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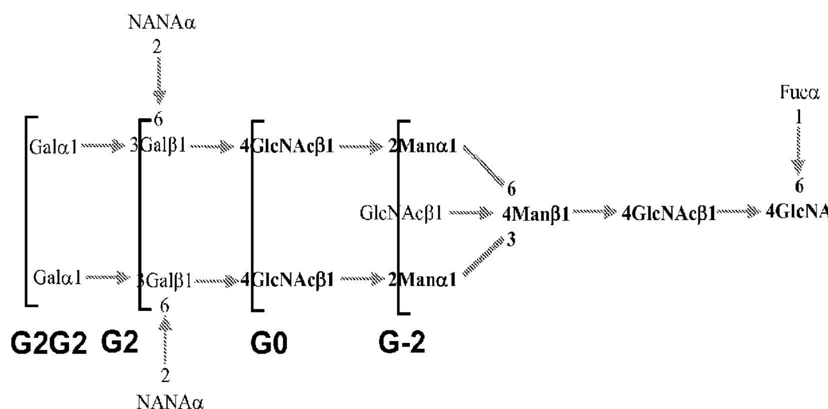
[Continued on next page]

(54) Title: GALACTOSE ALPHA (1-3) GALACTOSE COMPOSITIONS

Fig. 1



Fuc = fucosyl; Gal = galactosyl; Glc = glucosyl; GlcNAc = N-acetylglucosaminyl; Man = mannosyl; and NANA = sialyl (N-acetylneuraminyl); where NANA may be represented by one or more of 5-N-acetylneuraminic acid, (NeuAc) or 5-N-glycoly-1-neuraminic acid (NeuGc, NGNA).

(57) Abstract: An enzymatic method for synthesizing oligosaccharides comprising a terminal Gal-alpha(1,3)-Gal-beta(1-4)Glc-Nac is used to produce Fc-containing molecules with certain properties. The methods modify glycoproteins that interact with receptors or are processed in vivo and recognized as unique epitopes. In particular, the glycan groups on a therapeutic antibody capable of interaction with Fc-receptors are modified.

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— *as to the identity of the inventor (Rule 4.17(i))*

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GALACTOSE ALPHA(1-3) GALACTOSE COMPOSITIONS

BACKGROUND OF THE INVENTION

5 **Field of the Invention**

The invention is directed to a method of enzymatic synthesis of oligosaccharide structures. In particular, the invention provides a method for synthesizing glycoproteins including antibody compositions, comprising a terminal Gal-alpha(1,3)-Gal-beta(1-4)GlcNAc.

10 **Description of the Related Art**

The carbohydrate structure attached to a peptide chain is known as a "glycan." The specific glycan structure present on a protein affects the solubility, intra- and inter-polypeptide association (e.g., tendency for aggregation and ability to correctly fold), and therefore its functional or enzymatic activity. In addition, the glycan may 15 provide resistance to the peptide from proteolytic attack and the control of proteolysis leading to the conversion of inactive forms of the peptide to active forms or active forms into inactive forms. Importantly, terminal sialic acid residues present on the glycan molecule affect the half life of the peptide in the mammalian circulatory system. Thus, glycan structures provide methods to alter important pharmacokinetic properties of 20 recombinant protein therapeutics.

Antibodies are produced naturally and recombinantly as biopharmaceuticals in soluble glycoprotein form. All naturally produced antibodies possess glycans attached at conserved positions in the heavy chain constant regions, which position and structure vary with antibody isotype. Each isotype possesses a 25 distinct array of N-linked oligosaccharide structures, which variably affect protein assembly, secretion or functional activity (Wright, A., and Morrison, S. L., Trends Biotech. 15:26-32 (1997)). In the mature IgG isotype antibody, the two complex bi-antennary oligosaccharides attached to an asparagine residue of the heavy chain are buried between the CH2 domains, forming extensive contacts with the polypeptide

backbone. It has been found that their presence is essential for the antibody to mediate effector functions, such as ADCC (Lifely, M. R., et al., Glycobiology 5:813-822 (1995); Jefferis, R., et al., Immunol Rev. 163:59-76 (1998); Wright, A. and Morrison, S. L., *supra*). The major structures found in human IgG and other recombinantly-produced IgGs are the complex biantennary structures with or without exposed Gal residues (Fig. 1). The biological significance of terminal Gal containing structures on the antibody functions has been studied in detail. The extent of galactosylation of antibodies is affected by age, gender, and disease (Raju, T.S., et al. Glycobiology 2000. 10(5): 477-86). In general, oligosaccharide structures are somewhat species-specific and vary widely.

Typically, there is heterogeneous processing of the core oligosaccharide structures attached at a particular glycosylation site such that even monoclonal antibody oligosaccharides exist as multiple glycoforms. Likewise, it has been shown that major differences in antibody glycosylation occur between antibody-producing cell lines, and even minor differences are seen for a given cell line grown under different culture conditions.

Antibodies expressed in some rodent cell lines (such as rodent myeloma derived host cells NS/0 and SP2/0) often contain oligosaccharides terminated with alpha-galactose residues. The galactose residues are linked to the penultimate galactose residues at a hydroxyl of the third sugar carbon position, alpha(1-3) linkage. Neither human nor hamster cells express the active alpha-galactosyltransferase and humans have up to 1% of circulating antibodies directed against the enzymatic product of alpha 1,3-galactosyltransferase (Gal alpha 1-3Gal beta 1-4GlcNAc), also called Galili antigen (Galili, U., Clark, M. R., Shohet, S. B., Buehler, J., and Macher, B. A. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1369-1373). The absence of alpha 1-3Gal epitopes from human cells due to silencing of the gene for the enzyme alpha 1,3 galactosyltransferase, which participates in the glycosylation of cell membrane glycoconjugates in nonprimate mammals, prosimians, and New World monkeys, appears to have occurred in Old World primates 20-30 million years ago (Galili et al. 1988 J Biol Chem. 263(33):17755-62). The source of rejection of porcine organs transplanted to humans has also been traced to the alpha-Gal antigen.

Apart from the antigenic nature of the Gal alpha(1-3)Gal beta(1-4)GlcN Ac trisaccharide, the biological effect of alpha-galactosylated oligosaccharides on antibody function is unknown. Since oligosaccharides present in antibodies are highly heterogeneous, it is difficult to establish whether alpha-galactose present in therapeutic antibody preparations impacts the bioactivity. In one report, non-Fc-linked N-glycans present in the variable (antigen binding) region of a therapeutic antibody provided immunogenic (Chung et al. 2008 New Engl J Med 358:1109-17) and the reactive antigen was identified as Gal-alpha-1,3-Gal.

Therefore, a preparation of homogeneously alpha 1-3galactosylated antibodies that can be used to study the biological significance of alpha-galactose epitopes on antibody functions and pK would be of use in determining the biological impact of these glycans in therapeutic antibody preparations produced by non-primate host cells.

SUMMARY OF THE INVENTION

The invention provides a method for synthesis of Gal alpha(1-3)Gal beta(1-4)GlcN Ac containing oligosaccharides in a single reaction. The invention further provides substantially homogeneous preparations comprising Gal alpha(1-3)Gal beta(1-4)GlcN Ac containing oligosaccharide.

In one embodiment for forming an alpha-galactosylated oligosaccharide structure comprising a terminal Gal-alpha(1,3)-Gal-beta(1-4)GlcNac includes the steps of

(a) admixing the following ingredients in an aqueous medium within a single vessel to form an aqueous reaction medium:

i) a GlcNAc acceptor molecule;

ii) a source of UDP-Gal;

iii) a divalent metal selected from the group consisting of Mn^{2+}, Ca^{2+}, and Zn^{2+};

iv) an alpha(1-3)galactosyltransferase; and

v) a beta(1-4)galactosyltranferase; and

(b) maintaining said aqueous reaction medium at a pH value of about 5 to about 10 at a temperature of about 25°C to about 40°C for a time period sufficient for said acceptor to be glycosylated.

In one embodiment, the alpha-galactosylated oligosaccharide is a biantennary structure. In a specific embodiment, the alpha-galactosylated oligosaccharide biantennary structure is an N-glycan of a polypeptide. In an embodiment, the polypeptide is the heavy chain of an immunogloblulin.

The homogeneous preparations comprising Gal-alpha(1-3)Gal-beta(1-4)GlcNAc containing oligosaccharide may be used to study the antigenic nature of the terminal trisaccharide epitope and other biological responses to the presence of the epitope in various human and non-human systems. The preparations may be admixed to form a minor but defined component of the oligosaccharide preparation for such studies. The preparations may be used as starting material for preparations of oligosaccharides with greater complexity.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING

Fig. 1 shows the basic biantennary structure of major oligosaccharide structures found in either a naturally occurring and recombinant isolated IgG preparation, where the saccharide residues shown in bold face are core residues and those shown in normal font represent positions which vary based on the synthetic environment, such as the host cell origin, host cell nutritional environment, and post secretory processing or degradation: bisecting GlcNAc, alpha1-6 fucosylation of the core GlnNAc, and sialylation of galactosylated structures (alpha 2,6-sialylation).

Figs. 2A-2C show chromatograms from a normal phase HPLC separation of oligosaccharides released from the starting preparation of IgG (Fig. 2A); the IgG after reaction with UDP-Gal in the presence of beta1,4galactosyltransferase (Fig. 2B); or the IgG after reaction with UDP-Gal in the presence of beta1,4galactosyltransferase and alpha-galactosyltransferase (Fig. 2C).

Figs. 3A-3C show a tracing from MALDI-TOF-MS analysis of oligosaccharides released from IgG samples.

DETAILED DESCRIPTION OF THE INVENTION

Abbreviations

α1,3GT, α-1,3-galactosyltransferase; α2,3ST, α-2,3-sialyltransferase; β1,4GT, β-1,4-galactosyltransferase; ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-directed cytotoxicity; CMP-Sia, cytidine monophosphate, *N*-acetylneuraminic acid; fuc = fucosyl; gal = galactose; GalNac = N-acetylgalactose; Glc = glucosyl; IgG, immunoglobulin G; Man = mannosyl; MALDI-TOF-MS, matrix-assisted laser/desorption ionization time-of-flight mass spectrometry; MHX, mycophenolic acid, hypoxanthine, xanthine.; NANA, *N*-acetylneuraminic acid isomer of sialic acid; NGNA, *N*-glycolylneuraminic acid isomer of sialic acid; PNGase F, peptide *N*glycosidase F; RP-HPLC, reversed phase high-performance liquid chromatography; Sia, sialic acid; UDP-Gal, uridine diphosphate galactose; UDP-GlcNAc, uridine diphosphate *N*-acetylglucosamine.

Definitions

The terms "antibody," "immunoglobulin," or "IgG" is intended to encompass antibodies, digestion fragments, specified portions and variants thereof, including, without limitation, antibody mimetics or comprising portions of antibodies that mimic the structure and/or function of an antibody or specified fragment or portion thereof, and retain Fc-mediated functions, including but not limited to: ligand binding, binding to Fc-receptors (e.g. FcγRI (CD64) FcγRIIA (CD32A), FcγRIIIA (CD16A) and FcRn), binding complement (e.g. C1q), ADCC and CDC.

The term "Fc-containing protein" or "Fc-containing molecule" as used herein refers to a monomeric, dimeric or heterodimeric protein having at least an immunoglobulin CH2 and CH3 domain, and preferably a dimerization domain, such as an immunoglobuline hinge region. The CH2 and CH3 domains can form at least a part of the dimeric region of the protein/molecule (e.g., antibody), wherein an N-linked glycosylatation site is present on one of the CH2 domains.

"Glycosylation sites" refer to amino acid residues which are recognized by a eukaryotic cell as locations for the attachment of sugar residues. The amino acids where carbohydrate, such as oligosaccharide, is attached are typically asparagine (N-

linkage), serine (O-linkage), and threonine (O-linkage) residues. The specific site of attachment is typically signaled by a sequence of amino acids, referred to herein as a "glycosylation site sequence." The glycosylation site sequence for N-linked glycosylation is known as -Asn-X-Ser- or -Asn-X-Thr- (NXT), where X may be any of the conventional amino acids, other than proline. The predominant glycosylation site sequence for O-linked glycosylation is: -(Thr or Ser)-X-X-Pro-, where X is any conventional amino acid. The recognition sequence for glycosaminoglycans (a specific type of sulfated sugar) is -Ser-Gly-X-Gly, where X is any conventional amino acid. The terms "N-linked" and "O-linked" refer to the chemical group that serves as the attachment site between the sugar molecule and the amino acid residue. N-linked sugars are attached through an amino group; O-linked sugars are attached through a hydroxyl group. However, not all glycosylation site sequences in a protein are necessarily glycosylated; some proteins are secreted in both glycosylated and nonglycosylated forms, while others are fully glysosylated at one glycosylation site sequence but contain another glycosylation site sequence that is not glycosylated. Therefore, not all glycosylation site sequences that are present in a polypeptide are necessarily glycosylation sites where sugar residues are actually attached. The initial N-glycosylation during biosynthesis inserts the "core carbohydrate" or "core oligosaccharide" (Proteins, Structures and Molecular Principles, (1984) Creighton (ed.), W.H. Freeman and Company, New York, which is incorporated herein by reference).

The term "monoclonal antibody" as used herein is a specific form of Fc-containing fusion protein in which the ligand binding domain retains substantial homology to at least one of a heavy or light chain antibody variable domain of at least one species of animal antibody and the antibody is produced by single host cell type which may be a hybridoma or transfectoma but more typically, where the nucleic acids encoding the antibody have been recloned using standard recombinant methods and reintroduced into the host cell.

By "NANA" or "sialic acid" is meant a member of a family of nine-carbon carboxylated sugars. The most common member of the sialic acid family is N-acetyl neuraminic acid (2-keto-5-acetamido-3,5-dideoxy-D-glycero-D- galactononulopyranos-l-onic I acid (Neu5Ac, NeuAc, or NANA). A second member of the family is N-glycolyl-

neuraminic acid (NGNA, Neu5Gc or NeuGc), in which the N-acetyl group of NeuAc is hydroxylated. This form is prevalent in glycoproteins from rodent and microbial sources. A third sialic acid family member is 2-keto-3-deoxy-nonulosonic acid (KDN) (Nadano et al. (1986) J. Biol. Chem. 261: 11550-11557; Kanamori et al., J. Biol. Chem. 265: 21811-
5 21819 (1990)). Also included are 9-substituted sialic acids such as a 9-O-C -C6 acyl Neu5Ac like 9-O-lactyl-Neu5Ac or 9- O-acetyl-Neu5Ac, 9-deoxy-9-fluoro-Neu5Ac and 9 azido-9-deoxy-Neu5Ac. For review of the static acid family, see, e.g., Varki, Glycobiology 2: 25-40 (1992); Sialic Acids: Chemistry, Metabolism and Function, R. Schauer, Ed. (Springer Verlag, New York (1992)).

10 **Subject Glycans**

The invention relates to compositions which are oligosaccharide, also called "glycan" structures. Oligosaccharides are considered to have a reducing end and a non-reducing end, whether or not the saccharide at the reducing end is in fact a reducing sugar. In accordance with accepted nomenclature, oligosaccharides are depicted herein
15 with the non-reducing end on the left and the reducing end on the right. All oligosaccharides described herein are described with the name or abbreviation for the non-reducing saccharide (e.g., Gal), followed by the configuration of the glycosidic bond (α or β), the ring bond, the ring position of the reducing saccharide involved in the bond, and then the name or abbreviation of the reducing saccharide (e.g., GlcNAc). The
20 linkage between two sugars may be expressed, for example, as 1,3, 1→3, or (1-3). Each saccharide is a pyranose.

The oligosaccharide structures of the present invention occur on a protein, lipid or peptide expressed as N-linked oligosaccharides. "N-linked glycosylation" refers to the attachment of the carbohydrate moiety via GlcNAc to an asparagine residue in a
25 polypeptide or lipid chain. The N-linked oligosaccharides on mammalian antibodies contain a common Man alpha(1 –6)[Man alpha(1 - 3)]Manbeta(1-4)GlcNAcbeta(1-4)GIcNAcbeta-R "core structure" also referred to as G-2 (Fig. 1). Therefore, in the core structure described, R represents an asparagine residue of the produced glycoprotein linked to the first saccharide of the carbohydrate: 2-acetamido-N-(L-aspart-4-yl)-2-
30 deoxy-b-D-glucopyranosylamine, i.e. N^4-(N-acetyl-b-D-glucosaminyl)asparagine, which

is also abbreviated to (GlcNAc-)Asn (parentheses here around the carbohydrates placed next to the symbol for an the asparagine residue indicates substitution is on the N at the fourth atom which is the side chain amine). Oligosaccharides having branched chains are considered complex carbohydrates and the present invention relates to complex biantennary carbohydrate structures also referred to as the glycan portion of a glycoprotein, such as those attached to the CH2 domain of immunoglobulins.

The natural modification of polypeptides with oligosaccharides occurs in the golgi apparatus of eukaryotic cells, particularly eukaryotic cells capable of adding an N-linked "core oligosaccharide" containing at least one mannose residue and/or capable of adding an O-linked sugar, to at least one glycosylation site sequence in at least one polypeptide expressed in said cell, particularly, a secreted protein. Thus, cells capable of forming glycoproteins contain at least one glycosyltransferase that catalyzes the attachment of a sugar residue to a glycosylating site sequence in a protein or polypeptide. Mammalian cells are typically capable of glycosylating proteins while other eukaryotic cells, such as insect cells and yeast, may glycosylate secreted proteins but with alternative or truncated structures as compared to those produced by mammalian cells.

Compositions

The product of the method of the invention is a substantially homogeneous preparation comprising an alpha-galactosylated oligosaccharide structure comprising a terminal Gal-alpha(1,3)-Gal-beta(1-4)GlcNAc. The alpha-galactosylated oligosaccharide structure may be linked to proteins or lipids, through amine or hydroxyl functionalities present on proteins on the side chain of asparagine, serine, or threonine residues and hydroxyl groups of terpenoids or ceramide, sphingoid, such as prenyl phosphate.

The invention also relates to complex biantennary structures comprising α1,3-linkage Gal, optionally, with α2,6-linked NANA. In one embodiment, the structure produced by the method of the invention is shown in the formula below (I):

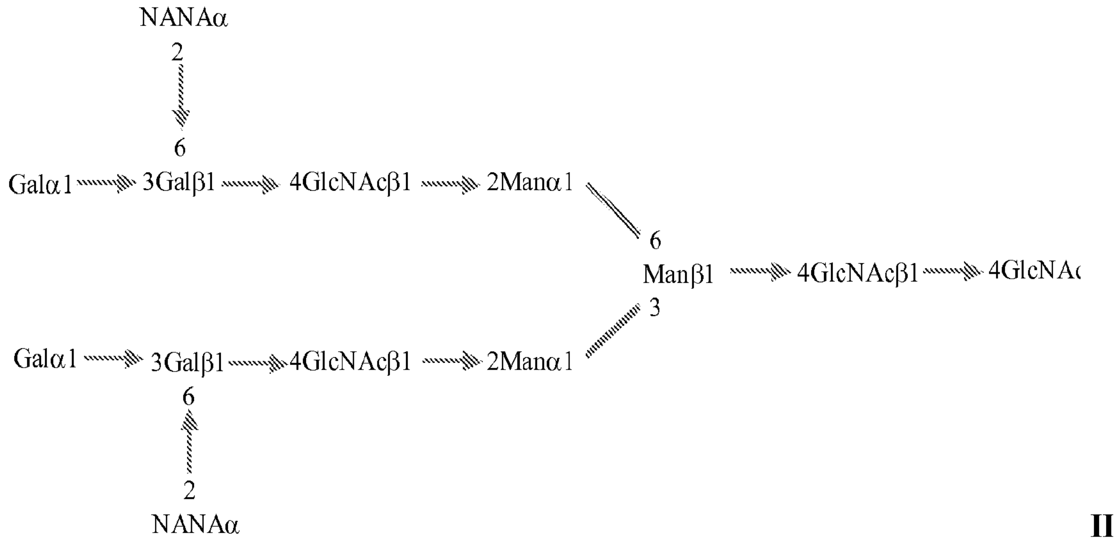
Galα1 → 3Galβ1 → 4GlcNAcβ1 → 2Manα1 ↘ 6

Manβ1 → 4GlcNAcβ1 → 4GlcNA(

Galα1 → 3Galβ1 → 4GlcNAcβ1 → 2Manα1 ↗ 3

I

In a different embodiment, the structure produced by the method of the invention is shown in the formula below (II):



II

5 It will be obvious to those skilled in the art that included within the present invention are variations of formula I and II that are possible along with variants, such as those depicted in Fig. 1, which include the presence of core fucose and bisecting GlcNAc.

Methods of Making the Compositions

10 A number of glycosyltransferases have been described and, in some cases, methods whereby the enzymes may be used concurrently instead of sequentially to affect the synthesis of a bisaccharide of stereo- and region-specificity. Over 200 glycsosyltransferases from various sources have been identified and the ability to select compatible combinations for the directed synthesis of specific oligosaccharide structures 15 has not been exhaustively explored. The invention describes that by selection of galactosyltransferase enzymes with predetermined specificity, it is possible to transfer two molecules of galactose in series in a single reaction to a substrate comprising a terminal GlcNac forming the specific trisaccharide structure Gal α(1-3)Galβ(1-4)GlcNAc.

20 In one embodiment for forming an alpha-galactosylated oligosaccharide structure comprising a terminal Gal-alpha(1,3)-Galbeta(1-4)GlcNAc includes the steps

of:

(a) admixing the following ingredients in an aqueous medium within a single vessel to form an aqueous reaction medium:

vi) an GlcNAc acceptor molecule;

5 vii) a source of UDP-Gal;

viii) a divalent metal selected from the group consisting of Mn^{2+}, Ca^{2+}, and Zn^{2+};

ix) an alpha(1-3)galactosyltransferase; and

x) a beta(1-4)galactosyltranferase; and

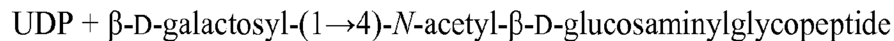
10 (b) maintaining said aqueous reaction medium at a pH of about 5 to about 10 at a temperature of about 25°C to about 40°C for a time period sufficient for said acceptor to be glycosylated.

In one embodiment the galactosyltransferase is isolated from a natural source. For example, bovine milk beta-1,4 galactosyltransferase is a common source of 15 commercially available enzyme. Recombinant forms of bovine, porcine, and other galactosyltransferases are also available. Recombinant alpha-1,3 galactosyltransferases have been previously expressed as complete proteins or as the soluble extracellular domain which is a fully active soluble enzyme (Henion, T. R., Macher, B. A., Anaraki, F., and Galili, U. (1994) Glycobiology 4, 193-201).

20 The divalent metal specificity for activating the alpha(1-3) and beta(1-4)-galactosyltransferases is similar or at least overlapping in vitro environments and includes Mn^{2+}, Zn^{2+}, and Co^{2+} (Zhang et al. 2001 J. Biol. Chem., 276(15): 11567-11574). The metal or metals are present at 1-25 mM.

Exemplary galactosyltransferases and glycosaminoglycan 25 galactosyltransferase of *Dictyostelium discoideum* (EC 2.4.1.74), mammalian glucosaminylgalactosylglucosylceramide β-galactosyltransferase (EC 2.4.1.86); β-*N*-acetylglucosaminyl-glycopeptide β-1,4-galactosyltransferase (E.C. No. 2.4.1.38) also called N-acetyllactosamine synthase (EC 2.4.1.22) capable of catalyzing the reaction

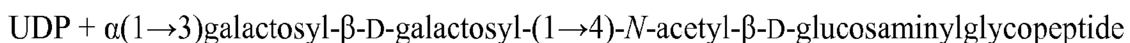
UDP-galactose + *N*-acetyl-β-D-glucosaminylglycopeptide =


UDP + β-D-galactosyl-(1→4)-*N*-acetyl-β-D-glucosaminylglycopeptide

In another embodiment, the galactosyltrasferease is also called N-acetyllactosamine synthase (EC 2.4.1.22) and is capable of catalyzing the transfer of galactose from UDP-galactose to N-acetylglucosamine.

The α(1,3) galactosyltransferase (E.C. No. 2.4.1.151) especially that of calf thymus (Blanken et al. J Biol Chem. 1985 Oct 25;260(24):12927-34) or porcine + β-D-galactosyl-*N*-acetylglucosamine- α(1,3)D-galactosyltransferase is capable of catalyzing the formation of the trisaccharide antigen, Gal α(1-3)Galβ(1-4)GlcNAc. The α(1,3)D-galactosyltransferases useful in the method of the invention are capable of catalyzing the reaction:

UDP-galactose + β-D-galactosyl-(1→4)-*N*-acetyl-β-D-glucosaminylglycopeptide =


UDP + α(1→3)galactosyl-β-D-galactosyl-(1→4)-*N*-acetyl-β-D-glucosaminylglycopeptide

For production of the structure of formula II, a NANA transferring enzyme can be used, such enzymes include Gal-β-1,4- GlcNAc α-2,6 sialyltransferase (See, Kurosawa et al. Eur. J. Biochem. 219: 375-381 (1994)) and US Pat. 7,220,555).

Other glucosyltransferases particularly useful in preparing oligosaccharides acceptor molecules of invention are the mannosyltransferases including α(1,2) mannosyltransferase, α(1,3) mannosyltransferase, β(1,4) mannosyltransferase, Dol-P-Man synthase, OCh1, and Pmt1.

Still other glucosyltransferases include N-acetylgalactosaminyltransferases including α(1,3) N-acetylgalactosaminyltransferase, β(1,4) N-acetylgalactosaminyltransferases (Nagata et al. J. Biol. Chem. 267:12082- 12089 (1992) and Smith et al. J. Biol Chem. 269:15162 (1994)) and polypeptide N-acetylgalactosaminyltransferase (Homa et al. J. Biol Chem. 268:12609 (1993)). Suitable N-acetylglucosaminyltransferases include GnTI (2.4.1.101, Hull et al., BBRC 176:608 (1991)), GnTII, and GnTIII (Ihara et al. J. Biolchem. 113:692 (1993)), GnTV (Shoreiban et al. J. Biol. Chem. 268: 15381 (1993)).

For those embodiments in which the method is to be practiced on a

commercial scale, it can be advantageous to immobilize the glycosyltransferase on a support. This immobilization facilitates the removal of the enzyme from the batch of product and subsequent reuse of the enzyme. Immobilization of glycosyltransferases can be accomplished, for example, by removing from the transferase its membrane-binding domain, and attaching in its place a cellulose-binding domain. One of skill in the art will understand that other methods of immobilization can also be used and are described in the available literature.

The glycosyltransferase used is specific for both the transferred glycosyl group and the acceptor to which the glycosyl group (Gal or GlcNAc) is transferred. When synthesizing oligosaccharides from scratch, the acceptor substrates can essentially be any monosaccharide or oligosaccharide having a terminal saccharide residue for which the particular glycosyltransferase exhibits specificity, and the substrate may be substituted at the position of its non-reducing end. Thus, the glycoside acceptor may be a monosaccharide, an oligosaccharide, a fluorescent-labeled saccharide, or a saccharide derivative, such as an aminoglycoside antibiotic, a ganglioside, a glycolipid, or a glycoprotein including antibodies and other Fc-containing proteins. In one group of preferred embodiments, the glycoside acceptor is an oligosaccharide, which when beta-galactosylated will comprise the disaccharide unit Galβ(1-4)GlcNAc, thereby acting as an acceptor for the alpha-galactosyltransfersas. The saccharide or oligosaccharide acceptor is preferably,

GlcNAc,

GlcNAcβ(1- 2)Man,

GlcNAcβ(1- 2)Manα(1-3)Man,

GlcNAcβ(1- 2)Manα(1,6)Man,

GlcNAcβ(1- 2)Manα(1,6)Man β(1- 4)GlcNAc,

GlcNAcβ(1- 2)Manα(1,6)Man β(1- 4)GlcNAc, β(1-4)GlcNac

GlcNAcβ(1- 2)Manα(1,6)Man β(1- 4)GlcNAc, β(1-4)GlcNac-R, or

GlcNAcβ(1- 2)Manα(1,6)[Galβ(1- 4)GlcNAcβ(1- 2)Manα(1-3)]Man β(1- 4)GlcNAc,

β(1-4)GlcNac-R.

In a particular embodiment, the oligosaccharide acceptor, is linked to R, where R is an asparagines residue within the CH2 domain of an Fc-containing protein. In another embodiment, the non-reducing terminal sugar may be substititute with a reporter group or be attached to a lipid such as an aminophospholipid.

The glycosyltransferase will also have specificity for the donor sugar nucleotide. In the case of the galactosyltransferases, the donor sugar nucleotide may be UDP-Gal. The use of activated sugar substrate, i.e., sugar-nucleoside phosphate, can be circumvented by using a regenerating reaction concurrently with the glycotransferase reaction (also known as a recycling system). For example, as taught in, e.g., U.S. Pat. 5,516,665; a uridine diphosphate recycling system that includes (a) UDP, UTP or both, (b) a phosphate donor, and (c) a kinase to transfer a phosphate group from the phosphate donor to UDP to form UTP, wherein each of the enzymes is present in a catalytic amount. Either or both of UDP and UTP can be present inasmuch as UDP is converted into UTP, and after the glycosyl transfer reaction, UDP is formed again. Because UDP and UTP interconvert and are reused, the total amount of one or the other is usually discussed rather than amounts for both. The phosphate donor of the regenerating system is a phosphorylated compound, the phosphate group of which can be used to phosphorylate UDP to form UTP. The only limitation on the selection of a phosphate donor is that neither the phosphorylated nor the dephosphorylated forms of the phosphate donor substantially interferes with any of the reactions involved in the formation of the glycosylated acceptor saccharide. Phosphate donors are phosphoenolpyruvate (PEP) and acetyl phosphate (AcOP).

Yet another system for forming UDP-gal is taught in US 5,728,554 and includes a donor substrate recycling system comprising at least 1 mole of glucose-1-phosphate per each mole of substrate oligosaccharide, a phosphate donor, a kinase capable of transferring phosphate from the phosphate donor to nucleoside diphosphates, and a pyrophosphorylase capable of forming UDP-glucose from UTP and glucose-1-phosphate and catalytic amounts of UDP and a UDP-galactose-4-epimerase. This system can be used with α(1,3) galactosyltransferase (E.C. No. 2.4.1.151) and β(1,4)

galactosyltransferase (E.C. No. 2.4.1.38).

An alternative method of preparing oligosaccharides is through the use of a glycosyltransferase and activated glycosyl derivatives as donor sugars obviating the need for sugar nucleotides as donor sugars as taught in U.S. Pat. 5,952,203. The
5 activated glycosyl derivatives act as alternates to the naturally-occurring substrates, which are expensive sugar-nucleotides, usually nucleotide diphosphosugars or nucleotide monophosphosugars in which the nucleotide phosphate is α-linked to the 1-position of the sugar.

Activated glycoside derivatives which are useful include an activated
10 leaving group, such as, for example, fluoro, chloro, bromo, tosylate ester, mesylate ester, triflate ester and the like. Preferred embodiments of activated glycoside derivatives include glycosyl fluorides and glycosyl mesylates, with glycosyl fluorides being particularly preferred. Among the glycosyl fluorides, α-galactosyl fluoride, α-mannosyl fluoride, α-glucosyl fluoride, α- fucosyl fluoride, α-xylosyl fluoride, α-sialyl fluoride,
15 alpha-N-acetylglucosaminyl fluoride, α-N-acetylgalactosaminyl fluoride, β-galactosyl fluoride, β-mannosyl fluoride, β-glucosyl fluoride, β-fucosyl fluoride, β-xylosyl fluoride, beta-sialyl fluoride, β-N-acetylglucosaminyl fluoride and β-N- acetylgalactosaminyl fluoride are most preferred.

Glycosyl fluorides can be prepared from the free sugar by first acetylating
20 the sugar and then treating it with HF/pyridine. Acetylated glycosyl fluorides may be deprotected by reaction with mild (catalytic) base in methanol (e.g., NaOMe/MeOH). In addition, many glycosyl fluorides are commercially available. Other activated glycosyl derivatives can be prepared using conventional methods known to those of skill in the art. For example, glycosyl mesylates can be prepared by treatment of the fully benzylated
25 hemiacetal form of the sugar with mesyl chloride, followed by catalytic hydrogenation to remove the benzyl groups.

Suitable analogs include, for example, nucleoside sulfates and sulfonates. Still other analogs include simple phosphates, for example, pyrophosphate.

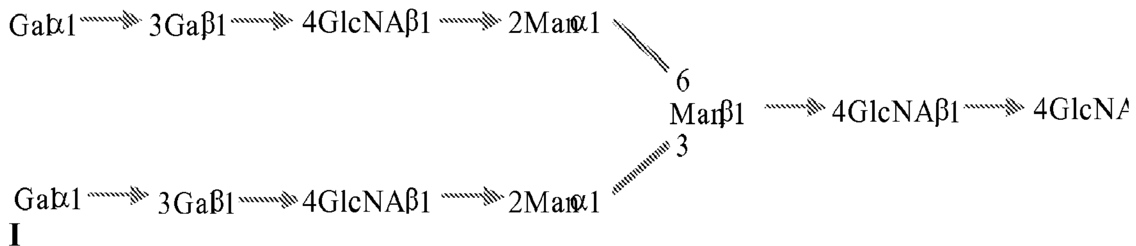
One procedure for modifying recombinant proteins produced, in e.g.,
30 murine cells wherein the hydroxylated form of sialic acid predominates (NGNA), is to

treat the protein with sialidase, to remove NGNA-type sialic acid, followed by enzymatic galactosylation using the reagent UDP-Gal and beta1,4 Galtransferase to produce highly homogeneous G2 glycoforms.

An alternative approach for preparing sublots of an Fc-containing protein that differ in α-galactose content of the oligosaccharides in the Fc region is to treat a portion of an Fc-containing protein preparation with sialidase enzyme, thereby removing sialic acids.

Methods of Using the Invention

The method the invention can be used to modifiy polypeptides having an consensus glycosylation sequence (NXT) having a core glycan structure known as G0 (Fig. 1) to structures containing beta Gal residues (G2) further comprising at least one alpha1-3 galactosylated saccharides (G2G1 or G2G2) as shown in Fig. 1 and below (I).



I

The invention further relates to preparations of IgG which comprise glycan structures which are substantially homogeneously in the form of G2G2 as shown in (I) which may further be fucosylated at the core GlcNac, or may have bisecting beta-1-4 N-acetyl aminoglucosylated at the core mannose of the structure, or may be sialylated at the same galactose residue which is alpha-galactosylated, by an alpha 2-6 linkage but not alpha-2-3 sialylated at the same galactose residue which is alpha-galactosylated.

The compositions prepared by the process of the invention, are useful as therapeutic compositions wherein a substantially homogeneous preparation of IgG molecules is desired having glycans in the G2G2 configuration. The method of the invention may be used to modify glycoproteins that interact with receptors. In particular, the invention relates to the modification of the glycan groups on a therapeutic antibody capable of interaction with Fc-receptors and producing modified therapeutic proteins,

e.g., antibodies, such that the composition of the oligosaccharide chains may be optimized for one or more biological activities in vivo.

The compositions prepared by the process of the invention may be subjected to further biologic or chemical processing or modification. For example, antibodies prepared with glycan structures in the G2G2 configuration can be modified to include alpha-2,6-sialylation. Higher order structures or modifications, such as PEGylation or lipidation, of one or more of the saccharide residues of compositions produced by the method of the enzymatic method of the invention are encompassed by the invention.

EXAMPLE 1: GALACTOSYLATION OF ANTIBODY SAMPLES

A method of preparation of IgG substantially in the G2 glycoform is described.

Bovine β-1,4-galactosyltransferase and UDP-Gal were obtained from Sigma Chemical Co. (St. Louis, MO). PNGase F was obtained from New England Biolabs (Beverly, MA) or from Prozyme (San Leandro, CA) or from Selectin BioSciences (Pleasant Hill, CA). NAP-5 and HiTrap protein A columns were obtained from Pharmacia Biotech (Piscataway, NJ). All other reagents were of analytical grade. Recombinant IgGs comprising a human Fc-domain were produced at Centocor Research & Development, Inc. (Radnor, PA).

The IgG samples in 100 mM MES buffer (pH 7.0) (approximately 10 mg in 1.0 mL of buffer) were treated with 50 milliunits of bovine β1,4-galactosyltransferase (from Sigma), 5 μmol of UDP-Gal, and 5 μmol of $MnCl_2$ at 37°C for 24 hours. Another aliquot of enzyme and UDP-Gal was added and the mixture was incubated for an additional 24 hours at 37°C. The alpha-galactosylated IgG samples were purified using a HiTrap protein A column. The oligosaccharides were released by treating IgGs with PNGase F and characterized the released oligosaccharides by MALDI-TOF-MS and by NP-HPLC (normal phase HPLC).

The MALDI-TOF-MS analysis of glycans released from starting IgG sample (control) showed the presence of 45% G0, 50% G1 and 5% G2 glycans along

with minor amounts of other glycans (Fig. 3A). The NP-HPLC analysis of glycans released from untreated IgG sample showed no appreciable amounts of sialylated glycans and confirmed the presence of G0, G1 and G2 as major glycans (Fig. 2A). Thus, after incubation of the samples with Bovine β-1,4-galactosyltransferase and UDP-Gal
5 (obtained from Sigma), both MALDI-TOF-MS and NP-HPLC analyses of glycans released from the galactosylated IgG sample showed the presence of only G2 glycan (Figs. 2B and 3B) and the absence of G0 and G1 glycans suggesting that the galactosylation was complete.

EXAMPLE 2: α-GALACTOSYLATION OF ANTIBODY SAMPLES

10 A homogeneous preparation containing Gal(alpha1-3)Galβ1,4 linkages in an IgG preparation comprising the glycan of formula I was prepared in a single reaction step using non-primate enzymes

The reagents were as described in Example 1 with the addition of recombinant porcine α-galactosyltransferase was obtained from Calbiochem (San Diego,
15 CA)

IgG samples in 100 mM MES buffer (pH 7.0) (~10 mg in 1.0 mL of buffer) were treated with 50 milliunits of each of bovine β1,4-galactosyltransferase (from Sigma) and recombinant rat liver α1,3-galactosyltransferase (from CalBiochem) in the presence of 5 µmol of UDP-Gal and 5 µmol of $MnCl_2$ at 37 °C. After 24 hr of incubation,
20 another aliquot of bovine β1,4-galactosyltransferase and recombinant rat liver α1,3-galactosyltransferas along with 5 µmol of UDP-Gal were added. The mixture was incubated for an additional 24 hr at 37 °C. The beta-galactosylated and α-galactosylated IgG samples were purified using a HiTrap protein A column. The oligosaccharides were released from IgGs by treating with PNGase F and characterized the released
25 oligosaccharides by MALDI-TOF-MS and by NP-HPLC.

Both MALDI-TOF-MS and NP-HPLC analyses of glycans released from the treated IgG sample showed the presence of only α-galactosylated structure (Figs. 2C and 3C) i.e., G2α2 (Formula 1) and the absence of G0, G1 and G2 structures suggesting that the α-galactosylation of IgG is complete.

WHAT IS TO BE CLAIMED:

1. A method of producing an alpha-galactosylated oligosaccharide structure on a glycoprotein comprising a terminal Gal-alpha(1,3)-Gal-beta(1-4)GlcNAc comprising:

   (a) admixing a saccharide acceptor molecule, an activated galactose, a divalent metal selected from the group consisting of Mn^{2+}, Ca^{2+}, and Zn^{2+}, an alpha(1-3)galactosyltransferase, and a beta(1-4)galactosyltranferase in an aqueous medium within a single vessel to form an aqueous reaction medium; and

   (b) maintaining said aqueous reaction medium at a pH of about 5 to about 10 at a temperature of about 25°C to about 40°C for a time period sufficient for said acceptor to be glycosylated.

2. The method of claim 1 wherein the alpha(1-3)galactosyltransferase is porcine α-galactosyltransferase.

3. The method of claim 1 or 2 wherein the divalent metal salt is Mn^{2+}.

4. The method of claim 1 wherein the activated galactose is uridine diphosphate-galactose (UDP-galactose).

5. The method of claim 1 wherein the beta(1-4)galactosyltransferase is a mammalian beta1-4, galactosyl transferase.

6. The method of claim 1 wherein the reaction temperature is about 37°C, the divalent metal is Mn^{2+} at a concentration of about 5 mM, the UDP- galactose concentration is about 5mM and the beta(1-4)galactosyltransferase concentration is about 50 mUnit/ml.

7. The method of claim 1 wherein the glycoprotein is an antibody.

8. The method of claim 7 wherein the antibody is an IgG.

9. The method of claim 1 wherein the saccharide acceptor is selected from the group consisting of a monosaccharide, an oligosaccharide, a fluorescent-labeled saccharide, and a saccharide derivative.

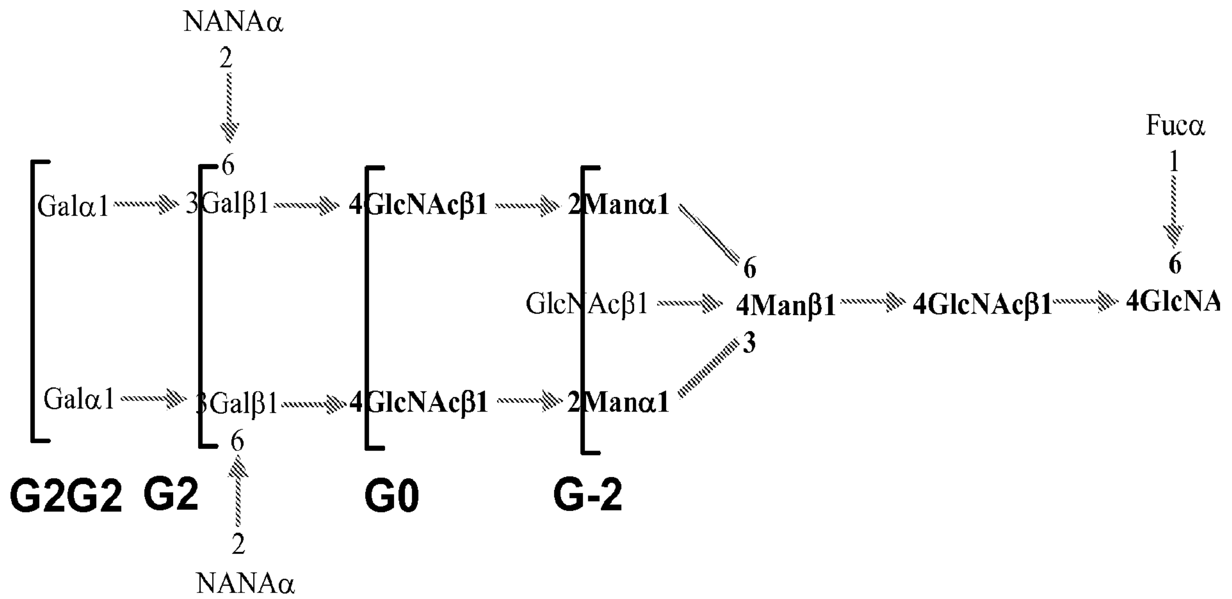
10. The method of claim 9 wherein the saccharide derivative is selected from the group consisting of an aminoglycoside antibiotic, a ganglioside, a glycolipid, and a glycoprotein.

11. The method of claim 1, wherein the saccharide acceptor is selected from the group consisting of GlcNAc, GlcNAcβ(1- 2)Man, GlcNAcβ(1- 2)Manα(1-3)Man, GlcNAcβ(1- 2)Manα(1,6)Man, GlcNAcβ(1- 2)Manα(1,6)Man β(1- 4)GlcNAc, GlcNAcβ(1- 2)Manα(1,6)Man β(1- 4)GlcNAc, β(1-4)GlcNac, GlcNAcβ(1- 2)Manα(1,6)Man β(1- 4)GlcNAc, β(1-4)GlcNac-R, and GlcNAcβ(1- 2)Manα(1,6)[Galβ(1- 4)GlcNAcβ(1- 2)Manα(1-3)]Man β(1- 4)GlcNAc, β(1-4)GlcNac-R.

12. The method of any of claims 1-11 wherein the glycan comprises alpha-2,6-sialic acid residues.

13. A glycoprotein modified by the method of any of claims 1-12.

Fig. 1



Fuc = fucosyl; Gal = galactosyl; Glc = glucosyl; GlcNAc = N-acetylglucosaminyl; Man = mannosyl; and NANA = sialyl (N-acetylneuraminyl); where NANA may be represented by one or more of 5-N-acetylneuraminic acid, (NeuAc) or 5-N-glycoly-1-neuraminic acid (NeuGc, NGNA).

Fig. 2A

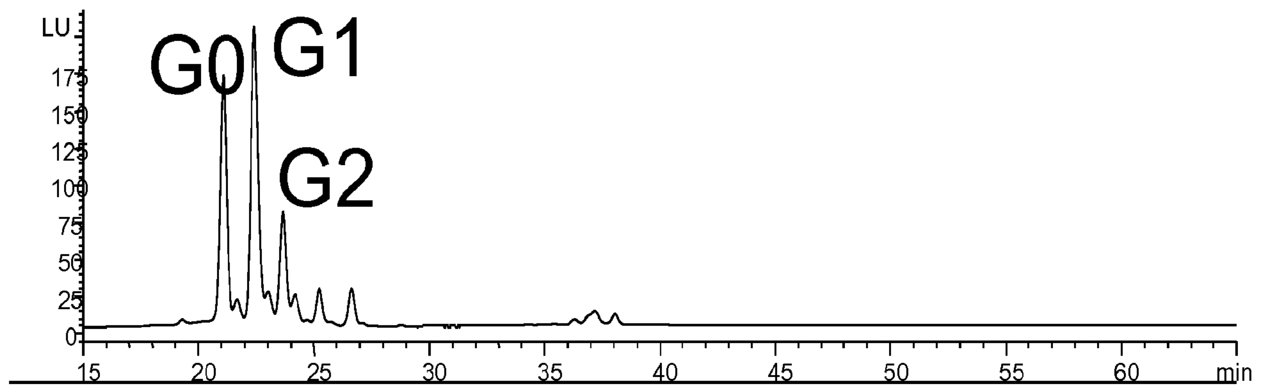


Fig. 2B

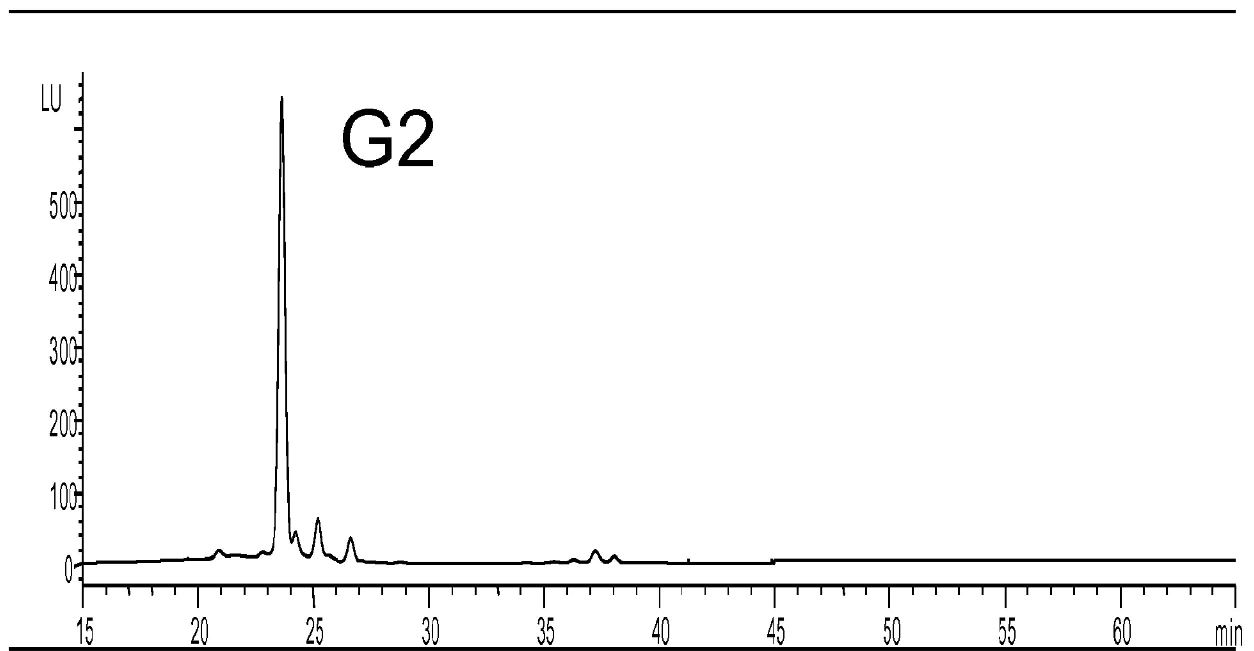


Fig. 2C

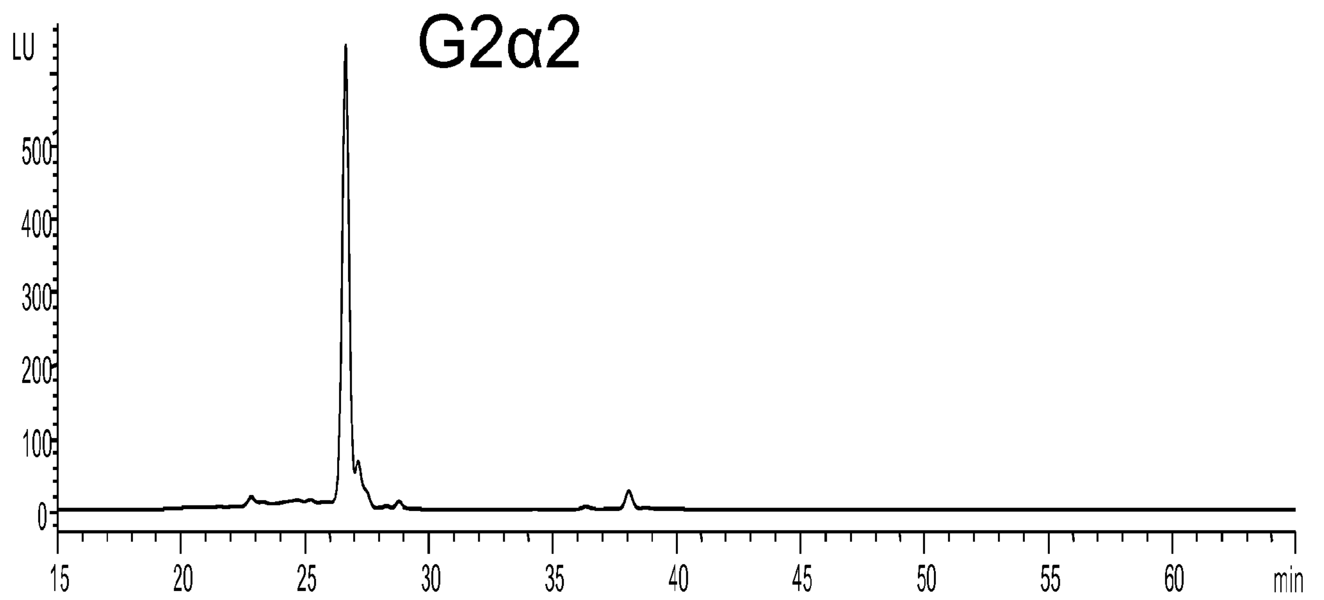


Fig. 3A

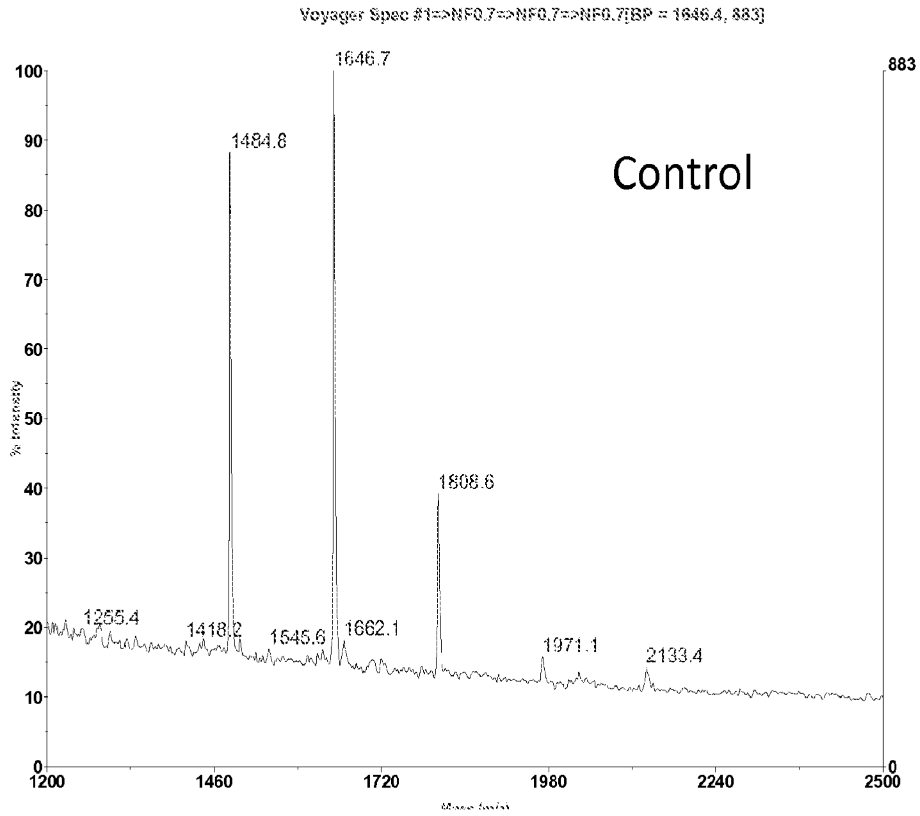


Fig. 3B

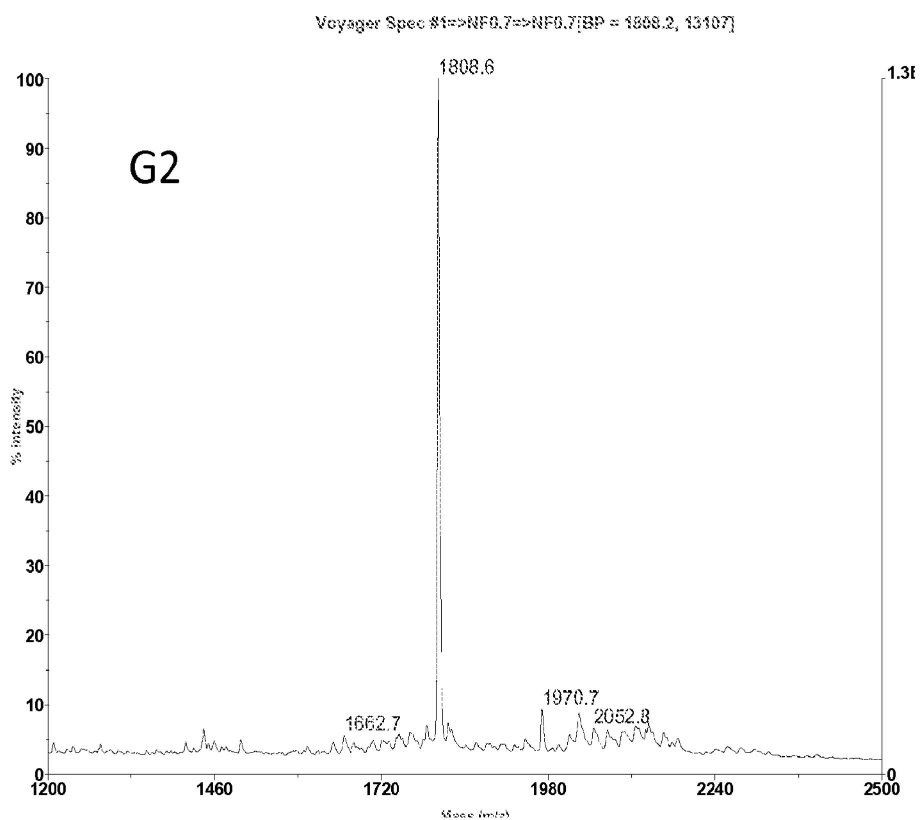
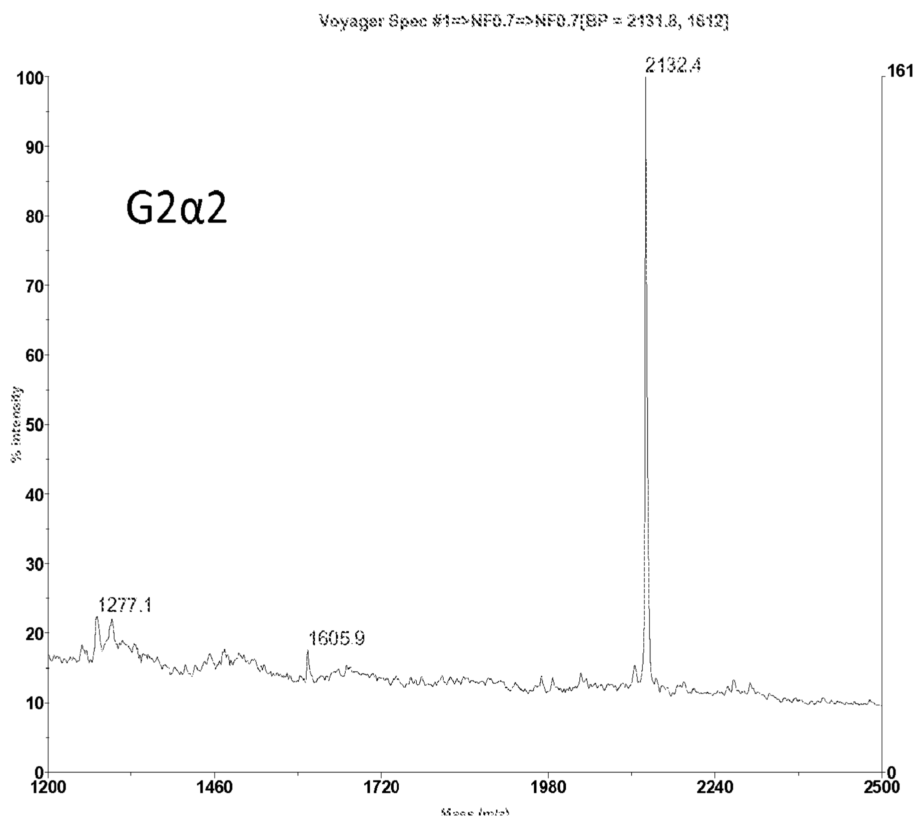


Fig. 3C



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 09/61810

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12P 19/18; C12N 9/24; C07G 3/00; C07H 1/00 (2009.01)
USPC - 435/97, 435/200, 435/72, 536/4.1, 536/123.1, 536/124
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC: 435/97, 435/200, 435/72, 536/4.1, 536/123.1, 536/124
IPC(8): C12P 19/18; C12N 9/24; C07G 3/00; C07H 1/00 (2009.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 435/193, 183, 74, 84, 100, 101

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic Databases Searched: PubWEST DB=PGPB,USPT,USOC,EPAB,JPAB; PLUR=NO; OP=ADJ, Google Scholar, Google Patent
Search Terms Used: oligosaccharid$, galactosyltransferase, UDP-galactose, IgG, GlcNac, mammalian, porcine, alpha-galactosylated oligosaccharides, Gal-alpha(1,3)-Gal-beta(1-4)GlcNac

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,922,577 A (Defrees et al.) 13 July 1999 (13.07.1999). Entire document, esp: abstract, col 1 ln 14-15; col 1 ln 51- col 2 ln 2; col 4 ln 13-17; col 9 ln 28-45; col 9 ln 52-55; col 9 ln 62-63; col 10 ln 46-58; col 10 ln 65 - col 11 ln 11; col 11 ln 19-29; col 11 ln 39-41; col 15 ln 52-59; col 21 ln 5-6; col 21 ln 11; col 23 ln 21.	1-11
A	US 6,406,894 B1 (Hoersch et al.) 18 June 2002 (18.06.2002) entire document.	1-11

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 November 2009 (22.11.2009)	09 DEC 2009

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 09/61810

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 12, 13
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.