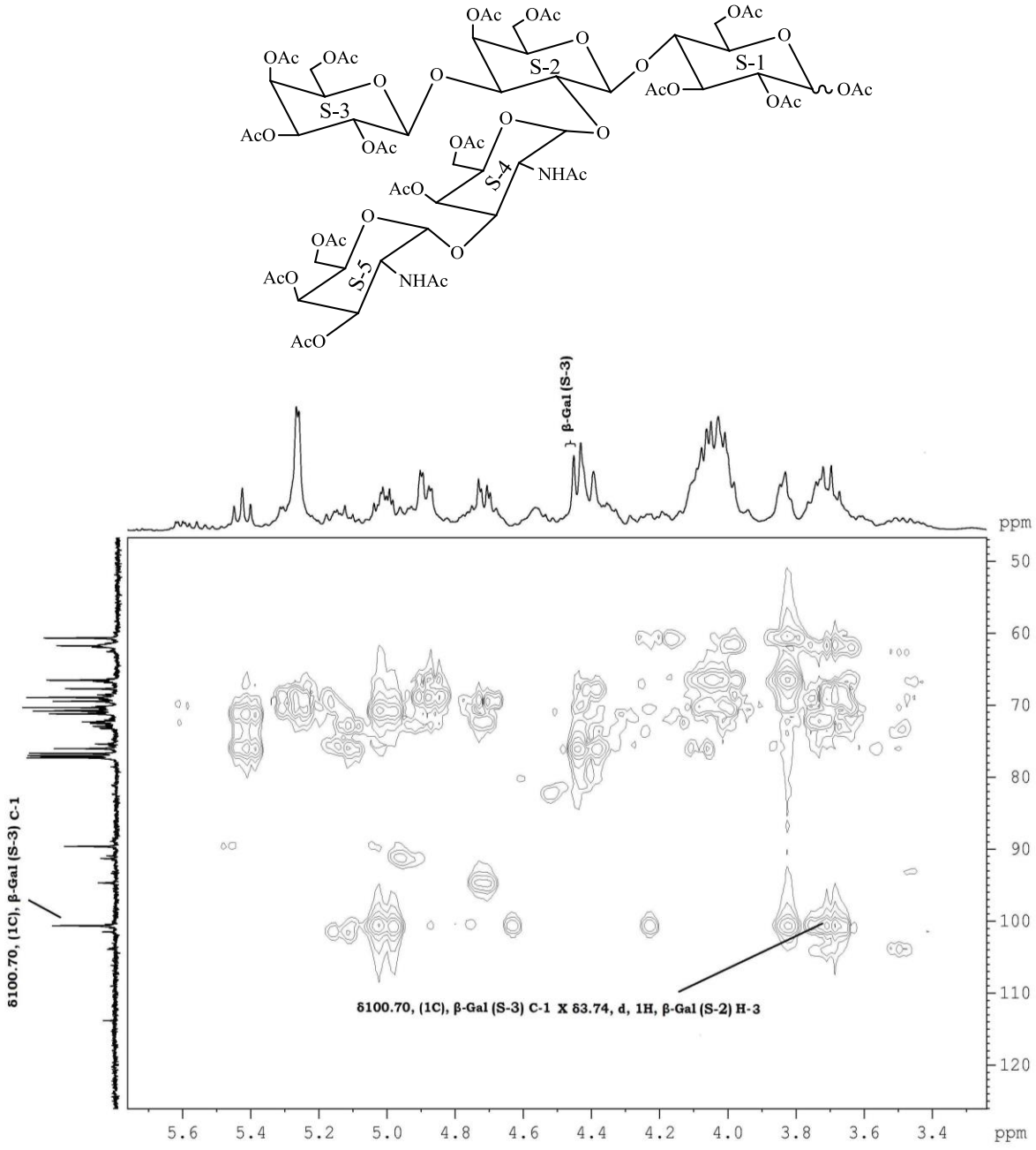


Fig. 3.17 ^{1}H-^{13}C HMBC spectrum of Murtiose acetate in $CDCl_3$ at 400 MHz

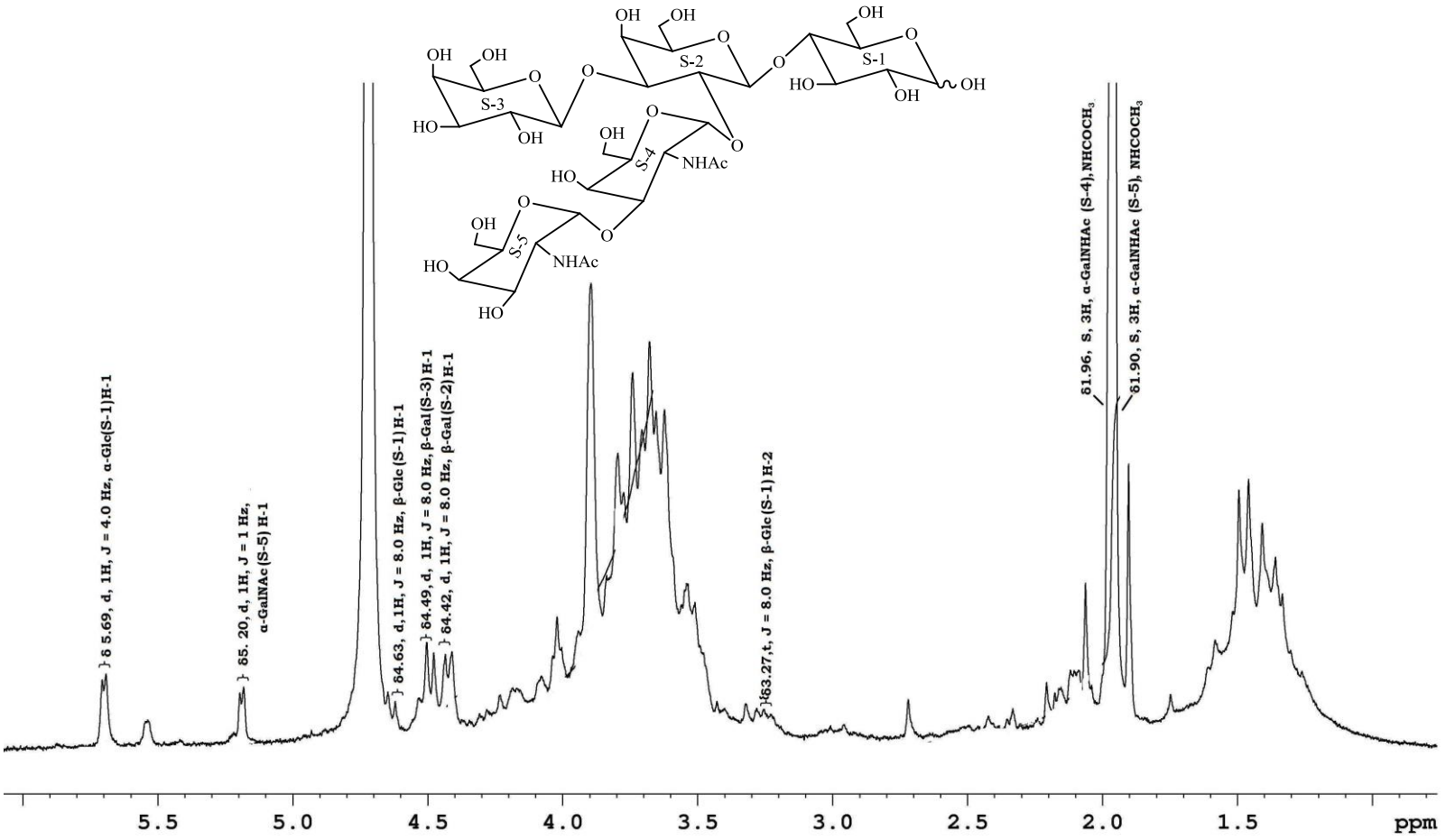
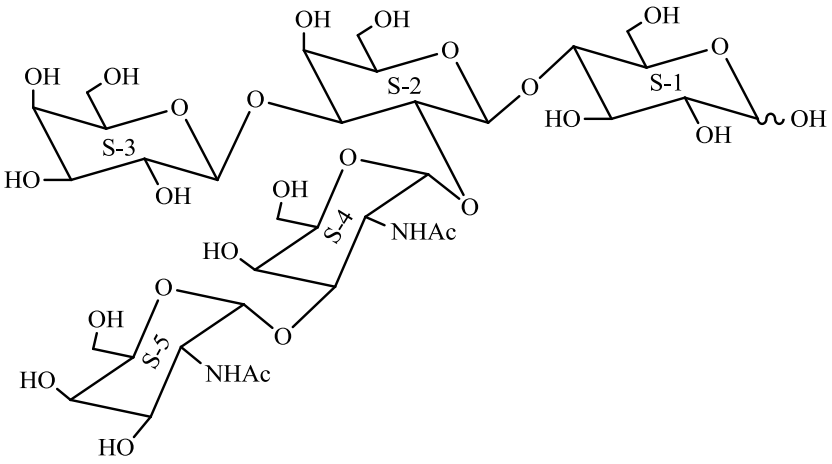


Fig. 3.18 ^{1}H NMR spectrum of Murtiose in D_2O at 300 MHz

The electrospray Mass spectrometry data of Murtiose not only confirmed the derived structure but also supported the sequence of monosaccharide in Murtiose (Fig.3.20). The highest mass ion peaks were recorded at m/z 972 assigned to $[M+Na+K]^+$ and m/z 949 assigned to $[M+K]^+$, it also contain the molecular ion peak at m/z 910 confirming the molecular weight as 910 which was in agreement with its molecular formula $C_{34}H_{58}O_{26}N_2$. 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 910 (I) fragmented to give mass ion at m/z 748(II) [910-S-3] which was tetrasaccharide (II), this fragment was arised due to the loss of 162 in Gal (S-3) moiety from pentasaccharide (I). It further fragmented to give mass ion peak at m/z 545 (III) which was a trisaccharide moiety (III) [748-S-5], due to loss of 203 in GalNAc (S-5) moiety from tetrasaccharide. This fragment of 545 further fragmented to give mass ion peak at m/z 342 (IV) [545-S-4] which was a disaccharide (IV), due to loss of 203 as GlcNAc (S-4) moiety from the tetrasaccharide. This disaccharide (IV) unit further fragmented to give mass ion peak at m/z 180 (V) [342-S-1], which was due to loss of 162 in Gal (S-1) moiety from disaccharide (Fig.3.21). These four mass ion peak II, III, IV, V were appeared due to the consequent loss of S-3, S-5, S-4, and S-1 from original molecule. The mass spectrum also contain the mass ion peak at are m/z 748, 545, 586, corresponds to the mass ion fragment A, B, C which confirm the position of S-1, S-2, S-3 ,S-4, S-5 (Fig.3.22).

The other fragmentation pathway in ES Mass spectrum of compound B Murtiose at m/z 910 shows the mass ion peaks at 875 [910-H_2O(18), -OH(17)], 851[910-CH_2OCHO(59)], 820 [851-CH_2OH(31)], 785 [820-OH(17), -H_2O(18)], 671 [748-CH_2OHCHO(60), OH(17)], 717 [748-CH_2OH(31)], 507 [545-2H_2O(36), $2H^+$(2)], 485 [545- CH_2OHCHO(60)], 487 [545-$NHCOCH_3$(58)], 342 [545-S-3], 484 [342-$NHCOCH_3$(58)], 325 [342-OH(17)], 281 [342-CH_2OHCHO(60), -H^+(1)] (Fig. 3.23).

Based on the above results obtained from chemical degradation / acid hydrolysis, chemical transformation, electrospray mass spectrometry and ^{1}H, ^{13}C NMR and 2D NMR, COSY, TOCSY and HSQC techniques the structure and sequence of isolated novel oligosaccharide molecule Murtiose was deduced as (Fig. 3.19)



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

Fig. 3.19 Structure of Murtiose

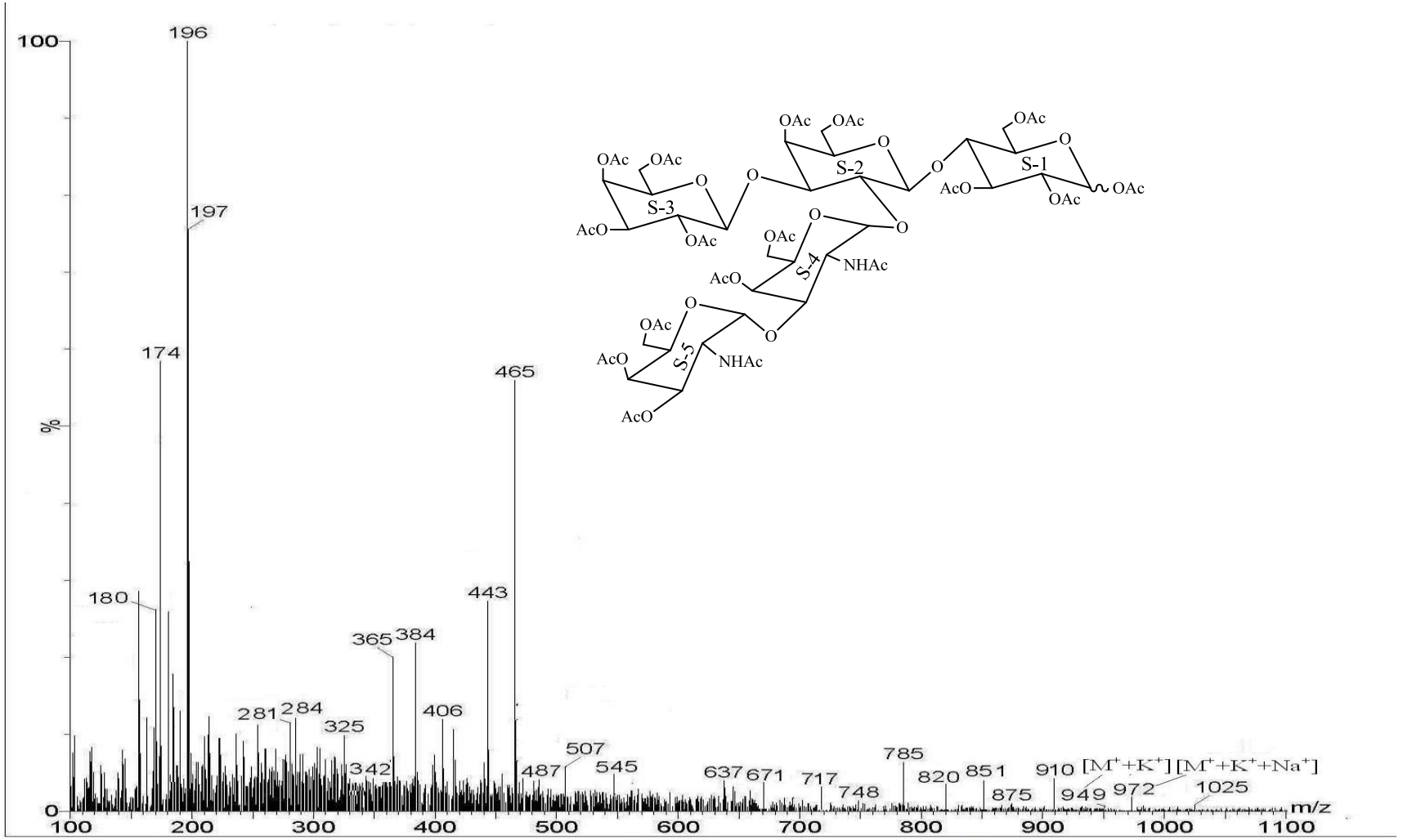


Fig. 3.20 ES Mass spectrum of compound Murtiose

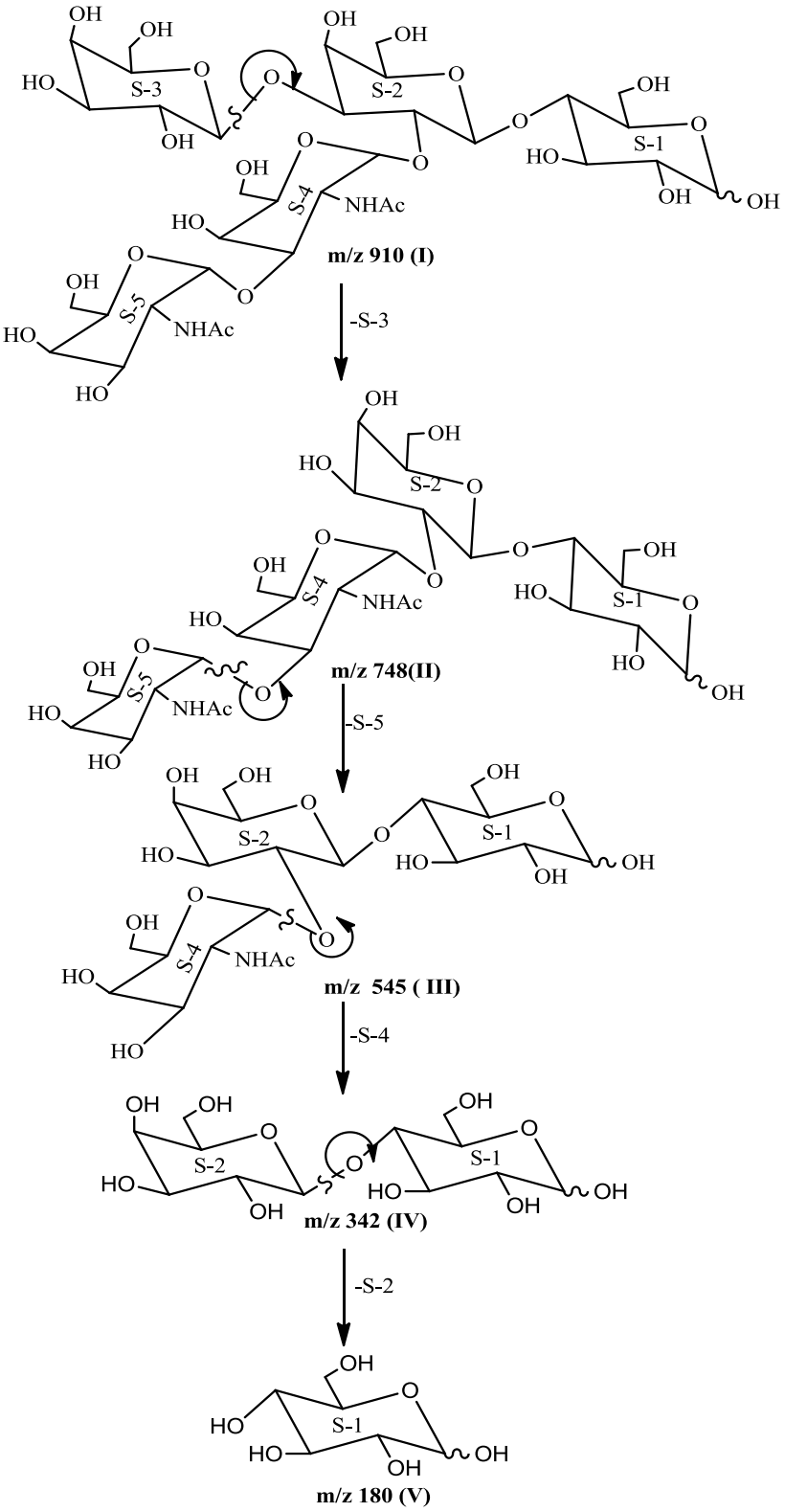


Fig. 3.21 Mass fragmentations of compound Murtiose

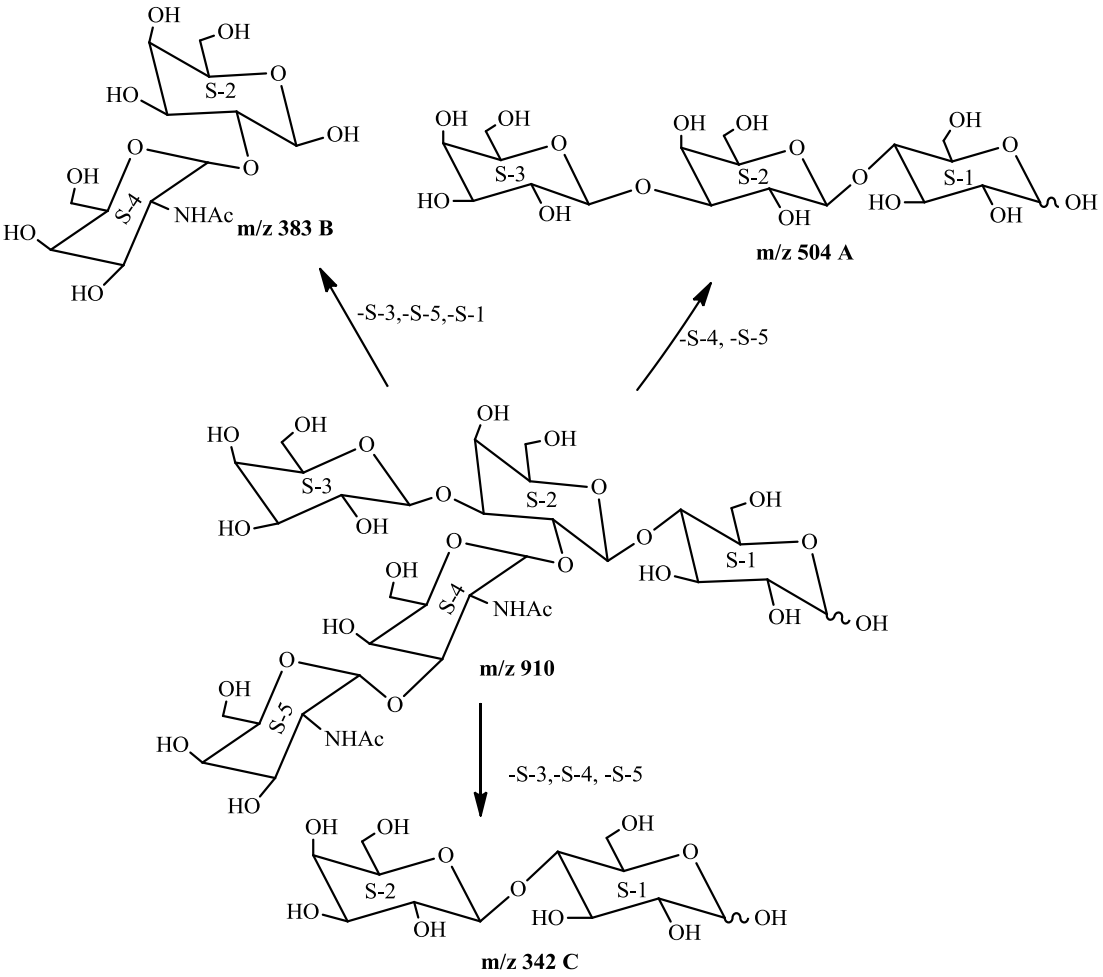


Fig. 3.22 ES-MS fragmentations of compound Murtiose

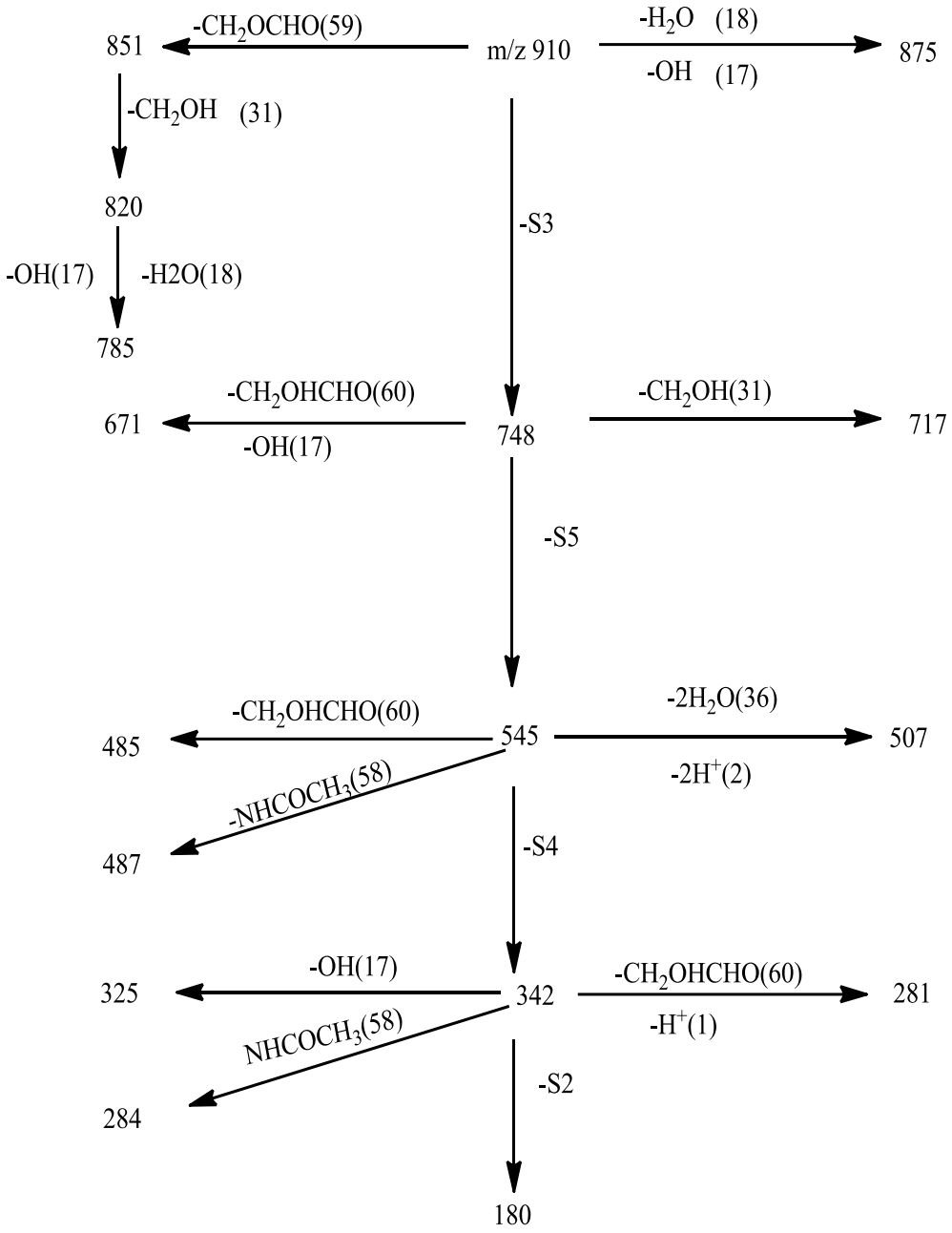


Fig. 3.23 Line diagram of ES-MS fragmentations of compound Murtiose

3.1.3 Structural elucidation of compound – C (Vediose)

Compound C, $C_{52}H_{88}O_{41}N_2$, gave positive phenol-sulphuric acid test (Dubois et.al., 1956), Fiegl test (Fiegl, 1975) and Morgon-Elson test (Gey et.al., 1996) showing the presence of normal and amino sugars moiety in the compound Vediose. The HSQC spectrum of acetylated Vediose showed the presence of nine cross peaks of anomeric protons and carbons in the anomeric region at δ 6.22 x 89.00, 5.65 x 91.08, 5.57 x 93.06, 5.34 x 90.00, 5.27 x 92.01, 4.67 x 96.01, 4.60 x 102.02, 4.54 x 102.02 and 4.46 x 102.02 suggesting the presence of nine anomeric protons and carbons in it (Fig. 3.24). The presence of nine anomeric protons were confirmed by the presence of nine anomeric proton doublets at δ 6.22 (J = 3.2 Hz), 5.65 (J = 8.4 Hz), 5.57 (J = 2.0 Hz), 5.34 (J = 3.0 Hz), 5.27 (J = 1.6 Hz), 4.67 (J = 6.0 Hz), 4.60 (J = 7.2 Hz), 4.54 (J = 8.4 Hz) and 4.46 (J = 7.8 Hz) in the ^{1}H NMR of acetylated compound C (Fig. 3.25). The presence of nine anomeric carbons were confirmed by the presence of nine anomeric carbon signals at δ 102.02 (3C), 96.01 (1C), 93.06(1C), 92.01 (1C), 91.08 (1C), 90.00 (1C), 89.00 (1C), in the ^{13}C NMR spectrum of acetylated Vediose. These data suggested that compound Vediose may be an octasaccharide in its reducing form. The ^{1}H NMR of Vediose in D_2O at 300 MHz shows doublet for anomeric protons at δ 5.70 (1H), 5.54 (1H), 5.20 (1H), 4.62 (1H), 4.52 (1H), 4.51 (1H), 4.43(1H). Further the ES Mass spectrum of Vediose showed the highest mass ion peaks at m/z 1458 assigned to $[M+Na+K]^+$ and m/z 1435 assigned to $[M+K]^+$, it also contain the molecular ion peak at m/z 1396 confirming the molecular weight as 1396 which was in agreement of derived composition $C_{52}H_{88}O_{41}N_2$. The reducing nature of compound Vediose was confirmed by methylglycosylation of compound Vediose by $MeOH/H^+$ followed by its acid hydrolysis which led to the isolation of α and β methyl glucosides, suggesting the presence of glucose at the reducing end. For convenience the eight monosaccharides present in compound Vediose have been designated as S-1, S-2, S-3, S-4, S-5, S-6, S-7 and S-8 respectively starting from the reducing end.

The monosaccharide constituents in compound Vediose were confirmed by Killiani hydrolysis (Killiani, 1930) under strong acidic conditions, followed by PC and TLC. In this hydrolysis four spots were found on PC and TLC which were found identical in glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) and by co-chromatography with authentic samples. Thus the octasaccharide contained four types of monosaccharide units i.e. Glc, Gal, GalNAc and GlcNAc in it. The presence of two anomeric proton signals at δ 5.20 (J = 3.2 Hz) and δ 4.62 (J = 7.8 Hz) in the ^{1}H NMR spectrum of Vediose in D_2O at 300 MHz were assigned for α and β anomers of glucose (S-1) confirming the presence of Glc (S-1) at the reducing end in compound Vediose. The ^{1}H NMR of Vediose also contain a triplet δ 3.26 (Stucture Reporter Groups) (Dua, and Bush, 1983) suggesting for presence of lactose type of structure at reducing end confirming the 1→4 linkage between β-Gal (S-2) and β-Glc (S-1), hence suggesting the presence of lactosyl moiety at the reducing end. Since the HSQC spectrum of Vediose acetate showed nine anomeric protons and carbon signal, confirming Vediose has octasaccharide in its reducing form. The anomeric signal for α and β anomers were detected at δ 6.22 (J = 3.2 Hz) and δ 5.65 (J = 8.4 Hz), respectively, which were assigned for reducing glucose. In the TOCSY spectrum of Vediose acetate the anomeric signal of β-Glc (S-1) at δ 5.65 (J = 8.4 Hz) gave cross peak at δ 5.65 x 3.52, 5.65 x 3.73, 5.65 x 5.00, out of which two peaks at δ 5.65 x 3.52 & 5.65 x δ 3.73, in the TOCSY spectrum of Vediose acetate in $CDCl_3$ showed that the two position in S-1 were available for glycosidic linkages, showing that the two hydroxyl groups of β-Glc (S-1) were involved in glycosidic linkages by other monosaccharide moieties (Fig. 3.26). These signals at δ 3.52 and δ 3.73 were identified for H-4 and H-3 of β-Glc (S-1) by the COSY spectrum of Vediose acetate suggesting that H-4 and H-3 of β-Glc (S-1) were available for glycosidic linkages by the next monosaccharide units (Fig. 3.27). In the ^{1}H NMR presence of another anomeric proton doublet at δ 4.67 (J = 6.0 Hz) of Vediose acetate in $CDCl_3$, confirming the presence of β-Gal (S-2) residue as the next monosaccharide. Since it was explained earlier by ^{1}H NMR of Vediose in D_2O at 300 MHz that due to the presence of anomeric proton signals at δ 5.20 (J = 3.2 Hz) and

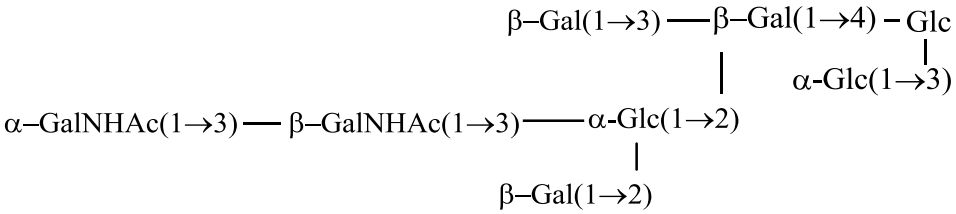
4.62 (J = 7.8 Hz) for reducing glucose along with a triplet at δ 3.26 suggesting a lactose type structure at the reducing end, hence further the ^{1}H NMR signal for H-4 of S-1 at δ 3.52 in the ^{1}H NMR of Vediose acetate confirmed H-4 of Glc (S-1) was glycosidically linked to Gal (S-2), concluded that Gal (S-2) must be glycosidically linked to H-4 of Glc (S-1). The coupling constant of anomeric proton signal at δ 4.67 β-Gal (S-2) with J value of 7.2 Hz confirmed the β-configuration of the β-Gal (S-2) moiety and hence β 1→4 glycosidic linkage between S-2 and S-1 was confirmed. The next anomeric proton signal appeared as doublet at δ 5.34 (J = 3 Hz) in the ^{1}H NMR spectrum of Vediose acetate in $CDCl_3$ at 400 MHz was due to the presence of α-Glc (S-6) moiety, since the Glc (S-1) possess H-3 and H-4 position for glycosidic linkage and position H-4 was already occupied by Gal (S-2). Hence, the leftover H-3 of β-Glc(S-1) was glycosically linkaged by the α-Glc (S-6) moiety. The anomeric position at δ 5.34 have a small coupling constant (J = 3.0 Hz) and it was confirmed that α-1→3 glycosidic linkage between S-6 and S-1. Since, none of methine proton of α-Glc (S-6) gave its cross peak in the linkage region i.e. δ 3-4 in the TOCSY spectrum of Vediose acetate confirms that α-Glc (S-6) was linked at the non reducing end. Further in the ^{1}H NMR of Vediose acetate presence of anomeric proton doublet at δ 4.67 (J = 6.0 Hz), assigned to β-Gal (S-2), in the TOCSY spectrum of Vediose acetate anomeric signal at δ 4.67 (J = 6.0 Hz) showed cross peak at δ 4.67 x 3.40, 4.67 x 3.50, 4.67 x 3.64, 4.67 x 3.73, out of which two cross peaks at δ 4.67 x 3.40 & 4.67 x 3.73 in the ^{1}H NMR of Vediose acetate in $CDCl_3$ showed their two position in S-2 were available for glycosidic linkages, showing that the two hydroxyl groups of β-Gal (S-2) were involved in glycosidic linkages by other monosaccharide moieties. These position of δ 3.40 and δ 3.73 were later confirmed as H-2 and H-3 of β-Gal (S-2) confirming that H-2 and H-3 of β-Gal (S-2) were available for glycosidic linkage by next monosaccharides. The next anomeric proton signal appeared as doublet at δ 4.60 (J = 7.2 Hz) in the ^{1}H NMR spectrum of Vediose acetate in $CDCl_3$ at 400 MHz was due to the presence of β Gal (S-7) moiety. The ^{1}H NMR signals at δ 4.60 of Vediose acetate gave its complementary signal at δ 102.2 in the HSQC spectrum of Vediose acetate. Further the anomeric signal of 102.2 shows its cross peak with the chemical

shift of ^{1}H NMR signal present at δ 3.73 and gave cross peak at 102.2 x 3.73 in the HMBC spectrum of Vediose acetate confirming that the Gal (S-7) was linked glycosidically with H-3 of S-2 confirming the 1→3 glycosidic linkage between S-7 and S-2. The anomeric proton signal of S-7 present at δ 4.60 (J = 7.2 Hz) showed a large coupling constant suggesting a β-glycosidic linkage between S-7 and S-2 confirming the 1→3 glycosidic linkage between S-7 and S-2 (Fig. 3.28). Since, none of methine proton of β-Gal (S-7) came in the linkage region in the TOCSY spectrum of Vediose acetate it confirmed that β-Gal (S-7) was linked at the non reducing end. The anomeric position at δ 4.60 have a large coupling constant (J = 7.2 Hz) and it was confirmed that β-(1→3) glycosidic linkage between S-7 and S-2. Since, none of methine proton of β-Gal (S-7) gave its cross peak in the linkage region i.e. δ 3-4 in the TOCSY spectrum of Vediose acetate confirms that β-Gal (S-7) was linked at the non reducing end. The next anomeric proton signal at δ 5.57 (J = 2.0 Hz) in the ^{1}H NMR spectrum of Vediose acetate was assigned to α-Glc (S-3), since it was already established earlier that H-3 of S-2 was glycosidically linked by Gal (S-7) and H-2 of S-2 was vacant therefore α-Glc (S-3) must be attached to H-2 of S-2. The anomeric proton signal of S-3 present at δ 5.57 (J = 2.0 Hz) showed a small coupling constant suggesting the α -glycosidic linkage between S-3 and S-2 confirming the α-1→3 glycosidic linkage between S-3 and S-2. The anomeric position at δ 5.57 have a small coupling constant (J = 2.0 Hz) and it was confirmed that α-1→2 glycosidic linkage between S-3 and S-2. Further the anomeric proton signal of α-Glc (S-3) at δ 5.57 (J = 2.0 Hz) in the TOCSY spectrum of Vediose acetate showed cross peak at 5.57 x 3.53, 5.57 x 3.70 in $CDCl_3$ at 400 MHz. These signals showed their two positions in S-3 were available for glycosidic linkages, showing that the two hydroxyl groups of α-Glc (S-3) were involved in glycosidic linkages by other monosaccharide moieties. These position of δ 3.53 and δ 3.70 were later confirmed as H-2 and H-3 of α-Glc (S-3) confirming that H-2 and H-3 of α-Glc (S-3) were available for glycosidic linkage by next monosaccharides. The next anomeric proton signal appeared as doublet at δ 4.54 (J = 8.4 Hz) in the ^{1}H NMR spectrum of Vediose acetate in $CDCl_3$ at 400 MHz was due to the presence of β Gal (S-8) moiety. The ^{1}H NMR signals at δ 4.54 in the ^{1}H

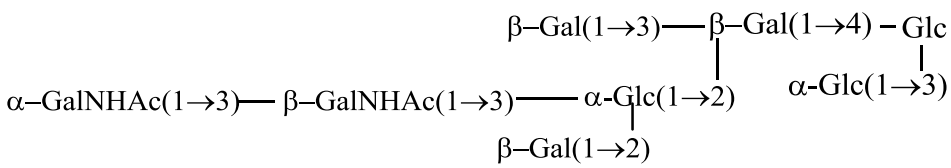
NMR of Vediose acetate gave its complementary signal at δ 102.2 in the HSQC spectrum. Further the anomeric signal of 102.2 shows its cross peak with the chemical shift of ^{1}H NMR signal present at δ 3.53, gave cross peak at 102.2 x 3.53 in the HMBC spectrum confirming that the Gal (S-8) was linked glycosidically with H-2 of S-3 confirming the β-1→2 glycosidic linkage between S-8 and S-3 (Fig. 3.28). The anomeric proton signal of S-8 present at δ 4.54 (J = 8.4 Hz) showed a large coupling constant suggesting a β-glycosidic linkage between S-8 and S-3 confirming β-1→2 glycosidic linkage between S-8 and S-3. Since, none of methine proton of β-Gal (S-8) came in the linkage region which also confirmed that β-Gal (S-8) was linked at the non reducing end. The next anomeric proton signal at δ 4.46 (J = 8.4 Hz) along with a singlet of amide methyl (-$NHCOCH_3$) at δ 1.96 in the ^{1}H NMR spectrum of Vediose acetate was assigned to β- GalNAc (S-4). It was already established earlier that H-2 of S-3 was already glycosidically linked by Gal (S-8) and H-3 of S-3 was vacant therefore β-GalNAc (S-4) must be attached to H-3 of S-3 confirming the 1→3 glycosidic linkage between S-4 and S-3. The anomeric proton signal of S-4 present at δ 4.46 (J = 8.4 Hz) showed a large coupling constant suggesting a β-glycosidic linkage between S-4 and S-3 confirming the β-1→3 glycosidic linkage between S-4 and S-3. Further the anomeric proton signal of β-GalNAc (S-4) at δ 4.46 (J = 8.4 Hz) in the TOCSY spectrum of Vediose acetate showed cross peak at 4.46 x 3.70, 4.46 x 4.13, 4.46 x 4.90, in $CDCl_3$ at 400 MHz, out of which one signals δ 4.46 x 3.70, showed in the linkage region, indicate their one position in S-4 were available for glycosidic linkages, which showed that the one hydroxyl groups of β-GalNAc (S-4) were involved in glycosidic linkages by other monosaccharide moieties. Other signal at δ 4.46 x 4.13 confirms the position of NAc group at H-2 position of S-4 moiety at δ 4.13. The position of δ 3.70 was later confirmed at H-3 of β-GalNAc (S-4) confirming that H-3 of β-GalNAc (S-4) was available for glycosidic linkage by next monosaccharides. The next anomeric proton signal at δ 5.27 (J = 1.6 Hz) in the ^{1}H NMR spectrum of Vediose acetate was assigned to α-GalNAc (S-5) and it was already established earlier that H-3 of S-4 was vacant, therefore, α-GalNAc (S-5) must be attached to H-3 of S-4. The next anomeric proton signal appeared as doublet at δ 5.27

(J = 1.6 Hz) along with a singlet of amide methyl (-NHCOCH$_3$) at δ 1.92 in the ^{1}H NMR spectrum of Vediose in CDCl$_3$ at 400 MHz was due to the presence of α-GalNAc (S-5) moiety. Hence the H-3 of β GalNAc (S-4) was glycosically linkaged by the α-GalNAc (S-5) moiety and hence α–(1→3) glycosidic linkage between S-5 and S-4 were confirmed. The small coupling constant of α-GalNAc (S-5) (J = 1.6 Hz) confirmed the α-glycosidic linkage between α-GalNAc (S-5) and β-GalNAc (S-4) and none of methine proton of α-GalNAc (S-5) came in the linkage region which also confirms that α-GalNAc (S-5) was linked at the non reducing end. The absence of methine protons in linkage region of α –GalNAc (S-5) confirm that α-GalNAc (S-5) was also present at non reducing end and also confirmed by the TOCSY and COSY spectrum.

All the ^{1}H NMR assignments for ring protons of monosaccharide units of Vediose were confirmed by COSY and TOCSY spectrum. The positions of glycosidation in the oligosaccharide were confirmed by position of anomeric signals, and comparing the signals in ^{1}H and ^{13}C NMR of acetylated oligosaccharide. The glycosidic linkages in Vediose were assigned by the cross peaks for glycosidically linked carbons with their protons in the HSQC spectrum of acetylated Vediose. The values of cross peaks appeared as- β-Glc (S-1) H-4 and C-4 at δ3.52 x 70.0 showed (1→4) linkage between S-2 and S-1, β-Glc (S-1) H-3 and C-3 at δ3.73 x 71.0 showed (1→3) linkage between S-6 and S-1, β-Gal (S-2) H-3 and C-3 at δ 3.73 x 71.0 showed (1→3) linkage between S-7 and S-2, β-Gal (S-2) H-2 and C-2 at δ 3.40 x 73.0 showed (1→2) linkage between S-3 and S-2, α-Glc (S-3) H-3 and C-3 at δ 3.70 x 72.0 showed (1→3) linkage between S-4 and S-3, α-Glc (S-3) H-2 and C-3 at δ 3.53 x 70.0 showed (1→2) linkage between S-8 and S-3, β-Gal (S-4) H-3 and C-3 at δ 3.64 x 80 showed (1→3) linkage between S-5 and S-4. All signals obtained in ^{1}H and ^{13}C NMR of compound Vediose were in conformity with the assigned structure and their position were confirmed by 2D NMR ^{1}H-^{1}H COSY, TOCSY and HSQC spectra. Thus based on the pattern of chemical shifts of ^{1}H, ^{13}C, COSY, TOCSY and HSQC NMR spectra it is interpreted that the compound has octasaccharide structure as-



The structure of Vediose derived from 2D NMR, was further confirmed the ^{1}H NMR of Vediose in D_2O at 300 MHz, which contains anomeric proton signal at δ 5.70 (1H), δ 5.54 (1H), δ 5.20 (1H), δ 4.62 (1H) and δ 4.52, (1H), δ 4.51, (1H) δ 4.43 (1H) i.e. the anomeric proton signal of glucose S-1 gave signal for α and β anomers at δ 5.20 (J = 3.2 Hz) and 4.62 (J = 7.8 Hz), respectively, while the anomeric proton signal of S-2 was not detected but its complementary signal as a triplet at δ 3.26 which is structure reporter group for the presence of lactose structure at the reducing end (Dua, and Bush, 1983) another anomeric proton signal at δ 5.54 (J = 3.0 Hz), was assigned as α-Glc (S-6) which is also linked with Glc (S-1), while the anomeric proton signal of S-2 was not detected but it also showed two linkage position which were already detected in Vediose acetate which linked with another two anomeric proton signal δ 5.70 (J = 2.0 Hz) and δ 4.52 (J = 7.4 Hz) was assigned as α-Glc (S-3) and β-Gal (S-7). Further another anomeric proton signal at δ 5.70 (J = 2.0 Hz), α-Glc (S-3) which was linked with other two anomeric proton signal δ 4.43 (J = 7.8 Hz) and δ 4.51 (J = 7.8 Hz) which were confirmed as β-GalNAc (S-4) along with a singlet of amide methyl signal at δ 1.96 and β-Gal (S-8). The anomeric proton signal for S-5 was not detected but its complementary signal of 1.92 for $NHCOCH_3$ group was present. The position of inter glycosidic linkage are already discussed earlier and were confirmed by 2D NMR. Thus based on the pattern of chemical shifts of ^{1}H, ^{13}C, COSY, TOCSY and HSQC NMR spectra it was interpreted that the compound was a octasaccharide having structure as



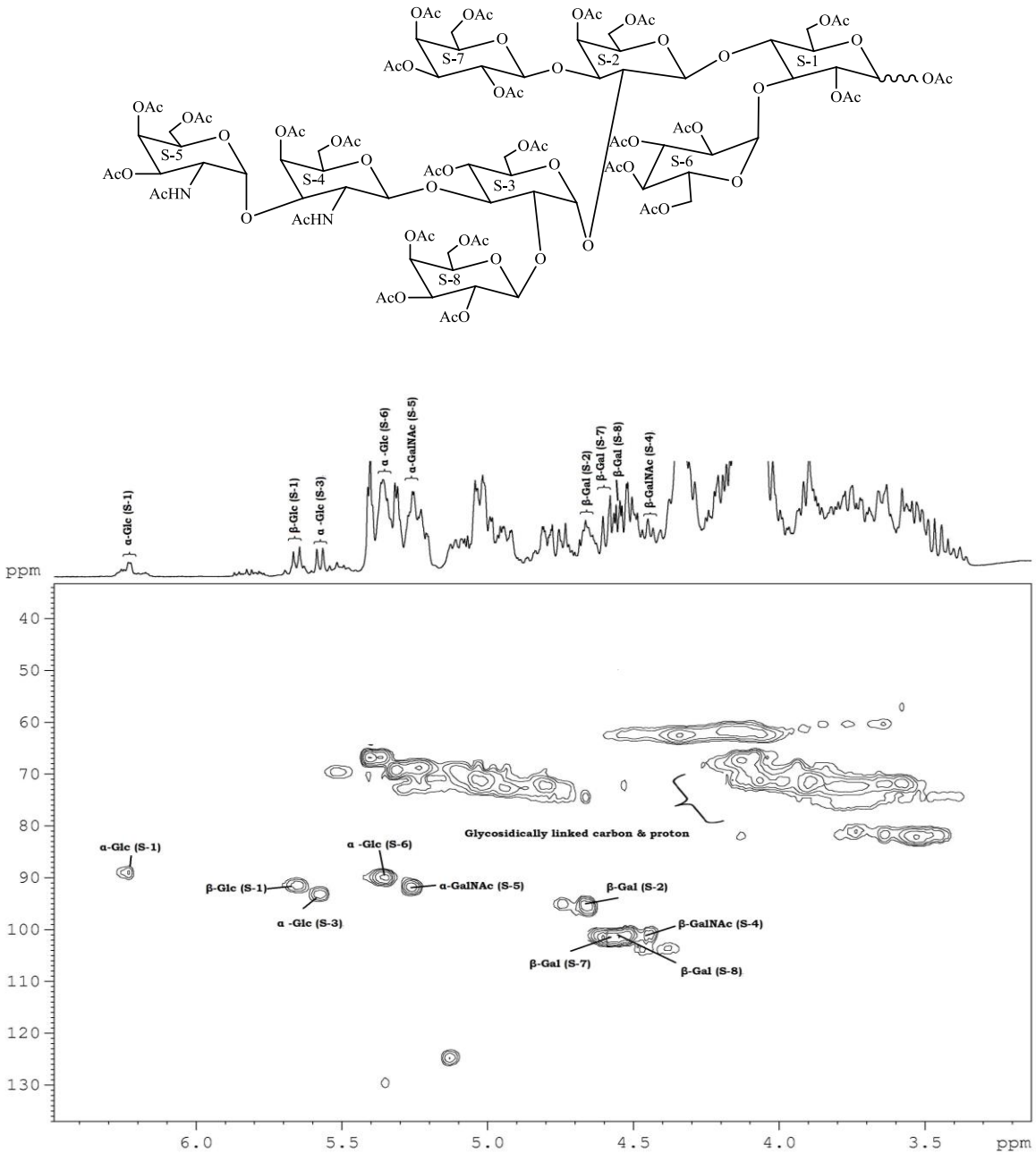


Fig. 3.24 ^{1}H-^{13}C HSQC spectrum of Vediose acetate in $CDCl_3$ at 400 MHz

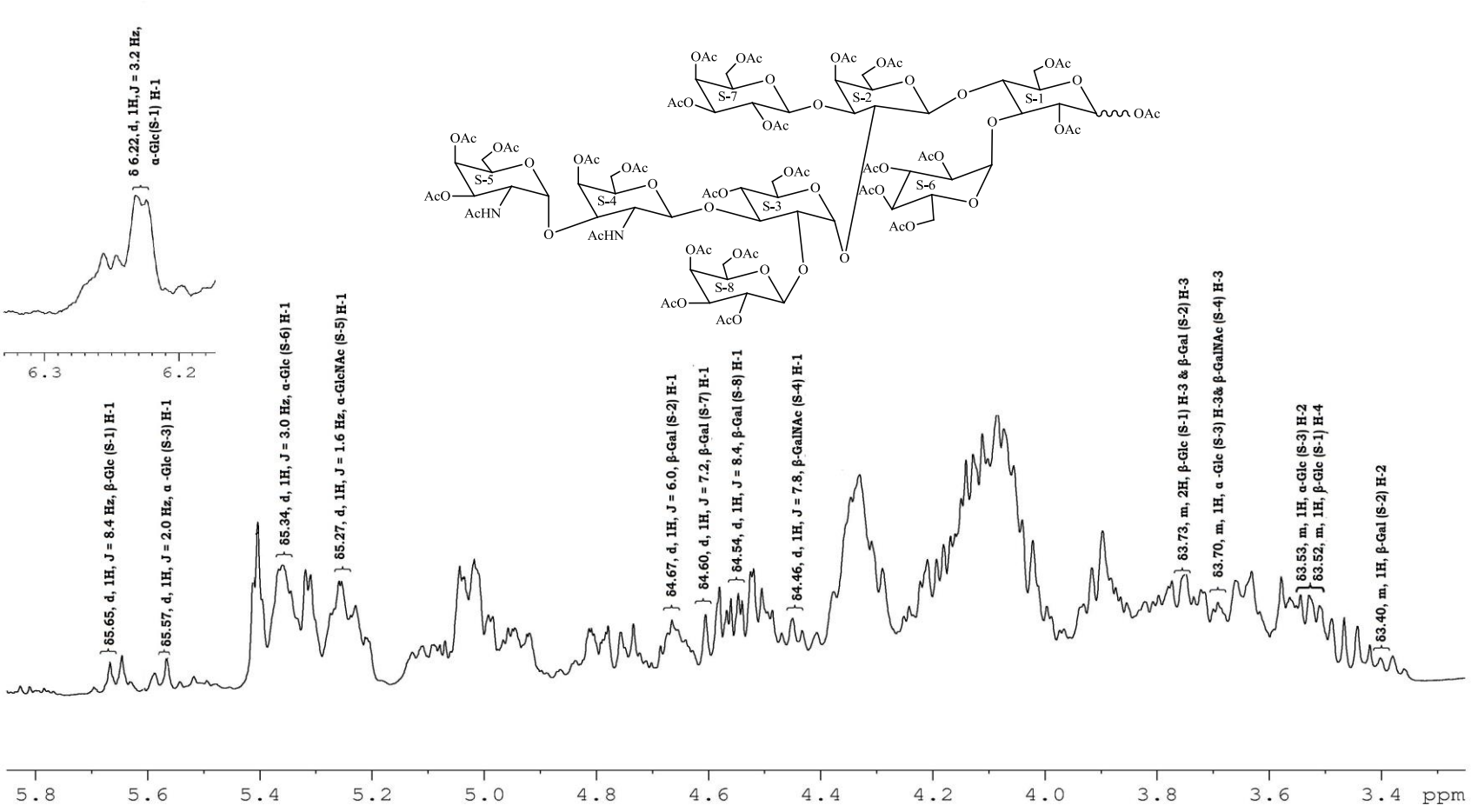


Fig. 3.25 ^{1}H NMR spectrum of Vediose acetate in $CDCl_3$ at 400 MHz

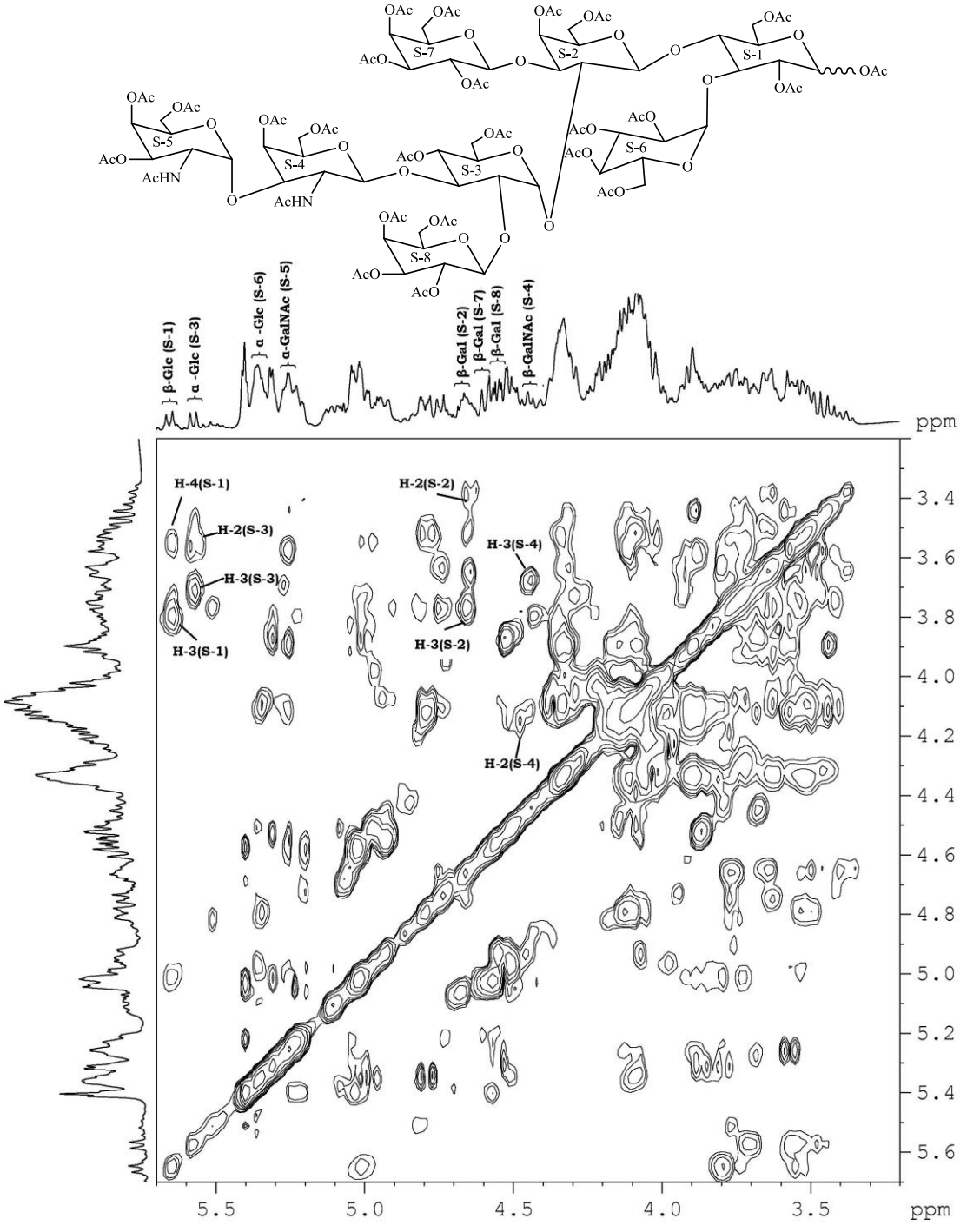


Fig. 3.26 TOCSY spectrum of Vediose acetate in $CDCl_3$ at 400 MHz

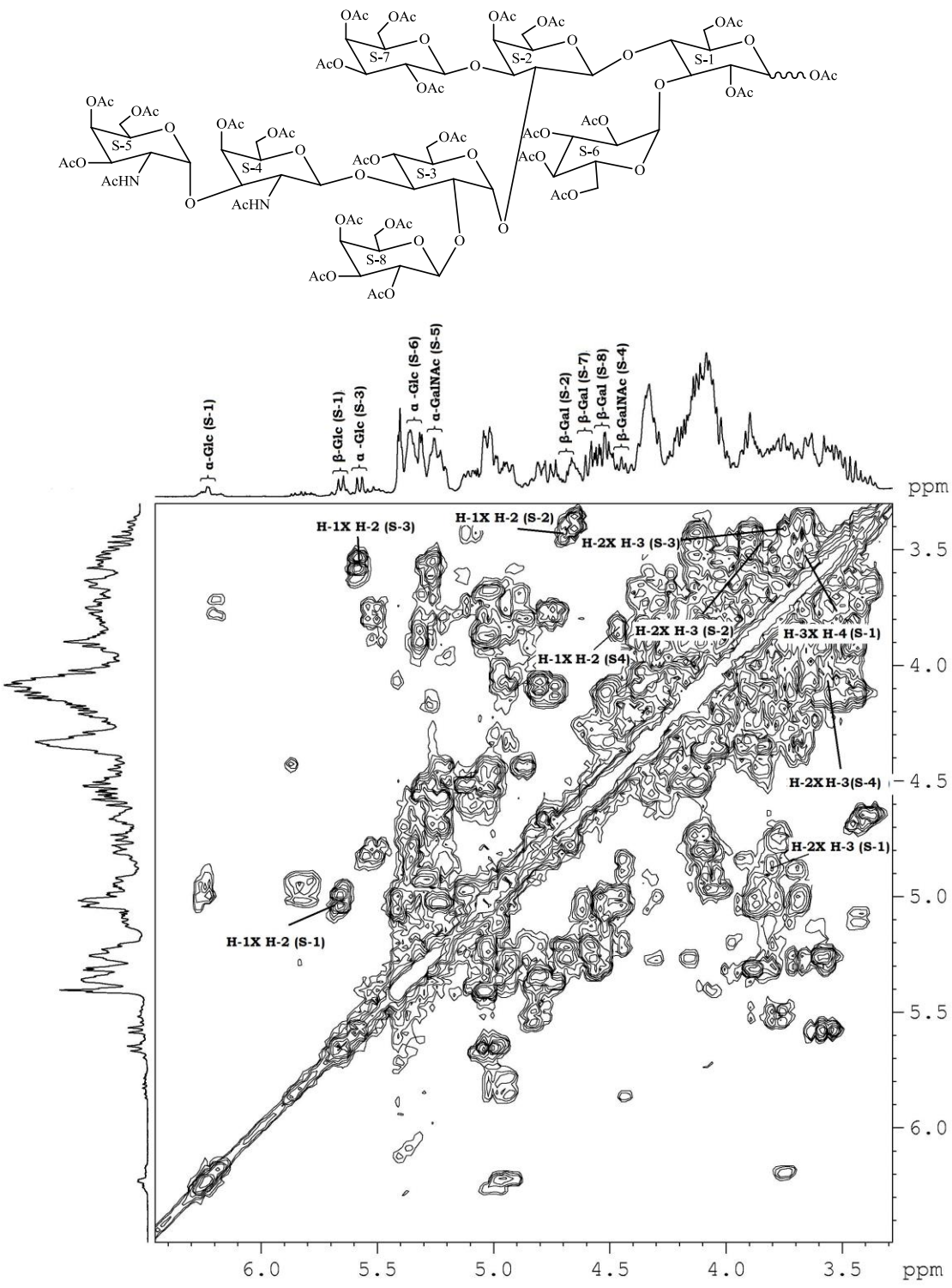


Fig. 3.27 COSY spectrum of Vediose acetate in $CDCl_3$ at 400 MHz

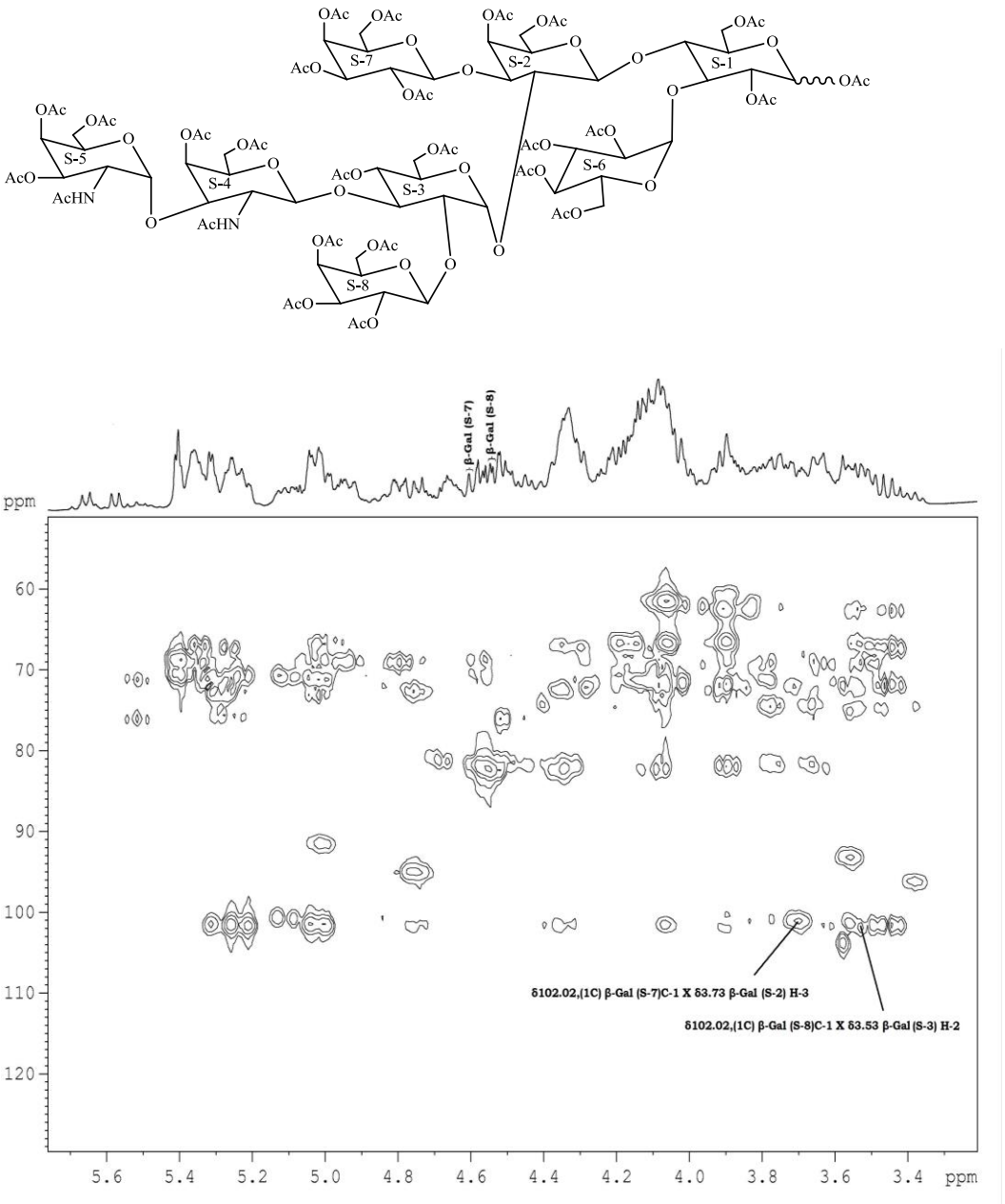


Fig. 3.28 ^{1}H-^{13}C HMBC spectrum of Vediose acetate in $CDCl_3$ at 400 MHz

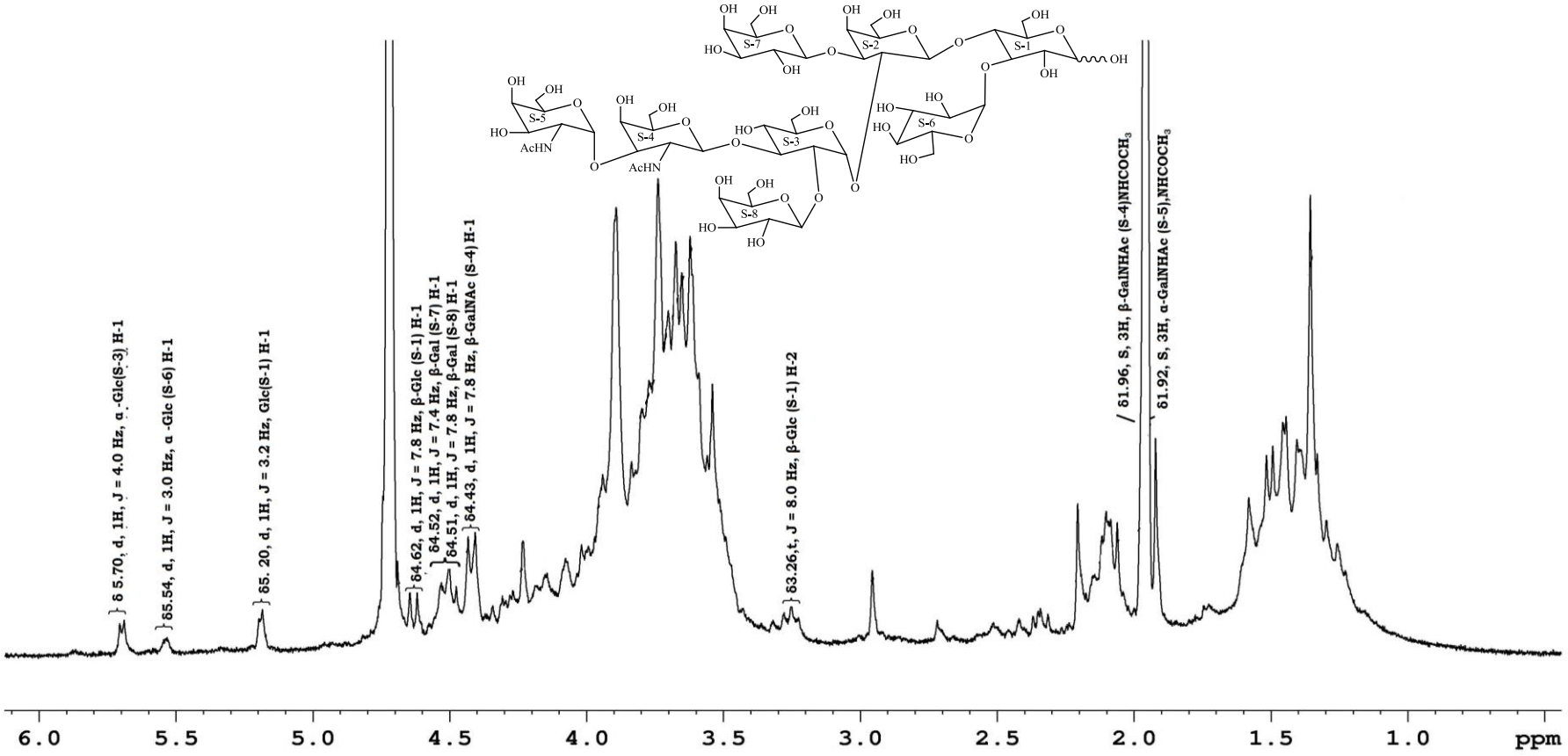


Fig. 3.29 ^{1}H NMR spectrum of Vediose in D_2O at 300 MHz

The electronspray Mass spectrometry data of Vediose not only confirmed the derived structure but also supported the sequence of monosaccharide in Vediose (Fig. 3.31). The highest mass ion peaks were recorded at m/z 1458 and 1435 which were due to [M+Na+K] and [M+K+], respectively. It also contains the molecular ion peak at m/z 1396 confirming the molecular weight of Vediose as 1396 and was in agreement with its molecular formula $C_{52}H_{88}O_{41}N_2$. 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 octasaccharide m/z 1396 (I) fragmented to give mass ion at m/z 1234(II) [1396-S-8], this fragment was arised due to the loss of terminal β-Gal (S-8) moiety from octasaccharide. It further fragmented from octasacharide to give mass ion peak at m/z 1072 (III) [1234-S-7] which was due to loss of Gal S-7 moiety from heptasaccharide. This fragment of [1072-S-6] further fragmented to give mass ion peak at m/z 910 (IV) hexasaccharide. It further fragmented from pentasaccharide [910-S-5] to give mass ion peak at m/z 707 (V) tetrasaccharide which was due to loss of GalNAc (S-5) moiety from the pentasaccharide. Further, fragmentation gives mass ion peak at m/z 504 [707-S-4] (VI) which was due to loss of GalNAc moiety from tetrasaccharide. This fragment of trisacharide at 504 further fragmented to give mass ion peak at m/z 342 (VII) [504-S-3], which was due to loss of Glc (S-3) moiety from trisaccharide. This disaccharide unit fragmented to give mass ion peak at m/z 180(VIII) [342-S-1], which was due to loss of Gal (S-1) moiety from disaccharide (Fig. 3.32). These eight mass ion peak II, III, IV, V, VI, VII and VIII, were appeared due to the consequent loss of S-8, S-7, S-6, S-5, S-4, S-3 and S-2 from original molecule. The mass spectrum also contain the mass ion peak at m/z 707, 586, 545, corresponding to the mass ion fragment A, B and C, which confirm the position of S-1, S-2, S-3, S-4, S-5, S-6, S-7, S-8 (Fig. 3.33).

The other fragmentation pathway in ES Mass spectrum of compound Vediose m/z 1396 shows the mass ion peak at 1297 [1396 -CH_2OHCHO, -CH_2OCHO], 1277 [1396 -CHO], 1234 [1396 -S-8], 1217 [1234-OH], 1205 [1234 -CHO], 1072 [1234 -S-7], 1041 [1072 -CH_2OH], 1013 [1072 -CH_2OCHO], 910 [1072 -S-6], 852 [910 -$NHCOCH_3$], 861 [910 -CH_2OH, H_2O], 835 [910 -CH_3, -CH_2OHCHO], 707 [910 -S-5], 504 [707 -S-4], 428 [504 -$NHCOCH_3$, H_2O], 473 [504 -CH_2OH], 486 [504 -H_2O], 342 [504 -S-3], 310 [342 -CH_2OH, -H^+], 283 [342 -CH_2OCHO], 180 [342 -S-2] (Fig. 3.34).

Based on the above results obtained from chemical degradation / acid hydrolysis, chemical transformation, electrospray mass spectrometry, ^{1}H, ^{13}C NMR and 2D NMR- COSY, TOCSY and HSQC spectra the structure and sequence of isolated novel oligosaccharide molecule Vediose was deduced as (Fig. 3.30).

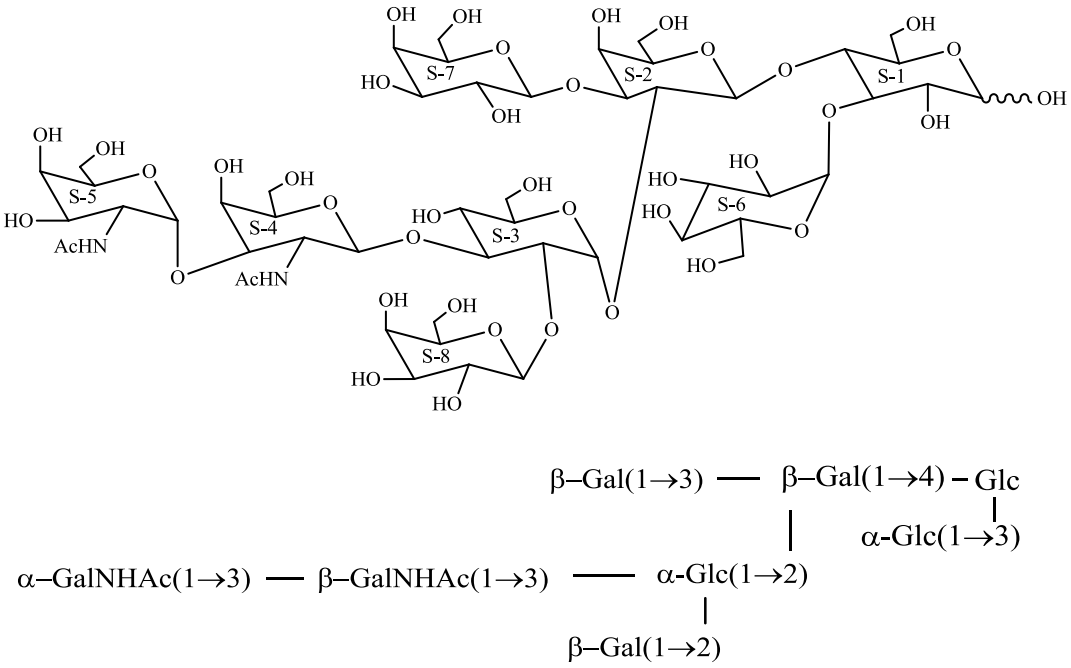


Fig. 3.30 Structure of Vediose

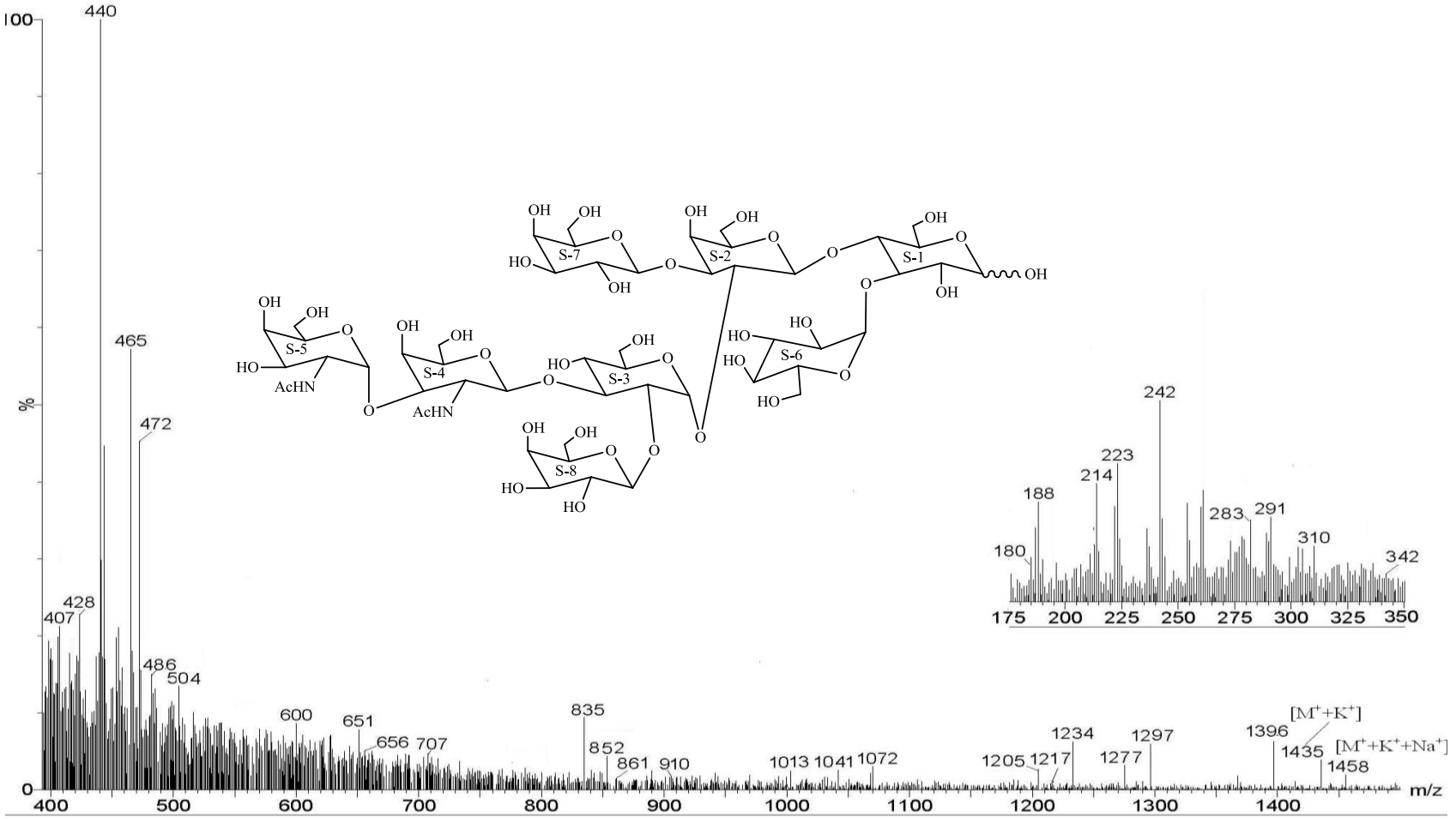
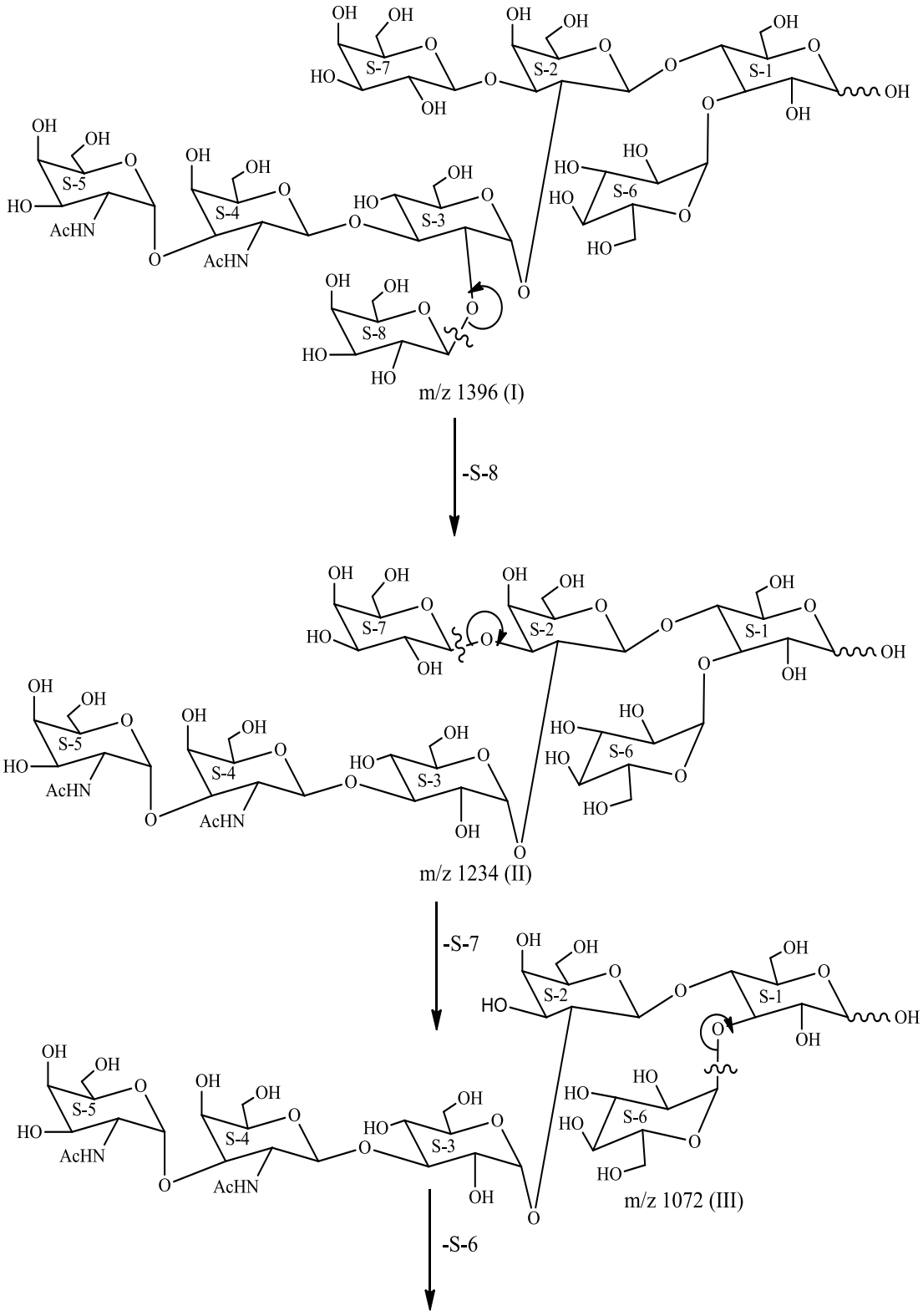


Fig. 3.31 ES Mass spectrum of compound Vediose



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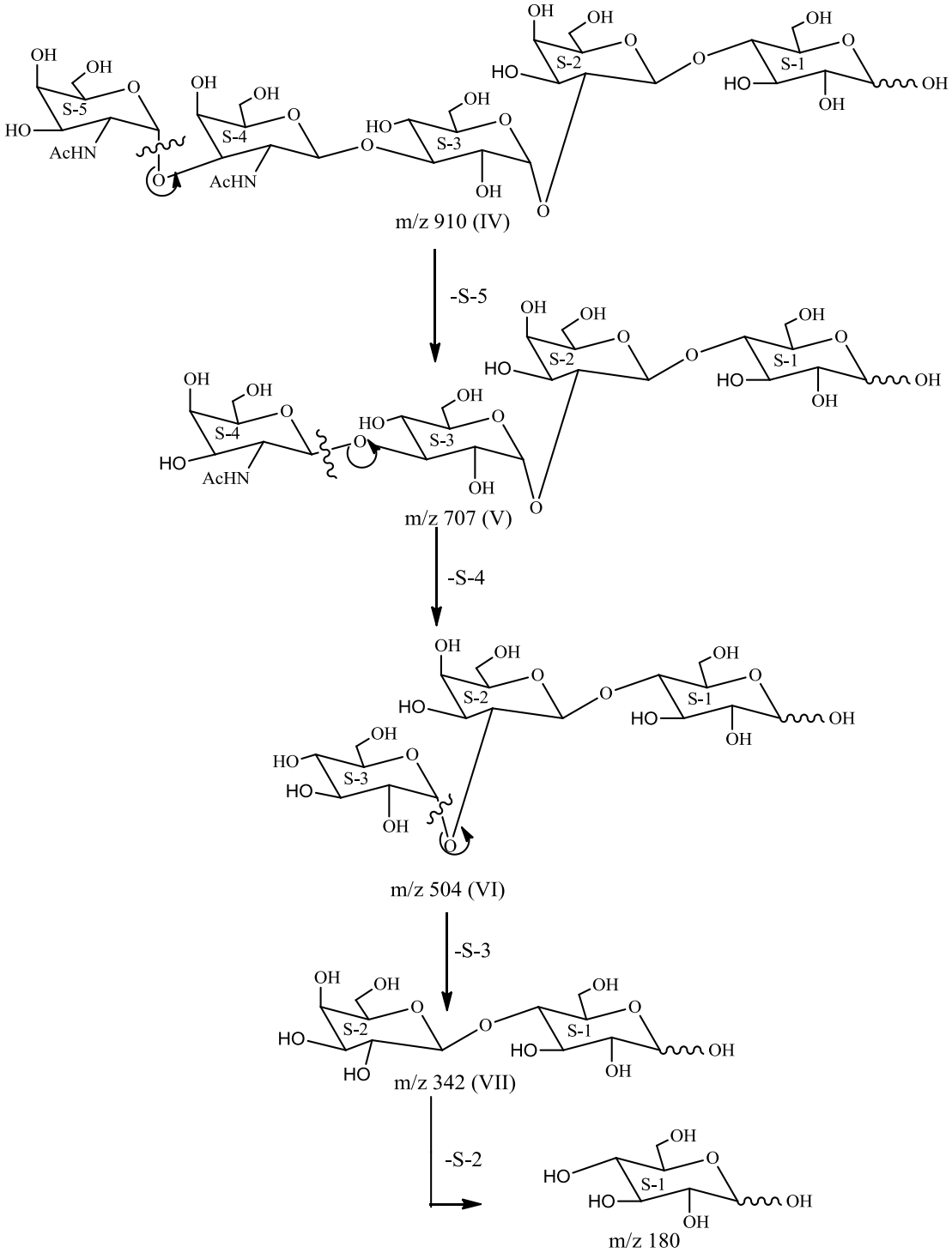


Fig. 3.32 Mass fragmentations of compound Vediose

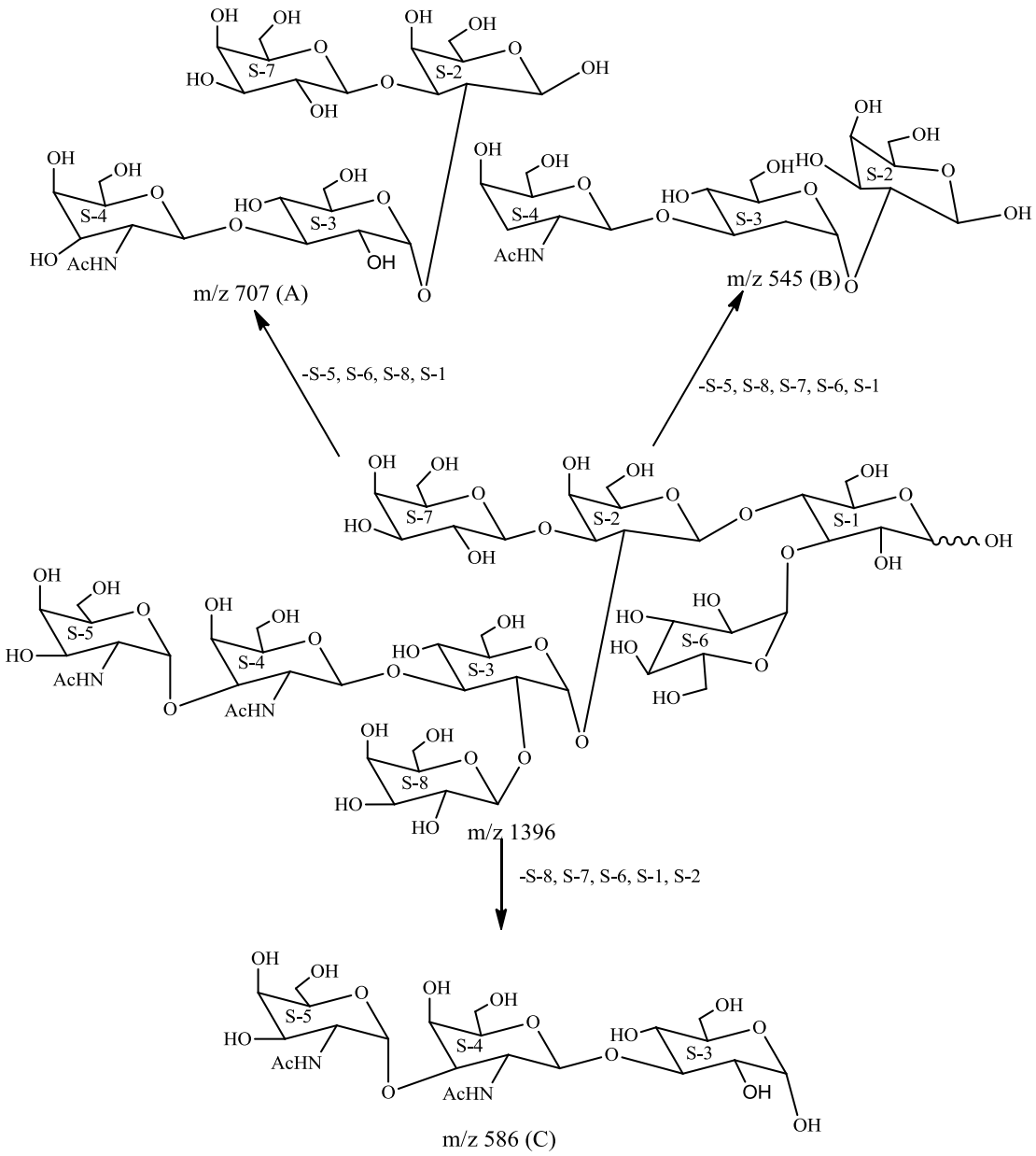


Fig. 3.33 ES-MS fragmentations of compound Vediose

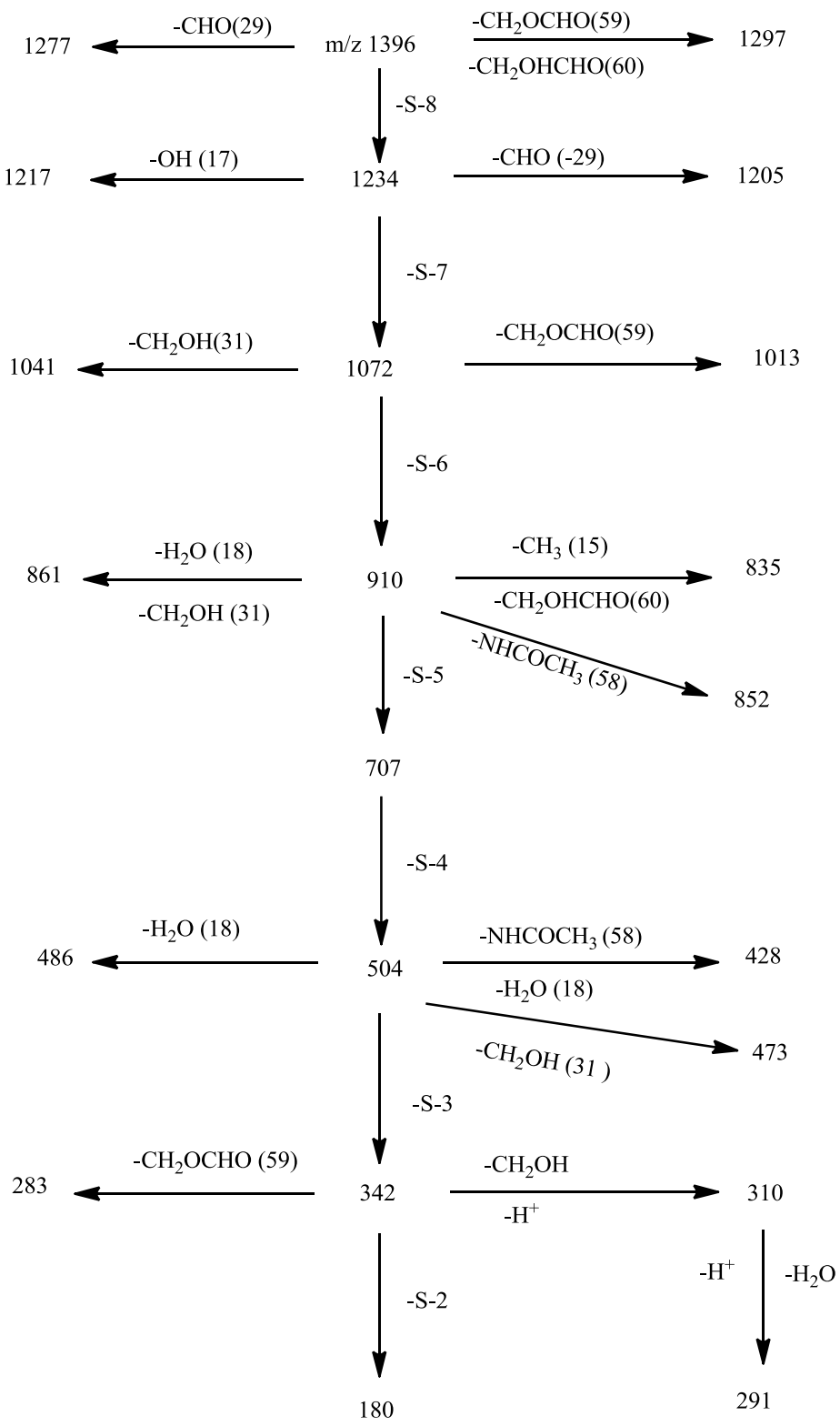


Fig. 3.34 Line diagram of ES-MS fragmentations of compound Vediose

3.1.4 Structural elucidation of compound – D (Bebiose)

Compound D, $C_{40}H_{68}O_{31}N_2$, gave positive Phenol-sulphuric acid test (Dubois et.al., 1956), Fiegl test (Fiegl, F., 1975) and Morgon-Elson test (Gey, et. al., 1996) showing the presence of normal and amino sugar moieties in the compound Bebiose. The HSQC spectrum of acetylated Bebiose showed the presence of seven cross peaks of anomeric protons and carbons in the respective region at δ 6.17 x 91.00, δ 5.69 x 91.08, δ 5.40 x 90.00, δ 4.72 x 96.00, δ 4.59 x 102.00, δ 4.52 x 102.00, δ 4.44 x 102.00 suggesting the presence of seven anomeric protons and carbons in it (Fig. 3.35). The presence of seven anomeric protons were further confirmed by the presence of seven anomeric doublets at δ 6.17 (1H), δ 5.69 (1H), δ 5.40 (1H), δ 4.72 (1H), δ 4.59 (1H), δ 4.52 (1H), δ 4.44(1H) in the ^{1}H NMR spectrum of acetylated Bebiose in $CDCl_3$ at 400 MHz (Fig. 3.36). The presence of seven anomeric carbons were also confirmed by seven anomeric carbon signals at δ 102.00 (3C), δ 96.00 (1C), δ 90.00 (1C), δ 91.08(1C) and δ 91.00 (1C) in the ^{13}C NMR spectrum of acetylated Bebiose in $CDCl_3$ at 400 MHz. Since all these spectrums contained downfield shifted α and β anomeric proton and carbon suggested that compound Bebiose may be a hexasaccharide in its reducing form. The reducing nature of compound Bebiose was further confirmed by methylglycosylation of compound Bebiose by $MeOH/H^+$ followed by its acid hydrolysis which led to the isolation of α and β-methyl glucosides, suggesting the presence of glucose at the reducing end. For convenience, six monosaccharide units present in Bebiose has been designated as S-1, S-2, S-3, S-4, S-5 and S-6, respectively, starting from glucose (S-1) the reducing end.

The monosachharide constituents in compound Bebiose was confirmed by its Killiani hydrolysis (Killiani, 1930) under strong acidic conditions, followed by PC and TLC. In its hydrolysis four spots were found on PC and TLC which were found identical with glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) by co-chromatography with authentic samples. Thus, confirming that the hexasaccharide 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 Bebiose 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 Bebiose. The presence of two anomeric proton signals at δ 5.69 (J = 4.0 Hz) and δ 5.20 in the ^{1}H NMR spectrum of Bebiose in D_2O at 300 MHz were assigned for α and β anomers of glucose (S-1) confirming the presence of Glc (S-1) at the reducing end in compound Bebiose. In the ^{1}H NMR presence of another anomeric proton doublet at δ 4.52 (J = 7.8 Hz) along with a triplet δ 3.25 (Structure Reporter Groups) (Dua, and Bush, 1983) of Bebiose in D_2O showed the presence of β-Gal(S-2) residue as the next monosaccharide, for presence of lactose type of structure at reducing end confirming the 1→4 glycosidic linkage between β-Gal (S-2) and β-Glc (S-1), hence confirming the presence of lactosyl moiety (Dua, and Bush, 1983) at the reducing end, since the HSQC spectrum of Bebiose acetate showed seven anomeric proton and carbon signal. The anomeric signal for α and β anomers were detected at δ 6.17 and δ 5.69, respectively, which were assigned for reducing glucose. In the TOCSY spectrum of Bebiose acetate the anomeric signal of β-Glc (S-1) at δ 5.69 gave cross peaks at 5.69 x 3.81, 5.69 x 5.07, 5.69 x 5.28 out of which one cross peak at δ 5.69 x 3.81 suggested that only one position in S-1 was available for glycosidic linkage (Fig. 3.37), which was assigned as H-4 of β-Glc (S-1) by the COSY spectrum of Bebiose acetate. This shows that reducing Glc (S-1) was 1→4 linked with as next monosaccharide unit i.e. galactose (Fig. 3.38). Another anomeric proton signal present at δ 4.52 (J = 8.4 Hz) was assigned for β-Gal (S-2). The large coupling constant of anomeric signal β-Gal (S-2) with J value of 8.4 Hz confirmed the β-configuration of glycosidic linkage of β-Gal (S-2). Further the anomeric proton signal of β-Gal (S-2) at δ 4.52 (J = 8.4 Hz) in the TOCSY spectrum of Bebiose acetate showed cross peak at δ 4.52 x 3.80, 4.52 x 4.13, 4.52 x 4.37, out of which two cross peaks at δ 4.52 x 3.80 and δ 4.52 x 4.13 that the two position in S-2 were available for glycosidic linkages, i.e. the two hydroxyl groups of β-Gal (S-2) were involved in glycosidic linkages were by other monosaccharide moieties, the position of these glycosidic linkages were confirmed by the COSY spectrum of Bebiose acetate confirmed that H-2 and H-3 of β-Gal (S-2) were available for glycosidic linkages by the next monosaccharide units. The next anomeric proton signal which appeared as a doublet at δ 4.59 (J = 8.0 Hz) in the ^{1}H NMR spectrum of Bebiose acetate in $CDCl_3$ at 400 MHz was assigned the presence of β Gal (S-3) moiety. The

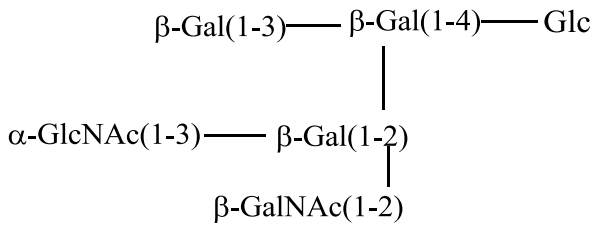
anomeric proton signal at δ 4.59 in the ^{1}H NMR spectrum of Bebiose acetate showed its complementary ^{13}C anomeric signal at δ102.00 in the HSQC spectrum of Bebiose acetate later this ^{13}C anomeric signal at 102.00 showed its cross peak at 102.00 x 3.80 in the HMBC spectrum of Bebiose acetate showing the glycosidic linkages, between S-3 and S-2 (Fig. 3.39). The signal of δ 3.80 was assigned for H-3 of S-2 by COSY spectrum confirming the 1→3 glycosidic linkage between S-3→S-2. The large coupling constant of anomeric signal at δ 4.59 (J = 8.0 Hz) of (S-3) confirmed β-configuration of the β-Gal (S-3) moiety. Therefore, the glycosidic linkage between S-3 and S-2 was confirmed as β 1→3. Since the anomeric proton of β-Gal (S-3) does not have any cross peak in to linkage region of TOCSY spectrum of Bebiose acetate hence confirming that none of methine proton of β-Gal (S-3) gave its signal into the linkage region confirming that β-Gal (S-3) was linked at the non reducing end. Another anomeric proton signal which appeared as a doublet at δ 4.72 (J = 8.0 Hz), in the ^{1}H NMR Bebiose acetate in $CDCl_3$ at 400 MHz, was assigned for the presence of β-Gal (S-4) moiety. Since it was ascertained by COSY and TOCSY spectrum of Bebiose acetate that the positions 2 and 3 of β-Gal (S-2) were available for glycosidic linkages and position 3 of β-Gal (S-2) was already linked with β-Gal (S-3), hence the leftover H-2 position of S-2 must be linked by β-Gal (S-4). The position of linkage between β-Gal (S-4) and β-Gal (S-2) was further confirmed by the appearance of H-2 signal of β-Gal δ 4.13 (S-2) in the ^{1}H NMR spectrum of Bebiose acetate which was also confirmed by COSY and TOCSY spectrum of Bebiose acetate at 400 MHz in $CDCl_3$. The large coupling constant of β-Gal (S-4) at δ 4.72 (J = 8.0 Hz) showed β-glycosidic linkage between β-Gal (S-4) and β-Gal (S-2). Further, anomeric proton signal which appeared as a doublet at δ 4.72 (J = 8.0 Hz) in the ^{1}H NMR of Bebiose acetate cross peaks at δ 4.72 x 3.64, 4.72 x 3.80, in the linkage region showed that the two position in S-4 were available for glycosidic linkages, showing that the two hydroxyl groups of β-Gal (S-4) were involved in glycosidic linkages by other monosaccharide moieties by the COSY spectrum of Bebiose acetate suggested that H-2 at δ 3.80 and H-3 at δ 3.64 of β-Gal (S-4) were available for glycosidic linkages by the next monosaccharide units. The next anomeric proton signal which appeared as doublet at δ 5.40 (J = 2.8 Hz) along with singlet at δ 1.97 for $NHCOCH_3$ in the ^{1}H

NMR spectrum of Bebiose in $CDCl_3$ at 400 MHz was suggested for the presence α-GlcNAc (S-5) moiety. The signal of δ 4.72 x 3.64 was assigned for H-3 of S-4 by COSY spectrum suggesting that the H-3 of S-4 was available for glycosidic linkage, which may be linked to S-5. The ^1H NMR signal of H-3 at δ 3.64 along with anomeric proton signal of S-5 at δ 5.40 with the small coupling constant of anomeric signal of S-5 (J = 2.8 Hz) confirmed 1→3 glycosidic linkage between S-5 and S-4 with α-configuration of the (S-5) moiety. Therefore the glycosidic linkage between S-5 and S-4 was confirmed as α-1→3. Since, none of methine proton of α-GlcNAc (S-5) came in the linkage region confirmed that α- GlcNAc (S-5) was linked at the non reducing end. Another anomeric proton signal which appeared as a doublet at δ 4.44 (J = 6.3 Hz) along with a signal of NAc at 1.96 in $CDCl_3$ at 400 MHz, was assigned for the presence of β-GalNAc (S-6) moiety. Since it was ascertained by COSY and TOCSY spectrum of Bebiose acetate that the positions 2 and 3 of β-Gal (S-4) were available for glycosidic linkages and position 3 of β-Gal(S-4) was already linked with α-GlcNAc (S-5), hence the leftover H-2 position of S-4 must be linked by β-GalNAc (S-6), which was confirmed by the chemical shift of H-2 of S-4 at δ 3.80 was assigned at H-2 of S-4 by COSY spectrum confirming the 1→2 glycosidic linkage between S-6 and S-4. The position of linkage between β-GalNAc (S-6) and β-Gal (S-4) was further confirmed by COSY and TOCSY spectrum of Bebiose acetate at 400 MHz in $CDCl_3$. The large coupling constant of β-GalNAc (S-6) J = 6.3 Hz confirmed β-glycosidic linkage between β-GalNAc (S-6) and β-Gal (S-4). Since the anomeric signal of β-GalNAc (S-6) present in the TOCSY spectrum of Bebiose acetate at δ 4.44 does not contain any methine protons in glycosidic linkage region i.e., δ 3-4 ppm, confirmed that none of -OH group of β- GalNAc (S-6) was involved in glycosidic linkages hence, confirming that β- GalNAc (S-6) were present at non-reducing end and none of their -OH group participated in glycosidic linkages.

All the ^1H NMR assignments for ring proton of monosaccharide units of Bebiose were confirmed by COSY and TOCSY spectra. The positions of glycosidation in the oligosaccharide were confirmed by position of anomeric signals, and comparing the signals in ^1H and ^{13}C NMR of acetylated Bebiose. The glycosidic linkages in Bebiose were also confirmed by the cross peaks for glycosidically linked

carbons with their protons in the HSQC spectrum of acetylated Bebiose. The values of these cross peaks appeared as Glc (S-1) H-4 x C-4 at δ 3.81 x 75 showed (1→4) linkage between S-2 and S-1, β-Gal (S-2) H-3 x C-3 at δ 3.80 x 75 showed (1→3) linkage between S-3 and S-2, β-Gal (S-2) H-2 x C-2 at δ 4.13 x 70 showed (1→2) linkage between S-4 and S-2, β -Gal (S-4) H-3 x C-3 at δ 3.64 x 71 showed (1→3) linkage between S-5 and S-4, β -Gal (S-4) H-2 x C-2 at δ 3.80 x 75 showed (1→2) linkage between S-6 and S-4. All signals obtained in ^{1}H and ^{13}C NMR of compound Bebiose were in conformed by 2D COSY, TOCSY and HSQC spectra.

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 'D' Bebiose is a hexasaccharides having the following structure as-



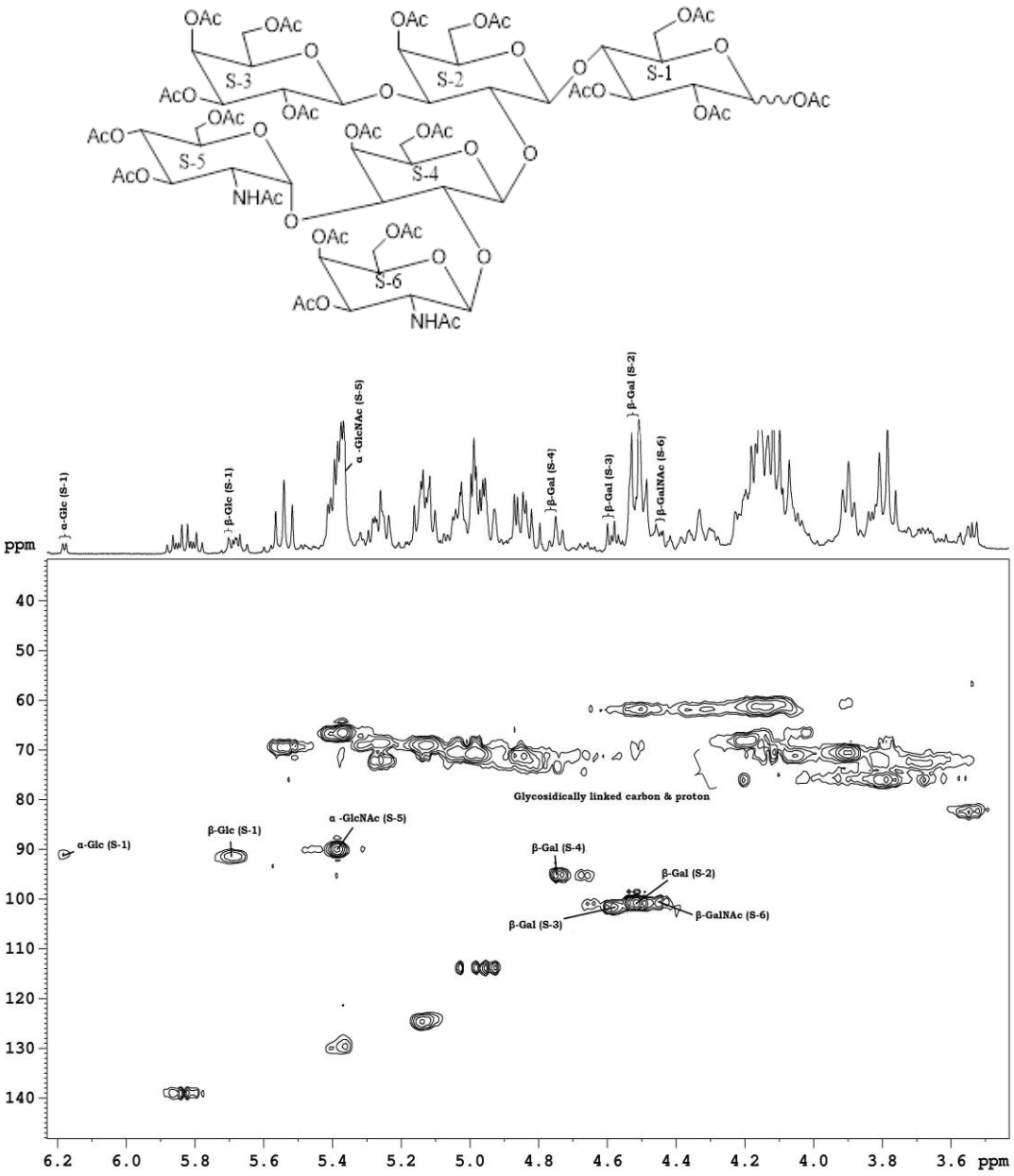


Fig. 3.35 ^{1}H-^{13}C HSQC spectrum of Bebiose acetate in $CDCl_3$ at 400 MHz

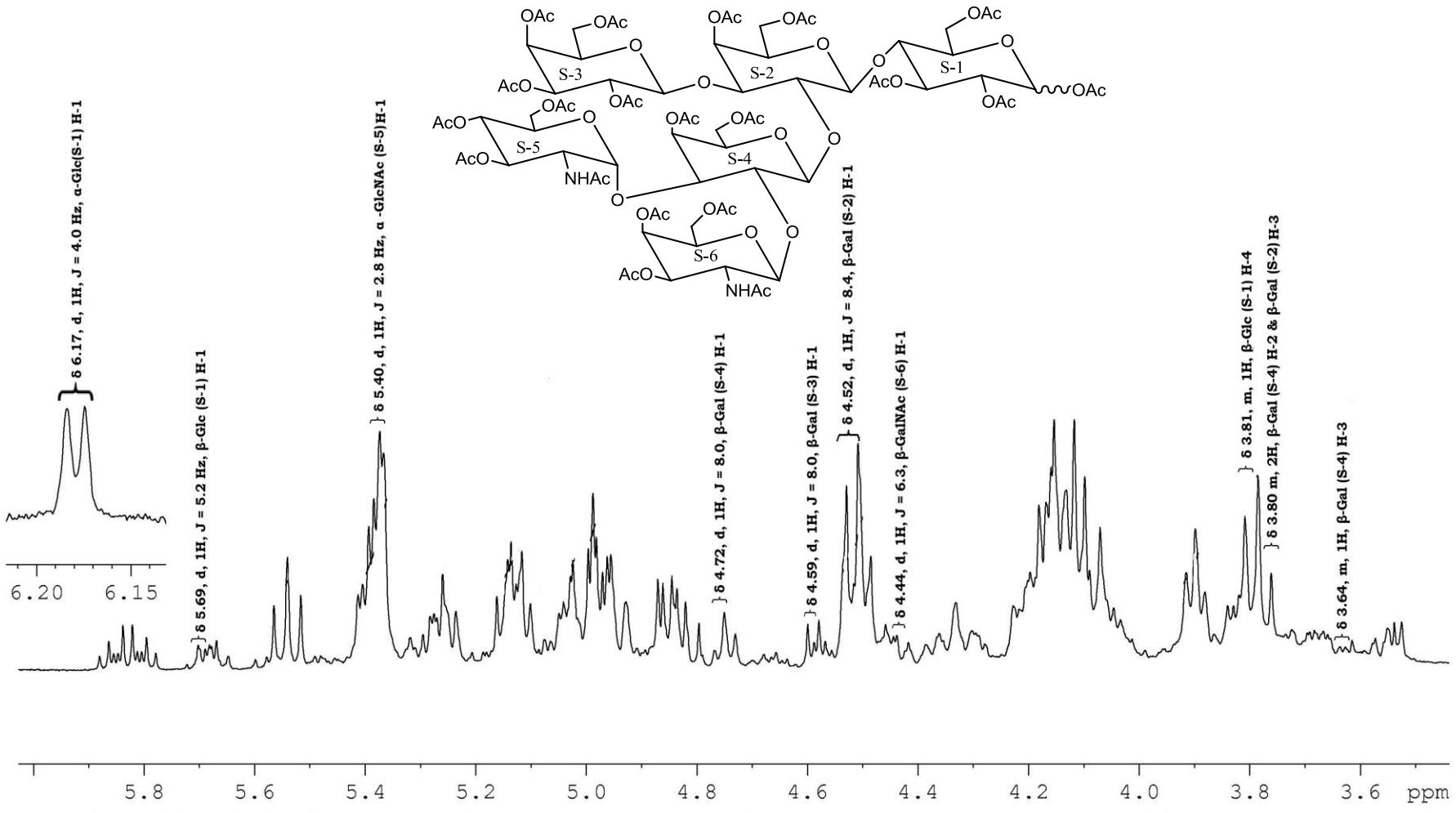


Fig. 3.36 ^{1}H NMR spectrum of Bebiose acetate in $CDCl_3$ at 400 MHz

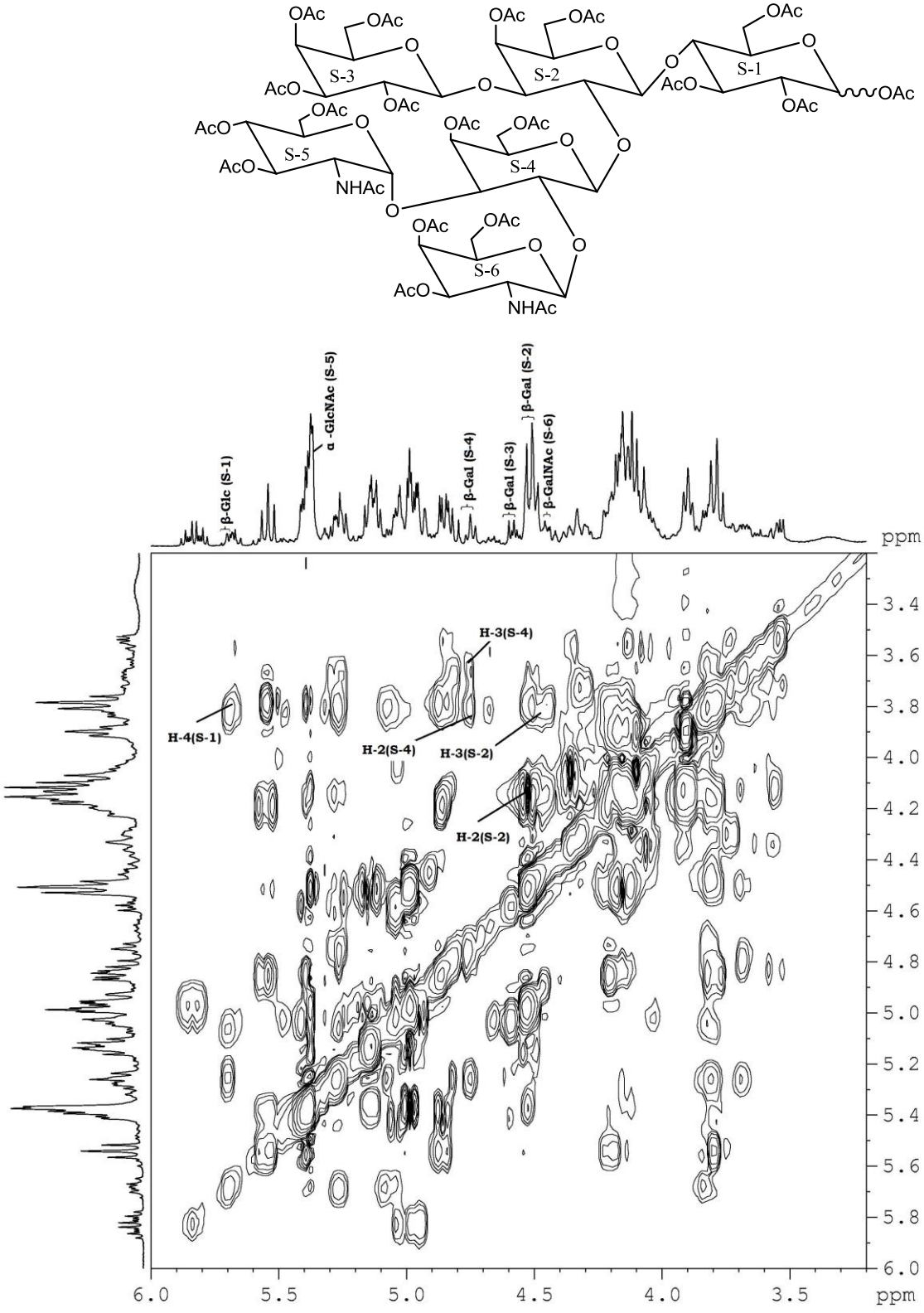


Fig. 3.37 TOCSY spectrum of Bebiose acetate in $CDCl_3$ at 400 MHz

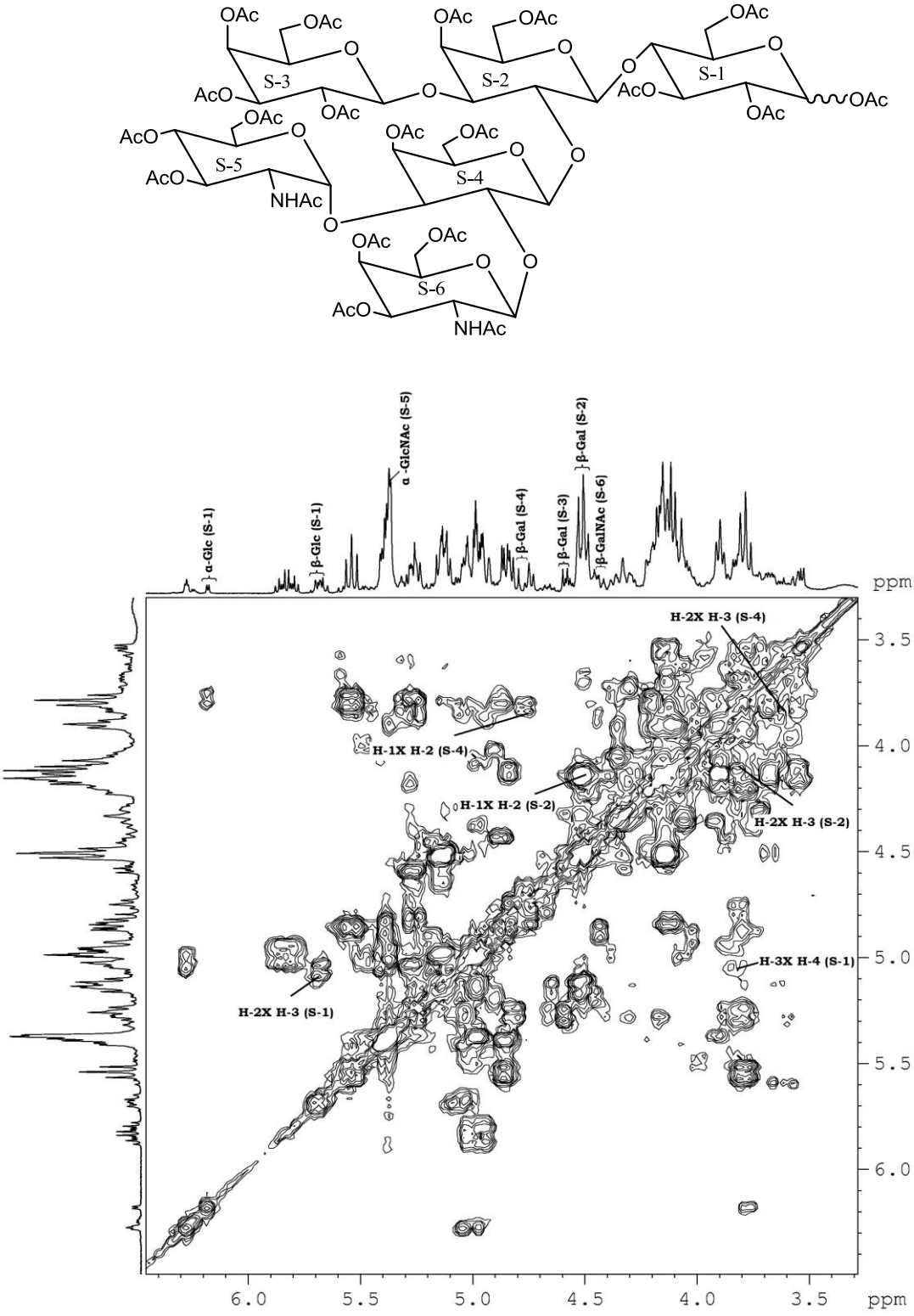


Fig. 3.38 COSY spectrum of Bebiose acetate in $CDCl_3$ at 400 MHz

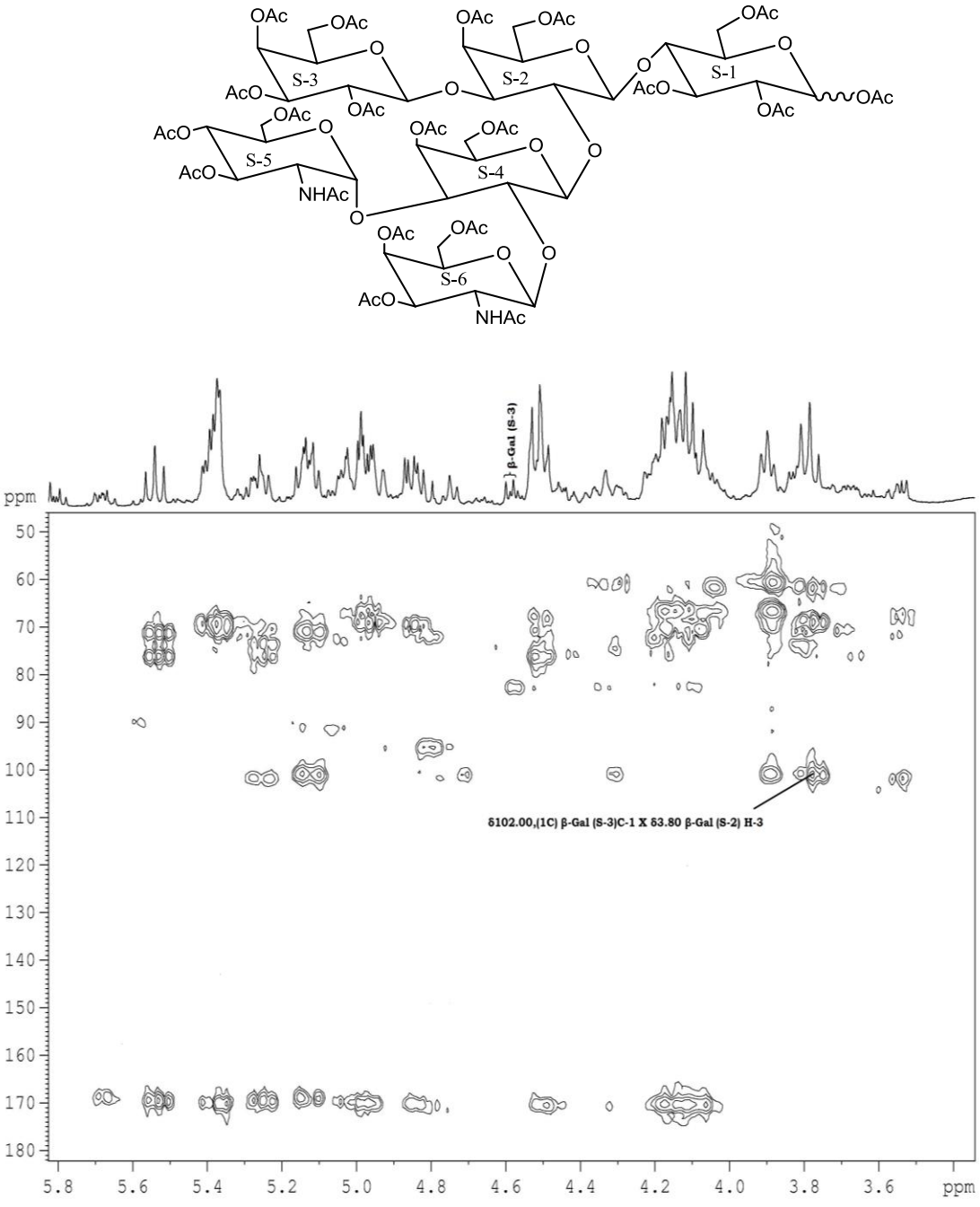


Fig. 3.39 ^{1}H-^{13}C HMBC spectrum of Bebiose acetate in $CDCl_3$ at 400 MHz

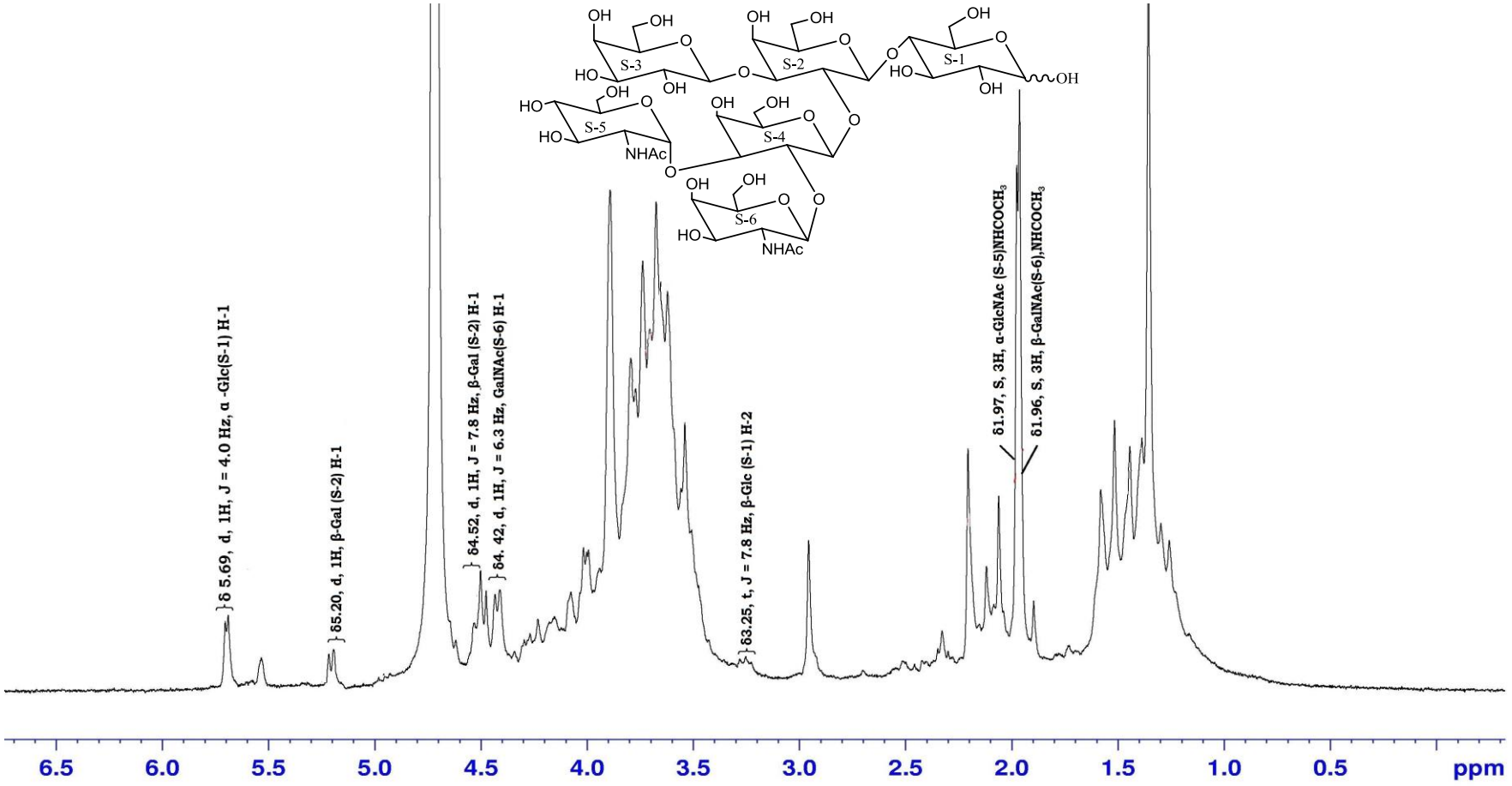


Fig. 3.40 ^{1}H NMR spectrum of Bebiose in D_2O at 300 MHz

The electronspray Mass spectrometry data of compound not only confirmed the derived structure but also supported the sequence of monosaccharide in Bebiose (Fig. 3.42). The highest mass ion peak was recorded at m/z 1095 and 1134 which was due [M+Na] and [M+Na+K] the other mass ion peak recorded at m/z 1072 was due to $[M]^+$ confirming the molecular weight of Bebiose as 1072 and was in agreement with its molecular formula $C_{40}H_{68}O_{31}N_2$. Further the mass fragments were formed by repeated H transfer in the oligosaccharide and was accompanied by the elimination of terminal sugar less -OH. The hexasaccharide m/z 1072 (I) fragmented to give mass ion at m/z 869 (II) [1072-S-6], this fragment was arised due to the loss of terminal GalNAc (S-6) moiety from hexasaccharide indicating the presence of GalNAc (S-6) at the non reducing end. It further fragmented to give mass ion peak at m/z 666 (III) [869-S-5], which was due to loss of another GalNAc (S-5) moiety from pentasaccharide. This further fragmented to give mass ion peak at m/z 504 (IV) [666-S-4], which was due to loss of Gal (S-4) moiety from tetrasaccharide. This further fragmented to give mass ion peak at m/z 342 (V) [504-S-3], which was due to loss of Gal (S-3) moiety from trisaccharide. This further fragmented to give mass ion peak at m/z 180 (VI) [342-S-1], which was due to loss of Gal (S-1) moiety from disaccharide (Fig. 3.43). These five mass ion peak II, III, IV, V and VI, were appeared due to the consequent loss of S-6, 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 504, 342 corresponding to the mass ion fragment A and B which confirm the position of S-1, S-2, S-3, S-4, S-5 and S-6 (Fig. 3.44).

The other fragmentation pathway in ES Mass spectrum of compound Bebiose m/z 1072 shows the mass ion peak at 1054 [1072 -H_2O,], 1013 [1072 -CH_2OCHO], 869 [1072 -S-6], 811 [869 -NHCOCH], 833 [869 -H_2O, H^+], 666 [869 -S-5], 648 [666 -H_2O], 606 [666 -CH_2OH, -CHO], 504 [666 -S-4], 473 [504 -CH_2OH], 467 [504 -H_2O, -OH, $2H^+$], 342 [504 -S-3], 324 [342 -H_2O], 180 [342 -S-2] (Fig. 3.45).

Based on the above results obtained from chemical degradation / acid hydrolysis, chemical transformation, electrospray mass spectrometry and ^{1}H, ^{13}C NMR and 2D NMR, COSY, TOCSY and HSQC spectrahe structure and sequence of isolated novel oligosaccharide molecule Bebiose was deduced as (Fig. 3.41) -

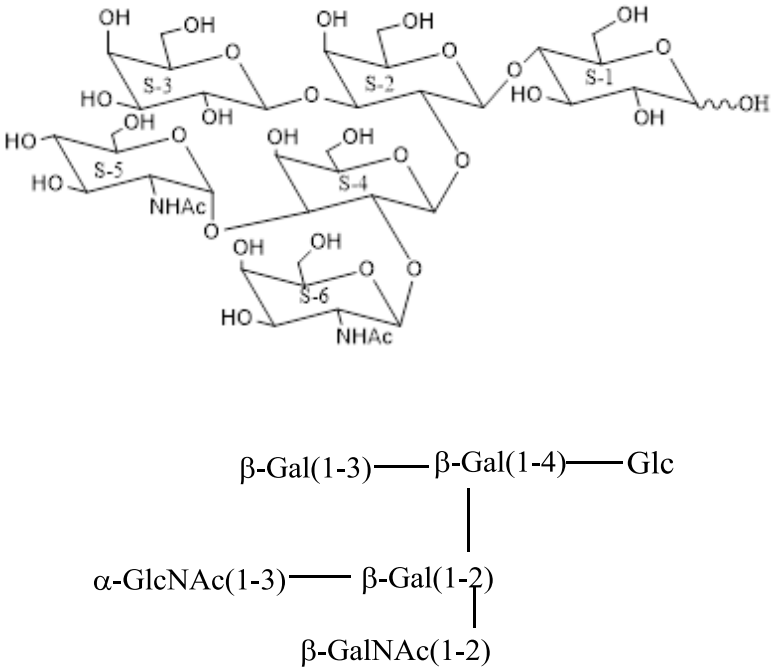


Fig. 3.41 Structure of Bebiose

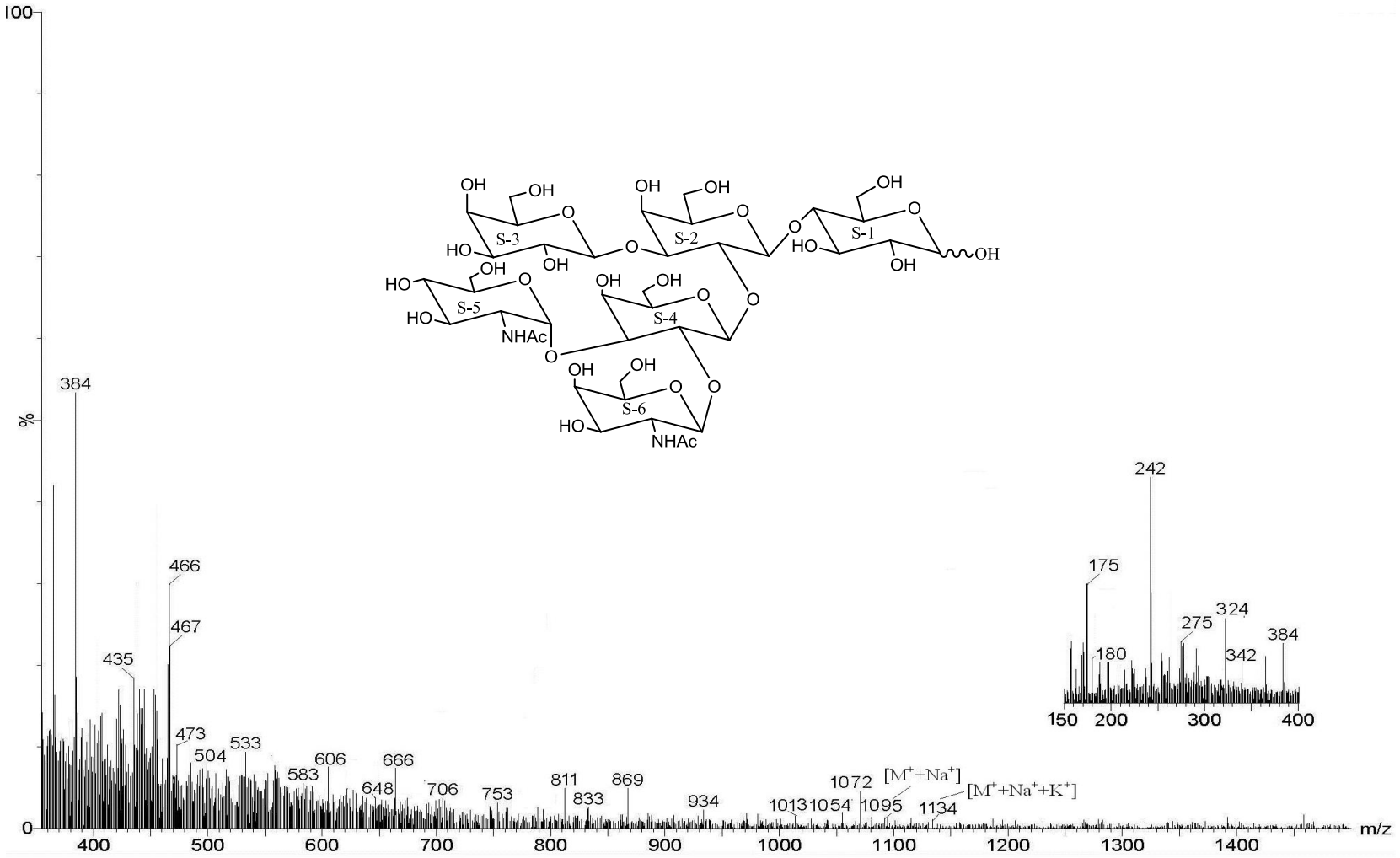
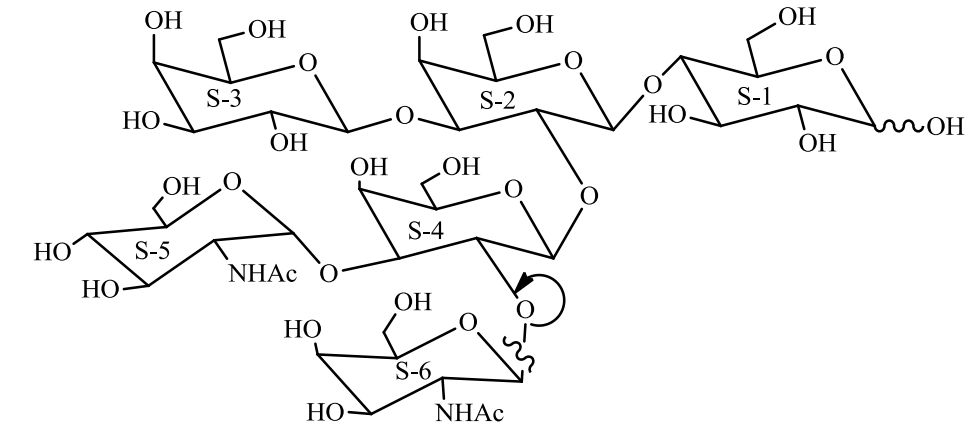
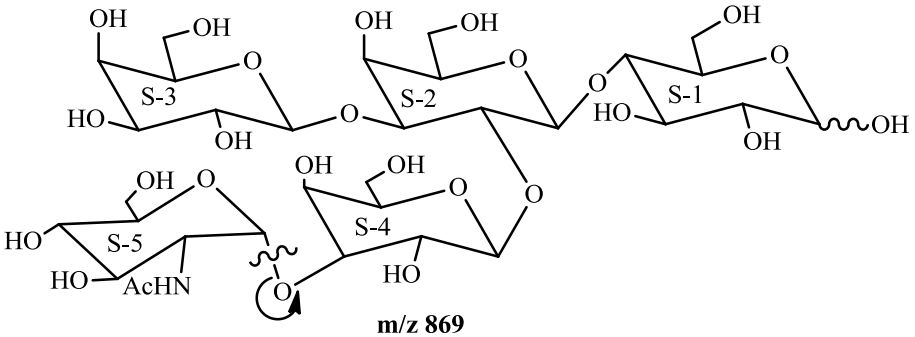


Fig. 3.42 ES Mass spectrum of compound Bebiose



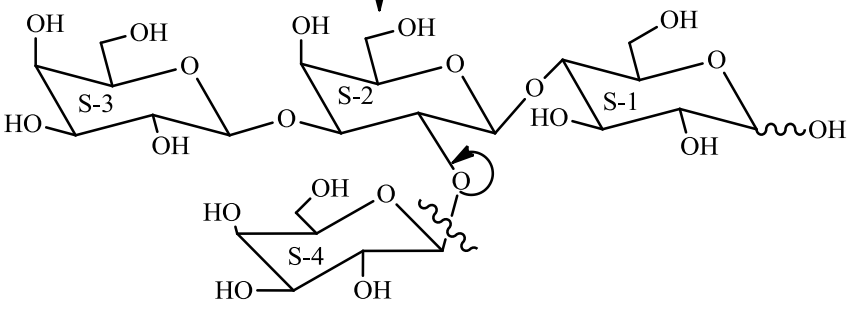
m/z 1072

-S6



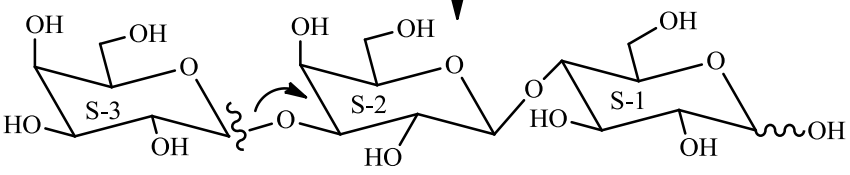
m/z 869

-S5



m/z 666

-S4



m/z 504(IV)

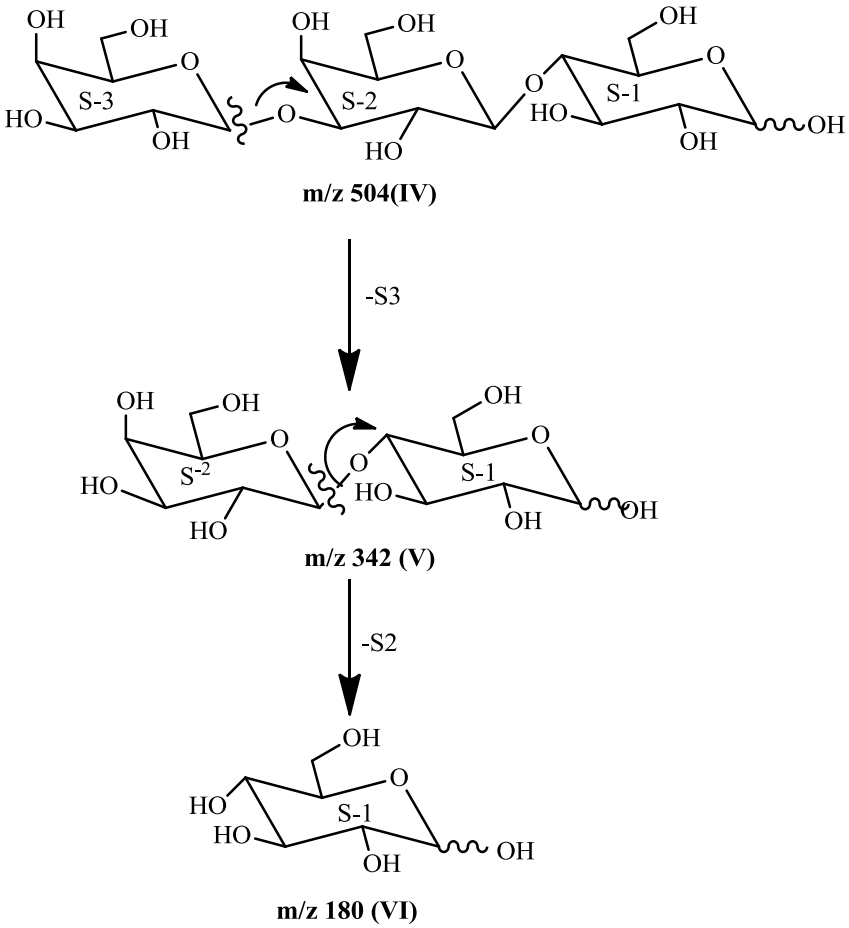


Fig. 3.43 Mass fragmentations of compound Bebiose

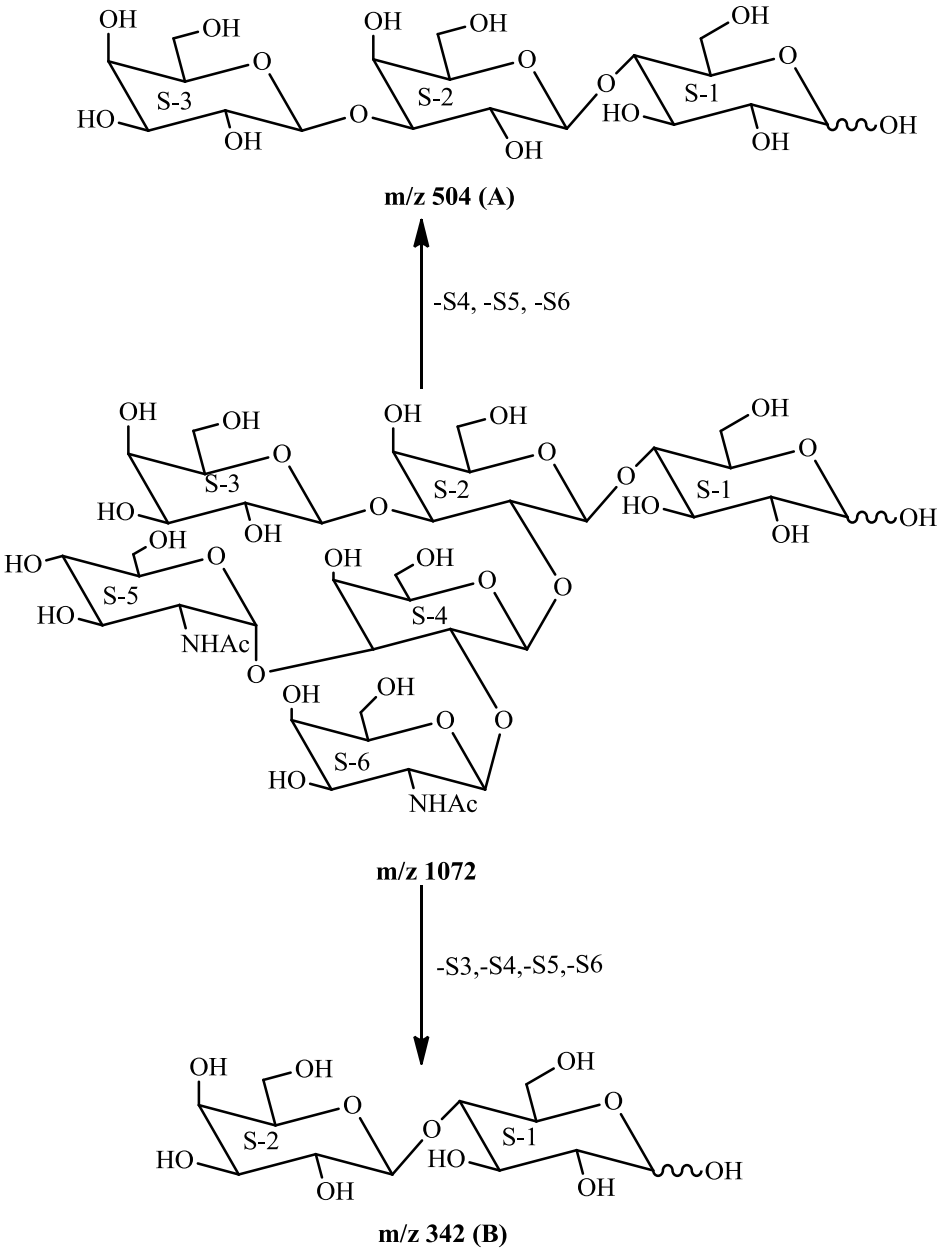


Fig. 3.44 ES-MS fragmentations of compound Bebiose

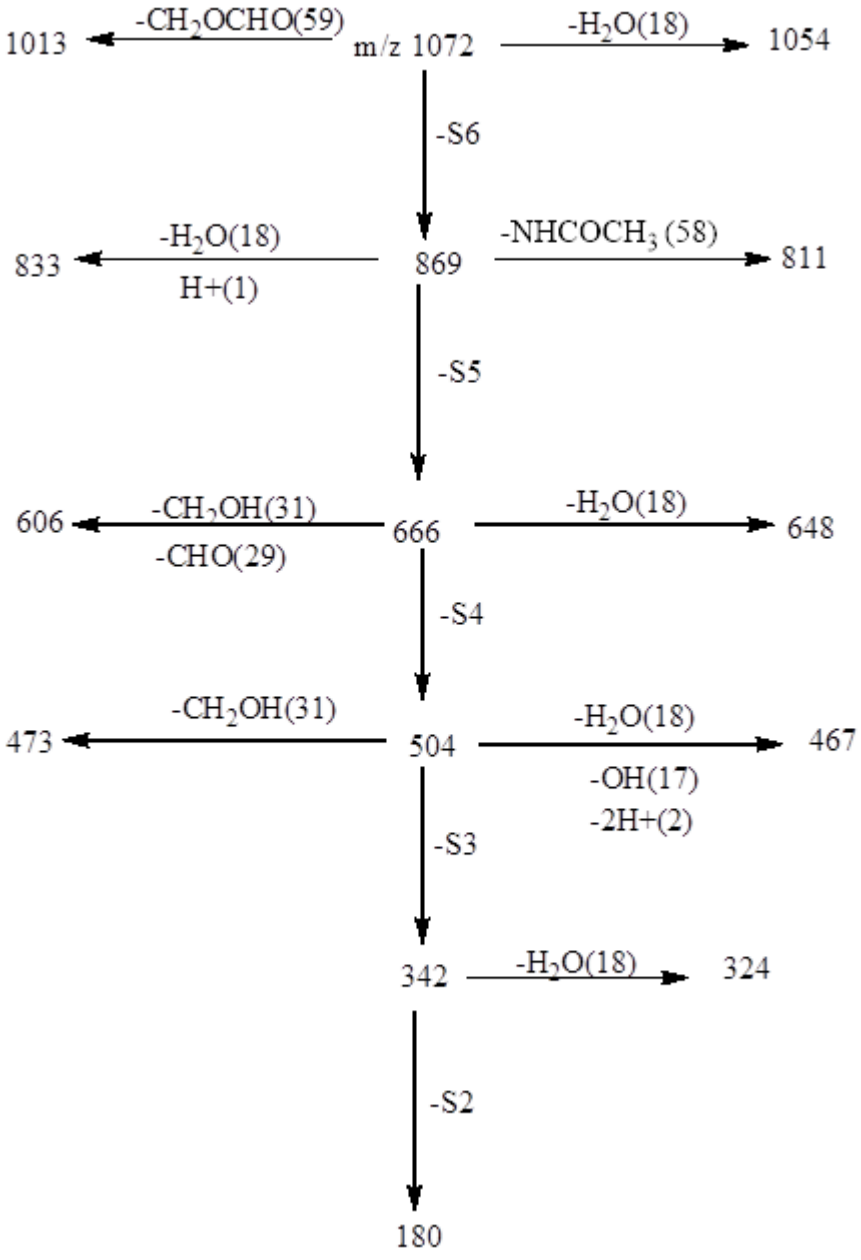


Fig. 3.45 Line diagram of ES-MS fragmentations of compound Bebiose

CHAPTER-4

EXPERIMENTAL

4.1 General procedure -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 25^0C on a Bruker AM 300 and 400 FT NMR spectrometer. The electrospray 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 print outs are averaged 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 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. Freeze drying of the compound was done with the help of CT 60e (HETO) lyophylizer and centrifuged by a cooling centrifuged (Remi instruments C-23 JJRCI 763). 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.

4.2 Chromatography-Following chromatographic techniques have been used for the isolation and identification of the compounds.

4.2.1 Paper chromatography (PC) - Paper chromatography was performed on Whatman paper No.1 by the use of a three solvent system of toluene, butanol and water and spots were detected by appropriate reagent for specific moieties.

4.2.2 Thin layer chromatography (TLC) - The glass plates coated with slurry of silica gel G (SRL) in water were used which dried at room temperature for about 24 h and activated at $100\text{-}110^0C$ and developed by soluble reagents.

4.3 Phenol-Sulphuric acid test for sugars - To each fraction (0.1ml) distilled water (1ml), 5% phenol (1ml) and conc. H_2SO_4 (25 ml) was added and the mixture was vigorously shaken. After 30 min the carbohydrate rich fractions showed yellow colour (Dubois, et. al., 1956).

4.4 Fiegl test for sugars - The test sample (0.1 mg) was placed in a micro-crucible and one drop of syrupy phosphoric acid was added. The crucible was covered with filter paper moistened with 10% solution of aniline in AcOH (10%). A small watch glass was used as a paper weight. The bottom of the crucible was cautiously heated for 30-60 min with micro burner, avoiding excess heating. A pink to red colour was imparted on filter paper for normal sugars and 6-deoxy sugars exhibited brown coloration. The 2-deoxy sugars did not give any colour under this test (Fiegl, 1975).

4.5 p-Dimethyl amino benzaldehyde test for proteins - The presence of proteins in a sample was tested by p-dimethyl amino benzaldehyde test. The sample (solid or in solution) was mixed in a micro-crucible with several drops of saturated glacial acetic acid solution of p-dimethylaminobenzaldehyde and one drop of fuming hydrochloric acid. A violet colour indicated the presence of proteins (Chaturvedi and sharma, 1988).

4.6 Partridge reagent for sugars - Freshly distilled aniline (0.93 g) in water saturated with butanol (100 ml) was mixed with phthalic acid (1.66 g) and was shaken till dissolve. The reagent was sprayed on the paper on which a spot of test sample was applied and then heated at 100-118°C for 3-5 min. A pink-brown colour indicated the presence of normal hexoses (Partridge, 1949).

4.7 Morgon-Elson test for sugars

Acetyl-acetone reagent I: Solution A- 0.5 ml of acetyl-acetone was dissolved in butanol (50 ml). Solution B- 50% (w/v) aq. KOH (5 ml) was dissolved in ethanol (20 ml). For formation of reagent I, 10 ml of Solution A was added in 0.5 ml of solution B (Gey, et.al., 1996).

p- Dimethyl amino benzaldehyde reagent II: 1 g of p-dimethyl amino benzaldehyde was dissolved in the mixture of 30 ml of ethanol and 30 ml of conc. HCl and solution was then diluted with 180 ml of distilled butanol to get reagent II.

Chromatograms were sprayed with reagent I and heated in the oven for 5 min at 105°C. The dry paper strips were then sprayed with reagent II and returned to the oven for 5 min at 90°C. The appearance of purple-violet colour indicated the presence of amino sugars.

4.8 Thiobarbituric acid assay (Warren assay) for sialic acid

Reagent A: 1.07 g of sodium metaperiodate was dissolved in 1.0 ml of water. It was added in 14.5 ml of concentrated orthophosphoric acid and water was added to make it 25 ml.

Reagent B: 10 g of sodium arsenite and 0.71 g of sodium sulphate was dissolved in 0.1M sulphuric acid (made by diluting 0.57 ml concentrated sulphuric acid with 100 ml of water) to a total volume of 10 ml.

Reagent C: 0.12 g of thiobarbituric acid and 142 g of sodium sulphate was dissolved in water and the volume was made up to 20ml.

In the sample (0.05µ mole in a volume of 0.2 ml) 0.1 ml of reagent A was added. The tube was shaken and allowed to stand at room temperature for 20 minutes. 1 ml of reagent B was then added and the tube was shaken until a yellow-brown colour disappears. 3 ml of reagent C was added in the tube with shaking; it was capped with a glass bead and then heated in a vigorously boiling water bath for 15 minutes. The tube was then removed and placed in cold water for 5 minutes. During cooling the colour faded and the solution becomes cloudy. From this solution, 1 ml was transferred to another tube which contained 1 ml of cyclohexanone. The tube was shaken and then centrifuged for 3 minutes. The clear upper cyclohexanone phase was red and the colour was more intense than it was when in water (Warren, 1959).

4.9 Bromocresol green test - Spraying reagent bromocresol green (0.04 g) was dissolved in ethanol (96%, 100 ml) drops of 0.1 N NaOH were added until a blue coloration just appeared. A spot of the test sample when applied on a paper moistened with this reagent (blue) give a yellow coloration which indicated the presence of a carboxylic group (Bryant and Overell,1953).

4.10 Acetylation of oligosaccharide mixture - 8.00 gm of pooled fractions which gave positive phenol-sulphuric acid test were acetylated with pyridine (8.00 ml) and acetic anhydride (8.00 ml) at 60°C and the solution was stirred overnight. The mixture was evaporated under reduced pressure and the viscous residue was taken in $CHCl_3$ (250 ml)

and it was washed in sequence with 2N-HCl (1 x 25 ml), ice cold 2N-$NaHCO_3$ (2 x 25 ml) and finally with H_2O (2 x 25 ml). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding the acetylated mixture (9.60 g).

4.11 Compound A (Meeniose)

4.11.1 Deacetylation of compound 'A'- Compound 'A' (44 mg) was obtained from eight column chromatography of acetylated oligosaccharide mixture. 30 mg of compound 'A' was dissolved in acetone (3 ml) and 3.5 ml of 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 (3 x 5 ml) $CHCl_3$ (to remove acetamide) and the water layer was finally freeze dried giving the deacetylated oligosaccharide A (29 mg).

4.11.2 Methyl glycosidation/Acid hydrolysis of compound A - Compound A (5 mg) was ref1uxed with absolute MeOH (2 ml) 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 methylglycoside of A, 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 $BaCO_3$ 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 on TLC and PC.

4.11.3 Kiliani hydrolysis of compound A-Compound A (5 mg) was dissolved in 2 ml Kiliani mixture (AcOH-H_2O-HCI, 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, GalNAc and GlcNAc on comparison with authentic samples of glucose, GalNAc and GlcNAc (Killiani, 1930).

4.12 Compound B (Murtiose)

4.12.1 Deacetylation of compound -B - Compound B (78 mg) was obtained from seventh column chromatography of acetylated oligosaccharide mixture. 50 mg of compound B was dissolved in acetone (2 ml) and 3 ml of NH_3 was added in it and was left overnight in a stoppered hydrolysis flask. After 24 h ammonia was removed under reduced pressure and

the compound was washed thrice with $CHCl_3$ (5 ml) (to remove acetamide) and the water layer was finally freeze dried giving the deacetylated oligosaccharide B (35 mg).

4.12.2 Methyl glycosidation/Acid hydrolysis of compound B - Compound B (8 mg) was ref1uxed with absolute MeOH (2 ml) 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 methylglycoside of B, 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 $BaCO_3$ and concentrated under reduced pressure to afford α–and β-methylglucosides along with the Glc, Gal, GalNAc and GlcNAc. Their identification was confirmed by comparison with authentic samples on TLC and PC.

4.12.3 Kiliani hydrolysis of compound B - Compound B (5 mg) was dissolved in 2 ml Kiliani mixture (AcOH-H_2O-HCI, 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 Glc, Gal, GalNAc and GlcNAc on comparison with authentic samples of Glc, Gal, GalNAc and GlcNAc.

4.13 Compound C (Vediose)

4.13.1 Deacetylation of compound C- Compound 'C' (34 mg) was obtained from fifth column chromatography of acetylated oligosaccharide mixture. 34 mg of compound C was dissolved in acetone (2 ml) and 3 ml of NH_3 was added in it and was left overnight in a stoppered hydrolysis flask. After 24 hrs ammonia was removed under reduced pressure and the compound was washed thrice with $CHCl_3$ (5 ml) (to remove acetamide) and water layer was finally freeze dried giving the deacetylated oligosaccharide C (31 mg).

4.13.2 Methyl glycosidation/Acid hydrolysis of compound C - Compound C (8 mg) was ref1uxed with absolute MeOH (2 ml) 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 methylglycoside of C, 1, 4-dioxane (1 ml), and 0.1N H_2S0_4 (1 ml) was added and the solution was warmed for 30 minute at 50°C. The hydrolysis was complete after 24 h. The hydrolysate was neutralized with freshly prepared $BaCO_3$ and concentrated under reduced pressure to afford α–and β-methylglucosides along with the Glc, Gal and

GlcNAc. Their identification was confirmed by comparison with authentic samples on TLC, and PC.

4.13.3 Kiliani hydrolysis of compound C - Compound C (5 mg) was dissolved in 2 ml Kiliani mixture (AcOH-H_2O-HCI, 7:11:2) and heated at 100^oC 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, Gal and GlcNAc on comparison with authentic samples of glucose, Gal and GlcNAc.

4.13 Compound D (Bebiose)

4.13.1 Deacetylation of compound D - Compound D (47 mg) was obtained from third column chromatography of acetylated oligosaccharide mixture. 25 mg of compound D was dissolved in acetone (3 ml) and 3.5 ml of NH_3 was added in it and was left overnight in a stoppered hydrolysis flask. After 24 hrs ammonia was removed under reduced pressure and the compound was washed thrice with $CHCl_3$ (5 ml) (to remove acetamide) and water layer was finally freeze dried giving the deacetylated oligosaccharide D (16 mg).

4.13.2 Methyl glycosidation/Acid hydrolysis of compound D - Compound D (8 mg) was ref1uxed with absolute MeOH (2 ml) 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 methylglycoside of D, 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^oC. The hydrolysis was complete after 24 h. The hydrolysate was neutralized with freshly prepared $BaCO_3$ and concentrated under reduced pressure to afford α- and β-methylglucosides along with the Glc, Gal and GlcNAc. Their identification was confirmed by comparison with authentic samples on TLC and PC.

4.13.3 Kiliani hydrolysis of compound D - Compound D (5 mg) was dissolved in 2 ml Kiliani mixture (AcOH-H_2O-HCI, 7:11:2) and heated at 100^oC 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, Gal and GlcNAc on comparison with authentic samples of glucose, Gal and GlcNAc

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APPENDIX-I

List of research publication-

Research Papers

1. Singh, M., Kumar, A., and Deepak, D., 2015. Sructure elucidation of a novel pentasaccharide Meeniose from *Babalus bubalis* colostrum. *Journal of Biological and Chemical Research,*Vol. 32 (1), pp. 438-446
2. Singh, M., Kumar. A., Srivastava, G., and Deepak, D., 2015 Isolation, structure elucidation and DFT study on three novel oligosaccharides from Yak milk. *Journal of molecular structure.* (Accepted)

International & National Symposium / Seminars / Conference / Educational Programs

1. International Symposium on "Recent Development & Application of Biomedical Magnetic Resonance" at CBMR, SGPGIMS, Lucknow, U.P. on 21-24 Feb. 2010.
2. National Seminar on "Organometallic Chemistry" at Department of Chemistry, University of Lucknow, Lucknow, U.P. on 31 Mar. 2010.
3. National Seminar on "Role of Bioscience to Save Environment" at Department of Chemistry, Kishan P.G. College, Bahraich, U.P. on 6-7 Oct. 2012.
4. BBDU Scholars Conclave on Advances in Research at BBD University, Lucknow, U.P. on 12 Jul. 2013.
5. National Seminar on "Newer Trends in Physico-chemical Techniques" at Department of Chemistry, University of Lucknow, Lucknow, U.P. on 7 Aug. 2013.
6. National Seminar on "Impact of Modern Agriculture on Environment" at R. S. Government College, Kanpur, U.P. on 15 Feb. 2015.