(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 July 2017 (20.07.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/124084 A1

(51) International Patent Classification:
C07K 16/00 (2006.01) *A61K 47/50* (2017.01)
C12P 21/00 (2006.01)

(21) International Application Number:
PCT/US2017/013734

(22) International Filing Date:
17 January 2017 (17.01.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/279,087 15 January 2016 (15.01.2016) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU,

[Continued on next page]

(54) Title: ENDO-S2 MUTANTS AS GLYCOSYNTHASES, METHOD OF MAKING AND USE FOR GLYCOENGINEERING OF GLYCOPROTEINS

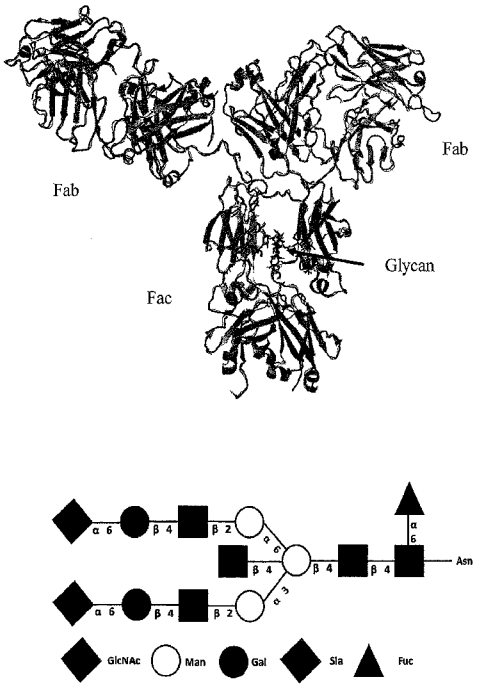

Figure 1

(57) Abstract: The present invention provides for recombinant Endo-S2 mutants (named Endo-S2 glycosynthases) that exhibit reduced hydrolysis activity and increased transglycosylation activity for the synthesis of glycoproteins wherein a desired sugar chain is added to a fucosylated or non-fucosylated GlcNAc-IgG acceptor. As such, the present invention allows for the synthesis and remodeling of therapeutic antibodies thereby providing for certain biological activities, such as, prolonged half-life time in vivo, less immunogenicity, enhanced in vivo activity, increased targeting ability, and/or ability to deliver a therapeutic agent.

WO 2017/124084 A1

TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

ENDO-S2 MUTANTS AS GLYCOSYNTHASES, METHOD OF MAKING AND USE FOR GLYCOENGINEERING OF GLYCOPRTEINS

GOVERNMENT RIGHTS IN INVENTION

[001] This invention was made with government support under Grant Numbers R01 GM096973 and R01 GM080374awarded by the National Institutes of Health. The government has certain rights in the invention.

CROSSREFERENCE TO RELATED APPLICATION

[002] The present application claims priority to co-pending U.S. Provisional Application No. 62/279,087 filed on January 15, 2016, the contents of the application is hereby incorporated by reference herein for all purposes.

BACKGROUND OF THE INVENTION

[003] Field of the Invention

[004] The invention relates to glycoprotein synthesis, and more particularly, to the use of a recombinant mutants Endo S2, an Endo-β-N-acetylglucosminidase from the strain NZ131 of serotype M49 of S*treptococcus pyogenes*, that possesses transglycosylation activity and limited hydrolyzing activity thereby providing for efficient glycosylation remodeling of antibodies.

[005] Description of the Related Art

[006] Monoclonal antibodies (mAbs) represent a major class of therapeutic proteins used for the treatment of cancers, inflammatory disorders, and infectious diseases [1-3]. Compelling experimental data have shown that glycosylation can have profound impacts on the stability, biological functions, and overall therapeutic efficacy of antibodies [4-7]. For example, core-fucosylation of Fc glycans could significantly reduce the antibody-dependent cellular cytotoxicity (ADCC), and antibodies with low content of core fucosylation have shown improved therapeutic efficacy in anti-cancer therapy [8,9]. On the other hand, it has

been reported that the terminal α-2,6-sialylated Fc glycoform, a minor component in the intravenous immunoglobulin (IVIG), is responsible for the anti-inflammatory activity of IVIG as demonstrated in animal models [10-13]. However, natural and recombinant antibodies are usually produced as heterogeneous glycoforms that are difficult to separate for further probing the structure-activity relationships of antibodies. Moreover, for a majority of anti-cancer mAbs on the market that rely on ADCC as a major mechanism of the therapeutic efficacy, the most active non-fucosylated glycoforms are usually present as minor fractions among the heterogeneous mixtures [8,9]. Thus, methods that can lead to the production of structural well defined, homogeneous glycoforms of antibodies are highly desirable for both functional studies and for the development of better antibody-based therapeutics. In parallel to the attempt to control glycosylation through host glycosylation pathway engineering [14-20], a chemoenzymatic glycosylation remodeling method, which involves endoglycosidase-catalyzed deglycosylation and subsequent transglycosylation of intact antibodies, has been emerging as a promising approach to obtain homogeneous antibody glycoforms [21]. It has been shown that the Fc glycans of recombinant IgG-Fc domain could be remodeled through the enzymatic deglycosylation - transglycosylation steps under the catalysis of an appropriate endoglycosidase, including Endo-A and Endo-D, without the need of denaturing the proteins [22-24]. In 2012, the first example of glycosylation remodeling of an intact therapeutic monoclonal antibody and IVIG, which was enabled by the discovery of glycosynthase mutants of Endo-S, an endoglycosidase from Streptococcus pyogenes [25]. In this approach, the heterogeneous Fc glycans of a monoclonal antibody such as rituximab are removed by Endo S catalyzed deglycosylation to yield the antibody protein backbone carrying only the α1,6- fucosylated GlcNAc acceptor at the glycosylation site. Then a desired N-glycan is transferred to the GlcNAc acceptor by an Endo-S glycosynthase mutant (Endo S-D233A or D233Q) in a site- and stereo-specific manner to reconstitute a homogeneous glycoform of the antibody. The Endo-S glycosynthase mutants have been used by several research groups for the synthesis of different homogeneous glycoforms of antibodies for structural and functional studies [26-31]. While the Endo-S glycosynthases were able to transfer biantennary complex type and modified Man3GlcNAc core, these mutants showed only marginal activity in transferring high-mannose type N-glycans. More recently, glycosynthase mutants from Endo-F3 have been generated, another GH18 family endoglycosidase [32]. It was found that the Endo-F3 glycosynthases, such as the D165A mutant, was able to transfer triantennary N-glycan to Fc domain of intact antibody, but they required α1,6-fucosylated GlcNAc moiety as the acceptor for transglycosylation and were unable to transfer to non-fucosylated GlcNAc

acceptors. These studies have demonstrated that the glycosynthases so far available still have limitations due to substrate specificity and also vary in transglycosylation efficiency.

[007] With an understanding of the shortcomings of other mutants, an effort to expand the scope of the glycosylation remodeling strategy, is described herein wherein attention is turned to Endo-S2, an endoglycosidase from Streptococcus pyogenes of serotype M49 [33,34]. Endo-S2 shows only 37% sequence identity to Endo-S from the same bacteria and demonstrates a broader glycan substrate specificity in Fc deglycosylation than Endo-S [34]. Data implicated that the wild type Endo-S2 can hydrolyze various types of N-glycans from antibody Fc domains than Endo-S. But it has been unknown whether Endo-S2 possesses transglycosylation activity and, if yes, whether Endo-S2 glycosynthases can be generated by site-directed mutagenesis.

[008] Thus, it would be advantageous to evaluate whether efficient glycosynthases could be generated from Endo-S2 and whether they would potentially have broader substrate specificity in transglycosylation than those previously reported.

SUMMARY OF THE INVENTION

[009] The present invention provides for recombinant Endo-S2 mutants (named Endo-S2 glycosynthases) that exhibit reduced hydrolysis activity and increased transglycosylation activity for the synthesis of glycoproteins wherein a desired sugar chain is added to a fucosylated or nonfucosylated GlcNAc-IgG acceptor. As such, the present invention allows for the synthesis and remodeling of therapeutic antibodies thereby providing for certain biological activities, such as, prolonged half-life time *in vivo*, less immunogenicity, enhanced *in vivo* activity, increased targeting ability, and/or ability to deliver a therapeutic agent. The mutant Endo-S2 glycosynthases of the present enable the glycosylation remodeling of therapeutic antibodies and Fc fragments thereof with increase number of diverse glycans such as high-mannose type and hybrid type glycans that cannot be achieved by the previously disclosed Endo-S mutants.

[0010] In one aspect, the present invention provides for transglycosylation activity of mutants of an endo-β-N-acetylglucosamindase of strain NZ131 of serotype M49 of *Streptococcus pyogenes* (Endo-S2) (SEQ ID NO: 1), wherein the mutants have at least 85%

homology thereto and exhibit transglycosylation activity on both fucosylated and nonfucosylated GlcNAc-IgG acceptors, wherein the endoglycosidases enable the transfer of an oligosaccharide (in the form of an activated sugar oxazoline) *en bloc* to a fucosylated or nonfucosylated GlcNAc-IgG to form a new glycoform of IgG.

[0011] In another aspect, the present invention provides for Endo-S2 mutant proteins that show remarkably enhanced transglycosylation efficiency and diminished or abrogated product hydrolytic activity relative to the wild type Endo S2. Mutants preferably include site-specific mutations including a mutation at Asp-184. The mutant proteins include, but are not limited to, D184A (SEQ ID NO: 2), D184N (SEQ ID NO: 3), D184Q (SEQ ID NO: 4), D184R (SEQ ID NO: 5), D184C (SEQ ID NO: 6), D184M (SEQ ID NO: 7), D184E (SEQ ID NO: 8), D184G (SEQ ID NO: 9), D184H (SEQ ID NO: 10), D184I (SEQ ID NO: 11), D184L(SEQ ID NO: 12), D184K(SEQ ID NO: 13), D184F (SEQ ID NO: 14), D184P(SEQ ID NO: 15), D184S(SEQ ID NO: 16), D184T (SEQ ID NO. 17); D184W(SEQ ID NO: 18), D184Y(SEQ ID NO: 19),D184V(SEQ ID NO: 20) or fragments thereof that include the catalytic domain and exhibit increased transglycosylation and reduced hydrolysis related to the wild type Endo-S2 protein. Preferably the mutant proteins include D184M (SEQ ID NO: 7), D184E (SEQ ID NO: 8) and D184Q (SEQ ID NO: 4).

[0012] Notably any Endo-S2 fragments and domains that carry a mutation at the D184 site are included in the present invention whereing any such domains and fragments may be fused to other proteins including, but not limited to, CPD, Fc, MBP, etc. The catalytic domain and the specific site of mutation are critical for the enzymatic activity, and thus, other sites on the enzyme may be modified or truncated, such as a terminus without affect much of the glycosynthase activity

[0013] In a further aspect, the present invention provides for a chemoenzymatic method for the preparation of a homogeneous fucosylated or nonfucosylated glycoforms of IgG antibodies, comprising:

a. providing an acceptor selected from the group consisting of a core fucosylated GlcNAc-IgG and nonfucosylated GlcNAc-IgG or corresponding IgG-Fc fragments; and

b. reacting the acceptor with a donor substrate including an activated oligosaccharide moiety, in the presence of a S. *pyogenes* Endo-S2 Asp-184 mutant protein or fragment thereof that includes the catalytic domain and exhibits increased transglycosylation and

reduced hydrolytic activity relative to the wild type Endo-S2 enzyme to transfer the activated oligosaccharide moiety to the acceptor and yield the homogeneous fucosylated or nonfucosylated glycoprotein.

[0014] In a still further aspect, the present invention provides a method for preparing a core-fucosylated IgG or IgG-Fc fragment having a predetermined oligosaccharide moiety, comprising:

a. providing a core-fucosylated acceptor protein comprising an asparagine-linked core-fucosylated N-acetylglucosamine (GlcNAc) residue; and

b. enzymatically reacting the core-fucosylated acceptor protein with an activated oligosaccharide donor in the presence of a Endoglycosidase-S2 D184 mutant protein or fragment thereof that includes the catalytic domain and exhibits increased transglycosylation and reduced hydrolytic activity relative to the wild type Endo-S2 enzyme, wherein the activated oligosaccharide donor carries an oligosaccharide moiety comprising a predetermined number and type of sugar residues, wherein the oligosaccharide moiety is covalently linked to the acceptor protein, thereby preparing the core-fucosylated IgG or IgG-Fc fragment having the predetermined oligosaccharide moiety.

[0015] In yet another aspect, the present invention provides for an activated oligosaccharide moiety, such as glycan or oligosaccharide oxazoline, glycosyl fluoride, glycosyl azide or an aryl glycoside, as a donor substrate for the synthesis of homogeneous core-fucosylated glycoproteins or nonfucosylated glycoproteins. Preferably the activated oligosaccharide moiety is an oligosaccharide oxazoline.

[0016] In a further aspect, the present invention relates to a chemoenzymatic method for the preparation of a homogeneous fucosylated or nonfucosylated glycoprotein, said method comprising:

a. providing an acceptor selected from core fucosylated or nonfucosylated GlcNAc-IgG or IgG-Fc fragments; and

b. reacting the acceptor with a donor substrate in the presence a S. *pyogenes* Endo-S Asp-184 mutant protein or fragment thereof that includes the catalytic domain and exhibits increased transglycosylation and reduced hydrolytic activity relative to the wild type Endo-S2 enzyme, wherein the donor substrate comprises a predetermined oligosaccharide component with a defined number and type of sugar residues and specific linkage types,

thereby providing the homogeneous fucosylated or nonfucosylated glycoprotein.

[0017] Notably the S. *pyogenes* Endo-S Asp-184 mutant protein or fragment thereof can include additional mutations in the amino acid residues but importantly carries a D184 mutation.

[0018] In one embodiment, the fucosylated GlcNAc containing protein is an alpha-1-6-fucosyl-GlcNAc- protein.

[0019] In another aspect, the present invention relates to a method of IgG or IgG-Fc glycoprotein remodeling with an oligosaccharide having a predetermined oligosaccharide component with a defined number and type of sugar residues and with specific linkage types, the method comprising:

a. providing a fucosylated glycoprotein substrate comprising Fc N-glycans;

b. treating the fucosylated glycoprotein substrate with an endo-enzyme to hydrolyze the bond between the two core GlcNAc residues in the N-glycans to yield a core-fucosylated or nonfucosylated GlcNAc-IgG or IgG-Fc fragments; and

c. attaching the oligosaccharide to the Asn-linked GlcNAc moiety in the presence of an Endo-S2 mutant having an amino acid sequence selected from the group consisting of SEQ ID NO: 2 to SEQ ID NO: 20 or fragment thereof that includes the catalytic domain and exhibits increased transglycosylation and reduced hydrolytic activity relative to the wild type Endo-S2 enzyme (SEQ ID NO: 1), thereby adding a predetermined the oligosaccharide component.

[0020] In a further aspect, the present invention relates to a method of fucosylated or nonfucosylated IgG or IgG-Fc fragment remodeling with an oligosaccharide having a predetermined oligosaccharide component with a defined number and type of sugar residues and with specific linkage types, the method comprising:

a. providing a fucosylated or nonfucosylated GlcNAc-IgG or IgG-Fc fragments obtained from natural or recombined sources carrying heterogeneous N-glycans;

b. treating the natural or recombinant IgG or IgG-Fc fragment with an endo-enzyme (a wild type endoglycosidase or a mutant endoglycosidase with efficient hydrolytic activity) to hydrolyze the bond between the two GlcNAc residues positioned closest to the peptide domain thereby forming a deglycosylated protein carrying the core fucosylated or

nonfucosylated GlcNAc-IgG or a IgG-Fc fragment; and

c. attaching the pre-determined oligosaccharide to the GlcNAc residue to reconstitute the natural beta-1,4-glycosidic bond through transglycosylation with a S. *pyogenes* Endo-S2 Asp-184 mutant enzyme or fragment thereof that includes the catalytic domain and exhibits increased transglycosylation and reduced hydrolytic activity relative to the wild type Endo-S2 enzyme, thereby adding a predetermined the oligosaccharide component.

[0021] Applicable oligosaccharide oxazolines include, but not limited to, high-mannose type, hybrid type, sialoglycan oxazoline and complex type N-glycan, as well as their selectively modified derivatives such as those with specific tags. Preferably, di-, tri-, tetra-, penta-, hexyl–, hepta-, octyl-, nona-, deca-, or undeca-saccharide oxazolines are utilized as donor substrates for a highly efficient chemoenzymatic synthesis of homogeneous core fucosylated or nonfucosylated IgG antibodies and IgG-Fc fragments.

[0022] In yet another aspect, the present invention relates to a method of synthesis of a modified antibody or fragment thereof, the method comprising;

a. using a naturally existing IgG antibody or a recombinant antibody or Fc domains carrying Fc N-glycans as precursors;

b. Fc deglycosylating using an endoglycosidase such as a wild Endo-S2 to deglycosylate the Fc domain to form a GlcNAc- acceptor; wherein the GlcNAc moiety is positioned on the Fc region of the antibody and the GlcNAc moiety is either core fucosylated or nonfucosylated; and

c. transglycosylating the GlcNAc moiety in the antibody with an oligosaccharide oxazoline or a sialoglycan oxazoline having a predetermined number of sugar residues under the catalysis of an enzyme selected from the group consisting of Endo-S2 mutants SEQ ID NO: 2 to SEQ ID NO: 20 or fragment thereof that includes the catalytic domain and exhibits increased transglycosylation and reduced hydrolytic activity relative to the wild type Endo-S2 enzyme to form the modified antibody with the predetermined number of saccharides.

[0023] In yet another aspect, the present invention provides a method of remodeling intravenous immunoglobulins (IVIG) exhibiting Fc-sialylated glycoforms, the method comprising:

a. providing an IVIG carrying Fc N-glycans;

b. Fc deglycosylating the Fc N-glycans using an endoglycosidase including Endo-S to form a GlcNAc- acceptors; wherein the GlcNAc-acceptors are positioned on the Fc region of the IVIG and the GlcNAc-acceptors are either fucosylated or nonfucosylated; and

c. transglycosylating the GlcNAc-acceptors with sialoglycan oxazoline having a predetermined number of sugar residues under the catalysis of an enzyme selected from the group consisting of Endo-S2 mutants SEQ ID NO: 2 to SEQ ID NO: 20 or fragment thereof that includes the catalytic domain and exhibits increased transglycosylation and reduced hydrolytic activity relative to the wild type Endo-S2 enzyme to form a sialylated IVIG.

[0024] Another aspect of the present invention provides for an IVIG preparation containing composition comprising at least 90% of homogeneous sialylated Fc glycoforms to increase anti-inflammatory activity, wherein the sialylated Fc glycoforms are synthesized using a *Streptococcus pyogenes* Endo-S2 Asp-184 mutant in combination with a GlcNAc moiety positioned on the Fc region of a deglycosylated IVIG and a sialoglycan oxazoline having a predetermined number of sugar residues.

[0025] In a still further aspect, the present invention relates to a method of synthesizing homogeneous core fucosylated or nonfucosylated IgG antibodies or IgG-Fc fragments, the method comprising:

a. providing a natural or recombinant IgG antibody or IgG-Fc fragment, wherein the recombinant IgG or IgG-Fc is produced from a typical protein expression system, including but not limited to yeast, insect, plant, and any mammalian expression system;

b. removing the N-glycans by an enzyme selected from the group consisting of Endo-H, Endo-A, Endo-S, Endo S2 (WT) and/or Endo-F3 to form a core fucosylated or nonfucosylated GlcNAc-containing protein;

c. providing a sugar oxazoline or sialoglycan oxazoline with a desired oligosaccharide component comprising a defined number and type of sugar residues in the chain; and

d. enzymatically transglycosylating the fucosylated or nonfucosylated GlcNAc-containing protein with a sugar oxazoline having a desired number of sugar residues or sialoglycan oxazoline having a desired number of sugar and sialic acid residues, with an endoglycosidase selected from the group consisting of a *Streptococcus pyogenes* Endo-S2 Asp-184 mutant enzyme or fragment thereof that exhibits increased transglycosylation and reduced hydrolytic activity relative to the wild type Endo-S2 enzyme, thereby forming a homogeneous core fucosylated or nonfucosylated IgG antibody or IgG-Fc fragment having an

extension of desired number of sugar residues and/or sialic acid.

[0026] It is envisioned that the oligosaccharide oxazoline or sialoglycan oxazoline having a predetermined oligosaccharide component with a defined number and type of sugar residues may further comprises an additional moiety or tag including, a therapeutic agent or drug such as for treating cancer, HIV or other viruses, substances that activates receptors on the cell plasma membrane, agents that affects intracellular chemistry, agents that affects cellular physics, genes, gene analogs, RNA, RNA analogs, DNA, DNA analogs, amino acid sequences of surface receptors such as CCR5 or CD4, antigenic structure having affinity for a specific antibody; amino acid sequences of receptor ligands such as gp120, gp41 or gp160, receptor antagonists, receptor blockers, enzymes, enzyme substrates, enzyme inhibitors, enzyme modulators, therapeutic proteins, protein analogs, metabolites, metabolite analogs, oligonucleotides, oligonucleotide analogs, antigens, antigen analogs, antibodies or fragments thereof, antibody analogs, an antibody different from the modified antibody which is reactive to another receptor bacteria, viruses, inorganic ions, metal ions, metal clusters, polymers, fluorescent compounds and any combinations thereof.

[0027] As such, the present invention further provides a delivery device for delivering a drug or therapeutic agent having biological activity to treat a condition, the delivery device comprising: a remodeled IgG or IgG-Fc fragment having a predetermined sugar chain or sialoglycan and a therapeutic agent or drug attached to the terminal sugar residue or sialic acid.

[0028] The present invention envisions modifying monoclonal antibodies related to HIV including, but not limited to 17b, 48d, A32, C11, 2G12, F240, IgG1b12, 19e, X5, TNX-355 and F91, all of which are commercially available.

[0029] Further antibodies related to cancer or other diseases may also be remodeled for individual fit to certain receptors thereby increasing biological activity, the monoclonal antibodies may include, but are not limited to, cetuximab, rituximab, muromonab-CD3, abciximab, daclizumab, basiliximab, palivizumab, infliximab, trastuzumab, gemtuzumab ozogamicin, alemtuzumab, ibritumomab tiuxetan, adalimumab, omalizumab, tositumomab, I-131 tositumomab, efalizumab, bevacizumab, panitumumab, pertuzumab, natalizumab, etanercept, IGN101 (Aphton), volociximab (Biogen Idec and PDL BioPharm), Anti-CD80

mAb (Biogen Idec), Anti-CD23 mAb (Biogen Idel), CAT-3888 (Cambridge Antibody Technology), CDP-791 (Imclone), eraptuzumab (Immunomedics), MDX-010 (Medarex and BMS), MDX-060 (Medarex), MDX-070 (Medarex), matuzumab (Merck), CP-675,206 (Pfizer), CAL (Roche), SGN-30 (Seattle Genetics), zanolimumab (Serono and Genmab), adecatumumab (Sereno), oregovomab (United Therapeutics), nimotuzumab (YM Bioscience), ABT-874 (Abbott Laboratories), denosumab (Amgen), AM 108 (Amgen), AMG 714 (Amgen), fontolizumab (Biogen Idec and PDL BioPharm), daclizumab (Biogent Idec and PDL BioPharm), golimumab (Centocor and Schering-Plough), CNTO 1275 (Centocor), ocrelizumab (Genetech and Roche), HuMax-CD20 (Genmab), belimumab (HGS and GSK), epratuzumab (Immunomedics), MLN1202 (Millennium Pharmaceuticals), visilizumab (PDL BioPharm), tocilizumab (Roche), ocrerlizumab (Roche), certolizumab pegol (UCB, formerly Celltech), eculizumab (Alexion Pharmaceuticals), pexelizumab (Alexion Pharmaceuticals and Procter & Gamble), abciximab (Centocor), ranibizimumab (Genetech), mepolizumab (GSK), TNX-355 (Tanox), or MYO-029 (Wyeth).

[0030] A still further aspect of the invention relates to a method of remodeling an antibody which initially includes a heterogeneous sugar chain, the method comprising:

a. removing the heterogeneous sugar chain from the antibody with an endoglycosidase to leave a single fucosylated- or nonfucosylated-GlcNAc moiety attached to an original glycosylation site; and

b. transferring a core oligosaccharide or sialoglycan oxazoline with at least one tag to the fucosylated- or –nonfucosylated GlcNAc moiety by an endoglycosidase catalyzed transglycosylation to yield a tagged antibody, wherein the endoglycosidase is selected from the group consisting of Endo-S2 mutants SEQ ID NO: 2 to SEQ ID NO: 20 or fragment thereof that includes the catalytic domain and exhibits increased transglycosylation and reduced hydrolytic activity relative to the wild type Endo-S2 enzyme.

[0031] The tag moiety may include, but is not limited to, antigens, therapeutic drugs such as for cancer or HIV, toxins, fluorescent probes, biotin, PEG species, lipids, or nucleotides.

[0032] In yet another aspect, the present invention provides for Antibody-drug conjugates or ADCs wherein the antibody can be modified according to the present invention and are designed as a targeted therapy for the treatment of people with cancer. Specifically, an ADC contains two parts: a monoclonal antibody and a small amount of a highly potent cytotoxic

drug, linked to the antibody. When the ADC's antibody binds with a particular receptor on the target cell's surface, the linkage breaks, and the ADC releases a lethal toxin into the cell. Specific monoclonal antibodies are described herein later and also possible cytotoxic drugs. Thus, the present invention provides for a modified antibody further comprising an additional moiety including, a therapeutic agent for treating cancer such as a chemokine and/or a cytokine thereby forming an Antibody-drug conjugate (ADC).

[0033] In another aspect, the present invention provides for a composition comprising at least one S*treptococcus pyogenes* Endo-S2 mutant selected from the group consisting of D184M (SEQ ID NO:7) and D184Q (SEQ ID No: 4).

[0034] In yet another aspect, the present invention provides a substantially homogeneous preparation of core fucosylated or nonfucosylated antibody or Fc fragment thereof having a predetermined oligosaccharide moiety, wherein the substantially homogeneous preparation is produced by any of the aforementioned methods. Also provided are compositions comprising such homogeneous preparations.

[0035] In another aspect, the present invention provides for a method of treatment using a remodeled antibody having a desired glycosylation state and/or sialylated form in an amount sufficient to modulate biological activity in the treated subject.

[0036] In a further aspect, the present invention provides for a kit including a least one S*treptococcus pyogenes* Endo-S2 mutant selected from the group consisting of SEQ ID NO: 2 to 20 and preferably D184M (SEQ ID NO:7), D184E (SEQ ID NO: 8) and D184Q (SEQ ID NO: 4).

[0037] Other aspects, features and embodiments of the invention will be more fully apparent from the ensuing disclosure and appended claims.

BRIEF DESCRIPTION OF THE FIGURES

[0038] Figure 1 shows the structures of a typical IgG antibody and the Fc N-glycans. a) Alpha backbone structure of human IgG showing functional regions (PDB code 1HZH): b)

The structure of a full-length bi-antennary complex type N-glycan attached to the Asn-297 in the Fc domain

[0039] Figure 2 shows the sequence alignment of Endo S2 (SEQ ID NO: 1) and Endo S (SEQ ID NO: 21). The aspartic acid residue (D233 of Endo-S and D184 of Endo-S2) critical for promoting oxazolinium ion formation in hydrolysis and the catalytic general acid/base residue (E235 of Endo-S and E186 of Endo-S2) are marked.

[0040] Figure 3 shows the scheme for glycosylation remodeling of fucosylated rituximab to homogeneous complex, high mannose, and hybrid glycoforms.

[0041] Figure 4 shows the SDS-PAGE and ESI-MS analysis of the glycosylation remodeling of rituximab. (A) SDS-PAGE analysis: Lane 1, commercial rituximab; Lane 2, EndoS2 de-glycosylated rituximab (**1**); Lane 3, transglycosylation product of complex glycoform (**5**) from the EndoS2-D184Q catalyzed reaction between (**1**) and sialo complex glycan oxazoline (**2**); Lane 4, transglycosylation product of Man9GlcNAc (**6**) from the EndoS2-D184A catalyzed reaction of (**1**) and (**3**); lane 5, the transglycosylation product of sialo hybrid glycoform (**7**) from the EndoS2-D184A catalyzed reaction between the deglycosylated rituximab (**1**) and hybrid glycan oxazoline (**4**); (B) ESI-MS (after deconvolution) of the light chain of the commercial rituximab; (C) ESI-MS (after deconvolution) of the heavy chain of the commercial rituximab; (D) ESI-MS of the de-glycosylated rituximab (**1**). (E) ESI-MS of the transglycosylation complex type product (**5**). (F) ESI-MS of the transglycosylation Man9 product (**6**). (G) ESI-MS of the transglycosylation sialo hybrid type product (**7**).

[0042] Figure 5 shows the scheme for enzymatic remodeling to non-fucosylated homogeneous complex, high mannose and hybrid glycoform of rituximab.

[0043] Figure 6 shows SDS-PAGE and ESI-MS analysis of glycoengineering of rituximab to the non-fucosylated G2 glycoform. (A) SDS-PAGE analysis: Lane 1, commercial rituximab; Lane 2, the EndoS2 de-glycosylated rituximab (**1**); Lane 3, the defucosylated product (**8**); Lane 4, the glycoengineered complex glycoform from the EndoS2-D184N catalyzed reaction (**9**); Lane 5, the glycoengineered Man9 glycoform from the EndoS2-D184N catalyzed reaction (**10**); Lane 6, the glycoengineered sialo hybrid glycoform from the EndoS2-D184A catalyzed reaction (**11**) (B) ESI-MS (after deconvolution) of the heavy chain of the

defucosylated rituximab (**8**). (C) ESI-MS of the heavy chain of the glycoengineered complex type rituximab (**9**). (D) ESI-MS of the heavy chain of the glycoengineered Man9 rituximab (**10**). (E) ESI-MS of the heavy chain of the glycoengineered sialo hybrid type rituximab (**11**).

[0044] Figure 7 shows the comparison of transglycosylation activity of alanine, asparagine, glutamine mutants of EndoS2 D184 of complex type glycan oxazoline to fucosylated rituximab.

[0045] Figure 8 show a schematic presentation of the hydrolysis and transglycosylation by Endo-S2 and its mutants using rituximab as the substrate. Fuc α1,6GlcNAc-rituximab, the deglycosylated rituximab carrying the core-fucosylated GlcNAc moiety at the glycosylation site; SCT-rituximab; the sialyl complex type glycoform of rituximab.

[0046] Figure 9 shows the evaluation of substrate specificity of Endo-S2 mutants on various glycans (HM, CT, and hybrid type). GlcNAc-rituximab, the rituximab glycoform carry only the first GlcNAc moiety at the Fc glycosylation site; Fuc α1,6GlcNAc-rituximab, the deglycosylated rituximab carrying the core fucosylated GlcNAc moiety at the glycosylation site; SCT-rituximab; the sialyl complex type glycoform of rituximab; HM-rituximab, the high-mannose type glycoform of rituximab; Hyb-rituximab, the hybrid type glycoform of rituximab.

[0047] Figure 10 shows the ESI-MS analysis of the glycosylation remodeling of rituximab using Endo-S2 D184M. A) ESI-MS (after deconvolution) of the heavy chain of the commercial rituximab; B) ESI-MS of the heavy chain of the Fuc α1,6GlcNAc-rituximab (**2**); C and D) ESI-MS of the heavy chain and light chain of transglycosylation product (**3**) (SCT-rituximab), respectively; E and F) ESI-MS of the heavy chain and light chain of transglycosylation product (**8**) (HM-rituximab); G and H) ESI-MS of the heavy chain and light chain of transglycosylation product (**6**) (Hyb-rituximab); I and J) ESI-MS of the heavy chain and light chain of transglycosylation product (**11**) (non-fucosylated rituximab).

[0048] Figure 11 shows a comparison of transglycosylation efficiency of different types of glycans by Endo-S2 D184M mutant. The transglycosylation reaction was carried out using deglycosylated rituximab (**2**) as the acceptor and different types of glycan oxazolines as the donor substrate under the catalysis of Endo-S2 D184M (0.05 mg/ml). The molar ratio of

donor to acceptor was 20:1. The data sets presented here are representative of two independent experiments.

[0049] Figure 12 show a comparison of the transglycosylation with SCT by Endo-S2 D184Q, Endo-S2 D184M, and Endo-S D233Q. The transglycosylation was performed using deglycosylated rituximab (**2**) as the acceptor and SCT glycan oxazoline (**4**) as the donor substrate under the catalysis of different endoglycosidase mutants at a fixed concentration of 0.05 mg/ml. The molar ratio of donor to acceptor was 20:1. The data sets presented here are representative of two independent experiments.

[0050] Figure 13 shows glycosylation remodeling of herceptin (trastuzumab) using a pair of Endo-S2 enzymes (Endo-S2 WT and Endo-S2 D184M mutant) and A) ESI-MS of the heavy chain of commercial trastuzumab (Herceptin); B) ESI-MS of the heavy chain of deglycosylated herceptin (**12**); C) ESI-MS of the heavy chain of transglycosylation product 13 (S2G2F-trastuzumab); D) ESI-MS of the light chain of transglycosylation product (**13**); E) ESI-MS of the heavy chain of transglycosylation product (**13**) after PNGase F catalyzed deglycosylation.

[0051] Figure 14 shows Fc glycoengineering of IVIG for improved anti-inflammatory activity of IVIG.

DETAILED DESCRIPTION OF THE INVENTION

[0052] The present invention provides for novel glycosynthase EndoS2 Asp 184 mutants that show remarkable transglycosylation efficiency capable of transferring complex, high mannose, and Hybrid type N-glycans from activated glycan oxazolines to deglycosylated intact antibodies without product hydrolysis. It has been found herein that the glycosynthase EndoS2 Asp 184 mutants acted efficiently on both core-fucosylated and nonfucosylated GlcNAc-Fc domain of intact antibodies to provide various defined IgG glycoforms. As described herein the Endo-S2 did possess potent transglycosylation activity, and the systematic site-directed mutagenesis led to the discovery of several glycosynthase mutants, including D184M and D184Q, that showed remarkable transglycosylation activity without apparent product hydrolysis activity. Moreover, it is shown herein that the Endo-S2 glycosynthases demonstrated remarkably relaxed substrate specificity, being capable of

transferring three major types (complex, high-mannose, and hybrid type) of N-glycans for antibody glycosylation remodeling. Further, as further described herein, it was found that the Endo-S2 glycosynthase mutants were much more active in general than the Endo-S mutants for transglycosylation. A highly efficient glycosylation remodeling of two therapeutic monoclonal antibodies, rituximab and trastuzumab (Herceptin), is described. Further, antibodies and intravenous immunoglobulins were transformed into Fc fully sialylated glycoforms having increased anti-inflammatory activity. Still further, the present invention provides for a homogeneous nonfucosylated glycoform having increased ADCC activity with enhanced FcγIIIa receptor-binding activity and azido-tagged glycoforms that can be further transformed into other glycoforms.

[0053] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of immunology, molecular biology, microbiology, cell biology and recombinant DNA, which are within the skill of the art. See, e.g., Sambrook, et al. MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel, et al. eds., (1987)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.): PCR 2: A PRACTICAL APPROACH (M. J. MacPherson, B. D. Hames and G. R. Taylor eds. (1995)), Harlow and Lane, eds. (1988) ANTIBODIES, A LABORATORY MANUAL, and ANIMAL CELL CULTURE (R. I. Freshney, ed. (1987)).

[0054] It is understood that aspects of the present invention described herein include "consisting" and/or "consisting essentially of" aspects.

[0055] Definitions

[0056] As used in the specification herein, “a” or “an” may mean one or more. As used herein in the claim(s), when used in conjunction with the word “comprising”, the words “a” or “an” may mean one or more than one. As used herein “another” may mean at least a second or more.

[0057] As used herein, “biological activity” refers to pharmacodynamic and pharmacokinetic properties including, for example, molecular affinity or resultant biochemical or physiological effect, receptor affinity or resultant biochemical or physiological effect, non-

receptor affinity or biochemical or physiological effect, efficacy, bioavailability, absorption, distribution, metabolism, or elimination.

[0058] As used herein, "sugar" refers to an oxidized or unoxidized carbohydrate-containing molecule, including, but not limited to, a monosaccharide, disaccharide, trisaccharide, oligosaccharide, or polysaccharide, including, for example, N-acetylglucosamine, mannose, galactose, N-acetylneuraminic acid (sialic acid), glucose, fructose, fucose, sorbose, rhamnose, mannoheptulose, N-acetylgalactosamine, dihydroxyacetone, xylose, xylulose, arabinose, glyceraldehyde, sucrose, lactose, maltose, trehalose, cellobiose or any combination thereof of the L- or D-isomer. Sugar further refers to, such molecules produced naturally, recombinantly, synthetically, and/or semi-synthetically.

[0059] As used herein, "homogenous" refers to core-fucosylated glycoproteins or nonfucosylated glycoproteins wherein the oligosaccharide component comprises at least 75%, more preferably at least 90%, and most preferably at least 95% of the same number and types of sugar residues.

[0060] As used herein, "protein" or "glycoprotein" is interchangeable with the terms peptide and glycopeptide.

[0061] As used herein, "homology" refers to amino acid sequence having substantial identity or similarity between two polypeptides and having at least 85%, and more preferably at least 95% similarity to a reference polypeptide. For polypeptides, the length of comparison to obtain the above-described percent homologies between sequences will generally be at least 25 amino acids, alternatively at least 50 amino acids, more likely at least 100 amino acids, and most likely 200 amino acids or more. Substantially identity or homologous polypeptides include additions, truncations, internal deletions or insertions, conservative and non-conservative substitutions, or other modifications located at positions of the amino acid sequence which do not destroy the function of the endoglycosidase. Those of skill in the art will recognize the numerous amino acids that can be modified or substituted with other chemically similar residues without substantially altering activity.

[0062] As used herein, "modulates" refers to an increase or decrease in "biological activity", as defined above, when comparing to a glycosylation-engineered antibody of the present invention to a non-glycosylation-engineered antibody.

[0063] As used herein, "immunoglobulin molecule" or "antibodies," refers to molecules that contain an antigen binding site which specifically binds an antigen or an Fc region that binds to cell receptors. Structurally, the simplest naturally occurring antibody (e.g., IgG) comprises four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. The natural immunoglobulins represent a large family of molecules that include several types of molecules, such as IgD, IgG, IgA, IgM and IgE. The term also encompasses hybrid antibodies, or altered antibodies, and fragments thereof, including but not limited to Fab fragment(s) and Fc fragment(s).

[0064] Antibodies can be fragmented using conventional techniques as described herein and the fragments screened for utility in the same manner as described for whole antibodies. A Fab fragment of an immunoglobulin molecule is a multimeric protein consisting of the portion of an immunoglobulin molecule containing the immunologically active portions of an immunoglobulin heavy chain and an immunoglobulin light chain covalently coupled together and capable of specifically combining with an antigen. Fab and Fc fragments can be prepared by proteolytic digestion of substantially intact immunoglobulin molecules with papain using methods that are well known in the art. However, a Fab or Fc fragment may also be prepared by expressing in a suitable host cell the desired portions of immunoglobulin heavy chain and immunoglobulin light chain using methods known in the art.

[0065] As used herein, with respect to antibodies, "substantially pure" means separated from those contaminants that accompany it in its natural state or those contaminants generated or used in the process of the obtaining the antibody. This term further includes the desired product having a single glycosylation state, whether or not this state includes glycosylation at a single site or multiple sites. Typically, the antibody is substantially pure when it constitutes at least 60%, by weight, of the antibody in the preparation. For example, the antibody in the preparation is at least about 75%, in certain embodiments at least about 80%, in certain embodiments at about 85%, in certain embodiments at least about 90%, in certain embodiments at least about 95%, and most preferably at least about 99%, by weight, of the

desired antibody. A substantially pure antibody includes a naturally, recombinantly, or synthetically produced antibody.

[0066] As used herein, "therapeutically effective amount" refers to an amount that results in an improvement or remediation of the symptoms of the disease or condition.

[0067] Antigens useful for attachment as a tag to a modified fucosylated or nonfucosylated glycoprotein of the present invention and more preferably an antibody or fragment thereof may be a foreign antigen, an endogenous antigen, fragments thereof, or variants having the same functional activity.

[0068] As used herein, "endogenous antigen" refers to a protein or part thereof that is naturally present in the recipient animal cell or tissue, such as a cellular protein, an immunoregulatory agent, or a therapeutic agent.

[0069] As used herein, "foreign antigen" refers to a protein or fragment thereof, which is foreign to the recipient animal cell or tissue including, but not limited to, a viral protein, a parasite protein, an immunoregulatory agent, or a therapeutic agent.

[0070] The foreign antigen may be a protein, an antigenic fragment or antigenic fragments thereof that originate from viral and parasitic pathogens.

[0071] Alternatively, the foreign antigen may be encoded by a synthetic gene and may be constructed using conventional recombinant DNA methods; the synthetic gene may express antigens or parts thereof that originate from viral and parasitic pathogens. These pathogens can be infectious in humans, domestic animals or wild animal hosts.

[0072] The foreign antigen can be any molecule that is expressed by any viral or parasitic pathogen prior to or during entry into, colonization of, or replication in their animal host.

[0073] The viral pathogens, from which the viral antigens are derived include, but are not limited to, Orthomyxoviruses, such as influenza virus (Taxonomy ID: 59771); Retroviruses, such as RSV, HTLV-1 (Taxonomy ID: 39015) and HTLV-II (Taxonomy ID: 11909); Herpes viruses, such as EBV (Taxonomy ID: 10295), CMV (Taxonomy ID: 10358) or herpes

simplex virus (ATCC #: VR-1487); Lentiviruses, such as HIV-1 (Taxonomy ID: 12721) and HIV-2 Taxonomy ID: 11709); Rhabdoviruses, such as rabies; Picornoviruses, such as Poliovirus (Taxonomy ID: 12080); Poxviruses, such as vaccinia Taxonomy ID: 10245); Rotavirus Taxonomy ID: 10912); and Parvoviruses, such as adeno-associated virus 1 (Taxonomy ID: 85106).

[0074] Examples of viral antigens include, but are not limited to, the human immunodeficiency virus antigens Nef (National Institute of Allergy and Infectious Disease HIV Repository Cat. # 183; GenBank accession # AF238278), Gag, Env (National Institute of Allergy and Infectious Disease HIV Repository Cat. # 2433; GenBank accession # U39362), Tat (National Institute of Allergy and Infectious Disease HIV Repository Cat. # 827; GenBank accession # M13137), Rev (National Institute of Allergy and Infectious Disease HIV Repository Cat. # 2088; GenBank accession # L14572), Pol (National Institute of Allergy and Infectious Disease HIV Repository Cat. # 238; GenBank accession # AJ237568) and T cell and B cell epitopes of gp120; the hepatitis B surface antigen (GenBank accession # AF043578); rotavirus antigens, such as VP4 (GenBank accession # AJ293721) and VP7 (GenBank accession # AY003871); influenza virus antigens, such as hemagglutinin (GenBank accession # AJ404627); nucleoprotein (GenBank accession # AJ289872); and herpes simplex virus antigens, such as thymidine kinase (GenBank accession # AB047378).

[0075] The bacterial pathogens, from which the bacterial antigens are derived, include but are not limited to, *Mycobacterium spp.*, *Helicobacter pylori*, *Salmonella spp.*, *Shigella spp.*, *E. coli*, *Rickettsia spp.*, *Listeria spp.*, *Legionella pneumoniae*, *Pseudomonas spp.*, *Vibrio spp.*, and *Borellia burgdorferi*.

[0076] Examples of protective antigens of bacterial pathogens include the somatic antigens of enterotoxigenic *E. coli*, such as the CFA/I fimbrial antigen and the nontoxic B-subunit of the heat-labile toxin; pertactin of *Bordetella pertussis*, adenylate cyclase-hemolysin of *B. pertussis*, fragment C of tetanus toxin of *Clostridium tetani*, OspA of *Borellia burgdorferi*, protective paracrystalline-surface-layer proteins of *Rickettsia prowazekii* and *Rickettsia typhi*, the listeriolysin (also known as "Llo" and "Hly") and/or the superoxide dismutase (also known as "SOD" and "p60") of *Listeria monocytogenes*; the urease of *Helicobacter pylori*, and the receptor-binding domain of lethal toxin and/or the protective antigen of *Bacillus anthrax* .

[0077] Example of antigens from biological weapons or pathogens include, but are not limited to, smallpox, anthrax, tularemia, plague, listeria, brucellosis, hepatitis, vaccinia, mycobacteria, coxsackievirus, tuberculosis, malaria, erhlichosis and bacterial meningitis.

[0078] The parasitic pathogens, from which the parasitic antigens are derived, include but are not limited to, *Plasmodium spp.*, such as Plasmodium falciparum (ATCC#: 30145); *Trypanosome spp.*, such as Trypanosoma cruzi (ATCC#: 50797); *Giardia spp.*, such as Giardia intestinalis (ATCC#: 30888D); *Boophilus spp.*; *Babesia spp.*, such as Babesia microti (ATCC#: 30221); *Entamoeba spp.*, such as Entamoeba histolytica (ATCC#: 30015); *Eimeria spp.*, such as Eimeria maxima (ATCC# 40357); *Leishmania spp.*, (Taxonomy ID: 38568); *Schistosome spp.*, such as Schistosoma mansoni (GenBank accession # AZ301495); *Brugia spp.*, such as Brugia malayi (GenBank accession # BE352806); *Fascida spp.*, such as Fasciola hepatica (GenBank accession # AF286903); *Dirofilaria spp.*, such as Dirofilaria immitis (GenBank accession # AF008300); *Wuchereria spp.*, such as Wuchereria bancrofti (GenBank accession # AF250996); and *Onchocerea spp;* such as Onchocerca volvulus (GenBank accession # BE588251).

[0079] Examples of parasite antigens include, but are not limited to, the pre-erythrocytic stage antigens of *Plasmodium spp.* such as the circumsporozoite antigen of *P. falciparum* (GenBank accession # M22982) *P vivax* (GenBank accession # M20670); the liver stage antigens of *Plasmodium spp*, such as the liver stage antigen 1 (as referred to as LSA-1; GenBank accession # AF086802); the merozoite stage antigens of *Plasmodium spp*; such as the merozoite surface antigen-1 (also referred to as MSA-1 or MSP-1; GenBank accession # AF199410); the surface antigens of *Entamoeba histolytica* , such as the galactose specific lectin (GenBank accession # M59850) or the serine rich *Entamoeba histolytica* protein; the surface proteins of *Leishmania spp*, such as 63 kDa glycoprotein (gp63) of *Leishmania major* (GenBank accession # Y00647 or the 46 kDa glycoprotein (gp46) of *Leishmania major*; paramyosin of *Brugia malayi* (GenBank accession # U77590; the triose-phosphate isomerase of *Schistosoma mansoni* (GenBank accession # W06781; the secreted globin-like protein of *Trichostrongylus colubriformis* (GenBank accession # M63263; the glutathione-S-transferases of *Fasciola hepatica* (GenBank accession # M77682; *Schistosoma bovis* (GenBank accession # M77682); *S. japonicum* (GenBank accession # U58012; and KLH of *Schistosoma bovis* and *S. japonicum* (Bashir, et al., *supra*).

[0080] Examples of tumor specific antigens include prostate specific antigen (PSA), TAG-72 and CEA; human tyrosinase (GenBank accession # M27160); tyrosinase-related protein (also referred to as TRP; GenBank accession # AJ132933); and tumor-specific peptide antigens.

[0081] Examples of transplant antigens include the CD3 molecule on T cells and histocompatibility antigens such as HLA A, HLA B, HLA C, HLA DR and HLA .

[0082] Examples of autoimmune antigens include IAS β chain, which is useful in therapeutic vaccines against autoimmune encephalomyelitis (GenBank accession # D88762); glatamic acid decarboxylase, which is useful in therapeutic vaccines against insulin-dependent type 1 diabetes (GenBank accession # NM013445); thyrotropin receptor (TSHr), which is useful in therapeutic vaccines against Grave's disease (GenBank accession # NM000369) and tyrosinase-related protein 1, which is useful in therapeutic vaccines against vitiligo (GenBank accession # NM000550).

[0083] HIV drugs that may be used in the construction of the tagged antibodies or fragments thereof include, but are not limited to antiviral agents such as nucleoside RT inhibitors, CCR5 inhibitors/antagonists, viral entry inhibitors and their functional analogs. Specifically, an antiviral agent may nucleoside RT inhibitors, such as Zidovudine (ZDV, AZT), Lamivudine (3TC), Stavudine (d4T), Didanosine (ddl), Zalcitabine (ddC), Abacavir (ABC), Emirivine (FTC), Tenofovir (TDF), Delaviradine (DLV), Efavirenz (EFV), Nevirapine (NVP), Saquinavir (SQV), Ritonavir (RTV), Indinavir (IDV), Nelfinavir (NFV), Amprenavir (APV), Lopinavir (LPV), Atazanavir, Combivir (ZDV/3TC), Kaletra (RTV/LPV), Trizivir (ZDV/3TC/ABC);

[0084] CCR5 inhibitors/antagonists, such as SCH-C, SCH-D, PRO 140, TAK 779, TAK-220, RANTES analogs, AK602, UK-427, 857, monoclonal antibodies; and viral entry inhibitors, such as Fuzeon (T-20) (enfuvirtide), NB-2, NB-64, T-649, T-1249, SCH-C, SCH-D, PRO 140, TAK 779, TAK-220, RANTES analogs, AK602, UK-427, 857; and functional analogs or equivalents thereof.

[0085] It is envisioned that many different fucosylated glycoproteins and nonfucosylated glycoproteins can be modified according to the methods of the present invention or used as a

therapeutic agent for conjugation to a terminal sugar including but not limited to, adrenocorticotropic hormone (ACTH); adrenocorticotropic hormone derivatives (e.g., ebiratide); angiotensin; angiotensin II; asparaginase; atrial natriuretic peptides; atrial sodium diuretic peptides; bacitracin; beta-endorphins; blood coagulation factors VII, VIII and IX; blood thymic factor (FTS); blood thymic factor derivatives; bombesin; bone morphogenic factor (BMP); bone morphogenic protein; bradykinin; caerulein; calcitonin gene related polypeptide (CGRP); calcitonins; CCK-8; cell growth factors (e.g., EGF; TGF-alpha; TGF-beta; PDGF; acidic FGF; basic FGF); cerulein; chemokines; cholecystokinin; cholecystokinin-8; cholecystokinin-pancreozymin (CCK-PZ); colistin; colony-stimulating factors (e.g. CSF; GCSF; GMCSF; MCSF); corticotropin-releasing factor (CRF); cytokines; desmopressin; dinorphin; dipeptide; dismutase; dynorphin; eledoisin; endorphins; endothelin; endothelin-antagonistic peptides; endotherins; enkephalins; enkephalin derivatives; epidermal growth factor (EGF); erythropoietin (EPO); follicle-stimulating hormone (FSH); gallanin; gastric inhibitory polypeptide; gastrin-releasing polypeptide (GRP); gastrins; G-CSF; glucagon; glutathione peroxidase; glutathio-peroxidase; gonadotropins (e.g., human chorionic gonadotrophin and .alpha. and .beta. subunits thereof); gramicidin; gramicidines; growth factor (EGF); growth hormone-releasing factor (GRF); growth hormones; hormone releasing hormone (LHRH); human artrial natriuretic polypeptide (h-ANP); human placental lactogen; insulin; insulin-like growth factors (IGF-I; IGF-II); interferon; interferons (e.g., alpha- beta- and gamma-interferons); interleukins (e.g. 1; 2; 3; 4; 5; 6; 7; 8; 9; 10; 11 and 12); intestinal polypeptide (VIP); kallikrein; kyotorphin; luliberin ; luteinizing hormone (LH); luteinizing hormone-releasing hormone (LH-RH); lysozyme chloride; melanocyte-stimulating hormone (MSH); melanophore stimulating hormone; mellitin; motilin; muramyl; muramyldipeptide; nerve growth factor (NGF); nerve nutrition factors (e.g. NT-3; NT-4; CNTF; GDNF; BDNF); neuropeptide Y; neurotensin; oxytocin; pancreastatin; pancreatic polypeptide; pancreozymin; parathyroid hormone (PTH); pentagastrin; polypeptide YY; pituitary adenyl cyclase-activating polypeptides (PACAPs); platelet-derived growth factor; polymixin B; prolactin; protein synthesis stimulating polypeptide; PTH-related protein; relaxin; renin; secretin; serum thymic factor; somatomedins; somatostatins derivatives; superoxide dismutase; taftsin; tetragastrin; thrombopoietin (TPO); thymic humoral factor (THF); thymopoietin; thymosin; thymostimulin; thyroid hormone releasing hormone; thyroid-stimulating hormone (TSH); thyrotropin releasing hormone TRH); trypsin ; tuftsin; tumor growth factor (TGF-alpha); tumor necrosis factor (TNF); tyrocidin; urogastrone; urokinase; vasoactive intestinal polypeptide; and vasopressin.

[0086] Fucosylated and nonfucosylated glycoproteins are important classes of biomolecules that play crucial roles in many biological events such as cell adhesion, tumor metastasis, pathogen infection, and immune response. As indicated previously herein, a major problem in structural and functional studies of fucosylated or nonfucosylated glycoproteins is their structural microheterogeneity. Natural and recombinant fucosylated or nonfucosylated glycoproteins are typically produced as a mixture of glycoforms that differ only in the structure of the pendent oligosaccharides.

[0087] The remodeled glycoproteins, such as antibodies can be subjected to any further structural modifications that are necessary or desired, including, without limitation, glycosyl transfer, and selective ligation (e.g., click chemistry, Staudinger reaction, etc.) to introduce the additional functional groups or tags. The functional groups can be of any suitable type, including, without limitation, toxins, special antigens (such as alpha-Gal), radioactive species, photoactive species, PEGs, etc. The glycoprotein can be catalytically reacted in a "click chemistry" cycloaddition reaction of the azide functionality of the glycoprotein with an alkyne bearing the functional moiety of interest. The azido and alkyne functional groups can be switched in the respective ligation components, and the glycoprotein can be functionalized with an alkynyl functionality and reacted with an azide-functionalized compound including the moiety of interest. It will also be appreciated that other ligation pairs can be devised for the click chemistry reaction.

[0088] The fucosylated and nonfucosylated glycoproteins, produced according to the methods described herein, can be used for diagnosis and therapeutics. Approximately two-thirds of therapeutic proteins used on the market and/or currently in clinical trials are glycoproteins. However, the structural heterogeneity in different glycoforms of natural and recombinant glycoproteins presents a major barrier in developing glycoprotein-based drugs, as different glycoforms may have different biological activities and controlling glycosylation to a homogeneous glycoform is extremely difficult during expression. The previous discovery of the transglycosylation activity of a class of endoglycosidases represents a major advance in the field for glycosylation engineering to enhance glycoproteins' therapeutic and diagnostic potentials and the Endo-S2 mutants of the present invention are able to transglycosylate fucosylated and nonfucosylated natural and recombinant glycoproteins without the negative aspects of hydrolysis.

[0089] The features and advantages of the present invention are more fully shown by the following non-limiting examples.

[0090] Examples

[0091] Generation of Endo-S2 Glycosynthase Mutants and Their Use for Glycosylation Remodeling of Intact Monoclonal Antibody Rituximab

[0092] Glycosynthases have been previously made from several GH85 endoglycosidases (ENGases), including EndoA, EndoM, EndoD and GH18 endoglycosidase EndoS, by site-directed mutagenesis of a key asparagine (Asn) in GH85 family or aspartic acid (Asp) residue in GH18 family responsible for promoting oxazolinium ion intermediate formation during hydrolysis. [36,38] Endo-S2 is an endoglycosidase belonging to the glycoside hydrolase family 18 (GH18) [33], which is in the same GH family as EndoS, EndoF1, EndoF2, and EndoF3 that were recently shown to have transglycosylation activity. Based on the assumption that EndoS2-catalyzed hydrolysis also proceeds by a substrate-assisted mechanism involving the formation of an oxazolinium ion intermediate, as demonstrated by other GH18 endoglycosidases such as EndoS, potential glycosynthases from Endo-S2 were created by identifying and mutating the residue responsible for promoting oxazolinium ion formation. Previous structural and mutagenesis studies on Endo-S have shown that an aspartic acid residue at position 233 (D233) is responsible for promoting oxazoline formation and that the E235 residue is the general acid/base for catalytic hydrolysis [47, 48]. Sequence alignment of EndoS2 with EndoS (Figure 2) led to the identification of two key residues in EndoS for catalysis: the D184 residue (corresponding to D233 in EndoS) responsible for promoting oxazolinium ion formation and the E186 residue (equivalent to E235 of EndoS) as the general acid/base residue in glycan hydrolysis as shown in Figure 2. Thus, on the assumption that the D184 is the critical residue that promotes oxazolinium ion intermediate formation in the hydrolysis via a substrate-assisted mechanism, nineteen specific mutants, D184A-Y (SEQ ID NO: 2-20) were generated by site-directed mutagenesis of Endo-S2 (SEQ ID NO:1). These mutants, as well as the wild-type EndoS2, were expressed in *Escherichia coli* in high yield (20–30 mg/L) as a CPD fusion protein and purified by Ni-NTA affinity chromatography.

[0093] Rituximab, a therapeutic monoclonal antibody, was used as a model mAb to examine the deglycosylation activity and potential transglycosylation activity of the enzymes. The major Fc glycans of commercial rituximab are core-fucosylated biantennary complex type oligosaccharides carrying 0–2 galactose moieties named G0F, G1F, and G2F glycoforms, respectively, as revealed in Figure 4C. Treatment of rituximab with the EndoS2–CPD fusion protein (here, referred as wild-type EndoS2 or EndoS2) resulted in a rapid deglycosylation to give the deglycosylated rituximab that bears the fucosylated GlcNAc disaccharide moiety (Fucα1,6GlcNAc) at the glycosylation sites (N297). These results confirm the remarkable Fc glycan-hydrolyzing activity of the wild-type EndoS2 on intact IgG, implicating its usefulness in the first step for glycosylation remodeling of mAbs. The transglycosylation potential of Endo-S2 mutants were then examined using the deglycosylated rituximab as the acceptor and the complex, high mannose, and hybrid glycan oxazolines as the donor substrates, as depicted in Figure 3. The glycosylation remodeling process was monitored by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and liquid chromatography mass spectrometry (LC-MS) analysis, as shown in Figure 4. The heavy chain and light chain of rituximab appeared at approximately 50 KDa and approximately 25 KDa, respectively, under reducing conditions (lane 1, in Figure 4A). After deglycosylation with wild-type EndoS2, the heavy chain appeared as a single band at approximately 48 KDa, suggesting the removal of the two N-glycans (each from a heavy chain) in rituximab (lane 2, in Figure 4A). Incubation of the deglycosylated rituximab (**1**) and the sialo complex type glycan oxazoline (**2**)(donor/acceptor, 20:1, molar ratio) with mutant EndoS2-D184Q gave a transglycosylation product (**5**), Figure 4E), the heavy chain of which appeared as a single band that was about 2 KDa larger than that of the deglycosylated rituximab (**1**) (Figure 4A, lane 3). This result suggests that a new N-glycan was attached to each of the Fc heavy chains. Interestingly, an essentially quantitative transglycosylation for the Fc domain of the intact antibody was achieved within 1h incubation.

[0094] The transglycosylation was further characterized by LC-MS analysis. The heavy chain and light chain of rituximab were separated under a LC-MS condition. Deconvolution of the light chain MS data gave a mass of 23 039 (Figure 4B), which was consistent with the calculated mass of rituximab light chain (M = 23 042 Da).[47] Deconvolution of the MS data of the heavy chain gave three distinct m/z species, 50508, 50669, and 50829, as shown in Figure 4C, which were in good agreement with the theoretical mass of heavy chain glycoforms: G0F, M = 50 515 Da; G1F, M = 50 677 Da; and G2F, M = 50 839 Da;

respectively.[47] The deconvoluted electron spray ionization mass spectrometry (ESI-MS) of the heavy chain of the deglycosylated rituximab (**1**) showed a single species at 49 411, as shown in graph c in Figure 4D, which matched well with a heavy chain carrying a Fucα1,6GlcNAc disaccharide moiety (calculated, M = 49 420 Da). After glycosylation remodeling, a single peak at 51 414 was observed from the heavy chain of the transglycosylation product with complex glycan(**5**), with an addition of 2003 Da to the deglycosylated heavy chain of the rituximab, as shown in graph in Figure 4E. This result indicates the attachment of a sialoglycan from the corresponding sugar oxazoline (**2**) to the heavy chain.

[0095] In addition to the sialylated complex type N-glycan oxazoline (**2**), the EndoS2 mutants were equally efficient to use the high mannose Man9GlcNAc core oxazoline (**3**) and the sialo hybrid type oxazoline (**4**) for rituximab glycoengineering, leading to the formation of the corresponding homogeneous glycoforms, (**6**) and (7), respectively (Figures 4F and Figure 4G). The deconvoluted ESI-MS of the heavy chain of the transglycosylation product (**6**) showed a single species at 51074, as shown in Figure 4F, which matched well with the calculated molecular mass (M = 51082 Da) of the rituximab heavy chain carrying a Man9GlcNAc2 glycan. Similarly, the deconvoluted ESI-MS of the heavy chain of transglycosylation product (7) showed a single species at 51080, as shown in Figure 4G, which was in good agreement with the calculated molecular mass (M = 50190 Da) of the rituximab heavy chain carrying a N3Man3GlcNAc2 glycan. The results set forth herein represents the first report of glycosylation remodeling of an intact IgG monoclonal antibody with an en bloc transfer of a full-size natural high mannose (Man9) and sialo hybrid type N-glycan to the Fc domain through a highly efficient deglycosylation–reglycosylation protocol enabled by the combined use of EndoS2 and EndoS2-based glycosynthase. After completion of the transglycosylation, the product could be purified by a simple protein A affinity chromatography, giving the well-defined homogeneous glycoform. Notably glycoengineering of intact rituximab with the transfer of biantennary complex type N-glycan to the Fc domain by using EndoS/EndoS-based glycosynthase has been reported. However, that system is inefficient in transfer high mannose and hybrid type glycan to antibody. The development of EndoS2/EndoS2-glycosynthethase system significantly expanded the scope of glycan specifies of chemoenzymatic glycoengineering of antibody.

[0096] *Glycoengineering of Rituximab To Provide Nonfucosylated complex, high mannose, and hybrid Glycoforms*

[0097] For anticancer therapy, nonfucosylated IgG glycoforms are desirable as it has been previously demonstrated that mAbs with low-fucose contents of Fc N-glycans showed enhanced ADCC activity *in vitro* and enhanced anticancer efficacy *in vivo*, particularly for those patients carrying the low affinity F158 allele of the FcγIIIa receptor. [8, 39, 40, 49]. However, no α-fucosidases is available to remove the α1,6-fucose in the intact rituximab. The α-1,6-fucose moiety might be shielded by the Fc domain and/or the complex N-glycan, making it inaccessible to α-fucosidases. It was theorized that, upon deglycosylation, the resulting Fuc(α1,6)GlcNAc glycoform of rituximab might be more accessible to α-fucosidases. Indeed, after deglycosylation with EndoS2, most of the α-1,6-fucose moiety could be removed by overnight incubation with α1,6-fucosidase from *Lactobacillus casei* to give the GlcNAc-containing rituximab (**8**). The result is confirmed with LC-MS (Figure 6B). Next, it was determined that the glycosynthases EndoS2-D184A, EndoS-D184Q, or EndoS-D184N were also efficient to recognize the nonfucosylated GlcNAc in (**8**) for transglycosylation with complex (**2**), high mannose (**3**) or hybrid (**4**) oxazoline to provide the relatively homogeneous, nonfucosylated complex (**9**), high mannose (**10**) and hybrid (**11**) type glycoform in an essentially quantitative conversion (Figure 5). The identity and purity of the glycoengineered product (**9**, **10, 11**) were confirmed by SDS-PAGE and LC-MS analysis, as shown in Figure 6. The defucosylated rituximab (**8**) showed a major peak at 49266 (Figure 6B), confirming the removal of the fucose (calcd. for the heavy chain of GlcNAc–rituximab, M = 49274 Da). The deconvoluted ESI-MS of the heavy chain of the transglycosylation product of complex type glycan (**9**) appeared as a major species at 51268 (Figure 6C), which matched well with the calculated molecular mass (M = 51276 Da) of the rituximab heavy chain carrying a sialylated biantennary complex type N-glycan, Sia2Gal2GlcNAc2Man3GlcNAc2. Similarly, LC-MS analysis of the heavy chain of transglycosylation product of the high mannose (**10**) and hybrid type glycan (**11**) appeared as a major species at 50930 (Figure 6D) and 50939 (Figure 6E), which matched well with the calculated molecular mass (50936 and 50944 Da), respectively. In the transfer of hybrid glycan oxazoline, approximately 33% of starting material is left untransferred. With optimization of reaction condition (increase of EndoS2 mutant concentration, addition of more oxazoline, etc.), the reaction should be able to push to completion. It just seems fucosylated rituximab (**1**) is a more preferred acceptor than non-fucosylated one (**8**). In a comparative study, it was also found that mutants D184A, D184N, and D184Q had a faster

transglycosylation reaction on the fucosylated GlcNAc–rituximab (**1**) than the nonfucosylated acceptor (**8**) (data not shown). Taken together, these experimental results revealed a combined enzymatic approach to making the nonfucosylated complex, high mannose and hybrid type homogeneous (or relatively homogeneous) glycoform from commercially available monoclonal antibodies. The resulting nonfucosylated rituximab is expected to gain improved ADCC and CDC effector functions as suggested by previously studies. [8, 42, 49]

[0098] *Comparison of the Transglycosylation Activity of Different Mutants of EndoS2 D184*

[0099] The activities of EndoS2-D184A, D184N, and D184Q were compared of transferring sialo bianntenary complex glycan oxazoline (**2**) to fucosylated GlcNAc-rituximab (**1**). With the same reactive conditions, the asparagine (N) mutant transferred more than 30% of oxazoline to the acceptor in five minutes while the alanine (A) and glutamine (Q) mutants transfer less than 20% (Figure 7). It seems the N mutant is more active than A and Q mutants in the transfer of complex type oxazoline.

[00100] *Cloning, Expression and Characterization of Endo-S2*

[00101] cDNA sequence encoding Endo-S2 (44-843 of SEQ ID NO: 1) (The Genbank accession number for the Endo-S2 gene (*ndoS2*) is ACI61688) was cloned into a pET22b-CPD vector, which adds to the C-terminus of the expressed protein the cysteine protease domain (CPD) of the *Vibrio cholerae* MARTX toxin and a 10x histidine tag [35] (SEQ ID NO 22). It was recently reported that a high level, soluble expression of Endo-F3 and its mutants could be achieved using this vector [32]. In the alternative, the cDNA sequence for encoding Endo-S2 may include (1, and 44-843 amino acids of SEQ ID NO: 1). Following a similar method, the Endo-S2 was successfully expressed in *E. coli* and was readily purified using immobilized metal ion affinity chromatography (IMAC) to obtain the soluble enzyme with a yield of more than 20 mg/L. The recombinant Endo-S2 showed high hydrolysis activity as demonstrated by its rapid deglycosylation of commercial rituximab, which was monitored by LC-MS analysis.

[00102] *Generation of glycosynthase mutants from Endo-S2*

[00103] Glycosynthase mutants from endoglycosidases of both GH85 and GH18 family were generated by site-directed mutation at a key residue that is responsible for promoting the formation of the oxazolinium ion intermediate during hydrolysis, which proceeds in a substrate-assisted mechanism. These include a key asparagine residue for

endoglycosidases Endo-A (Asn171) [36], Endo-M (Asn175) [37,38], and Endo-D (Asn322) [24] of the GH85 family, or a key aspartic acid residue for the GH18 family endoglycosidases Endo-S (Asp233) [25] and Endo-F3 (Asp-165) [32]. Sequence alignment of Endo-S2 and Endo-S revealed that the Asp-184 of Endo-S2 was the residue equivalent to the Asp233 of Endo-S essential for promoting oxazolinium ion formation in hydrolysis (Figure 2). To generate efficient glycosynthase mutants from Endo-S2, the Asp-184 was systematically replaced in the truncated expressed protein comprising the residues of 44 to 843) with other 19 natural amino acid residues using site-directed mutagenesis. Notably, it is envisioned that unnatural proteins may also be used in the substitution. The resulting 19 D184 mutants were also expressed as soluble proteins in the pET22bCPD vector and purified using immobilized metal ion affinity chromatography, in the same way as demonstrated for the wild type enzyme. The expression of the mutant enzymes gave comparable yields (15-20 mg/L) as the expression of the wild type enzyme.

[00104] Notably, it is envisioned that unnatural proteins may also be used in the substitution. Examples of unnatural amino acids that can be used by the translation system include: an unnatural analogue of a tyrosine amino acid; an unnatural analogue of a glutamine amino acid; an unnatural analogue of a phenylalanine amino acid; an unnatural analogue of a serine amino acid; an unnatural analogue of a threonine amino acid; an alkyl, aryl, acyl, azido, cyano, halo, hydrazine, hydrazide, hydroxyl, alkenyl, alkynl, ether, thiol, sulfonyl, seleno, ester, thioacid, borate, boronate, phospho, phosphono, phosphine, heterocyclic, enone, imine, aldehyde, hydroxylamine, keto, or amino substituted amino acid, or any combination thereof.

[00105] *Comparative study on the hydrolysis and transglycosylation activity of 19 mutants and WT*

[00106] The hydrolysis activities on Fc N-glycans in an intact antibody and the transglycosylation activities with glycan oxazoline were assessed by following the scheme as shown in Figure 8. The results indicate that most of the mutants at the D184 residue led to significantly reduced or completely diminished hydrolysis activity on Fc N-glycan. Among them, the D184F, D184H, D184K, D184R, and D184W mutants were completely devoid of hydrolytic activity, while several other mutants, including D184C, D184E, D184G, D184N, D184S, D184Y, were found to still retain significant hydrolysis activity (Table 1). On the other hand, the evaluation of transglycosylation indicated that almost all the mutants

possessed transglycosylation activity when using the deglycosylated rituximab as the acceptor and a biantennary complex type glycan oxazoline as the donor substrate, but the activities varied significantly among different mutants (Figure 8, Table 2). Among others, the D184C, D184M, D184G, D184E, D184Y, D184S, and D184A were found to be the most active mutants. However, the D184C, D184G, D184E, D184Y, D184S, and D184A also demonstrated significant residual hydrolytic activity. The most interesting mutant is D184M, which retained only marginal hydrolytic activity but showed extraordinarily high transglycosylation activity (only second to D184C), making it one of the best glycosynthase mutants to choose for glycosylation remodeling.

[00107] *Endo-S2 glycosynthases show remarkably broad substrate specificity in transglycosylation*

[00108] Rituximab, a therapeutic monoclonal antibody, was used as a model to examine the transglycosylation activity of the Endo-S2 D184 mutants. The major Fc glycans of commercial rituximab are core-fucosylated bi-antennary complex type oligosaccharides carrying 0–2 galactose moieties named G0F, G1F, and G2F glycoforms, respectively. The general glycosylation remodeling approach was presented in Figure 9 and the reaction products were assessed by LC-MS analysis (Figure 10). Treatment of rituximab (**1**) with wild-type Endo-S2 resulted in complete deglycosylation of rituximab, as demonstrated by the conversion of the glycoform mixtures (G0F, G1F, and G2F) found in commercial rituximab (Figure 10A) to the Fucα1,6GlcNAc-glycoform (**2**) of rituximab (Figure 10B). The Fucα1,6GlcNAc-rituximab (**2**) was purified away from the WT endoglycosidase and released glycans by Protein A affinity chromatography and used as the acceptor in the transglycosylation reactions. It was found that EndoS2 D184M was able to efficiently transfer the sialylated biantennary complex-type (SCT) N glycan from the corresponding glycan oxazoline (**4**) to the Fucα1,6GlcNAc-rituximab acceptor (2) to form the S2G2F glycoform of rituximab (**3**). The reaction could be readily pushed to completion with 20 molar equivalent (i.e., 10 molar equivalent per monomeric Fc domain) of the glycan oxazoline. The reaction yield was estimated by LC-MS analysis to be over 95% as almost no starting material was detected, which confirmed the completion of the transglycosylation. LC-MS analysis of the transglycosylation products (**3**) carrying the SCT N-glycan revealed that the heavy chain of (**3**) appeared as a single species at 51412 (deconvolution data), which is in good agreement with the calculated molecular mass (M= 51421 Da) for the heavy chain carrying a SCT N-glycan (with core-fucose), respectively (Figure 10C).

[00109] In addition to the complex-type N-glycan, the specificity of Endo-S2 was further tested with high-mannose-type (HM) Man9GlcNAc oxazoline (**7**) and sialo hybrid-type (Hyb) Neu5AcGalGlcNAcMan5GlcNAc oxazoline (**5**) as donor substrate in the transglycosylation reactions. The reactions led to the formation of the corresponding homogeneous glycoforms, (**8**) and (**6**), respectively (Figure 10E, 10G). The deconvoluted ESI-MS of the heavy chain of the transglycosylation product (**8**) showed a single species at 51074, as shown in Figure 10E, which matched well with the calculated molecular mass (M = 51081 Da) of the rituximab heavy chain carrying a Man9GlcNAc2 glycan. Similarly, the deconvoluted ESI-MS of the heavy chain of transglycosylation product (**6**) showed a single species at 51082, as shown in Figure 10G, which was in good agreement with the calculated molecular mass (M = 51090 Da) of the rituximab heavychain carrying a Neu5AcGalGlcNAcMan5GlcNAc2 glycan. Under the same conditions, it was found that the previously reported Endo-S mutants, including D233A and D233Q mutants of Endo-S [25] showed only marginal transglycosylation activity with high mannose and hybrid type N-glycans, although they could efficiently transfer biantennary complex type N-glycan. In addition, the recently reported D165A mutant of Endo-F3 [32] was unable to transfer the high-mannose or hybrid type Nglycans but could work on bi- and tri-antennary complex type sugars. Thus, these Endo-S2 derived mutants represent the first glycosynthases that can efficiently transfer high-mannose and hybrid type N-glycans to core-fucosylated GlcNAc acceptor in an intact antibody. It should be mentioned that the Endo-A mutant (N171A and N171Q) could transfer high-mannose type N-glycan to GlcNAcFc domain, but they were unable to use corefucosylated GlcNAc-Fc as an acceptor [22, 23, 36]. These studies also show that the combined use of wild type Endo-S2 and Endo-S2 glycosynthase mutants provides a particularly efficient glycosylation remodeling approach to various homogeneous glycoforms of antibodies starting from a single precursor.

[00110] *Endo-S2 based glycosylation remodeling for making nonfucosylated glycoforms*

[00111] For anticancer therapy, nonfucosylated IgG glycoforms are desirable as it has been previously demonstrated that mAbs with low-fucose contents of Fc glycosylation showed enhanced ADCC activity *in vitro* and enhanced anticancer efficacy *in vivo*, particularly for those patients carrying the low affinity F158 allele of the FcγIIIa receptor [8, 9, 39, 40]. To test whether Endo-S2 can glycosylate non-fucosylated IgG, Fucα1,6GlcNAc-

rituximab (**2**) was incubated with a recombinant α1,6-fucosidase from *Lactobacillus casei* [41] to give GlcNAc-rituximab (**9**) lacking the core-fucose. Endo-S2 D184M catalyzed transglycosylation of GlcNAc-rituximab (**9**) was carried out with a sialo biantennary complex-type (CT) N-glycan oxazoline (**10**). It was found that the D184M mutant could efficiently transfer the N-glycan to the GlcNAc acceptor in the antibody to give the fully galactosylated and nonfucosylated glycoform (**11**) in an essentially quantitative conversion. The deconvoluted ESI-MS of the heavy chain of transglycosylation product (**11**) showed a single species at 50684, as shown in Figure 10I, which was in good agreement with the calculated molecular mass (M = 50693 Da) of rituximab heavy chain carrying a fully galactosylated biantennary complex type N-glycan without the core-fucose. In addition to the confirmation of site-specific glycosylation of the heavy chain by LC-MS analysis combined with enzymatic transformation, the light chain of the transglycosylation products (**3, 6, 8, 11**) also appeared as a single species at 23034, which matches the calculated molecular mass of the light chain of rituximab (M = 23039 Da) (Figure 10D, F, H, J) without any modifications. These results indicated that there were no non-enzymatic modifications occurring on the heavy and light chains except the attachment of the transferring Nglycan at the GlcNAc acceptor at the Fc domain during the Endo-S2 catalyzed glycosylation remodeling processes. It should be pointed out that the fully galactosylated and non-fucosylated glycoform of rituximab (**11**) was previously shown to have at least 20-fold enhanced affinity for the FcγIIIA receptor in comparison with the commercial rituximab, which is an indication of a significantly enhanced ADCC [25].

[00112] *Comparison of transglycosylation efficiency on different N-glycan substrates by Endo-S2 D184M mutant*

[00113] To further characterize the N-glycan substrate preference of Endo-S2 D184M, three parallel transglycosylation reactions were carried out with the N-glycan oxazolines of the complex (SCT) type, high-mannose (HM) type, and hybrid (Hyb) type, respectively. The reaction progresses were monitored by LC-MS analysis of reaction aliquots taken at multiple time-points and the results were summarized in Figure 11. Under the same reaction conditions, the transglycosylation reaction with SCT-oxazoline (**4**) was completed within 20 minutes to give S2G2F-rituximab (**3**), while the transglycosylation with HM-oxazoline (**7**) and Hyb-oxazoline (**5**) were much slower. These results suggest that Endo-S2 D184M prefers complex-type over the high-mannose-type and hybrid-type N-glycan despite of having a remarkably relaxed N-glycan specificity.

[00114] *Comparison of the transglycosylation efficiency of typical Endo-S2 and Endo-S mutants*

[00115] Endo-S glycosynthase mutants D233Q and D233A were previously generated that transglycosylate rituximab efficiently with complex-type N-glycan oxazoline [25]. The EndoS D233 mutants have been recently used to generate homogeneous monoclonal antibodies for structural and functional studies [26-31]. To compare the transglycosylation efficiency of the glycosynthase mutants from Endo-S2 and Endo-S, the Endo-S D233Q mutant was selected, the equivalent Endo-S2 D184Q mutant and the Endo-S2 D184M mutant to catalyze three parallel transglycosylation reactions. The time-course of the transglycosylation reactions was monitored by LC-MS analysis and was summarized in Figure 12. Under the reaction conditions, the D184M mutant of Endo-S2 showed remarkably potent transglycosylation activity, and reached completion of the glycan transfer within 10 min. The other Endo-S2 mutant could also transfer the glycan smoothly and reached the completion within one hour. However, the corresponding Endo-S mutant (D233Q) was much less efficient, reaching about 10% of transglycosylation at one hour under the same conditions (Figure 12). As demonstrated in a separate experiment, much more (10-fold) of Endo-S D233Q mutant and a larger excess of the glycan oxazoline were required to achieve the same level of the transglycosylation catalyzed by the Endo-S2 D184Q mutant, and the D184M mutant of Endo-S2 was even much more efficient than the D184Q mutant. These studies suggest that the newly discovered Endo-S2 D184 mutants are superior to the previously reported Endo-S mutants for antibody glycosylation remodeling in both efficiency of reactions and the breadth of substrate diversity.

[00116] *Glycosylation remodeling of trastuzumab (Herceptin) using a pair of Endo-S2 enzymes*

[00117] To demonstrate that the observed enzymatic properties of Endo-S2 and its mutants are generally applicable to antibody glycosylation remodeling, glycosylation remodeling of another monoclonal antibody was performed, trastuzumab (Herceptin), which is widely used for treatment of breast cancer. The glycosylation remodeling was assessed by the synthesis of a sialylated glycoform of trastuzumab (Figure 13). Treatment of trastuzumab with wild-type Endo-S2 resulted in complete deglycosylation, as demonstrated by the conversion of the glycoform mixtures (G0F, G1F, and G2F) found in commercial trastuzumab (Figure 13A) to the Fucα1,6GlcNAc-glycoform (**12**) of trastuzumab (Figure

13B). The deglycosylated trastuzumab was purified away from the endoglycosidase and released glycans by Protein A affinity chromatography and used as the acceptor in the transglycosylation reactions. Incubation of the Fucα1,6GlcNAc-trastuzumab (**12**) with the donor substrate SCT-oxazoline (**4**) in the presence of Endo-S2 D184M mutant resulted in rapid conversion of the deglycosylated trastuzumab (**12**) to the fully glycosylated trastuzumab (**13**). The reaction was essentially quantitative to give a single transglycosylation product (**13**). LC-MS analysis of the transglycosylation products (**13**) revealed that the heavy chain of (**13**) appeared as a single species at 51500 Da (deconvolution data) (Figure 13C), indicating the attachment of a single sialylated N-glycan on the heavy chain. On the other hand, the light chain of the transglycosylation product (**13**) appeared as a single species at 23438, which is in a good agreement with the light chain of trastuzumab without any additional modifications (Figure 13D).

[00118] To confirm that the N-glycan was attached specifically to the Asn-297 N-glycosylation site of the Fc domain, instead of other sites of the polypeptide backbone that might occur by nonenzymatic reactions as implicated in a recent publication [30], the transglycosylation product (**13**) was treated with PNGase F and examined the protein portion by mass spec analysis. PNGase F was highly specific and could release the Nglycans only when they were attached to the Asn side chain in an N-glycosylamide linkage at the conserved glycosylation site in N-glycoproteins. LC-MS analysis of the heavy chain of the PNGase F treated transglycosylation product (**13**) gave a single species of 49150 (deconvolution data), which corresponds to the polypeptide backbone of the heavy chain without any additional modifications (Figure 13E). Taken together, these results clearly indicated that a single sialylated biantennary N-glycan was conjugated to the antibody heavy chain, and the intact N-glycan was attached at the conserved N-glycosylation site without any non-enzymatic glycations of the antibody. The highly efficient transformation catalyzed by the Endo-S2 glycosynthase mutants allowed much less reaction time and the use of much less excess of glycan oxazoline to achieve a quantitative conversion.

[00119] Described herein is the discovery of a new class of glycosynthases derived from an endoglycosidase (Endo-S2) of the *Streptococcus pyogenes* M49 serotype, which showed broader substrate specificity and much more potent transglycosylation activity for antibody glycosylation remodeling than the previously reported glycosynthases, such as those derived from Endo-A, Endo-M, Endo-S, and Endo-F3. These findings were enabled by a

systematic mutagenesis at the critical residue D184, coupled with comparative analysis of the hydrolysis and transglycosylation activities of the resulting 19 mutants. The experimental data also revealed remarkable difference in both the hydrolysis and transglycosylation activities among the 19 mutants (Tables 1 and 2), which would be difficult to predict without this comparative study. Several notable mutants, including the D184M and D184Q mutants, were identified that showed high transglycosylation activity for glycosylation remodeling but retained only marginal hydrolysis activity.

[00120] Comparison of the hydrolysis and transglycosylation activities of the mutants reveals several interesting features. First, most of the mutants that showed high transglycosylation activity, including the D184C, D184G, D184E, D184Y, D184S, and D184A mutants, also possessed relatively high residual hydrolysis activity. An exception is the D184M mutant, which demonstrated remarkable transglycosylation activity but retained only residual hydrolysis activity, making it the most efficient glycosynthase for glycosylation remodeling. Secondly, most the mutants with the D184 residue being replaced by amino acids with positively charged side chains (K, R, H) or bulky hydrophobic side chains (I, L, F, W) showed very low activities in both transglycosylation and hydrolysis. But, interestingly, the D184Y mutant retained the highest hydrolytic activity among all the mutants and also possessed a relatively high transglycosylation activity.

[00121] Another important discovery is the findings of the much broader substrate specificity of the Endo-S2 derived glycosynthase mutants than that of the previously reported glycosynthases. It was found that the D184M and D184Q mutants, two notable Endo-S2 glycosynthases identified, were able to efficiently transfer all three major types of N-glycans including high-mannose type, complex type, and hybrid type, in antibody glycosylation remodeling. In addition, the Endo-S2 glycosynthases could recognize both core-fucosylated GlcNAc or nonfucosylated GlcNAc moiety at the Fc domain as an acceptor for transglycosylation. These findings significantly expand the scope of the glycosylation remodeling strategy. For example, the previously reported Endo-S and Endo-F3 are specific for complex type N-glycans and are unable to efficiently transfer high-mannose and hybrid type N-glycans, and the Endo-F3 is efficient only for core-fucosylated GlcNAc acceptor [32]. A direct comparison of the transglycosylation activity of the typical Endo-S2 and Endo-S glycosynthase mutants reveals that the Endo-S2 mutants are generally much more active than the corresponding Endo-S mutant. By an estimate of the initial rate, the D184Q mutant of

Endo-S2 was at least 10-fold more active than the corresponding D233Q mutant of Endo-S, and the best Endo-S2 mutant, D184M, was estimated to be 100-fold better than the Endo-S D233Q mutant in glycosylating the deglycosylated rituximab (Figure 12). Finally, in addition to the remodeling of rituximab, the highly efficient glycosylation remodeling of trastuzumab (Herceptin) by using a pair of Endo-S2 (the wild type and the D184M mutant) enzymes to give a single homogeneous glycoform without side reactions (Figure 13) showcases the power of the newly discovered glycosynthases. It is expected that these highly efficient glycosynthases, which show also remarkably relaxed substrate specificity, will find a wide range of applications for producing various homogeneous glycoforms of antibodies for structural and functional studies, and for developing more effective antibody-based therapeutics as well.

[00122] Figure 14 shows the use of the present invention to provide the desired homogeneous glycoforms of antibodies. IVIG is widely used for the treatment of Rheumatoid arthritis, but usually requires high doses such as (1-1.5g/kg). However as shown in Figure 14 the homogenous glycoform provides the benefits of reduced doses, enhance efficacy and reduced side effects. Such effects are due to a remodeled IVIG with greater than 95% sialylation due the use of the Endo S2 mutants of the present invention. Such improvement in glycosylation is applicable to glycoprotein hormones, cytokines (IL-2, interferons, etc.) and enzyme replacement therapy (lysosomal diseases).

EXPERIMENTAL PROCEDURES

[00123] Materials—Monoclonal antibodies rituximab and trastuzumab (Herceptin) were products from Genentech Inc., (South San Francisco, CA). A sialo and sialoglycan complex type oxazoline was synthesized following the previously reported procedure [42]. The high mannose-type (HM) glycan (Man9GlcNAc) was prepared from soy bean flour by the previously described procedure [43]. The synthesis of hybrid type (Hyb) glycan (Neu5AcGalGlcNAcMan5GlcNAc) was achieved by a sequential enzymatic glycosylation of the Man5GlcNAc [43] under the catalysis of a β-1,2- GlcNAc transferase (GnT1) [44], a β-1,4-galactosyltransferase [45] and an α-2,6-Sialyltransferase [46]. The HM and Hybrid glycan oxazolines were synthesized following the previously described one-pot transformation procedure [42]. Endo-S D233Q from *Streptococcus pyogenes* was overexpressed and purified following our previous procedure [25].

[00124] *Site-directed Mutagenesis and Expression and Purification of Recombinant Endo-S2*

[00125] cDNA encoding amino acids 44-843 of Endo-S2 from Streptococcus pyogenes NZ131 (serotype M49) was amplified by PCR and cloned into the pCPDLasso vector (a pET22b-CPD derivative) (35), wherein the sequence for the CPD (cysteine protease domain of the *Vibrio cholerae* MARTX toxin and histidine tag is set forth in SEQ ID NO: 22. For saturation mutagenesis of Asp-184 residue, a forward primer, 5'-CGTAAATTCGTGCTCAATNNNAATATCTAGTCCATCGACACCACGATCAGTT-3', (SEQ ID NO: 23) and a reverse primer, 5'-AACTGATCGTGGTGTCGATGGACTAGATATTNNNATTGAGCACGAATTTACG-3' (SEQ ID NO: 24), were used. Mutations were confirmed by DNA sequencing. The plasmids containing mutated Endo-S2 genes were transformed into *E. coli* BL21 (DE3). For simultaneous production of 20 Endo-S2 D184 variants, the transformants were cultured in 20 mL 2xYT broth media supplemented with 100 μg/mL carbenicillin. Cultures were grown at 37°C until the cells reached an OD600 of 0.8-1.0. Then 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture to induce protein overproduction at 20 °C. After 24 h the cells were harvested by centrifugation. The cell pellets were lysed by Bacterial Cell Lysis Buffer (Gold Biotechnology, Inc.) following manufacturer's instructions. 10x histidine (His10)-tagged EndoS2/CPD fusion proteins were purified with NiNTA Spin Columns (Qiagen). The purified EndoS2 proteins were desalted into PBS (pH 7.4) using centrifugal diafiltration with Amicon ultra filtration (10 kDa, Millipore). The protein purity was confirmed by SDS-PAGE and the concentration was measured on a NanoDrop 2000c using absorbance at 280 nm. For large-scale purification of selected Endo-S2 variants, 1 L culture is used. The cell lysate was applied to a HisTrap HP column (GE) and washed with PBS with 0.5 M NaCl and 20 mM imidazole (pH 7.4). Bound His-tagged protein was eluted with a gradient of 0-250 mM imidazole in PBS buffer. Eluted fractions containing Endo-S2 protein were pooled, concentrated and further purified by size exclusion chromatography through a HiPrep 16/60 Sephacryl S-200 HR column (GE).

[00126] *Liquid Chromatography Mass Spectrometry (LC-ESI-MS) of IgG*

[00127] The LC-MS analysis was performed on an Exactive Plus Orbitrap (Thermo Scientific). For intact antibody, the analysis was performed with a Waters XBridgeTM BEH300 C4 column (3.5 μm, 2.1 x 50 mm) with a linear gradient of 5-90% MeCN

containing 0.1 % formic acid within 9 min at a flow rate of 0.4 ml/min. For analysis of antibody light chain and heavy chain, the IgG antibody were treated with 50 mM TCEP and heated at 37 °C for 20 min then subjected to LC-MS analysis with an Agilent Poroshell 300SB-C8 column (5μm, 75 x 1 mm). The analysis was performed at 60 °C eluting with a linear gradient of 25-35% MeCN containing 0.1% formic acid within 6 min at a flow rate of 0.40 mL/min. The LC-MS analysis of PNGase F treated antibody glycoforms was performed in the same manner but included a 3-hr incubation with PNGaese F prior to TCEP treatment. Raw data was deconvoluted using MagTran (Amgen).

[00128] *Deglycosylation of Rituximab by WildType Endo-S2 To Give (Fucα1,6)GlcNAc–Rituximab*

[00129] Commercial rituximab in its original buffer was incubated with wild-type Endo-S2 for one hour at 37 °C with an antibody-to-enzyme ratio of 500:1 (by weight). LC-MS analyses indicated the complete cleavage of the N-glycans on the heavy chain. The deglycosylated rituximab was purified by protein A chromatography. LC-MS: calculated for the heavy chain of (Fucα1,6)GlcNAc-rituximab (2), M = 49420 Da; found (m/z), 49412 (deconvolution data).

[00130] *Defucosylation of (Fucα1,6)GlcNAc–Rituximab by Bacterial α-Fucosidase*

[00131] A solution of (Fucα1,6)GlcNAc–rituximab (2) in a Tris-HCl buffer (50 mM, pH 7.4) was incubated with the α-fucosidase AlfC from *Lactobacillus casei* at 37 °C, with an antibody-to-enzyme ratio of 50:1. After 16 h incubation, LC-MS monitoring indicated the complete defucosylation of (Fucα1,6)GlcNAc rituximab (**2**) to give the product, GlcNAc–rituximab (**9**). The defucosylated rituximab was purified by protein A chromatography. LC-MS: calculated for the heavy chain of GlcNAc–rituximab (**9**) carrying a GlcNAc moiety, M = 49274 Da; found (m/z), 49265 (deconvolution data).

[00132] *Enzyme Assay*

[00133] Assay for the hydrolysis activity of each Endo-S2 variant (0.1 μg) was performed at 30°C with pure sialo-complex-type (S2G2F) rituximab 3 (10 μg, 7.0 μM) as substrate in PBS buffer (pH 7.4, 10 μl). An aliquot of each reaction mixture was diluted in 0.1% formic acid to stop the reaction and analyzed by LC-MS. Relative amount of the substrate and the hydrolysis products were quantified after deconvolution of the raw data and integration of the corresponding MS peaks using MagTran. The transglycosylation reaction

with synthetic sugar oxazoline was assayed as follows: (Fucα1,6)GlcNAc-rituximab (100 μg, 69 μM), SCTox (1.38 mM, 20 equivalents) was incubated with 0.1 μg of each Endo-S2 variants at 30°C in PBS (pH 7.4, 10 μl). The reactions were stopped and analyzed as in the hydrolysis assay stated above. Experiment of each mutant was repeated at least twice to ensure the consistency of the results.

[00134] *Transglycosylation of Fuc1,6 GlcNAc Rituximab with SCT-ox, Hyb-ox and HM-ox by Endo-S2 D184M: Synthesis of 3, 6, 8*

[00135] A solution of Fuc1,6GlcNAc-Rituximab (1 mg, 69 μM) (**2**) and SCTox (1.38 mM, 20 eq) (**4**) was incubated with Endo-S2 D184M (5 μg) at 30 °C in 100 μl of 100 mM Tris Buffer (pH 7.4) for 15 min. LC-MS analysis indicated the completion of the transglycosylation reaction. The product (**3**) was purified using protein A chromatography. LC-MS: calculated for the heavy chain of (**3**) carrying the fully sialylated bi-antennary N-glycan, M=51421 Da; found (m/z), 51412 (deconvolution data).

[00136] A solution of Fuc1,6GlcNAc-Rituximab (1 mg, 69 μM) (**2**) and Hybox (1.38 mM, 20 eq) (**5**) was incubated with Endo-S2 D184M (5 μg) at 30 °C in 100 μl of 100 mM Tris Buffer (pH 7.4) for 30 min. Then another 10 eq of Hybox (**5**) was added and the reaction was monitored by LC-MS of aliquots. When LC-MS analysis indicated the near completion of the transglycosylation reaction, the product (**6**) was purified using protein A chromatography. LC-MS: calculated for the heavy chain of (**5**) carrying the sialylated hybrid-type Nglycan, M=51090 Da; found (m/z), 51082 (deconvolution data).

[00137] A solution of Fuc1,6GlcNAc-Rituximab (1 mg, 69 μM) (**2**) and HMox (1.38 mM, 20 eq) (**7**) was incubated with Endo-S2 D184M (5 μg) at 30 °C in 100 μl of 100 mM Tris Buffer (pH 7.4) for 30 min. Then another 10 eq of HMox (**7**) was added and the reaction was monitored by LC-MS of aliquots. When LC-MS analysis indicated the completion of the transglycosylation reaction, the product (**8**) was purified using protein A chromatography. LC-MS: calculated for the heavy chain of (**12**) carrying the high-mannose-type (Man9GlcNAc2) N-glycan, M=51081 Da; found (m/z), 51074 (deconvolution data).

[00138] Transglycosylation of GlcNAc-Rituximab with CT-ox by Endo-S2 D184M: Synthesis of 11

[00139] A solution of GlcNAc-Rituximab (1 mg, 69 μM) (**9**) and CTox (1.38 mM, 20 eq) (**10**) was incubated with Endo-S2 D184M (5 μg) at 30 °C in 100 μl of 100 mM Tris Buffer (pH 7.4) for 30 min. LC-MS analysis indicated the completion of the transglycosylation reaction. The product (**11**) was purified using protein A chromatography. LC-MS: calculated for the heavy chain of (**11**) carrying the bi-antennary N-glycan, M=50693 Da; found (m/z), 50684 (deconvolution data).

[00140] *Comparison of transglycosylation activity of Endo-S2 D184M mutant using SCT-, HM- and Hyb-oxazoline as donor substrates*

[00141] In three separate reactions, Fuc1,6GlcNAc-Rituximab (0.2 mg, 69 μM) (**2**) together with SCTox (**4**), Hyb-ox (**5**) or HM-ox (**7**) (1.38 mM, 20 eq) was incubated with Endo-S2 D184M (1 μg) at 30 °C in 20 μl of 100 mM Tris Buffer (pH 7.4). An aliquot of reaction (0.5 μl) was taken out at several time points and dilute in 0.1 % formic acid to stop the reaction. All aliquots were analyzed by LC-MS and % transglycosylation was calculated from the deconvoluted data.

[00142] *Comparison of transglycosylation activity of Endo-S2 D184M, D184Q mutants and Endo-S D233Q mutant using SCT-oxazoline as the donor substrate*

[00143] In three separate reactions, Fuc1,6GlcNAc-Rituximab (0.2 mg, 69 μM) (**2**) and SCTox (**4**) (1.38 mM, 20 eq) was incubated with Endo-S2 D184M, Endo-S2 D184Q or Endo-S D233Q (1 μg) at 30 °C in 20 μl of 100 mM Tris Buffer (pH 7.4). An aliquot of reaction (0.5 μl) was taken out at several time points and dilute in 0.1 % formic acid to stop the reaction. All aliquots were analyzed by LC-MS and % transglycosylation was calculated from the deconvoluted data.

[00144] *Transglycosylation of Fuc1,6GlcNActrastuzumab with SCT-ox by Endo-S2 D184M*

[00145] Commercial trastuzumab (lyophilized powder) was dissolved in water and deglycosylated with wildtype Endo-S2 following the same method for rituximab. For transglycosylation, a solution of Fuc1,6GlcNAc- trastuzumab (**12**) (1 mg, 69 μM) and SCTox (**4**) (1.38 mM, 20 eq) was incubated with Endo-S2 D184M (5 μg) at 30 °C in 100 μl of 100 mM Tris Buffer (pH 7.4) for 15 min. LC-MS analysis indicated the completion of the transglycosylation reaction. The product (**13**) was purified using protein A chromatography.

[00146] References

[00147] The contents of all references cited herein are hereby incorporated by reference herein for all purposes.

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[00195] 48. Collin M, Olsen A: EndoS, a novel secreted protein from Streptococcus pyogenes with endoglycosidase activity on human IgG. *EMBO J.* 2001, 20:3046-3055.

[00196] 49 Strome SE, Sausville EA, Mann D: A mechanistic perspective of monoclonal antibodies in cancer therapy beyond target-related effects. *Oncologist* 2007, 12:1084-1095.

Table 1. Comparison of the specific hydrolysis activities of the Endo-S2 D184 mutants using a synthetic complex glycoform of rituximab as the substrate.

Mutant	Specific hydrolysis activity	Percentage of specific hydrolysis activity
	**100 (umole/min/mg)*	%
WT	11.185	100.000
D184A	0.750	6.701
D184C	1.386	12.392
D184E	0.879	7.855
D184F	0.000	0.000
D184G	1.249	11.168
D184H	0.000	0.000
D184K	0.000	0.000
D184R	0.000	0.000
D184I	0.033	0.295
D184L	0.045	0.405
D184M	0.158	1.410
D184N	0.842	7.526
D184P	0.340	3.043
D184Q	0.049	0.441
D184S	1.387	12.398
D184T	0.212	1.899
D184V	0.409	3.659
D184W	0.000	0.000
D184Y	5.526	49.406

Table 2. Comparison of the specific transglycosylation activity of Endo-S2 D184 mutants using sialoglycan oxazoline as donor substrate and deglycosylated rituximab as the acceptor substrate.

Mutants	Specific transglycosylation activity	Percentage of specific transglycosylation activity
	**10 (umol/min/mg)*	*%*
WT	1.712	44.31
D184A	1.023	26.46
D184C	3.864	100.00
D184E	2.006	51.907
D184F	0.044	1.14
D184G	2.270	58.73
D184H	0.026	0.68
D184K	0.021	0.54
D184R	0.022	0.57
D184I	0.066	1.71
D184L	0.099	2.55
D184M	2.866	74.18
D184N	0.879	22.75
D184P	0.160	4.13
D184Q	0.383	9.92
D184S	1.164	30.13
D184T	0.828	21.42
D184V	0.147	3.79
D184W	0.046	1.20
D184Y	1.198	30.99

CLAIMS

That which is claimed is:

1. A method of preparing a fucosylated or nonfucosylated glycoprotein having a predetermined oligosaccharide moiety, the method comprising:

providing a fucosylated or nonfucosylated acceptor protein comprising a fucosylated GlcNAc-protein or nonfucosylated GlcNAc-protein; and

enzymatically reacting the fucosylated or nonfucosylated GlcNAc acceptor with a S*treptococcus pyogenes* (serotype M49)Endoglycosidase-S2 Asp184 mutant enzyme or fragment thereof that includes the catalytic domain and exhibits increased transglycosylation and reduced hydrolytic activity relative to the wild type Endo-S2 enzyme, wherein the activated oligosaccharide donor carries an oligosaccharide moiety comprising a predetermined number and type of sugar residues, wherein via an enzymatic reaction, the activated oligosaccharide moiety is covalently linked to the fucosylated or nonfucosylated GlcNAc acceptor, thereby preparing the fucosylated or nonfucosylated glycoprotein having the predetermined oligosaccharide moiety.

2. The method of claim 1, wherein the fucosylated or nonfucosylated acceptor protein is an antibody or Fc-containing fragment thereof.

3. The method of any one of claims 1 or 2, wherein the activated oligosaccharide component is a synthetic oligosaccharide oxazoline or natural N-glycan (complex, high mannose, or hybrid) oxazoline.

4. The method of any of one of the preceding claims, wherein the synthetic oligosaccharide oxazoline is a di-, tri-, tetra-, penta-, hexyl-, hepta-, octyl-, nona-, deca- or undeca-saccharide oxazoline.

5. The method of any of one of the preceding claims, wherein the activated oligosaccharide component further comprises an additional biologically active agent or a tag.

6. The method of claim 5, wherein the additional biologically active agent or tag is a drug, toxin, fluorescent probe, biotin, a PEG, lipid, or polypeptide.

7. The method of any one of the preceding claims, wherein the fucosylated acceptor protein is an alpha-1-6-fucosyl-GlcNAc-protein.

8. The method of any of the preceding claims, wherein the fucosylated or nonfucosylated glycoprotein is a monoclonal antibody selected from the group consisting of 17b, 48d, A32, C11, 2G12, F240, IgG1b12, 19e, X5, TNX-355, cetuximab, rituximab, muromonab-CD3, abciximab, daclizumab, basiliximab, palivizumab, infliximab, trastuzumab, gemtuzumab ozogamicin, alemtuzumab, ibritumomab tiuxetan, adalimumab, omalizumab, tositumomab, I-131 tositumomab, efalizumab, bevacizumab, panitumumab, pertuzumab, natalizumab, etanercept, IGN101, volociximab, Anti-CD80 mAb, Anti-CD23 mAb, CAT-3888 , CDP-791, eraptuzumab, MDX-010, MDX-060, MDX-070, matuzumab, CP-675,206, CAL, SGN-30, zanolimumab, adecatumumab, oregovomab, nimotuzumab, ABT-874, denosumab, AM 108, AMG 714, fontolizumab, daclizumab, golimumab, CNTO 1275, ocrelizumab, HuMax-CD20, belimumab, epratuzumab, MLN1202, visilizumab, tocilizumab, ocrerlizumab, certolizumab pegol, eculizumab, pexelizumab, abciximab, ranibizimumab, mepolizumab and MYO-029.

9 The method of claim 2, wherein the antibody further comprises an additional moiety selected from a group consisting of a therapeutic agent for treating cancer, a therapeutic agent for HIV; a toxin, an antibody different from the modified antibody which is reactive to another receptor, an antigen, a chemokine and a cytokine.

10. The method of any one of the preceding claims, wherein the Endoglycosidase-S2 mutant is selected from a mutant library comprising D184A (SEQ ID NO: 2), D184N (SEQ ID NO: 3), D184Q (SEQ ID NO: 4), D184R (SEQ ID NO: 5), D184C (SEQ ID NO: 6), D184M (SEQ ID NO: 7), D184E (SEQ ID NO: 8), D184G (SEQ ID NO: 9), D184H (SEQ ID NO: 10), D184I (SEQ ID NO: 11), D184L(SEQ ID NO: 12), D184K(SEQ ID NO: 13), D184F (SEQ ID NO: 14), D184P(SEQ ID NO: 15), D184S(SEQ ID NO: 16), D184T (SEQ ID NO. 17); D184W(SEQ ID NO: 18), D184Y(SEQ ID NO: 19), D184V(SEQ ID NO: 20) or fragments thereof that includes the catalytic domain and exhibit increased transglycosylation and reduced hydrolysis related to the wild type Endo-S2 protein.

11. The method of any one of the preceding claims, wherein the Endoglycosidase-S2

mutant is D184M (SEQ ID NO:7), D184E (SEQ ID NO: 8) or D184Q (SEQ ID NO: 4).

12. A method of synthesizing a homogeneous fucosylated or nonfucosylated glycoprotein, the method comprising:

(a) providing a natural or recombinant glycoprotein;

(b) removing the heterogeneous or undesired N-glycans by an enzyme selected from the group Endo-H, Endo-F3, Endo S, or Endo-A to form a homogeneous fucosylated or nonfucosylated GlcNAc-containing protein;

(c) providing an oligosaccharide containing oxazoline with a desired oligosaccharide component comprising a N-glycan having a defined number and type of sugar residues;

(d) enzymatically transglycosylating the fucosylated or nonfucosylated GlcNAc-containing protein with the oligosaccharide containing oxazoline using a S*treptococcus pyogenes* Endo-S2 Asp184 mutant protein or fragment thereof that includes the catalytic domain and exhibits increased transglycosylation and reduced hydrolytic activity relative to the wild type Endo-S2 enzyme, thereby forming homogeneous fucosylated or nonfucosylated glycoproteins having the defined number and type of sugar residues.

13. The method of claim 12, wherein the oligosaccharide containing oxazoline is a di-, tri-, tetra-, penta-, hexyl-, hepta-, octyl-, nona-, deca-, or undeca-saccharide oxazoline.

14. The method of any one of claims 12 or 13, wherein the core-fucosylated GlcNAc containing protein is an alpha-1-6-fucosyl-GlcNAc-protein.

15. A method of synthesizing a modified antibody or Fc-fragment thereof, the method comprising;

providing an antibody or Fc fragment comprising a fucosylated or nonfucosylated N-acetylglucosamine (GlcNAc) moiety to form a GlcNAc-protein acceptor; wherein the fucosylated or nonfucosylated N-acetylglucosamine (GlcNAc) moiety is positioned on the Fc region of the antibody;

transglycosylating an oligosaccharide oxazoline having a predetermined number of saccharides and the GlcNAc-peptide acceptor under the catalysis of a S*treptococcus pyogenes* Endo-S2 Asp 184 mutant enzyme or fragment thereof that includes the catalytic domain and exhibits increased transglycosylation and

reduced hydrolytic activity relative to the wild type Endo-S2 enzyme, to form the modified antibody or Fc fragment with the predetermined number of saccharides.

16. The method of claim 15, wherein the modified antibody further comprises an additional moiety including, a therapeutic agent for treating cancer, a therapeutic agent for HIV; a toxin, an antibody different from the modified antibody which is reactive to another receptor, an antigen, a therapeutic polypeptide, a chemokine and/or a cytokine attached to the oligosaccharide oxazoline.

17. A delivery device for delivering a drug having biological activity to treat a condition, the delivery device comprising: a remodeled antibody comprising a recombinant fucosylated or nonfucosylated antibody having a predetermined number of sugar residues and a therapeutic agent attached to a terminal sugar or sialylated group, wherein the remodeled antibody is synthesized according to the method of claim 15.

18. The delivery device of claim 17, wherein the remodel antibody is a monoclonal antibody selected from the group consisting of 17b, 48d, A32, C11, 2G12, F240, IgG1b12, 19e, X5, TNX-355, cetuximab, rituximab, muromonab-CD3, abciximab, daclizumab, basiliximab, palivizumab, infliximab, trastuzumab, gemtuzumab ozogamicin, alemtuzumab, ibritumomab tiuxetan, adalimumab, omalizumab, tositumomab, I-131 tositumomab, efalizumab, bevacizumab, panitumumab, pertuzumab, natalizumab, etanercept, IGN101, volociximab, Anti-CD80 mAb, Anti-CD23 mAb, CAT-3888 , CDP-791, eraptuzumab, MDX-010, MDX-060, MDX-070, matuzumab, CP-675,206, CAL, SGN-30, zanolimumab, adecatumumab, oregovomab, nimotuzumab, ABT-874, denosumab, AM 108, AMG 714, fontolizumab, daclizumab, golimumab, CNTO 1275, ocrelizumab, HuMax-CD20, belimumab, epratuzumab, MLN1202, visilizumab, tocilizumab, ocrerlizumab, certolizumab pegol, eculizumab, pexelizumab, abciximab, ranibizimumab, mepolizumab and MYO-029.

19. The delivery device of claim 17, wherein the therapeutic agent is selected from an agent for treating cancer, a therapeutic agent for HIV; a toxin, an antibody different from the modified antibody which is reactive to another receptor, an antigen, a chemokine and a cytokine.

20. The delivery device of claim 15, wherein the modified antibody further comprises an

additional moiety including, a therapeutic agent for treating cancer, a therapeutic agent for HIV; a toxin, an antibody different from the modified antibody which is reactive to another receptor, an antigen, a therapeutic polypeptide, a chemokine and/or a cytokine thereby forming an Antibody-drug conjugate (ADC).

21. A method of synthesizing intravenous immunoglobulin (IVIG) preparation exhibiting Fc-sialylated glycoforms, the method comprising:

providing an IVIG carrying Fc N-glycans;

deglycosylating the Fc N-glycans using an endoglycosidase-S enzyme or another endoglycosidase such as Endo-F3 to form a GlcNAc- acceptor; wherein the GlcNAc moiety is positioned on the Fc region of the IVIG and the GlcNAc moiety is either fucosylated or nonfucosylated; and

transglycosylating the GlcNAc moiety in the IVIG with a sialoglycan oxazoline having a predetermined number of sugar residues under the catalysis of an enzyme selected from the group of Endo-S2 mutants SEQ ID NO: 2 to SEQ ID NO: 20 or fragment thereof that exhibits increased transglycosylation and reduced hydrolytic activity relative to the wild type Endo-S2 enzyme, to form a sialylated IVIG.

22. An IVIG containing composition comprising at least 90% of homogeneous sialylated Fc glycoforms to increase anti-inflammatory activity of the composition, wherein the sialylated Fc glycoforms are synthesized using a S*treptococcus pyogenes* Endo-S2 Asp-184 mutant or fragment thereof that includes the catalytic domain and exhibits increased transglycosylation and reduced hydrolytic activity relative to the wild type Endo-S2 enzyme in combination with a GlcNAc moiety positioned on the Fc region of a deglycosylated IVIG and a sialoglycan oxazoline having a predetermined number of sugar residues.

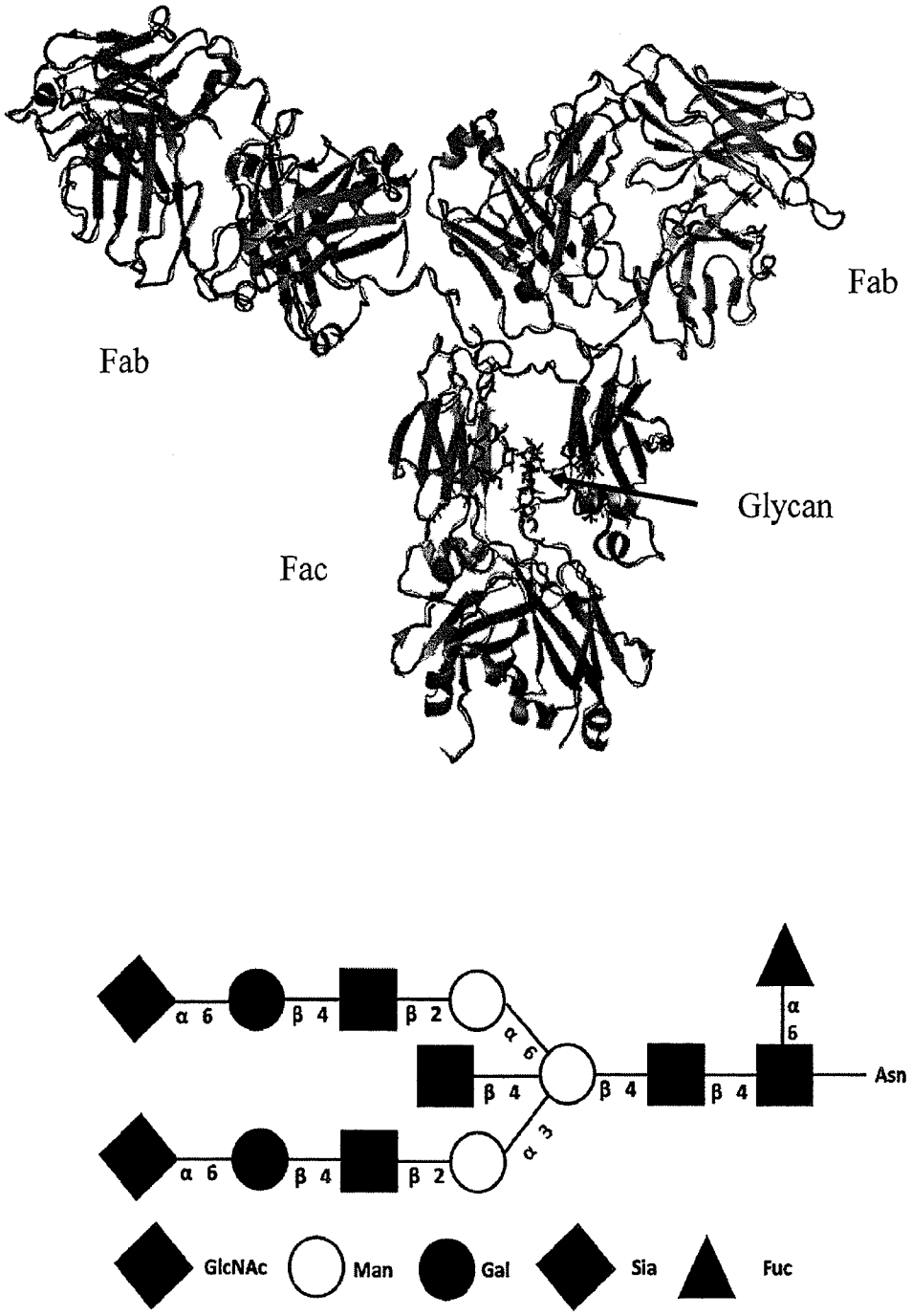
Fab
Fab
Glycan
Fac
α 6
β 4
β 2
α 6
β 4
β 4
β 4
α 6
Asn
α 6
β 4
β 2
α 3
GlcNAc
Man
Gal
Sia
Fuc

Figure 1

```
Endo S2     WHDRASTGIDGKQQHPENTMAEVPKEVDILFVFHDHTASDSPFWSELKDSYVHKLHQQGT 133
Endo S      WHDKTSDPT---EKDKVNSMGELPKEVDLAFIFHDWTKDYSLFWKELATKHVPKLNKQGT 177
            ***::*      ::.  *:*.*:*****: *:*** * . * **.**  .:* **.:***

Endo S2     ALVQTIGVNELNGR-----TGLSKDYPDTPEGNKALAAAIVKAFVTDRGVDGLDIDIEHE 188
Endo S      RVIRTIPWRFLAGGDNSGIAEDTSKYPNTPEGNKALAKAIVDEYVYKYNLDGLDVDVEHD 237
             :::**  . * *      :  :..**:********* ***. :* .  :****:*:**:

Endo S2     FTNKRTPEE----DARALNVFKEIAQLIGKNGSDKSKLLIMDTTLSVENNPIFKGIAEDL 244
Endo S      SIPKVDKKEDTAGVERSIQVFEEIGKLIGPKGVDKSRLFIMDSTYMADKNPLIERGAPYI 297
               *   :*      *:::**:**.:*** :* ***:*:***:*  .::**:::  *  :
```

Figure 2

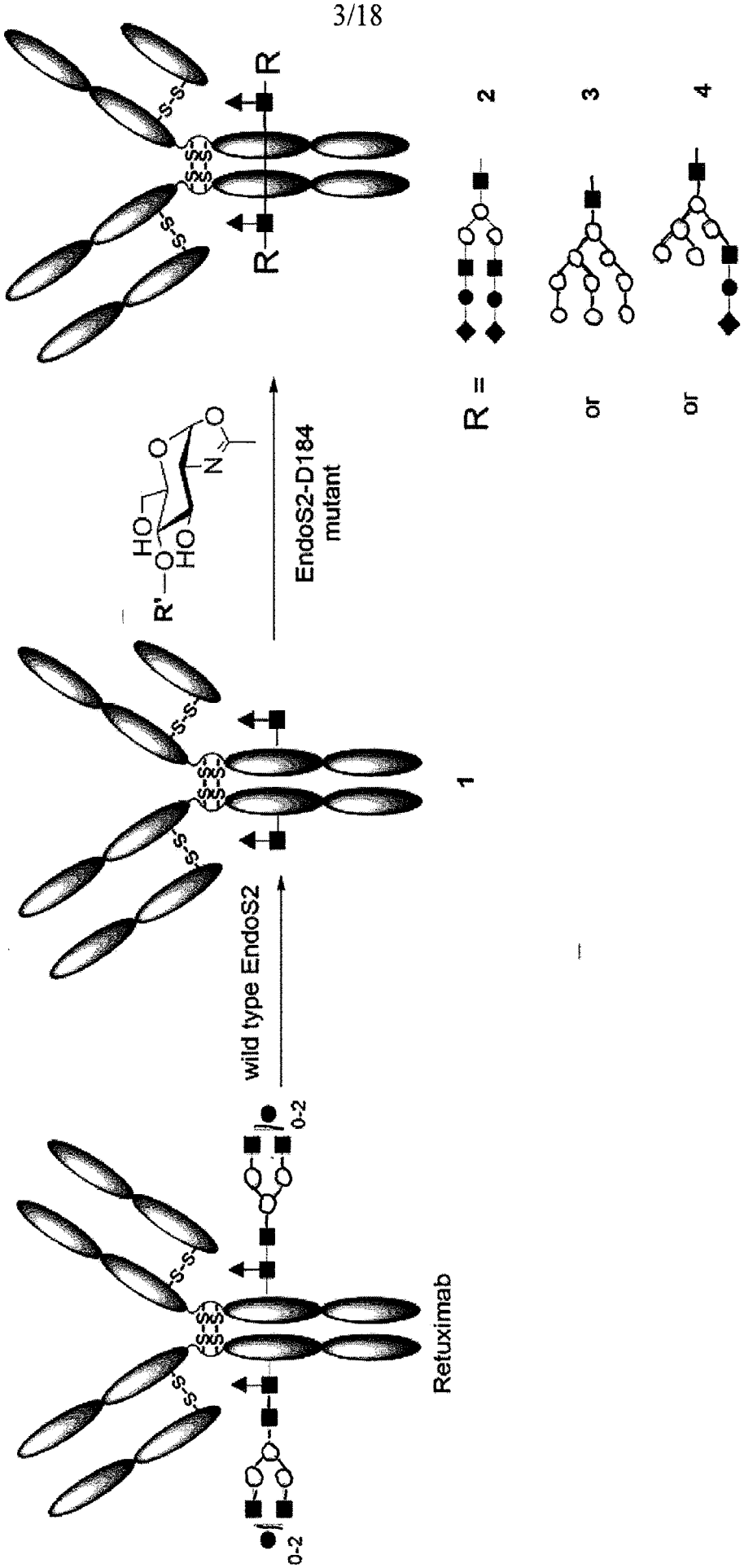
Retuximab
0-2
wild type EndoS2
1
EndoS2-D184 mutant
R'
HO
HO
O
N
R
R
S-S
R =
2
or
3
or
4

Figure 3

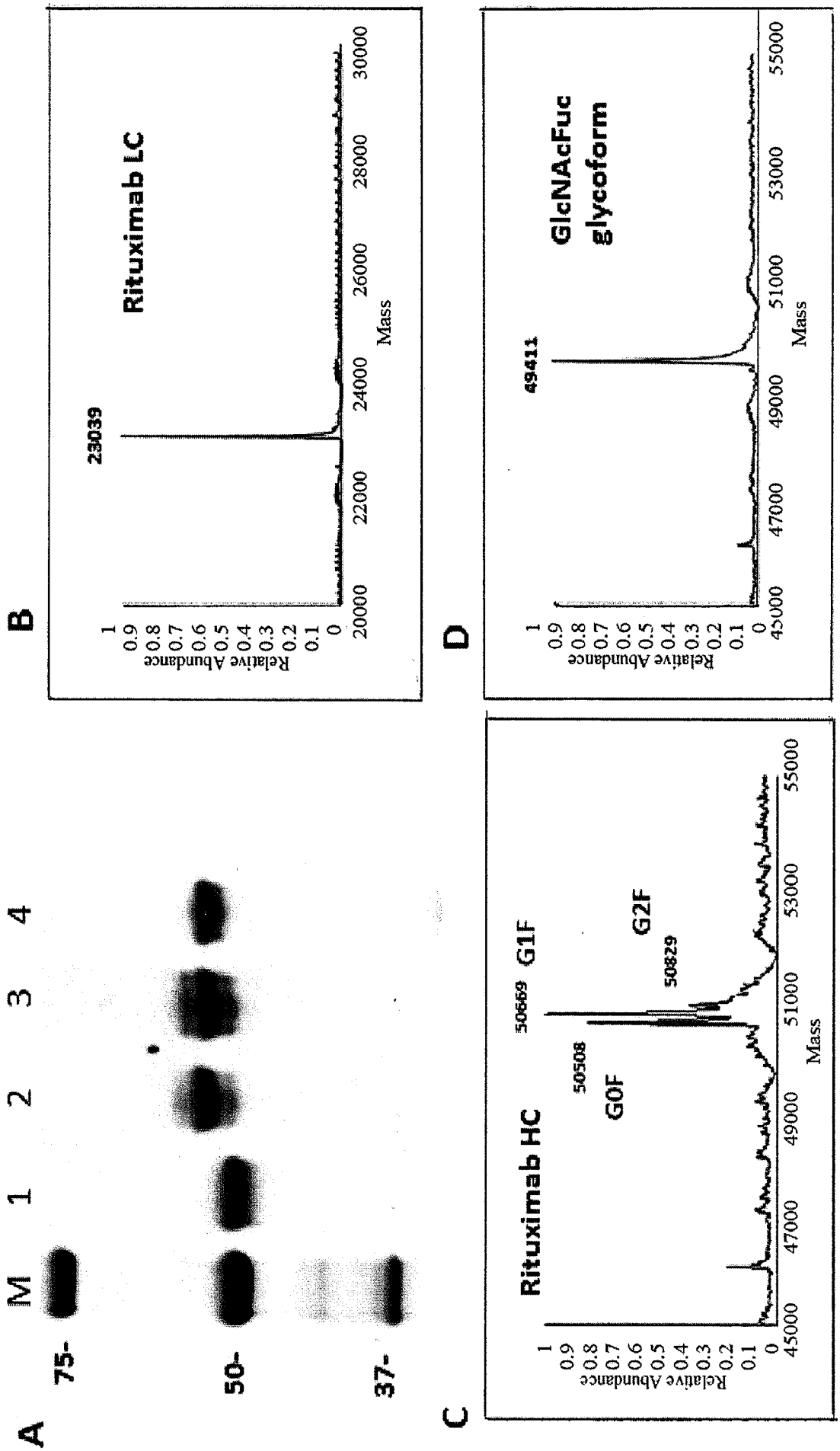
A
M
1
2
3
4
75-
50-
37-
B
Rituximab LC
23039
Relative Abundance
Mass
C
Rituximab HC
G0F
50508
G1F
50669
G2F
50829
Relative Abundance
Mass
D
GlcNAcFuc glycoform
49411
Relative Abundance
Mass

Figure 4

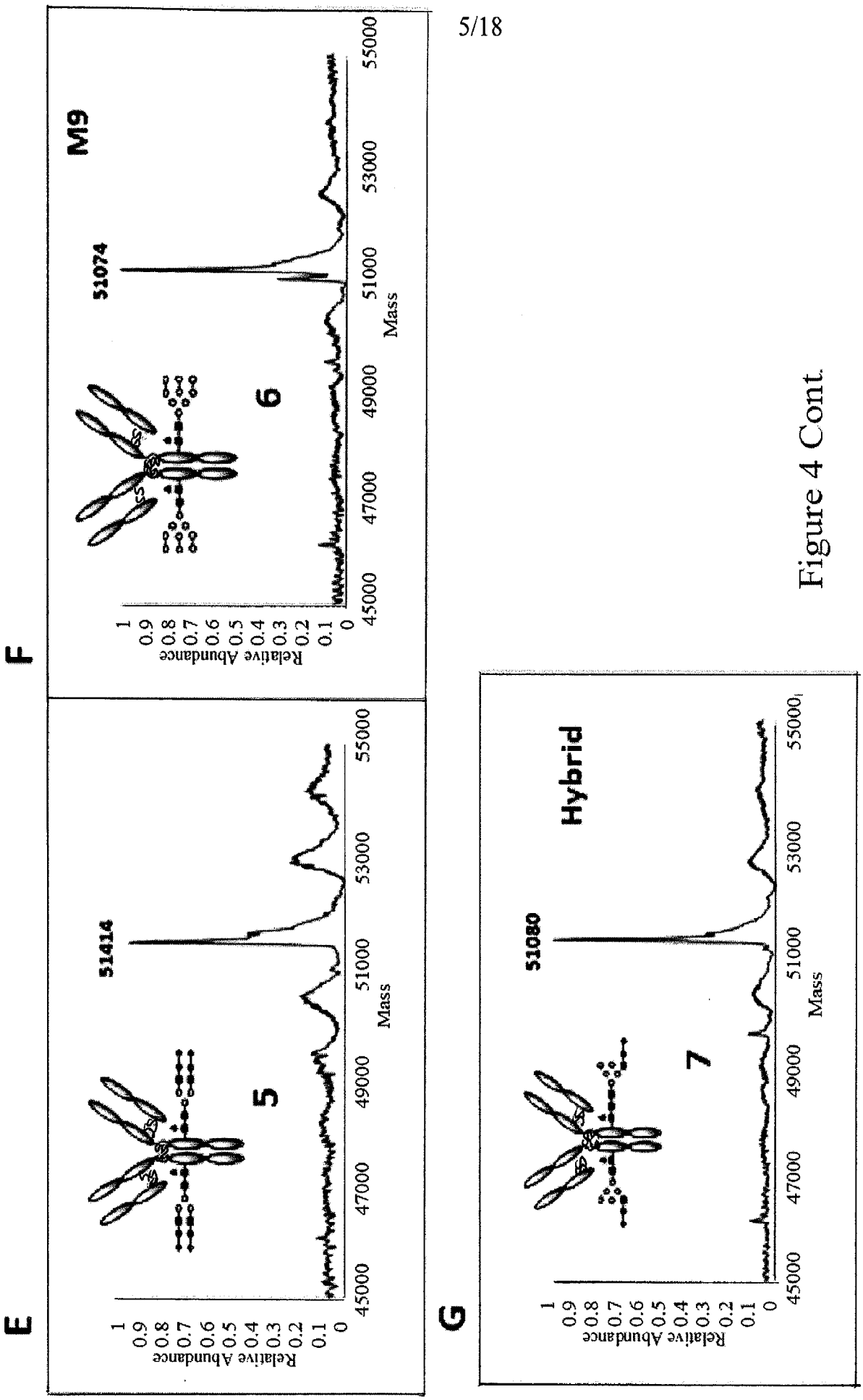

Figure 4 Cont.

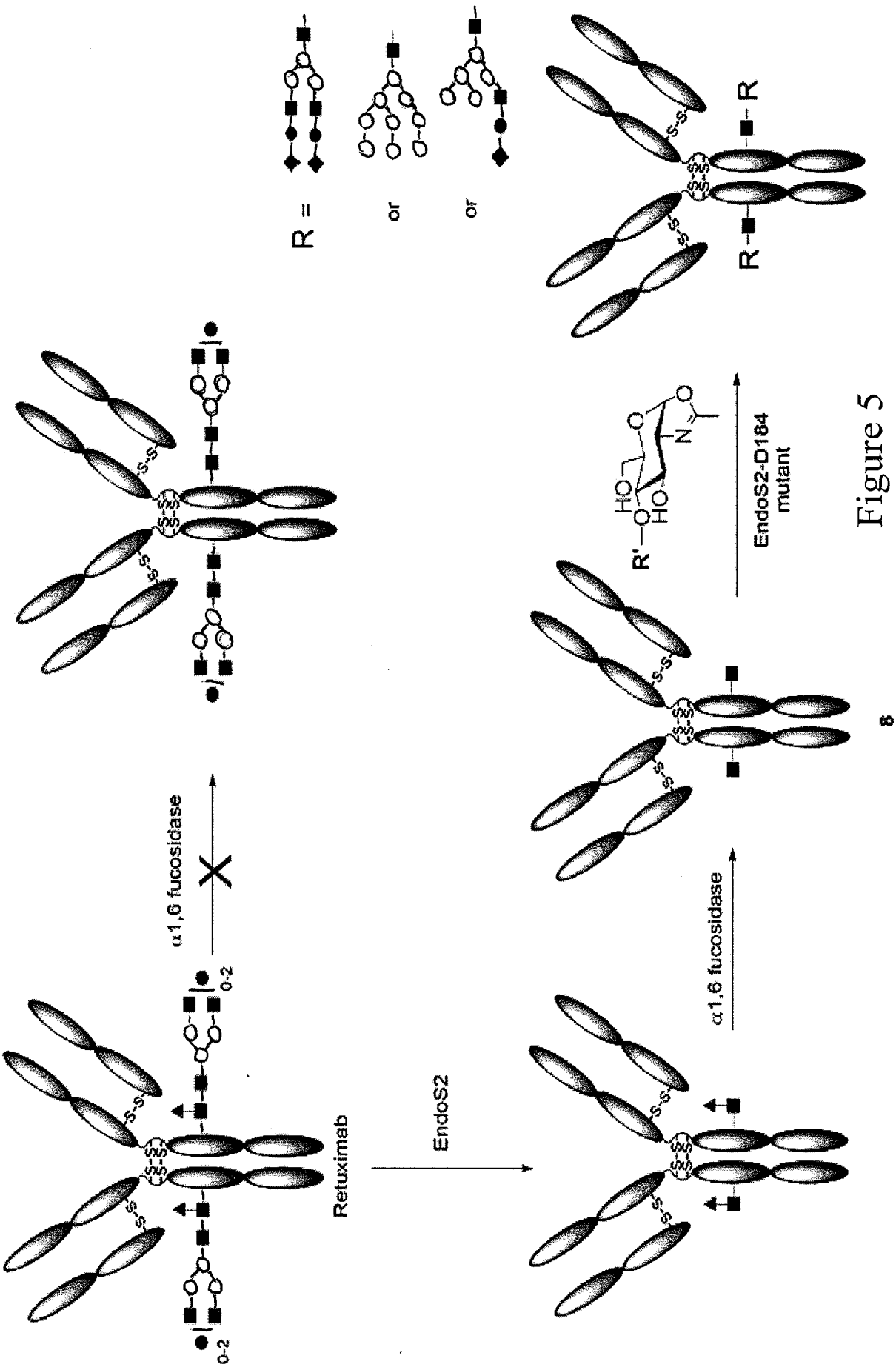
R =
or
or
R
R
Retuximab
EndoS2
α1,6 fucosidase
α1,6 fucosidase
EndoS2-D184 mutant
R'
0-2
0-2

Figure 5

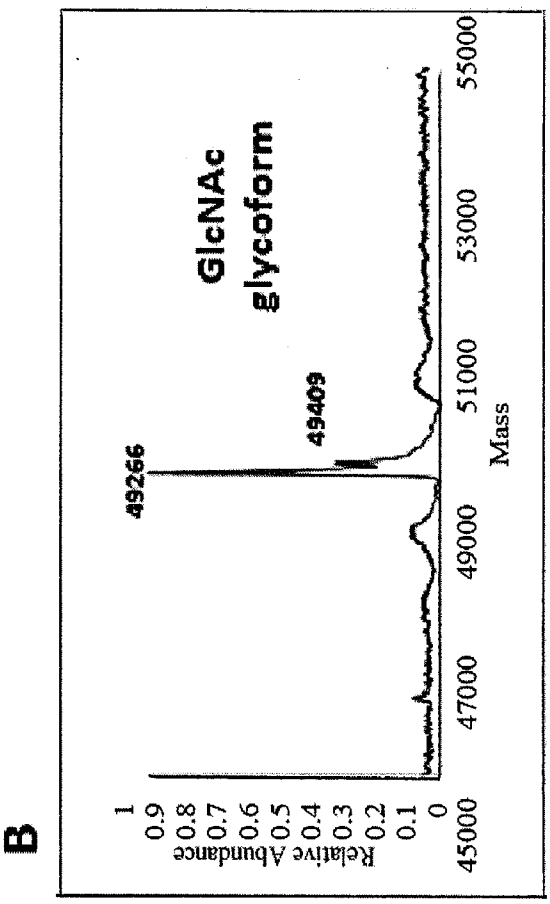
B
GlcNAc
glycoform
49266
49409
Relative Abundance
Mass
45000
47000
49000
51000
53000
55000
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
A
M
1
2
3
4
5
75-
50-
37-

Figure 6

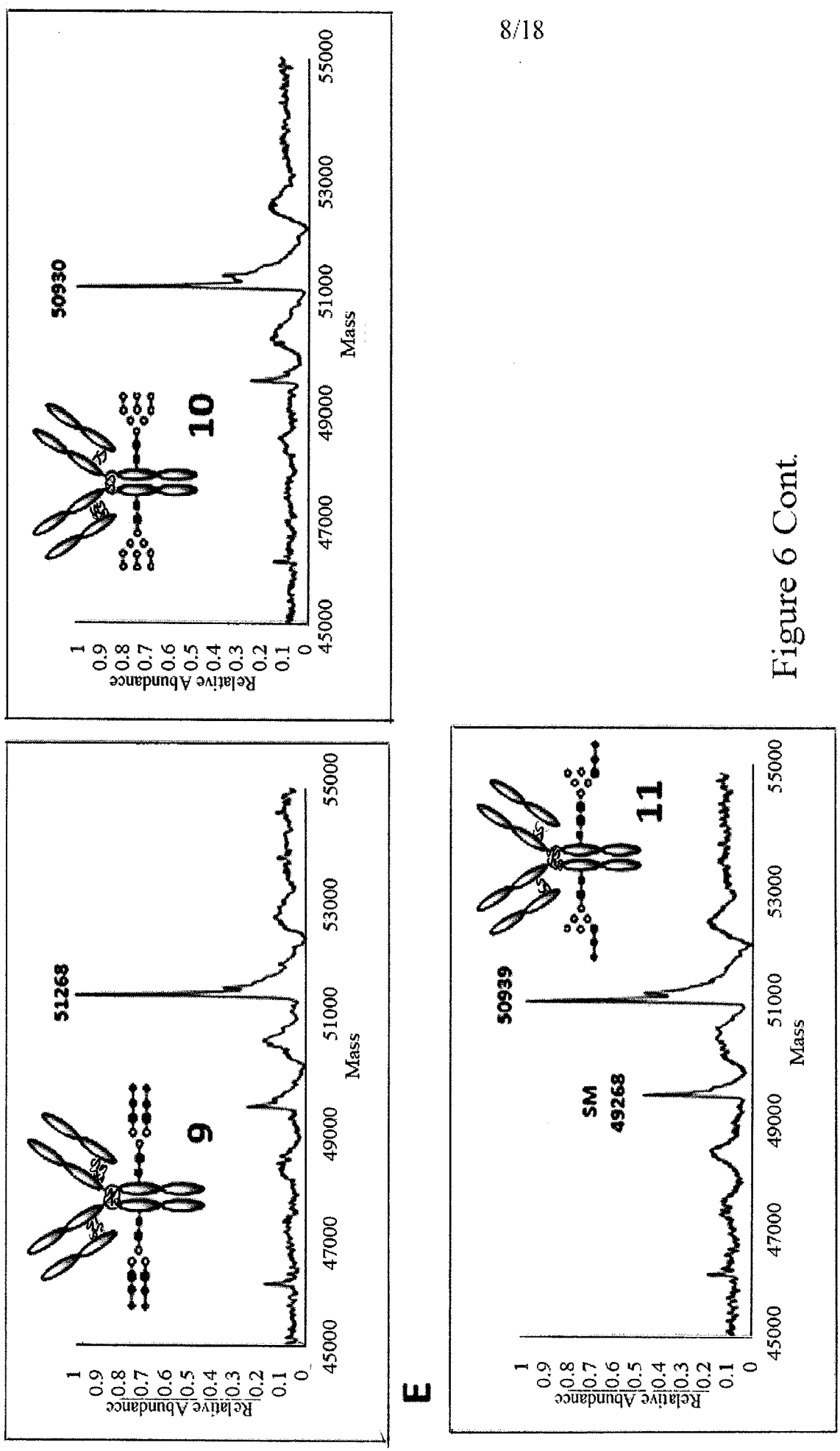

Figure 6 Cont.

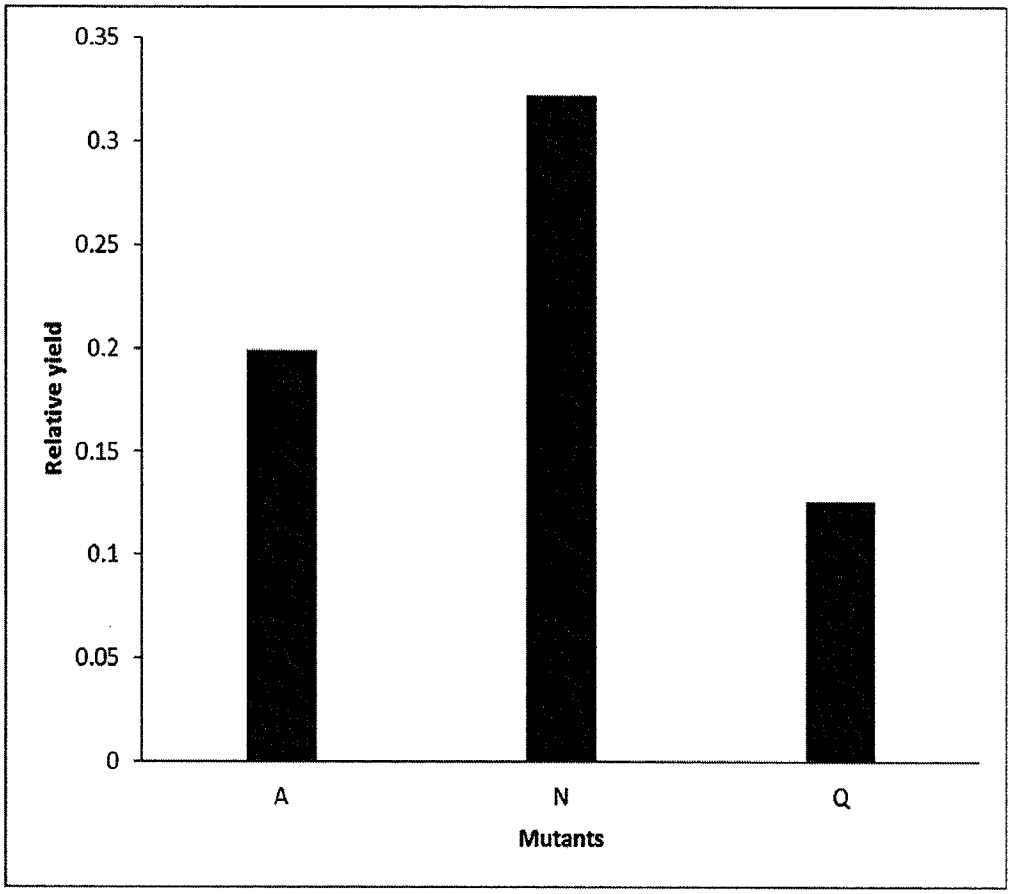

Figure 7

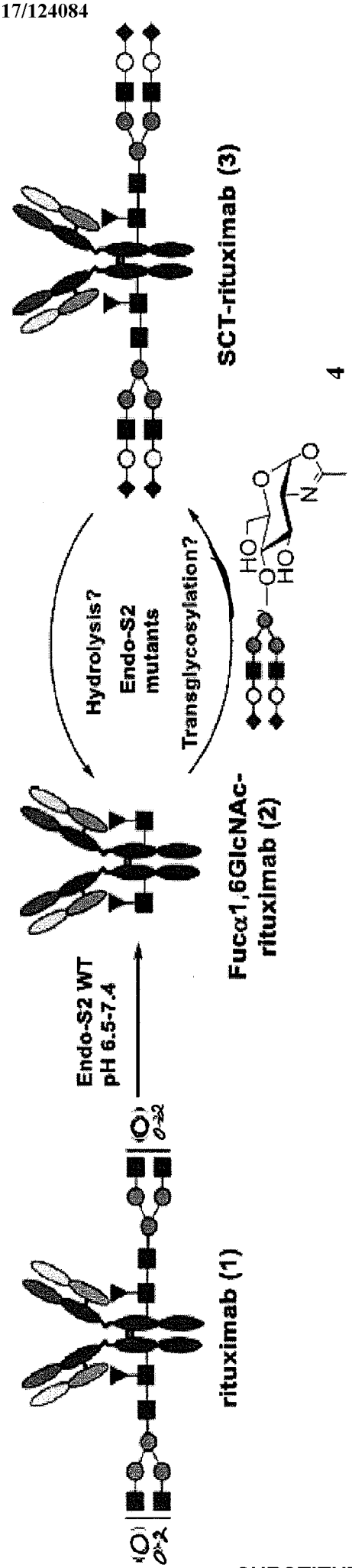
rituximab (1)
Endo-S2 WT
pH 6.5-7.4
Fucα1,6GlcNAc-
rituximab (2)
Hydrolysis?
Endo-S2
mutants
Transglycosylation?
SCT-rituximab (3)
4

Figure 8

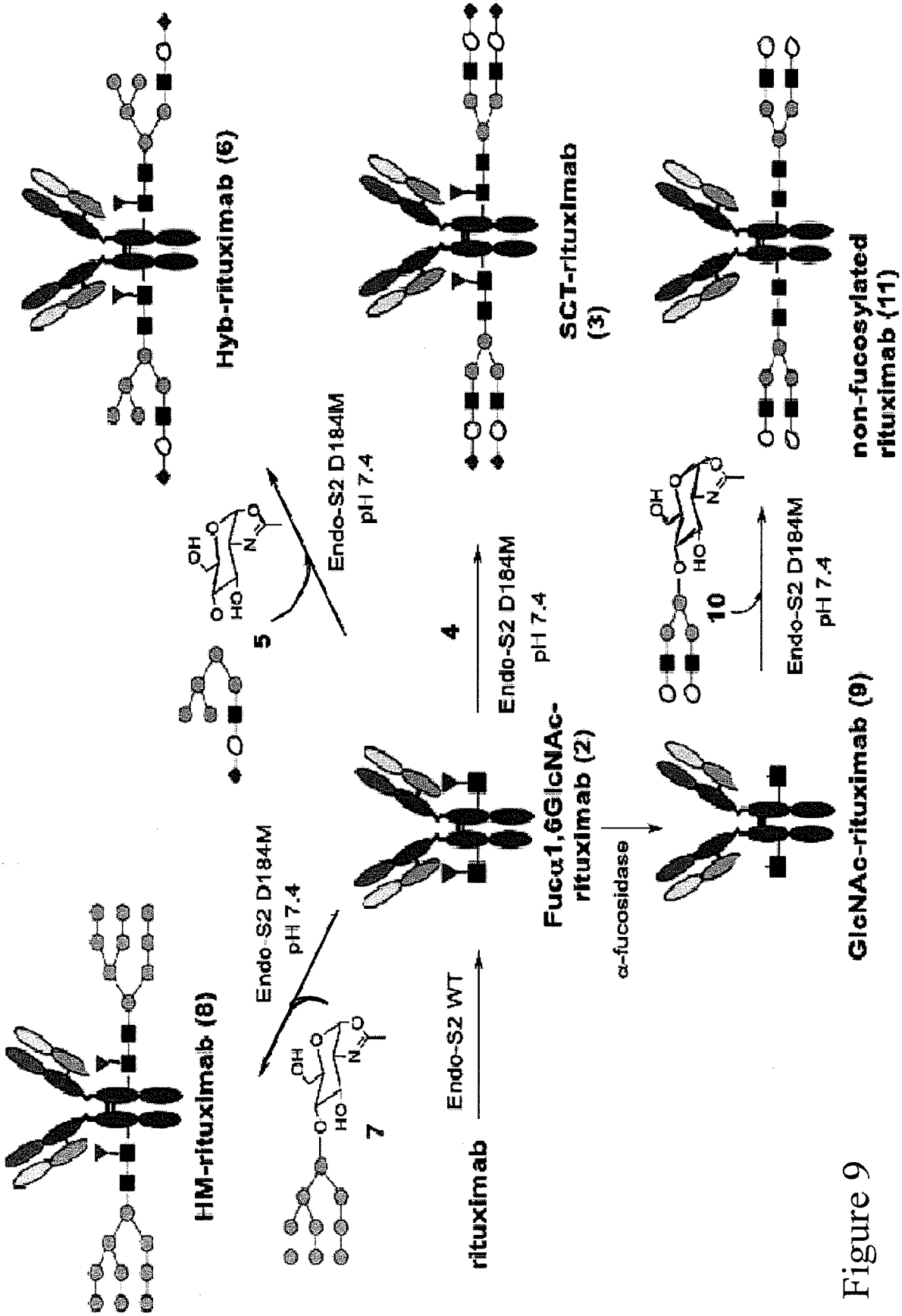
HM-rituximab (8)
Endo-S2 D184M
pH 7.4
7
Endo-S2 WT
rituximab
Hyb-rituximab (6)
5
Endo-S2 D184M
pH 7.4
4
Endo-S2 D184M
pH 7.4
Fucα1,6GlcNAc-rituximab (2)
α-fucosidase
SCT-rituximab (3)
10
Endo-S2 D184M
pH 7.4
GlcNAc-rituximab (9)
non-fucosylated rituximab (11)

Figure 9

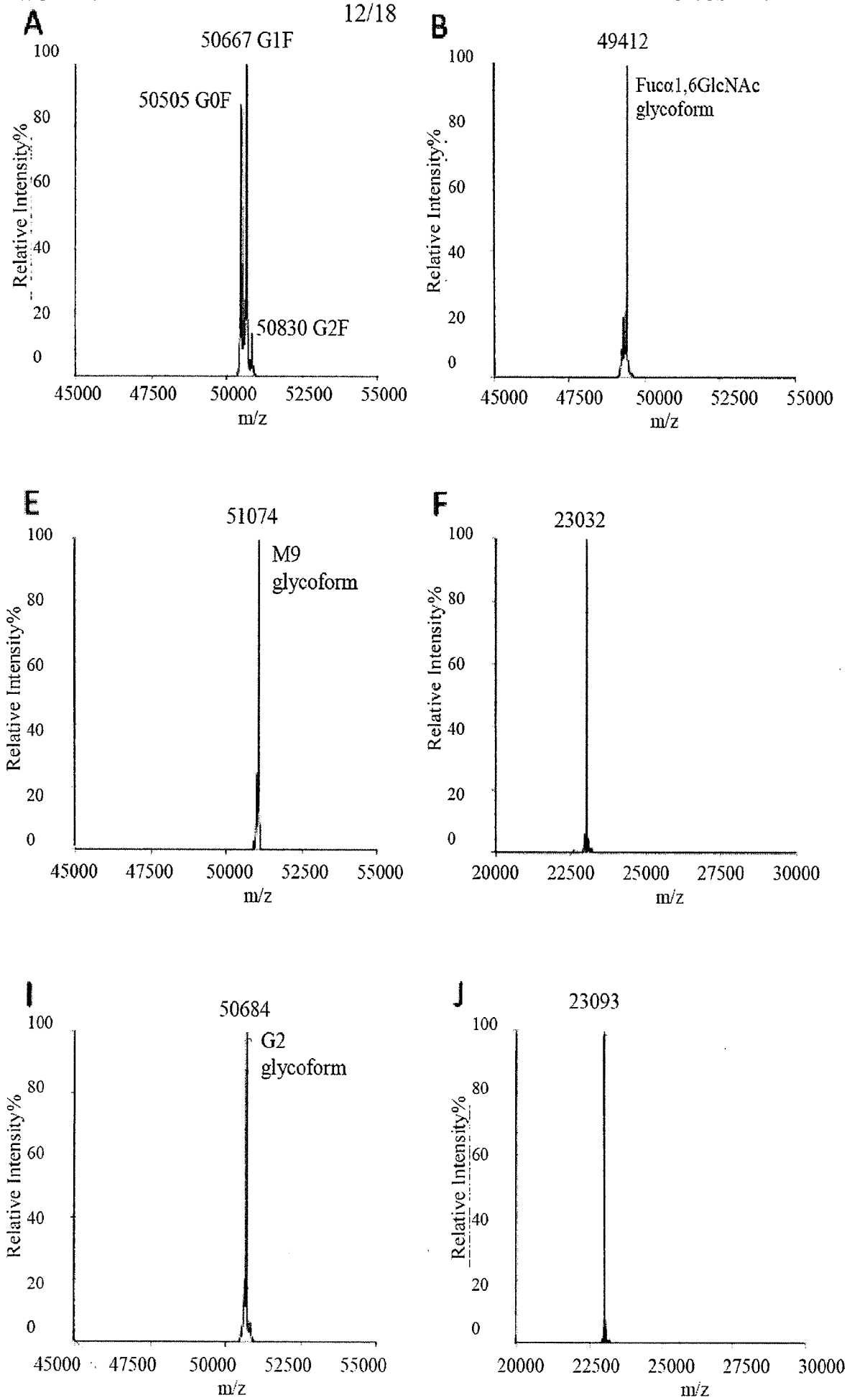
A
50667 G1F
50505 G0F
50830 G2F
B
49412
Fucα1,6GlcNAc
glycoform
E
51074
M9
glycoform
F
23032
I
50684
G2
glycoform
J
23093
Relative Intensity%
m/z

Figure 10

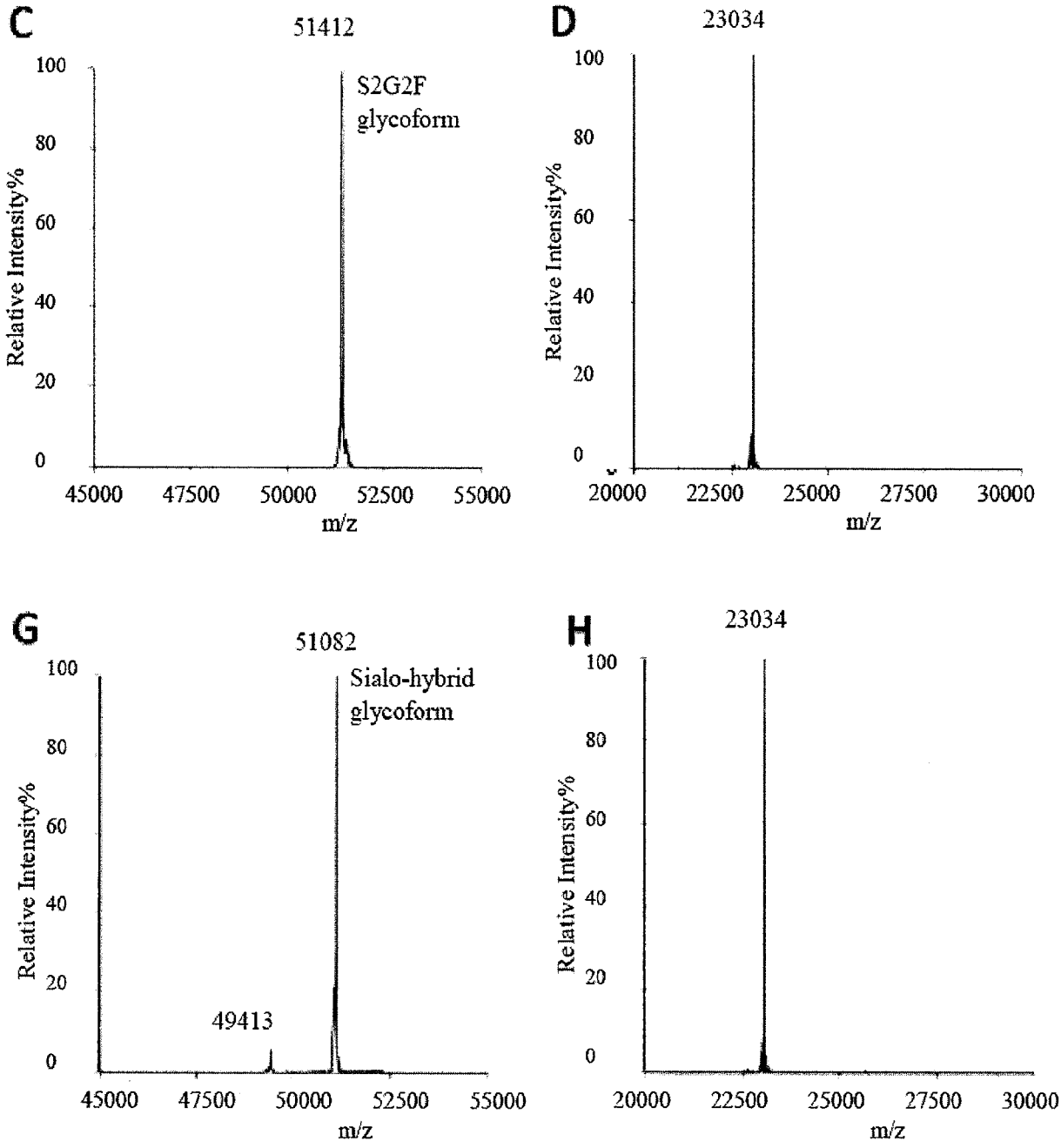

Figure 10 Cont.

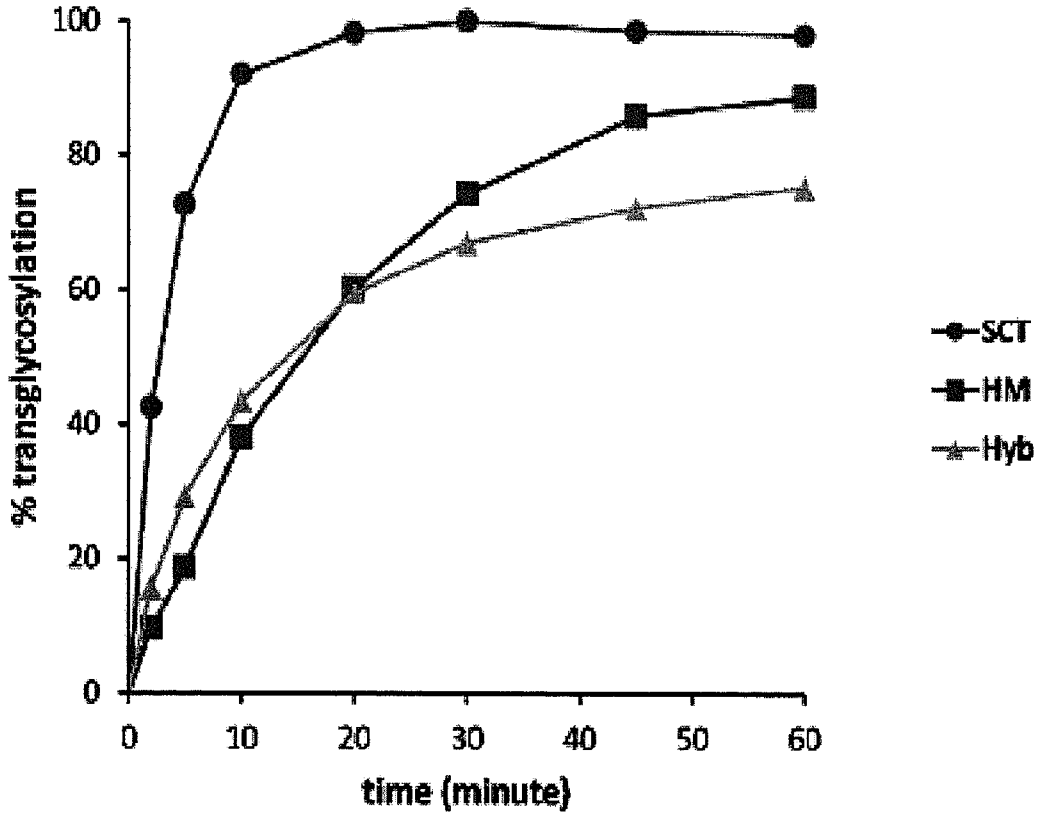
100
80
60
40
20
0
% transglycosylation
0
10
20
30
40
50
60
time (minute)
SCT
HM
Hyb

Figure 11

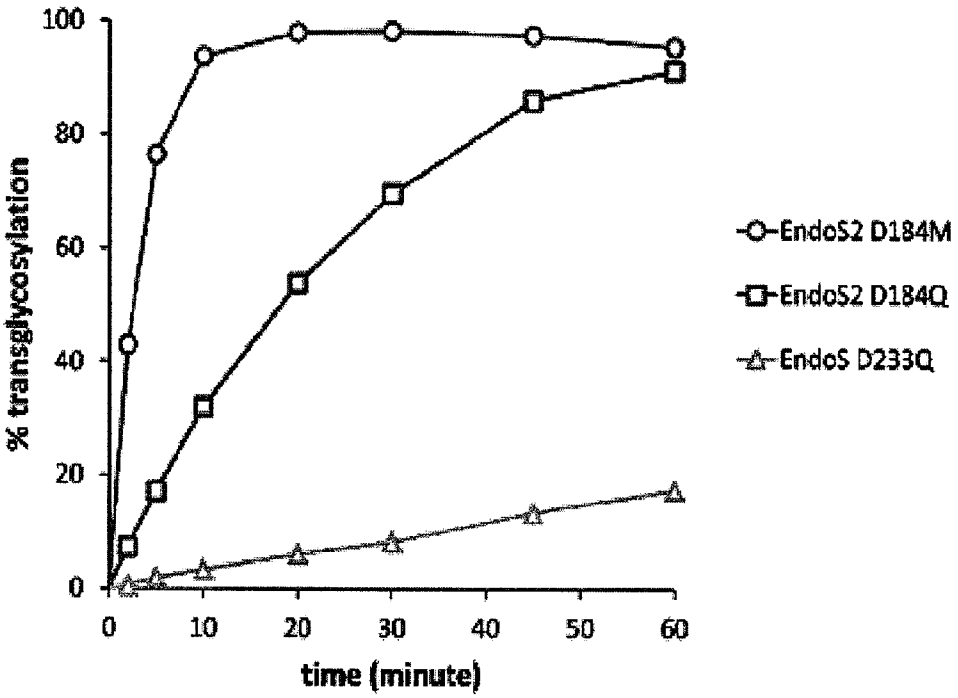
100
80
60
40
20
0
% transglycosylation
0
10
20
30
40
50
60
time (minute)
EndoS2 D184M
EndoS2 D184Q
EndoS D233Q

Figure 12

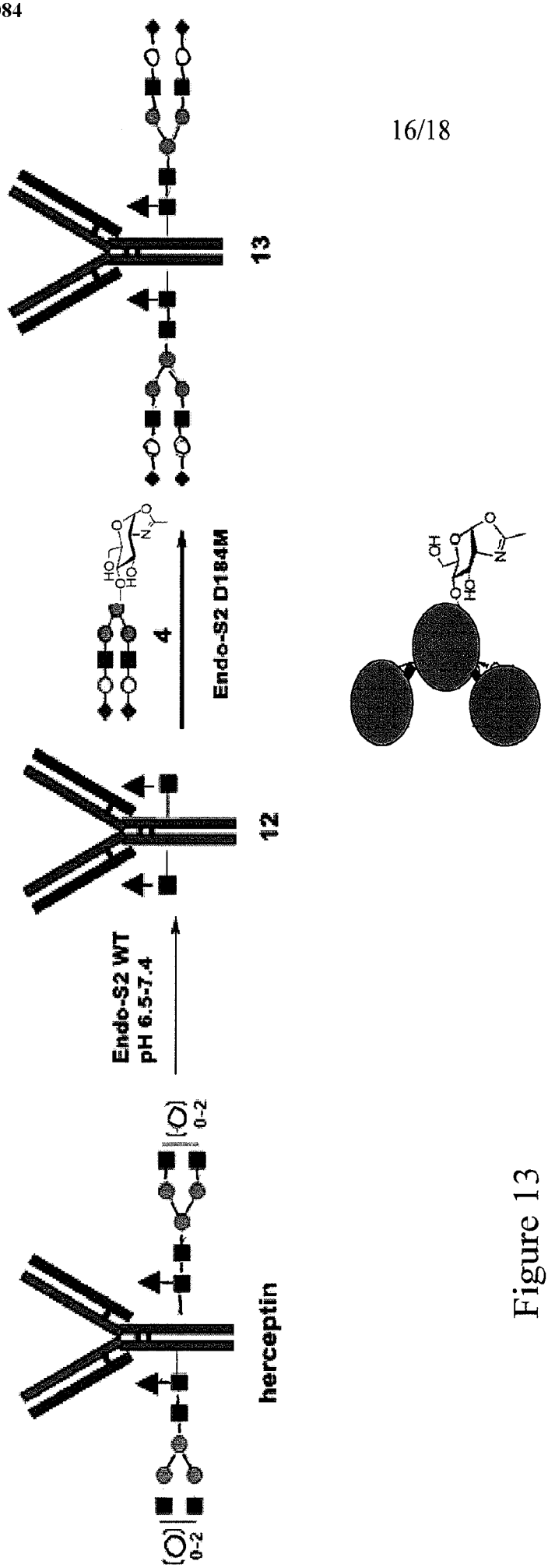

Figure 13

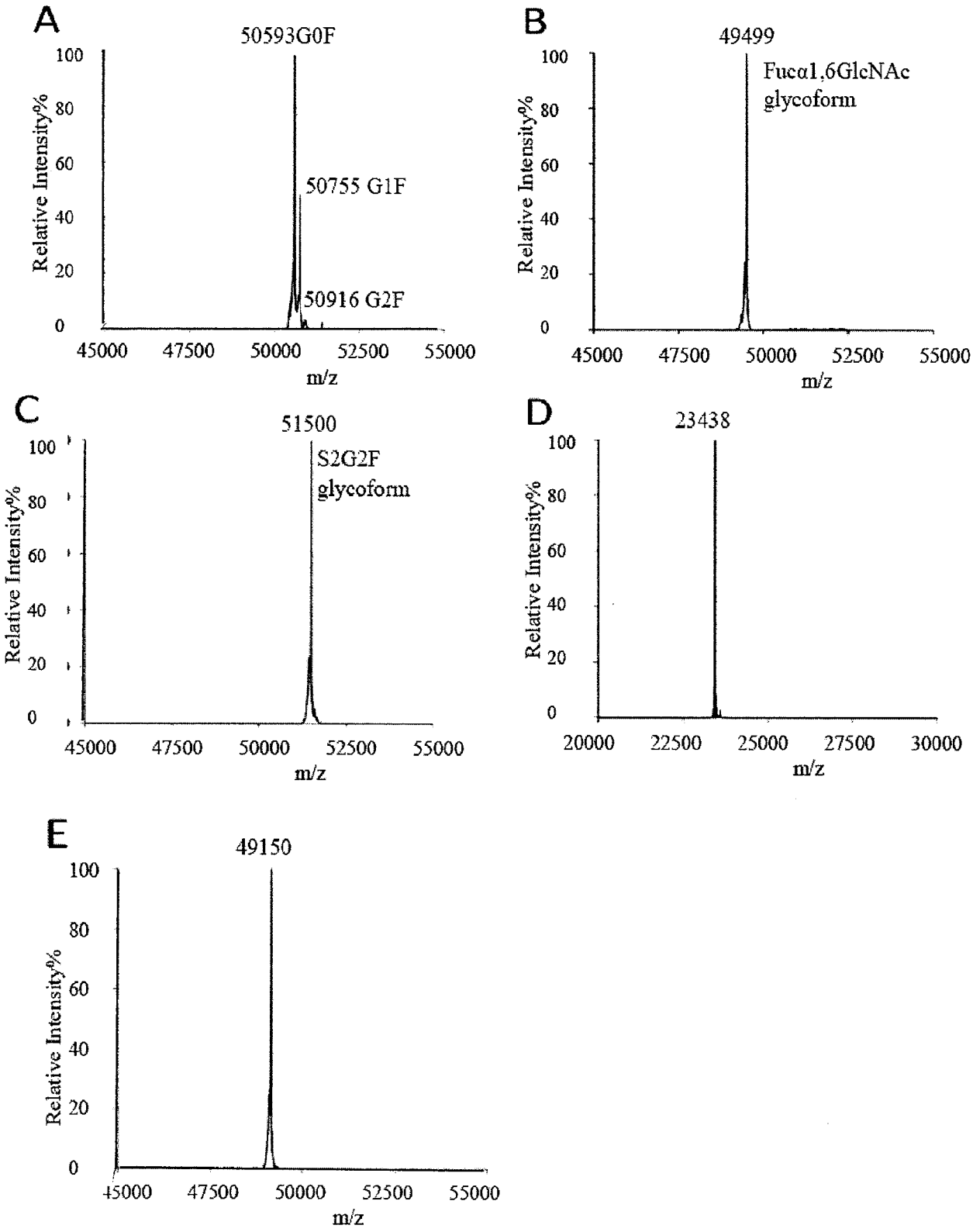

Figure 13 Cont.

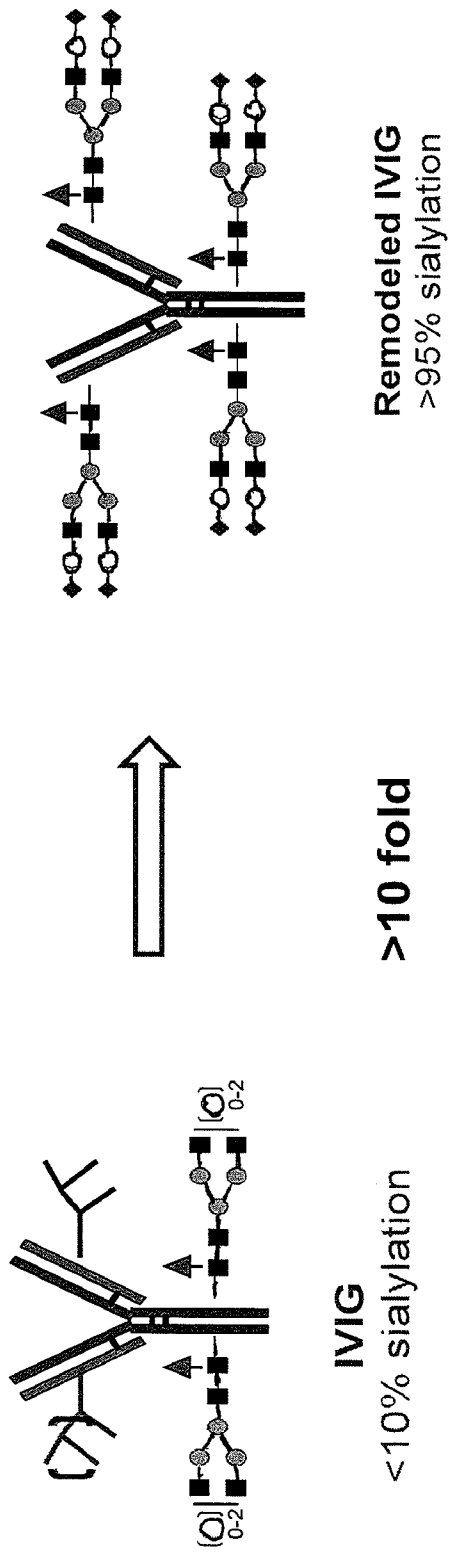

Figure 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/013734

A. CLASSIFICATION OF SUBJECT MATTER

C07K 16/00(2006.01)i, C12P 21/00(2006.01)i, A61K 47/50(2017.01)i

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)
C07K 16/00; C07K 16/28; A61K 39/395; C12P 21/00; C12P 21/08; G01N 33/50; C12N 9/24; A61K 47/50

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: GlcNAc protein, oligosaccharide oxazoline, antibody, Streptococcus pyogenes, Endo-S2 Asp mutant

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2015-0087814 A1 (UNIVERSITY OF MARYLAND, BALTIMORE) 26 March 2015 See paragraphs [0016], [0028]; claims 1-17, 20; Figure 3A.	17-19,22
A		1-3,9,12-16,20-21
A	WO 2015-057066 A1 (SYNAFFIX B.V.) 23 April 2015 See the whole document.	1-3,9,12-22
A	US 2015-0176045 A1 (CALIBER BIOTHERAPEUTICS, LLC) 25 June 2015 See the whole document.	1-3,9,12-22
A	GARRIDO et al., 'Endo-beta-N-acetylglucosaminidases from infant gut-associated bifidobacteria release complex N-glycans from human milk glycoproteins' Molecular and Cellular Proteomics, Vol.11, No.9, pages 775-785 (2012) See the whole document.	1-3,9,12-22
A	COLLIN et al., 'EndoS, a novel secreted protein from Streptococcus pyogenes with endoglycosidase activity on human IgG' The EMBO Journal, Vol.20, No.12, pages 3046-3055 (2001) See the whole document.	1-3,9,12-22

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* 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
17 April 2017 (17.04.2017)	**17 April 2017 (17.04.2017)**

Name and mailing address of the ISA/KR
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/013734

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	LI et al., 'Glycosynthase mutants of endoglycosidase S2 show potent transglycosylation activity and remarkably relaxed substrate specificity for antibody glycosylation remodeling' Journal of Biological Chemistry, Vol.291, No.32, pages 16508-16518 (07 June 2016) See pages 16508-16516.	1-3,9,12-22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/013734

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.: 6
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:
Claim 6 refers to one of unsearchable claims which do not comply with PCT Rule 6.4(a).

3. ☒ Claims Nos.: 4-5,7-8,10-11
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 addtional 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 an additional fees, this Authority did not invite payment of any 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.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2017/013734

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		EP 3083966 A1	26/10/2016
		JP 2017-502694 A	26/01/2017
		WO 2015-095037 A1	25/06/2015