

TITLE OF THE INVENTION

EFFICIENT PRODUCTION OF HETEROLOGOUS PROTEINS USING MANNOSYL
TRANSFERASE INHIBITORS

5 BACKGROUND OF THE INVENTION

Glycoproteins mediate many essential functions in humans and other mammals, including catalysis, signaling, cell-cell communication, and molecular recognition and association. Glycoproteins make up the majority of non-cytosolic proteins in eukaryotic organisms (Lis and Sharon, 1993, *Eur. J. Biochem.* 218:1-27). Many glycoproteins have been
10 exploited for therapeutic purposes, and during the last two decades, recombinant versions of naturally-occurring glycoproteins have been a major part of the biotechnology industry. Examples of recombinant glycosylated proteins used as therapeutics include erythropoietin (EPO), therapeutic monoclonal antibodies (mAbs), tissue plasminogen activator (tPA), interferon- β (IFN- β), granulocyte-macrophage colony stimulating factor (GM-CSF), and human
15 chorionic gonadotrophin (hCG) (Cumming *et al.*, 1991, *Glycobiology* 1:115-130). Variations in glycosylation patterns of recombinantly produced glycoproteins have recently been the topic of much attention in the scientific community as recombinant proteins produced as potential prophylactics and therapeutics approach the clinic.

In general, the glycosylation structures of glycoprotein oligosaccharides will vary
20 depending upon the host species of the cells used to produce them. Therapeutic proteins produced in non-human host cells are likely to contain non-human glycosylation which may elicit an immunogenic response in humans—e.g. hypermannosylation in yeast (Ballou, 1990, *Methods Enzymol.* 185:440-470); α (1,3)-fucose and β (1,2)-xylose in plants, (Cabanès-Macheteau *et al.*, 1999, *Glycobiology*, 9: 365-372); N-glycolylneuraminic acid in Chinese hamster ovary cells
25 (Noguchi *et al.*, 1995, *J. Biochem.* 117: 5-62); and, Gal α -1,3Gal glycosylation in mice (Borrebaeck, *et al.*, 1993, *Immun. Today*, 14: 477-479). Carbohydrate chains bound to proteins in animal cells include N-glycoside bond type carbohydrate chains (also called N-glycans; or N-linked glycosylation) bound to an asparagine (Asn) residue in the protein and O-glycoside bond type carbohydrate chains (also called O-glycans; or O-linked glycosylation) bound to a serine
30 (Ser) or threonine (Thr) residue in the protein.

Because the oligosaccharide structures of glycoproteins produced by non-human mammalian cells tend to be more closely related to those of human glycoproteins, most commercial glycoproteins are produced in mammalian cells. However, mammalian cells have

several important disadvantages as host cells for protein production. Besides being costly, processes for producing proteins in mammalian cells produce heterogeneous populations of glycoforms, have low volumetric titers, and require both ongoing viral containment and significant time to generate stable cell lines.

5 It is well recognized that the particular glycoforms on a protein can profoundly affect the properties of the protein, including its pharmacokinetic, pharmacodynamic, receptor-interaction, and tissue-specific targeting properties (Graddis *et al.*, 2002. *Curr Pharm Biotechnol.* 3: 285-297). For example, it has been shown that different glycosylation patterns of Igs are associated with different biological properties (Jefferis and Lund, 1997, *Antibody Eng. Chem.* 10 *Immunol.*, 65: 111-128; Wright and Morrison, 1997, *Trends Biotechnol.*, 15: 26-32). It has further been shown that galactosylation of a glycoprotein can vary with cell culture conditions, which may render some glycoprotein compositions immunogenic depending on the specific galactose pattern on the glycoprotein (Patel *et al.*, 1992. *Biochem J.* 285: 839-845). However, because it is not known which specific glycoform(s) contribute(s) to a desired biological 15 function, the ability to enrich for specific glycoforms on glycoproteins is highly desirable. Because different glycoforms are associated with different biological properties, the ability to enrich for glycoproteins having a specific glycoform can be used to elucidate the relationship between a specific glycoform and a specific biological function of the glycoprotein. Also, the ability to enrich for glycoproteins having a specific glycoform enables the production of 20 therapeutic glycoproteins having particular specificities. Thus, production of glycoprotein compositions that are enriched for particular glycoforms is highly desirable.

While the pathway for N-linked glycosylation has been the subject of much analysis, the process and function of O-linked glycosylation is not as well understood. However, it is known that in contrast to N-linked glycosylation, O-glycosylation is a posttranslational event, 25 which occurs in the *cis*-Golgi (Varki, 1993, *Glycobiol.*, 3: 97-130). While a consensus acceptor sequence for O-linked glycosylation like that for N-linked glycosylation does not appear to exist, a comparison of amino acid sequences around a large number of O-linked glycosylation sites of several glycoproteins show an increased frequency of proline residues at positions -1 and +3 relative to the glycosylated residues and a marked increase of serine, threonine, and alanine 30 residues (Wilson *et al.*, 1991, *Biochem. J.*, 275: 529-534). Stretches of serine and threonine residues in glycoproteins, may also be potential sites for O-glycosylation.

One gene family that has a role in O-linked glycosylation are the genes encoding the Dol-P-Man:Protein (Ser/Thr) Mannosyl Transferase (Pmt). These highly conserved genes

have been identified in both higher eukaryotes such as humans, rodents, insects, and the like and lower eukaryotes such as fungi and the like. Yeast such as *Saccharomyces cerevisiae* and *Pichia pastoris* encode up to seven *PMT* genes encoding Pmt homologues (reviewed in Willer *et al.* Curr. Opin. Struct. Biol. 2003 Oct;13(5): 621-30.). In yeast, O-linked glycosylation starts by the addition of the initial mannose from dolichol-phosphate mannose to a serine or threonine residue of a nascent glycoprotein in the endoplasmic reticulum by one of the seven O-mannosyl transferases genes. While there appear to be seven *PMT* genes encoding Pmt homologues in yeast, O-mannosylation of secreted fungal and heterologous proteins in yeast is primarily dependent on the genes encoding Pmt1 and Pmt2, which appear to function as a heterodimer. *PMT1* and *PMT2* and their protein products, Pmt1 and Pmt2, respectively, appear to be highly conserved among species.

Tanner *et al.* in U. S. Patent No. 5,714,377 describes the *PMT1* and *PMT2* genes of *Saccharomyces cerevisiae* and a method for making recombinant proteins having reduced O-linked glycosylation that uses fungal cells in which one or more of *PMT* genes have been genetically modified so that recombinant proteins are produced, which have reduced O-linked glycosylation.

Ng *et al.* in U.S. Published Patent Application No. 20020068325 discloses inhibition of O-glycosylation through the use of antisense or cosuppression or through the engineering of yeast host strains that have loss of function mutations in genes associated with O-linked glycosylation, in particular, one or more of the *PMT* genes.

UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetyl galactosaminyl-transferases (GalNAc-transferases) are involved in mucin type O-linked glycosylation found in higher eukaryotes. These enzymes initiate O-glycosylation of specific serine and threonine amino acids in proteins by adding N-acetylgalactosamine to the hydroxy group of these amino acids to which mannose residues can then be added in a step-wise manner. Clausen *et al.* in U. S. Patent No. 5,871,990 and U.S. Published Patent Application No. 20050026266 discloses a family of nucleic acids encoding UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetyl galactosaminyl-transferases (GalNAc-transferases). Clausen in U.S. Published Patent Application No. 20030186850 discloses the use of GalNAc-beta-benzyl to selectively inhibit lectins of polypeptide GalNAc-transferases and not serve as substrates for other glycosyltransferases involved in O-glycan biosyntheses, thus inhibiting O-glycosylation.

Inhibitors of O-linked glycosylation have been described. For example, Orchard *et al.* in U.S. Patent No. 7,105,554 describes benzylidene thiazolidinediones and their use as

antimycotic agents, e.g., antifungal agents. These benzylidene thiazolidinediones are reported to inhibit the Pmt1 enzyme, preventing the formation of the O-linked mannoproteins and compromising the integrity of the fungal cell wall. The end result is cell swelling and ultimately death through rupture.

5 Konrad *et al.* in U.S. Published Patent Application No. 20020128235 disclose a method for treating or preventing diabetes mellitus by pharmacologically inhibiting O-linked protein glycosylation in a tissue or cell. The method relies on treating a diabetic individual with (Z)-1-[N-(3-Ammoniopropyl)-N-(n-propyl)amino] diazen-ium-1,2-diolate or a derivative thereof, which binds O-linked N-acetylglucosamine transferase and thereby inhibits O-linked
10 glycosylation.

Kojima *et al.* in U. S. Patent No. 5,268,364 disclose therapeutic compositions for inhibition of O-glycosylation using compounds such as benzyle- α -N-acetylgalactosamine, which inhibits extension of O-glycosylation leading to accumulation of O- α -GalNAc, to block expression of SLe^x or SLe^a by leukocytes or tumor cells and thereby inhibit adhesion of these
15 cells to endothelial cells and platelets.

Boime *et al.* U. S. Patent No. 6,103,501 disclose variants of hormones in which O-linked glycosylation was altered by modifying the amino acid sequence at the site of glycosylation.

The invention is directed to novel inhibitors of Pmt proteins, which are useful for
20 production of recombinant proteins with reduced O-linked glycosylation. This enables O-linked glycosylation of proteins produced from fungi and yeast cells to be controlled.

SUMMARY OF THE INVENTION

Compounds and methods are described for producing protein compositions
25 having reduced amounts of O-linked glycosylation. The method includes producing the protein in cells cultured in the presence of certain benzylidene thiazolidinediones inhibitors of Pmt-mediated O-linked glycosylation.

BRIEF DESCRIPTION OF THE DRAWINGS

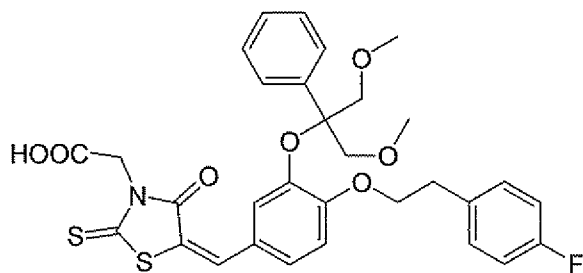
30 Figure 1 illustrates the effect of Pmt inhibitors including the novel inhibitor shown as Example 4 on O-glycosylation of secreted recombinant monoclonal antibody in *Pichia pastoris*. The chemical inhibitors of Pmt reduced O-glycosylation in a dose-dependent fashion. Western blotting using an anti-human H+ L antibody was used to detect heavy (Hc) and light

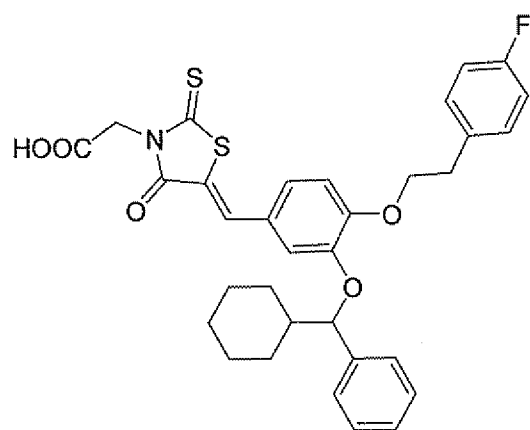
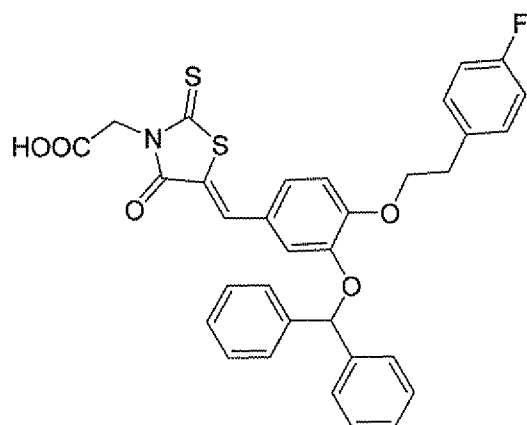
(Lc) chains in the growth media of strains treated with increasing amounts of Pmt inhibitor (higher doses in left lanes). The slowest migrating band (marked with asterisk) indicates the O-glycosylated Hc which is eliminated by Pmt inhibitor treatment.

5 DETAILED DESCRIPTION OF THE INVENTION

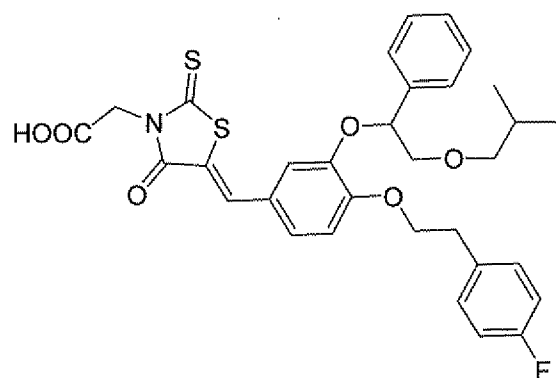
The present invention provides novel compounds and methods for expressing a recombinant protein (includes polypeptides and glycoproteins), which is susceptible to O-linked glycosylation in a particular host cell, having a reduced amount of O-linked glycosylation (including no O-linked glycosylation) in that cell type. The method involves inducing expression
 10 of a protein of interest in a host cell in which the protein is susceptible to O-linked glycosylation in the host cell in the presence of one or more novel compounds of the invention which are inhibitors of the activity of one or more of the Dol-P-Man:Protein (Ser/Thr) Mannosyl Transferase (Pmt) proteins involved in the transfer of mannose to a serine or threonine residue of the protein in the cell, optionally with one or more α 1,2-mannosidases as described in
 15 Bobrowicz et al. U.S. Published Application No. 2007061631, at the time expression of the protein is induced. The protein that is expressed in the presence of the inhibitor has a reduced amount of O-linked glycosylation compared to the amount of O-linked glycosylation that would have been present on the protein if it had been produced in the absence of the inhibitor. The method is particularly useful because it provides a means for producing therapeutically relevant
 20 proteins where it is desired that the protein have a reduced amount of O-glycosylation in host cells such as lower eukaryotes, for example yeast, and bacteria, which would normally produce proteins with O-linked glycans, having a reduced number of O-linked glycans. However, while the method is especially suitable for expressing proteins with reduced O-linked glycosylation in lower eukaryotic organisms, the method can also be practiced in higher eukaryotic organisms and
 25 bacteria.

The Pmt inhibitors of the invention are selected from the following group:





and



or a salt thereof.

The methods described herein are an improvement over certain prior art methods for producing proteins having reduced O-linked glycosylation in host cells in which the proteins are susceptible to O-linked glycosylation. For example, Tanner *et al.* in U. S. Patent No. 5,714,377 describes a method for making recombinant proteins having reduced O-linked glycosylation using fungal cells such as yeast cells in which one or more of *PMT* genes encoding

the Pmt protein have been genetically modified so that recombinant proteins are produced which have reduced O-linked glycosylation. While deletion of the *PMT1*, *PMT2*, or *PMT4* genes in a fungal host cell enables production of a recombinant protein having reduced O-linked glycosylation in the fungal host cell, expression of the *PMT* genes are important for host cell growth and either deletion alone also adversely affects the ability of the fungal host cell to grow thus making it difficult to produce a sufficient quantity of host cells or recombinant protein with a reduced amount of O-linked glycosylation. Deletion of *PMT2* plus either *PMT1* or *PMT4* appears to be lethal to the fungal host cell. Therefore, genetic elimination of the *PMT* genes in a host cell would appear to be an undesirable means for producing recombinant proteins having reduced O-linked glycosylation.

In contrast, the *PMT* genes in the host cells used in the methods of the present invention have not been modified or deleted, which enables the host cell to O-glycosylate those proteins that are important for cell growth until which time the activity of the Pmt proteins is inhibited. In general, this enables the host cells to be grown to higher levels than the levels that could be obtained if the *PMT* genes had been deleted. In addition, in particular embodiments, expression of the recombinant protein in the host cell is controlled by an inducible promoter and the Pmt activity in the host cell is not inhibited until expression of the recombinant protein is induced. This enables large quantities of host cells containing a nucleic acid encoding a recombinant protein to be produced in culture before inducing expression of the recombinant protein and adding the Pmt inhibitor. This can enable production of larger amounts recombinant protein having reduced O-linked glycosylation to be produced in the culture in a shorter period of time than would occur for host cells which have had one or more *PMT* genes deleted and grow poorly in culture.

The Pmt inhibitors described herein are improved over those described previously in Orchard *et al.* (U.S. Patent No. 7,105,554) and Bobrowicz *et al.* (U.S. Published Application No. 2007061631) based on increased potency as shown in Example 5. The increased potency allows for the use of smaller amounts of inhibitor to reduce fungal O-glycosylation to acceptable levels and/or increases the potential for complete elimination of O-glycans.

The methods described herein facilitate the production of glycoproteins having reduced O-linked glycosylation in host cells that have been genetically modified to produce glycoproteins having predominantly a particular N-linked glycan structure but which also O-glycosylate the glycoprotein. Methods for producing a wide variety of glycoproteins having

predominantly particular N-linked glycoforms have been disclosed in U.S. Patent No. 7,029,872 and U.S. Published Application Nos. 20050170452, 20050260729, 20040230042, 20050208617, 20050208617, 20040171826, 20060160179, 20060040353, and 20060211085. Any one of the host cells described in the aforementioned patent and patent applications can be used to produce a glycoprotein having predominantly a particular N-linked glycan structure and having reduced O-linked glycosylation using the method disclosed herein. It has been found that some host cells that have been genetically modified to produce glycoproteins having predominantly a particular N-linked glycan structure can grow less well in culture under particular conditions than host cells that have not been modified. For example, particular fungal and yeast cells in which genes involved in hypermannosylation have been deleted and other genes needed to produce particular mammalian or human like N-linked glycan structures have been added, can grow less well than fungal or yeast cells that do not the genetic modifications. In some of these genetically modified fungal or yeast cells, further introducing deletions of the *PMT* genes either is lethal to the cells or adversely affects the ability of the cells to grow to sufficient quantities in culture. The methods herein avoid the potential deleterious effects of deleting the *PMT* genes by allowing the cells to grow to sufficient quantities in culture before inducing expression of the recombinant glycoprotein and adding an inhibitor of the activity of the Pmt proteins, optionally with one or more α 1,2-mannosidases, to produce the recombinant glycoprotein having predominantly particular N-linked glycan structures and reduced O-linked glycosylation.

An aspect of the methods described herein is that it provides for a glycoprotein composition comprising reduced O-linked glycosylation and a predominantly a specific N-linked glycoform in which the recombinant glycoprotein may exhibit increased biological activity and/or decreased undesired immunogenicity relative to compositions of the same glycoprotein produced from mammalian cell culture, such as CHO cells. An additional advantage of producing the glycoprotein composition comprising reduced O-linked glycosylation and a predominant N-linked glycoform is that it avoids production of undesired or inactive glycoforms and heterogeneous mixtures, which may induce undesired effects and/or dilute the more effective glycoform. Thus, therapeutic pharmaceutical composition of glycoprotein molecules comprising, for example, predominantly Man₅GlcNAc₂, Man₃GlcNAc₂, GlcNAcMan₅GlcNAc₂, GlcNAcMan₃GlcNAc₂, GlcNAc₂Man₃GlcNAc₂, GalGlcNAcMan₅GlcNAc₂, Gal(GlcNAc)₂Man₅GlcNAc₂, (GalGlcNAc)₂Man₅GlcNAc₂, NANAGalGlcNAcMan₃GlcNAc₂, NANA₂Gal₂GlcNAcMan₃GlcNAc₂, and GalGlcNAcMan₃GlcNAc₂ glycoforms and having

reduced O-linked glycosylation may well be effective at lower doses, thus having higher efficacy/potency.

In general, the method for producing proteins having reduced O-linked glycosylation comprises transforming a host cell with a nucleic acid encoding a recombinant or heterologous protein in which it is desirable to produce the protein having reduced O-linked glycosylation. The nucleic acid encoding the recombinant protein is operably linked to regulatory sequences that allow expression of the recombinant protein. Such regulatory sequences include an inducible promoter and optionally an enhancer upstream, or 5', to the nucleic acid encoding the fusion protein and a transcription termination site 3' or down stream from the nucleic acid encoding the recombinant protein. The nucleic acid also typically encodes a 5' UTR region having a ribosome binding site and a 3' untranslated region. The nucleic acid is often a component of a vector replicable in cells in which the recombinant protein is expressed. The vector can also contain a marker to allow recognition of transformed cells. However, some cell types, particularly yeast, can be successfully transformed with a nucleic acid lacking extraneous vector sequences.

Nucleic acids encoding desired recombinant proteins can be obtained from several sources. cDNA sequences can be amplified from cell lines known to express the protein using primers to conserved regions (see, for example, Marks *et al.*, J. Mol. Biol. 581-596 (1991)). Nucleic acids can also be synthesized de novo based on sequences in the scientific literature. Nucleic acids can also be synthesized by extension of overlapping oligonucleotides spanning a desired sequence (see, e.g., Caldas *et al.*, Protein Engineering, 13, 353-360 (2000)).

In one aspect, the nucleic acid encoding the protein is operably linked to an inducible promoter, which allows expression of the protein to be induced when desired. In another aspect, the nucleic acid encoding the protein is operably linked to a constitutive promoter. To facilitate isolation of the expressed protein, it is currently preferable that the protein include a signal sequence that directs the protein to be excreted into the cell culture medium where it can then be isolated. In the first aspect, the transformed host cells are cultured for a time sufficient to produce a desired multiplicity of host cells sufficient to produce the desired amount of protein before adding one or more inhibitors of Pmt-mediated O-linked glycosylation to the culture medium. The inducer and inhibitor can be added to the culture simultaneously or the inducer is added to the culture before adding the one or more Pmt inhibitors or the one or more Pmt inhibitors is added to the culture before adding the inducer. The induced protein is produced having reduced O-linked glycosylation and can be recovered

from the culture medium or for proteins not having a signal sequence, from the host cell by lysis. In the second aspect, wherein the nucleic acid encoding the protein is operably linked to a constitutive promoter, the one or more inhibitors of Pmt-mediated O-linked glycosylation is added to the culture medium at the same time the culture is established and the protein, which is produced having reduced O-linked glycosylation, can be recovered from the culture medium or for proteins not having a signal sequence, from the host cell by lysis.

Chemicals or compositions that inhibit the activity one or more of the Pmt proteins useful for producing proteins with reduced O-linked glycosylation are described herein. When the host cell is a lower eukaryote such as fungi or yeast, it is desirable that the inhibitor inhibit at least the activity of Pmt1 or Pmt2, or both. In higher eukaryotes, it is desirable that the inhibitor inhibit activity of the homologue in the higher eukaryote that corresponds to the Pmt1 or Pmt2.

The compounds of the invention are shown to be effective in producing recombinant proteins having reduced O-linked glycosylation in *Pichia pastoris* strains that had intact, functional *PMT* genes. Table 1 and Figure 1 of Example 5 show that any one of the above four Pmt chemical inhibitors added to a culture of recombinant *Pichia pastoris* having intact, functional *PMT* genes and transformed with a nucleic acid encoding a recombinant, human anti-Her2 antibody protein operably linked to an inducible promoter at the time of expression of the recombinant protein was induced, produced a recombinant protein having a level of reduced O-linked glycosylation that was improved relative to the level of O-linked glycosylation seen for *Pichia pastoris* cells treated with Pmt inhibitor Pmti-3 described in Orchard *et al.* (Bioorgan & Med Chem Letters (2004) 14:3975-3978) and patent publications by the same authors including EP 1313471 B1 and Bobrowicz *et al.* U.S. Published Application No. 2007061631.

Host Cells

The term "cells" as used herein refers to host cells described as follows. While host cells for the method herein includes both higher eukaryote cells and lower eukaryote cells, lower eukaryote cells, for example filamentous fungi or yeast cells, are currently preferred for expression of proteins because they can be economically cultured, give high yields of protein, and when appropriately modified are capable of producing proteins having suitable glycosylation patterns. Lower eukaryotes include yeast, fungi, collar-flagellates, microsporidia, alveolates (e.g., dinoflagellates), stramenopiles (e.g, brown algae, protozoa), rhodophyta (e.g., red algae), plants (e.g., green algae, plant cells, moss) and other protists. Yeast and fungi include, but are

not limited to: *Pichia* sp. (for example, *Pichia pastoris*, *Pichia finlandica*, *Pichia trehalophila*, *Pichia koclamae*, *Pichia membranaefaciens*, *Pichia minuta* (*Ogataea minuta*, *Pichia lindneri*), *Pichia opuntiae*, *Pichia thermotolerans*, *Pichia salictaria*, *Pichia guercuum*, *Pichia pijperi*, *Pichia stiptis*, *Pichia methanolica*), *Saccharomyces* sp. (for example *Saccharomyces cerevisiae*), *Hansenula polymorpha*, *Kluyveromyces* sp. (for example, *Kluyveromyces lactis*), *Candida albicans*, *Aspergillus* sp (for example, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*), *Trichoderma reesei*, *Chrysosporium lucknowense*, *Fusarium* sp. (for example, *Fusarium gramineum*, *Fusarium venenatum*), *Physcomitrella patens* and *Neurospora crassa*. Yeast, in particular, are currently preferred because yeast offers established genetics allowing for rapid transformations, tested protein localization strategies, and facile gene knock-out techniques. Suitable vectors have expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences, and the like as desired.

Various yeasts, such as *K. lactis*, *Pichia pastoris*, *Pichia methanolica*, and *Hansenula polymorpha* are currently preferred for cell culture because they are able to grow to high cell densities and secrete large quantities of recombinant protein. Likewise, filamentous fungi, such as *Aspergillus niger*, *Fusarium* sp, *Neurospora crass*, and others can be used to produce recombinant proteins at an industrial scale.

Lower eukaryotes, in particular filamentous fungi and yeast, can be genetically modified so that they express proteins or glycoproteins in which the glycosylation pattern is human-like or humanized. This can be achieved by eliminating selected endogenous glycosylation enzymes and/or supplying exogenous enzymes as described by Gerngross *et al.* in U.S. Patent No. US7029872, and U. S. Published Patent Application Nos. 20040018590, 20050170452, 20050260729, 20040230042, 20050208617, 20040171826, 20050208617, 20060160179, 20060040353, and 20060211085. Thus, a host cell can additionally or alternatively be engineered to express one or more enzymes or enzyme activities, which enable the production of particular N-glycan structures at a high yield. Such an enzyme can be targeted to a host subcellular organelle in which the enzyme will have optimal activity, for example, by means of signal peptide not normally associated with the enzyme. Host cells can also be modified to express a sugar nucleotide transporter and/or a nucleotide diphosphatase enzyme. The transporter and diphosphatase improve the efficiency of engineered glycosylation steps, by providing the appropriate substrates for the glycosylation enzymes in the appropriate compartments, reducing competitive product inhibition, and promoting the removal of

nucleoside diphosphates. See, for example, Gerngross *et al.* in U.S. Published Patent Application No.20040018590 and Hamilton, 2003, Science 301: 1244-46 and the aforementioned U.S. patent and patent applications.

By way of example, a host cell (for example, yeast or fungal) can be selected or engineered to be depleted in 1,6-mannosyl transferase activities, which would otherwise add mannose residues onto the N-glycan of a glycoprotein, and to further include a nucleic acid for ectopic expression of an α -1,2 mannosidase activity, which enables production of recombinant glycoproteins having greater than 30 mole percent Man5GlcNAc₂ N-glycans. When a glycoprotein is produced in the host cells according to the method described herein, the host cells will produce a glycoprotein having predominantly a Man5GlcNAc₂ N-glycan structure and reduced O-glycosylation compared to the glycoprotein produced in the cell otherwise. In a further aspect, the host cell is engineered to further include a nucleic acid for ectopic expression of GlcNAc transferase I activity, which enables production of glycoproteins having predominantly GlcNAcMan5GlcNAc₂ N-glycans. When a glycoprotein is produced in the host cells according to the method described herein, the host cells will produce a glycoprotein having predominantly a GlcNAcMan5GlcNAc₂ N-glycan structure and reduced O-glycosylation compared to the glycoprotein produced in the cell otherwise. In a further still aspect, the host cell is engineered to further include a nucleic acid for ectopic expression of mannosidase II activity, which enables production of glycoproteins having predominantly GlcNAcMan₃GlcNAc₂ N-glycans. When a glycoprotein is produced in the host cells according to the method described herein, the host cells will produce a glycoprotein having predominantly a GlcNAcMan₃GlcNAc₂ N-glycan structure and reduced O-glycosylation compared to the glycoprotein produced in the cell otherwise. In a further still aspect, the host cell is engineered to further include a nucleic acid for ectopic expression of GlcNAc transferase II activity, which enables production of glycoproteins having predominantly GlcNAc₂Man₃GlcNAc₂ N-glycans. When a glycoprotein is produced in the host cells according to the method described herein, the host cells will produce a glycoprotein having predominantly a GlcNAc₂Man₃GlcNAc₂ N-glycan structure and reduced O-glycosylation compared to the glycoprotein produced in the cell otherwise. In further still aspects, the above host cells can be further engineered to produce particular hybrid or complex N-glycan or human-like N-glycan structures by further including one or more higher eukaryote genes involved in N-linked glycosylation, in any combination, that encode for example, sialyltransferase activities, class II and III mannosidase activities, GlcNAc

transferase II, III, IV, V, VI, IX activity, and galactose transferase activity. It is currently preferable that the cells further include one or more of nucleic acids encoding UDP-specific diphosphatase activity, GDP-specific diphosphatase activity, and UDP-GlcNAc transporter activity.

5 Plants and plant cell cultures may be used for expression of proteins and glycoproteins with reduced O-linked glycosylation as taught herein (*See*, for example, Larrick & Fry, 1991, *Hum. Antibodies Hybridomas* 2: 172-89); Benvenuto *et al.*, 1991, *Plant Mol. Biol.* 17: 865-74); Durin *et al.*, 1990, *Plant Mol. Biol.* 15: 281-93); Hiatt *et al.*, 1989, *Nature* 342: 76-8). Preferable plant hosts include, for example, *Arabidopsis*, *Nicotiana tabacum*, *Nicotiana rustica*,
10 and *Solanum tuberosum*.

 Insect cell culture can also be used to produce proteins and glycoproteins proteins and glycoproteins with reduced O-linked glycosylation, as taught herein for example, baculovirus-based expression systems (*See*, for example, Putlitz *et al.*, 1990, *Bio/Technology* 8: 651-654).

15 Although not currently as economical to culture as lower eukaryotes and prokaryotes, mammalian tissue cell culture can also be used to express and produce proteins and glycoproteins with reduced O-linked glycosylation as taught herein (*See* Winnacker, *From Genes to Clones* (VCH Publishers, NY, 1987). Suitable hosts include CHO cell lines, various COS cell lines, HeLa cells, preferably myeloma cell lines or the like or transformed B-cells or hybridomas.
20 Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer (Queen *et al.*, 1986I, *mmunol. Rev.* 89:49-68), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Expression control sequences are promoters derived from immunoglobulin genes, SV40, Adenovirus, bovine Papilloma Virus,
25 cytomegalovirus and the like. Generally, a selectable marker, such as a neoR expression cassette, is included in the expression vector.

 The nucleic acid encoding the protein to be expressed can be transferred into the host cell by conventional methods, which vary depending on the type of cellular host. For example, calcium phosphate treatment, protoplast fusion, natural breeding, lipofection, biolistics,
30 viral-based transduction, or electroporation can be used for cellular hosts. Tungsten particle ballistic transgenesis is preferred for plant cells and tissues. (*See*, generally, Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Press, 1982))

Once expressed, the proteins or glycoproteins having reduced O-linked glycosylation can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (See, generally, Scopes, R., Protein Purification (Springer-Verlag, N.Y., 1982)). Substantially
5 pure glycoproteins of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity most preferred, for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the proteins can then be used therapeutically (including extracorporeally) or in developing and performing assay procedures, immunofluorescent stainings, and the like. (See, generally, Immunological Methods, Vols. I and II (Lefkovits and Pernis, eds., Academic Press,
10 NY, 1979 and 1981).

Therefore, further provided are glycoprotein compositions comprising a predominant species of N-glycan structure and having reduced O-linked glycosylation compared to compositions of the glycoprotein which have been produced in host cells have not been incubated in the presence of an inhibitor of Pmt-mediated O-linked glycosylation or an α -1,2-mannosidase capable of trimming more than one mannose residue from a glycans structure or
15 both. In particular aspects, the glycoprotein composition comprises a glycoprotein having a predominant N-glycan structure selected from the group consisting of Man₅GlcNAc₂, Man₃GlcNAc₂, GlcNAcMan₅GlcNAc₂, GlcNAcMan₃GlcNAc₂, GlcNAc₂Man₃GlcNAc₂, GalGlcNAcMan₅GlcNAc₂, Gal(GlcNAc)₂Man₅GlcNAc₂, (GalGlcNAc)₂Man₅GlcNAc₂,
20 NANAGlcNAcMan₃GlcNAc₂, NANA₂Gal₂GlcNAcMan₃GlcNAc₂, and GalGlcNAcMan₃GlcNAc₂ glycoforms.

Pharmaceutical Compositions

Proteins and glycoproteins having reduced O-linked glycosylation can be
25 incorporated into pharmaceutical compositions comprising the glycoprotein as an active therapeutic agent and a variety of other pharmaceutically acceptable components (See, Remington's Pharmaceutical Science (15th ed., Mack Publishing Company, Easton, Pennsylvania, 1980). The preferred form depends on the intended mode of administration and therapeutic application. The compositions can also include, depending on the formulation
30 desired, pharmaceutically-acceptable, non-toxic carriers or diluents, which are defined as vehicles commonly used to formulate pharmaceutical compositions for animal or human administration. The diluent is selected so as not to affect the biological activity of the

combination. Examples of such diluents are distilled water, physiological phosphate-buffered saline, Ringer's solutions, dextrose solution, and Hank's solution. In addition, the pharmaceutical composition or formulation can also include other carriers, adjuvants, or nontoxic, nontherapeutic, nonimmunogenic stabilizers, and the like.

5 Pharmaceutical compositions for parenteral administration are sterile, substantially isotonic, pyrogen-free and prepared in accordance with GMP of the FDA or similar body. Glycoproteins can be administered as injectable dosages of a solution or suspension of the substance in a physiologically acceptable diluent with a pharmaceutical carrier that can be a sterile liquid such as water oils, saline, glycerol, or ethanol. Additionally, auxiliary substances,
10 such as wetting or emulsifying agents, surfactants, pH buffering substances and the like can be present in compositions. Other components of pharmaceutical compositions are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, and mineral oil. In general, glycols such as propylene glycol or polyethylene glycol are preferred liquid carriers, particularly for injectable solutions. Glycoproteins can be administered in the
15 form of a depot injection or implant preparation which can be formulated in such a manner as to permit a sustained release of the active ingredient. Typically, compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection can also be prepared. The preparation also can be emulsified or encapsulated in liposomes or micro particles such as polylactide, polyglycolide, or
20 copolymer for enhanced adjuvant effect, as discussed above (*See* Langer, Science 249, 1527 (1990) and Hanes, Advanced Drug Delivery Reviews 28, 97-119 (1997).

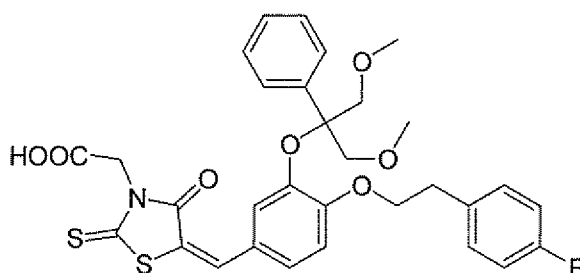
 The term "or a salt thereof" refers to salts prepared from acceptable bases including inorganic or organic bases. Salts derived from inorganic bases include aluminum, ammonium, calcium, copper, ferric, ferrous, lithium, magnesium, manganic salts, manganous,
25 potassium, sodium, zinc, and the like. Salts in the solid form may exist in more than one crystal structure, and may also be in the form of hydrates. Salts derived from bases include salts of primary, secondary, and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines, and basic ion exchange resins, such as arginine, betaine, caffeine, choline, N,N'-dibenzylethylene-diamine, diethylamine, 2-diethylaminoethanol, 2-
30 dimethylaminoethanol, ethanolamine, ethylenediamine, N-ethyl-morpholine, N-ethylpiperidine, glucamine, glucosamine, histidine, hydrabamine, isopropylamine, lysine, methylglucamine, morpholine, piperazine, piperidine, polyamine resins, procaine, purines, theobromine, triethylamine, trimethylamine, tripropylamine, tromethamine, and the like.

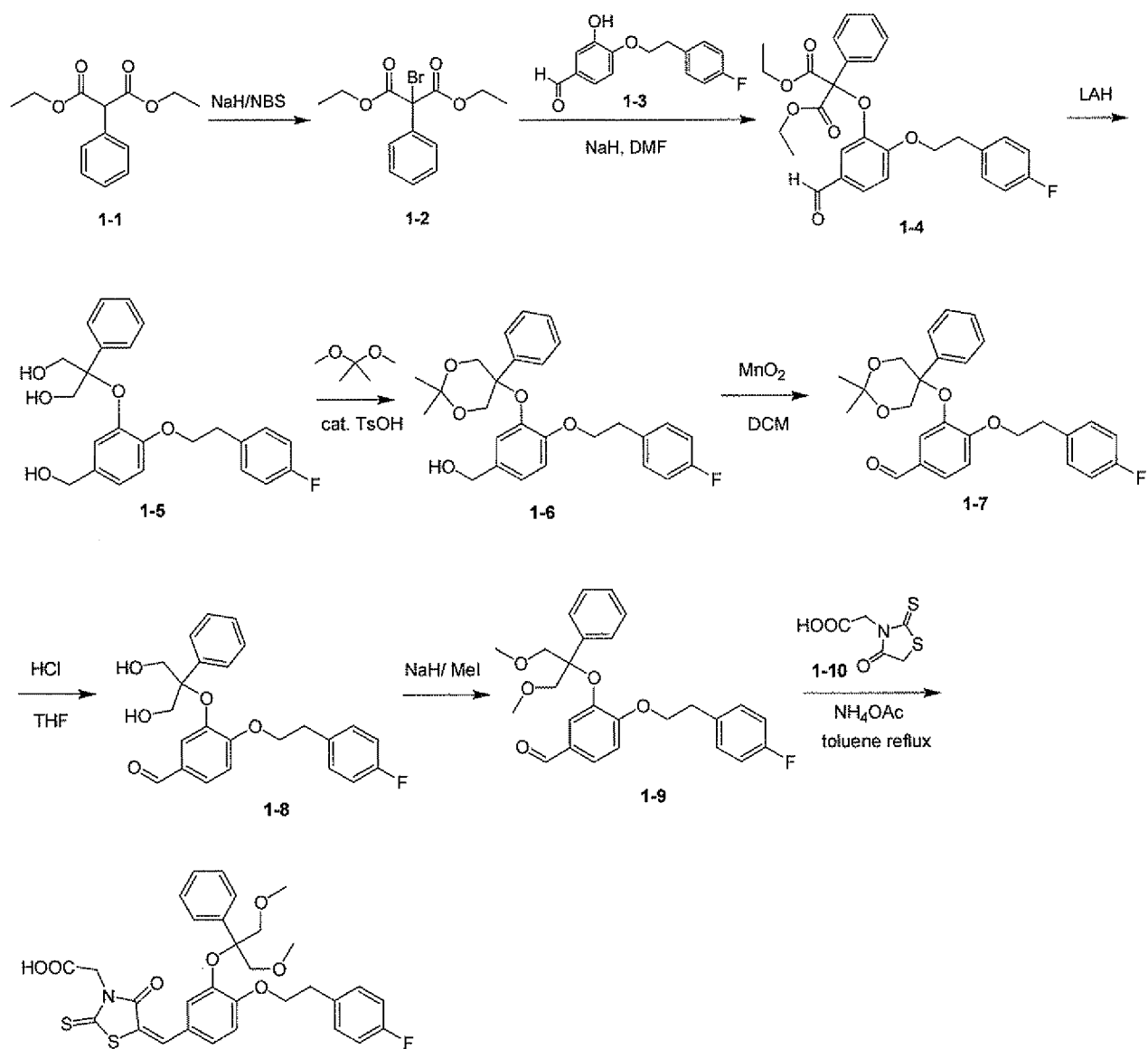
The compounds of the present invention may contain one or more asymmetric centers and can thus occur as racemates and racemic mixtures, single enantiomers, diastereomeric mixtures and individual diastereomers. The present invention is meant to comprehend all such isomeric forms of these compounds.

Unless otherwise defined herein, scientific and technical terms and phrases used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include the plural and plural terms shall include the singular. Generally, nomenclatures used in connection with, and techniques of biochemistry, enzymology, molecular and cellular biology, microbiology, genetics and protein and nucleic acid chemistry and hybridization described herein are those well known and commonly used in the art. The methods and techniques of the present invention are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. See, for example, Sambrook et al. *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); Ausubel *et al.*, *Current Protocols in Molecular Biology*, Greene Publishing Associates (1992, and Supplements to 2002); Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1990); Taylor and Drickamer, *Introduction to Glycobiology*, Oxford Univ. Press (2003); Worthington Enzyme Manual, Worthington Biochemical Corp., Freehold, NJ; *Handbook of Biochemistry: Section A Proteins*, Vol I, CRC Press (1976); *Handbook of Biochemistry: Section A Proteins*, Vol II, CRC Press (1976); *Essentials of Glycobiology*, Cold Spring Harbor Laboratory Press (1999).

The following examples provide methods for preparing various Pmt inhibitors of the invention.

EXAMPLE 1

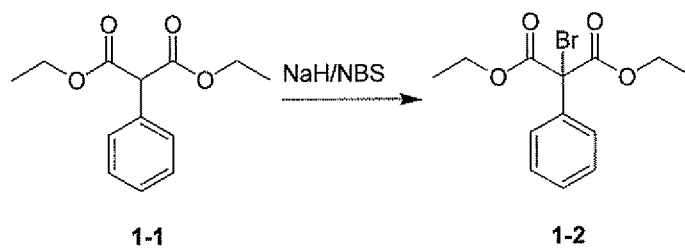




EXAMPLE 1

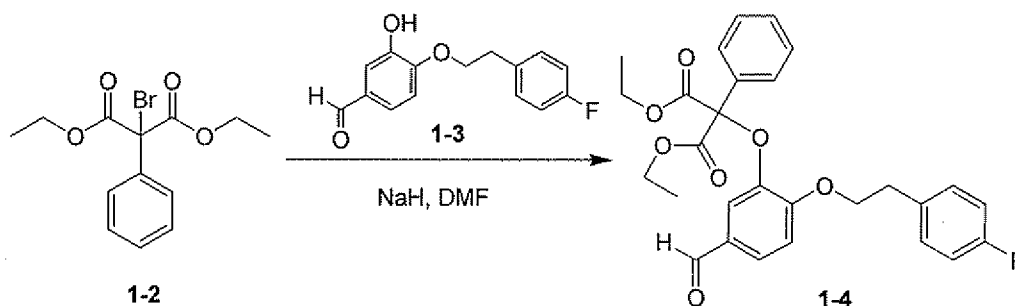
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Step A



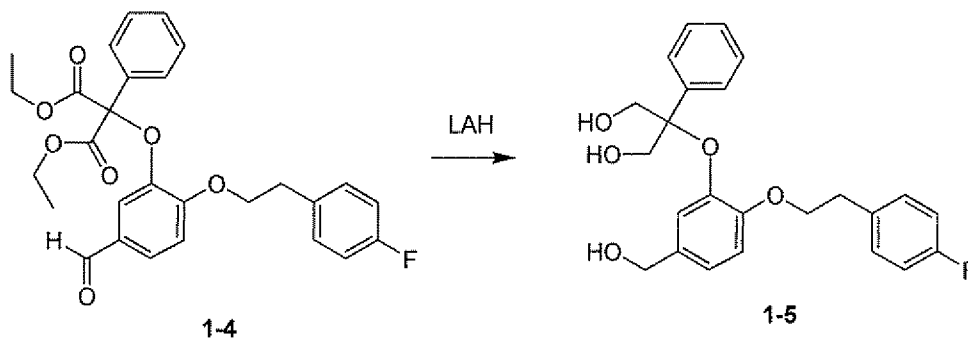
To a solution of **1-1** (5.90 g, 25 mmol) in THF (200 mL) was added NaH (2.0 g, 50 mmole) portionwise. The resulting mixture was stirred at 0 °C for half an hour. Then NBS (4.86 g, 27.5 mmol) was added into and stirred for 15 minutes. The white solid was filtered and the filtration was concentrated to give a residue, which was dissolved in CHCl₃ and dried over Na₂SO₄. The solvent was evaporated to give **1-2** (5 g, yield 63 %), which was used for next step without further purification.

Step B



To a solution of **1-2** (1.8 g, 6 mmol) and **1-3** (1.3 g, 5 mmol) in dried DMF (5 mL) was added NaH (2.4g g, 10 mmol) and then stirred at room temperature overnight. The resulting mixture was partitioned between water and EtOA. The combined organic layer were washed with brine, dried over sodium sulfate and concentrated. The residue was purified by prep-TLC to give **1-4** (800 mg, yield 40 %). ¹H-NMR (400 MHz, CDCl₃) δ 9.66 (s, 1H), 7.74~7.71 (m, 2H), 7.49~7.46 (m, 1H), 7.37~7.33 (m, 5H), 7.20 (d, *J*=2.02 Hz, 1H), 6.97~6.92 (m, 3H), 4.28~4.19 (m, 6H), 3.16 (t, *J*=6.6 Hz, 2H), 1.11 (m, 6H).

Step C



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Step D



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¹H-NMR (400 MHz, MeOD) δ ppm 7.52 (d, *J*=8.05 Hz, 2H), 7.40~7.15 (m, 5H), 6.90~6.85 (m, 2H), 6.80~6.75 (m, 2H), 4.25 (s, 2H), 4.23 (m, 6H), 3.0 (m, 2H), 1.48 (t, 6H).

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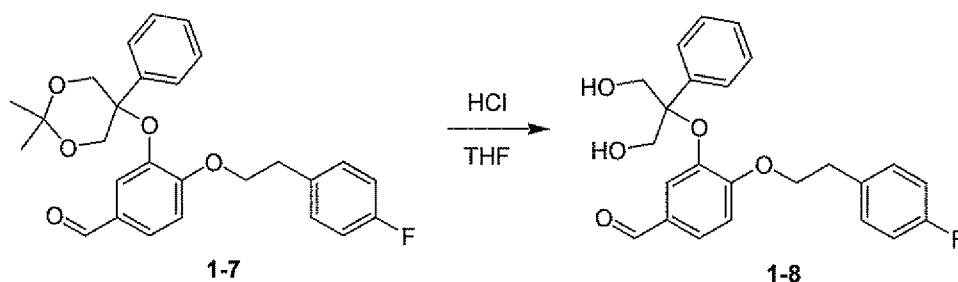


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(t, $J=8.6$ Hz, 2H), 6.89 (m, $J=8.6$ Hz, 2H), 6.76 (d, $J=8$ Hz, 1H), 4.23 (m, 6H), 3.0 (m, 2H), 1.48 (t, 6H).

Step F

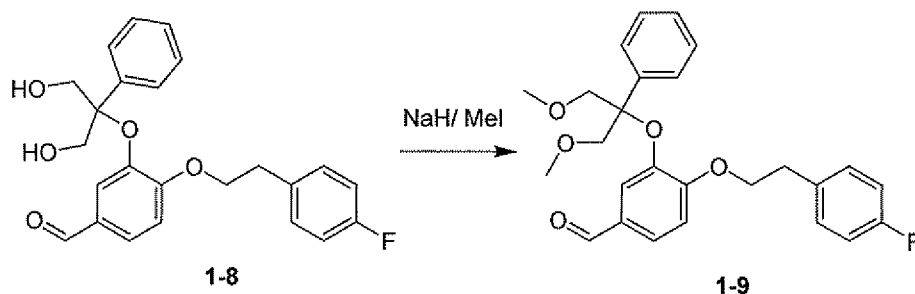
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To a solution of **1-7** (80 mg, 0.17 mmol) in THF was added 6 N HCl and the resulting mixture was stirred at room temperature for 2 hours. The solvent was removed in vacuo to afford the crude **1-8** (60 mg, 82% yield), which was subjected to next step without further purification.

10

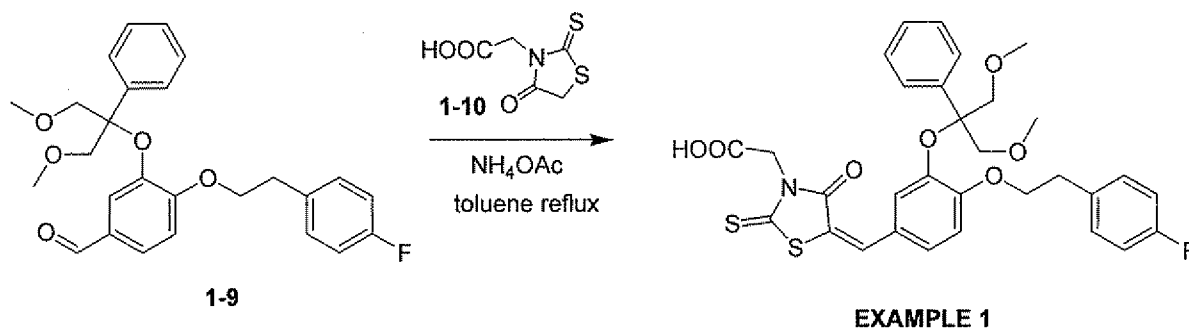
Step G



To a solution of **1-8** (60 mg, 0.146 mmol) in DMF (6mL) was added NaH (24 mg, 1 mmol) at °C and the resulting mixture was stirred at the same temperature for about 30 min and then CH₃I (0.2 mL) was added into. The mixture was stirred at the same temperature for another one hour. The mixture was then partitioned between H₂O and EtOAc. The combined organic layers were washed with brine, dried over sodium sulfate, and concentrated. The residue was purified by prep-TLC to give **1-9** (40 mg, yield 63.0 %). ¹H-NMR (400MHz, MeOD) δ ppm 9.6 (s, 1H), 7.52 (d, $J=8.08$ Hz, 2H), 7.43 (m, 1H), 7.36~7.30 (m, 5H), 7.16 (d, $J=2.02$ Hz, 1H), 7.0 (t, $J=8.6$ Hz, 2H), 6.89 (m, $J=8.6$ Hz, 2H), 4.23 (t, $J=6.56$ Hz, 2H), 3.95~3.85 (m, 4H), 3.30 (s, 6H), 3.13 (dd, $J_1 = 6.26$ Hz, $J_2 = 6.26$ Hz, 2H).

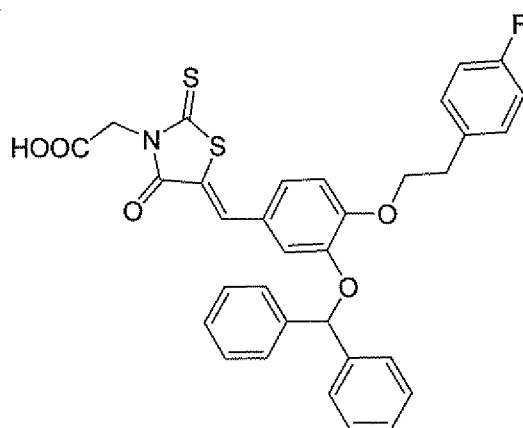
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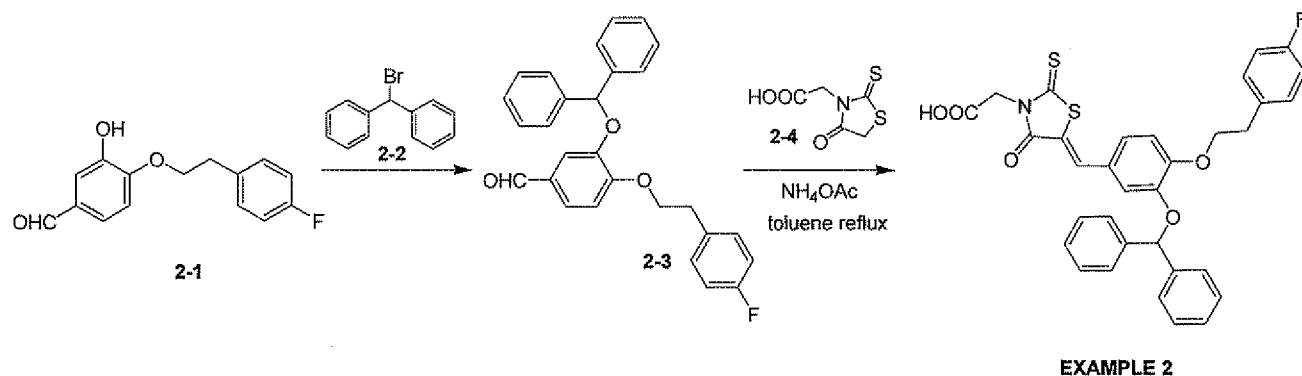
Step H



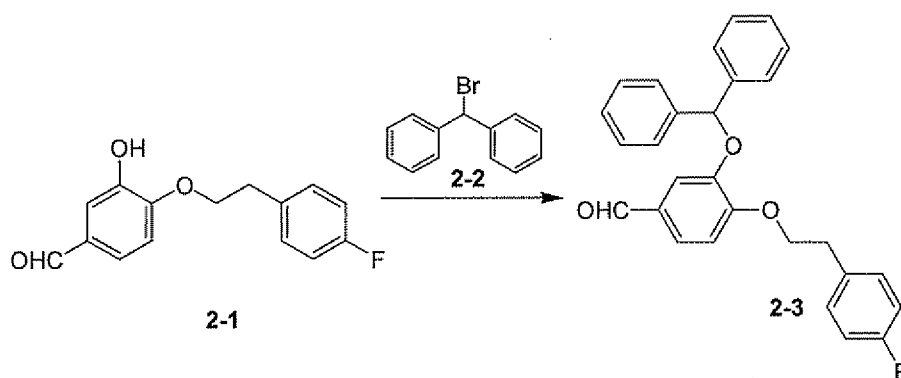
A mixture of **1-9** (40 mg, 0.09 mmol), **1-10** (19 mg, 0.09 mmol) and NH_4OAc (70 mg, 0.9 mmol) in toluene (10 mL) was heated under nitrogen to reflux for about 3 hrs. After cooling to room temperature, the resulting mixture was treated by diluted aq. HCl until pH=4~5 and then partitioned between H_2O and EtOAc. The combined organic layers were washed by brine, dried over Na_2SO_4 and concentrated. The residue was purified by prep-HPLC to give **EXAMPLE 1** (30 mg, yield 50 %) as a yellow solid. $^1\text{H-NMR}$ (400MHz, MeOD) δ ppm 7.46~7.53 (m, 3H), 7.31~7.40 (m, 5H), 7.15~7.18 (m, 1H), 6.92~7.06 (m, 4H), 4.71 (s, 2H), 4.25~4.30 (m, 2H), 3.85~3.94 (m, 4H), 3.26~3.29 (m, 6H), 3.10~3.13 (dd, $J = 6.26$ Hz, $J = 6.26$ Hz, 2H). MS m/z 612($M+1$) $^+$.

EXAMPLE 2





Step A

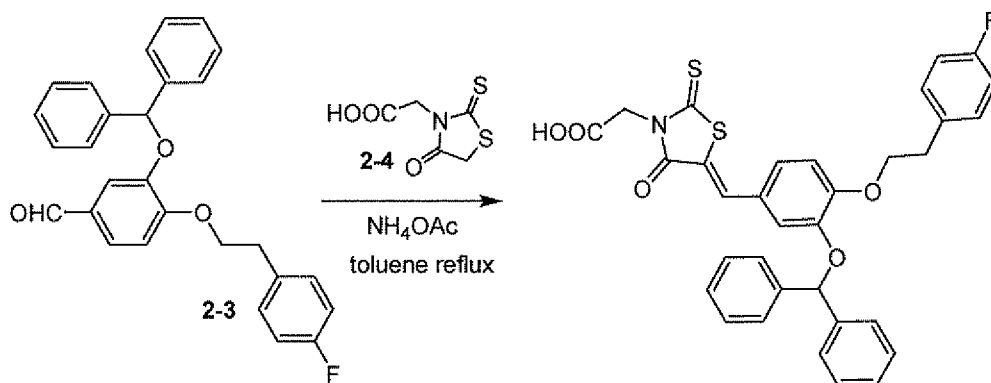


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To a solution of compound **2-1** (150 mg, 0.58 mmol, 1.0 eq), compound **2-2** (214 mg, 0.87 mmol, 1.5 eq) in DMF (10 mL) was added Cs_2CO_3 (161 mg, 0.49 mmol, 0.85 eq). The mixture was then stirred at room temperature overnight and then heated to 80 °C for 1.5 hrs. The resulting mixture was then partitioned between H_2O and EtOAc. The combined organic layers were washed with brine and dried over Na_2SO_4 , concentrated and purified by prep-TLC to give **2-3** (122 mg, yield 49.4 %).

10

Step B

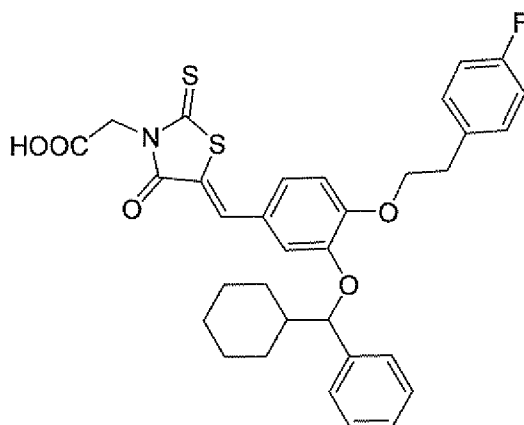


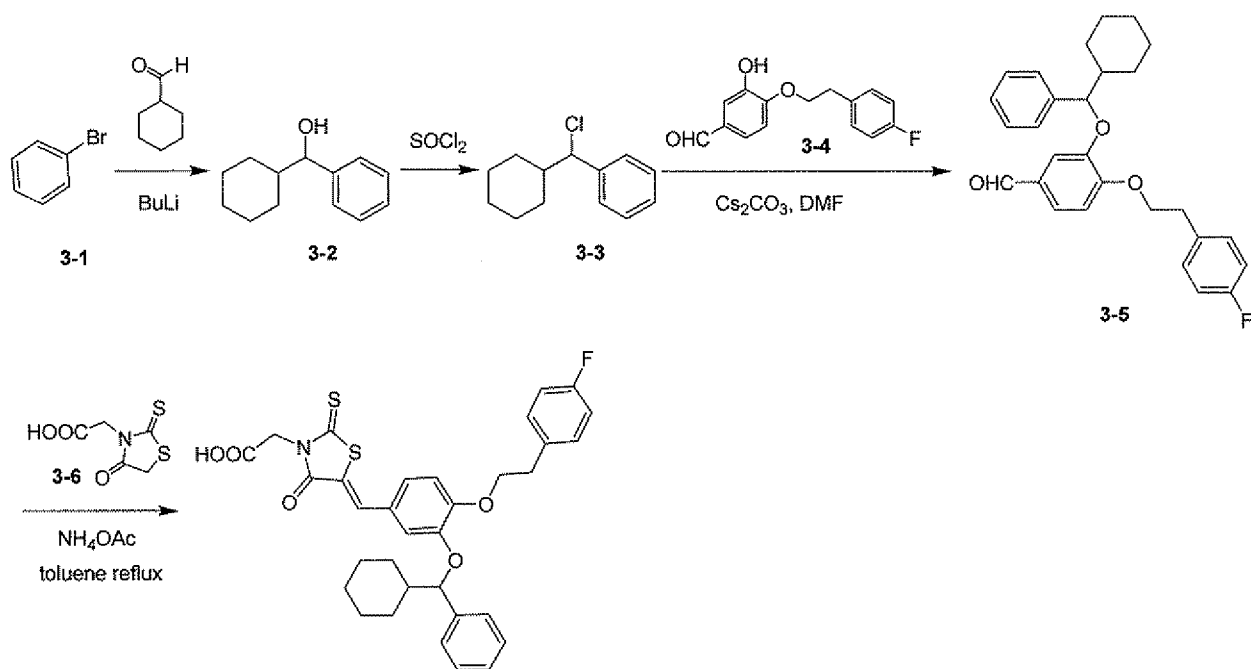
EXAMPLE 2

A mixture of compound **2-3** (122 mg, 0.29 mmol, 1.0 eq), **2-4** (57 mg, 0.30 mmol, 1.05 eq) and NH_4OAc (223 mg, 2.90 mmol, 10.0 eq) in toluene (15 mL) was heated under nitrogen to reflux for about 3 h. The resulting mixture was treated by 10 % HCl acid to pH=4-5, and then partitioned between H_2O and EtOAc. The combined organic layers were washed with brine and dried over Na_2SO_4 , concentrated and purified by prep-TLC to give **EXAMPLE 2** (52 mg, yield 29.9 %). $^1\text{H-NMR}$ (400 MHz, MeOD) δ 7.41-7.47 (m, 3H), 7.19-7.35 (m, 10H), 6.99-7.07 (m, 3H), 6.87-6.92 (m, 2H), 6.22 (s, 1H), 4.75 (s, 2H), 4.24-4.29 (m, 2H), 3.72-3.13 (m, 2H). **MS**

10 m/z 600 $(M+1)^+$

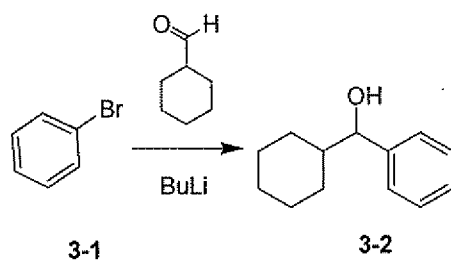
EXAMPLE 3





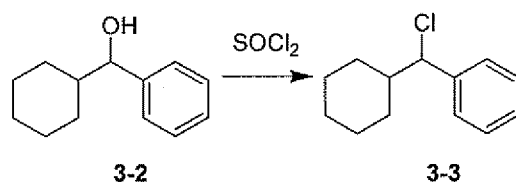
EXAMPLE 3

Step A



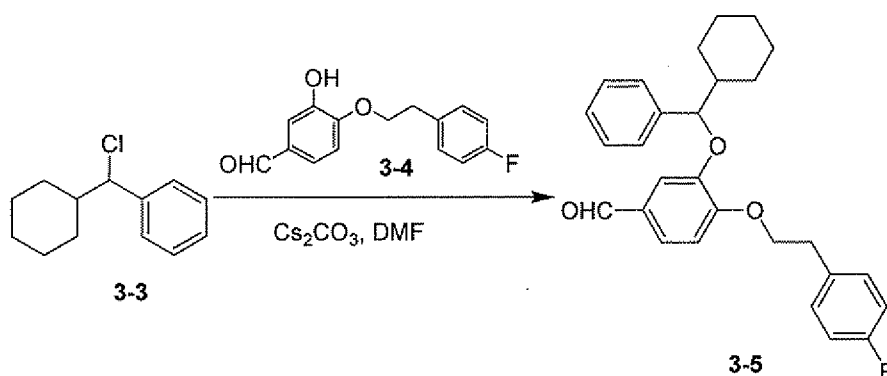
- 5 To a solution of **3-1** (1 g, 6.37 mmol) in dry THF (20 mL) was added BuLi (3 mL, 7.68 mmol) dropwise at -70 °C and then the mixture was stirred for 30 min at room temperature. After cooling to -70 °C again, a solution of cyclohexanecarbaldehyde (0.72 g, 6.43 mmol) in dry THF (5 mL) was added dropwise. After stirring for 30 min, saturated aqueous NH₄Cl was added into the mixture and the resulting mixture was extracted with EtOAc. The organic layers were
- 10 combined and dried over Na₂SO₄, concentrated to give **3-2** (1.52 g, 14.3 %) as a crude oil.

Step B



To the mixture of **3-2** (600 mg, 3.1 mmol) in dry DCM (15 mL) was added SOCl_2 (2 mL) dropwise at 0 °C. Then the resulting mixture was stirred for 2 h at room temperature. The mixture was concentrated under reduced pressure and then diluted with EtOAc. The organic layer was washed with saturated aqueous NaHCO_3 , brine, dried over Na_2SO_4 , and then concentrated to give **3-3** (500 mg, crude).

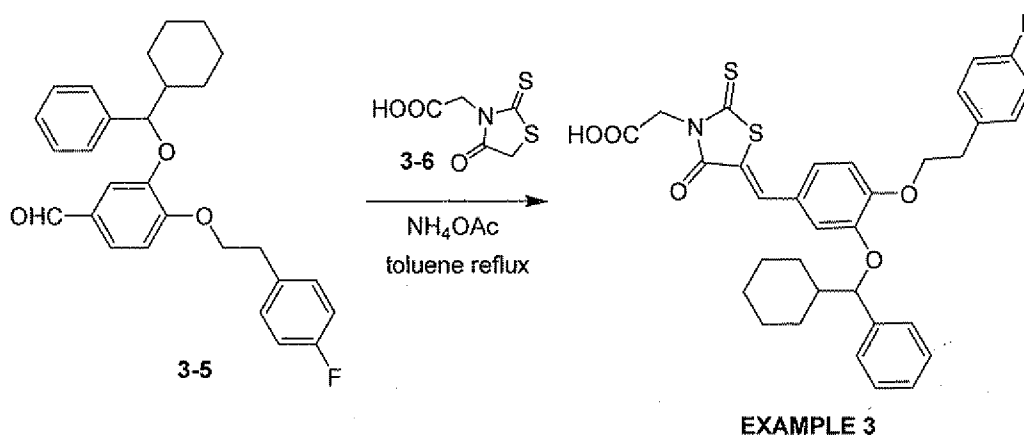
10 Step C



To a solution of **3-3** (450 mg, crude) and **3-4** (375 mg, 1.44 mmol) in DMF (10 mL) was added Cs_2CO_3 (399 mg, 1.22 mmol) in one portion and then the mixture was heated to 110 °C for 18 h.

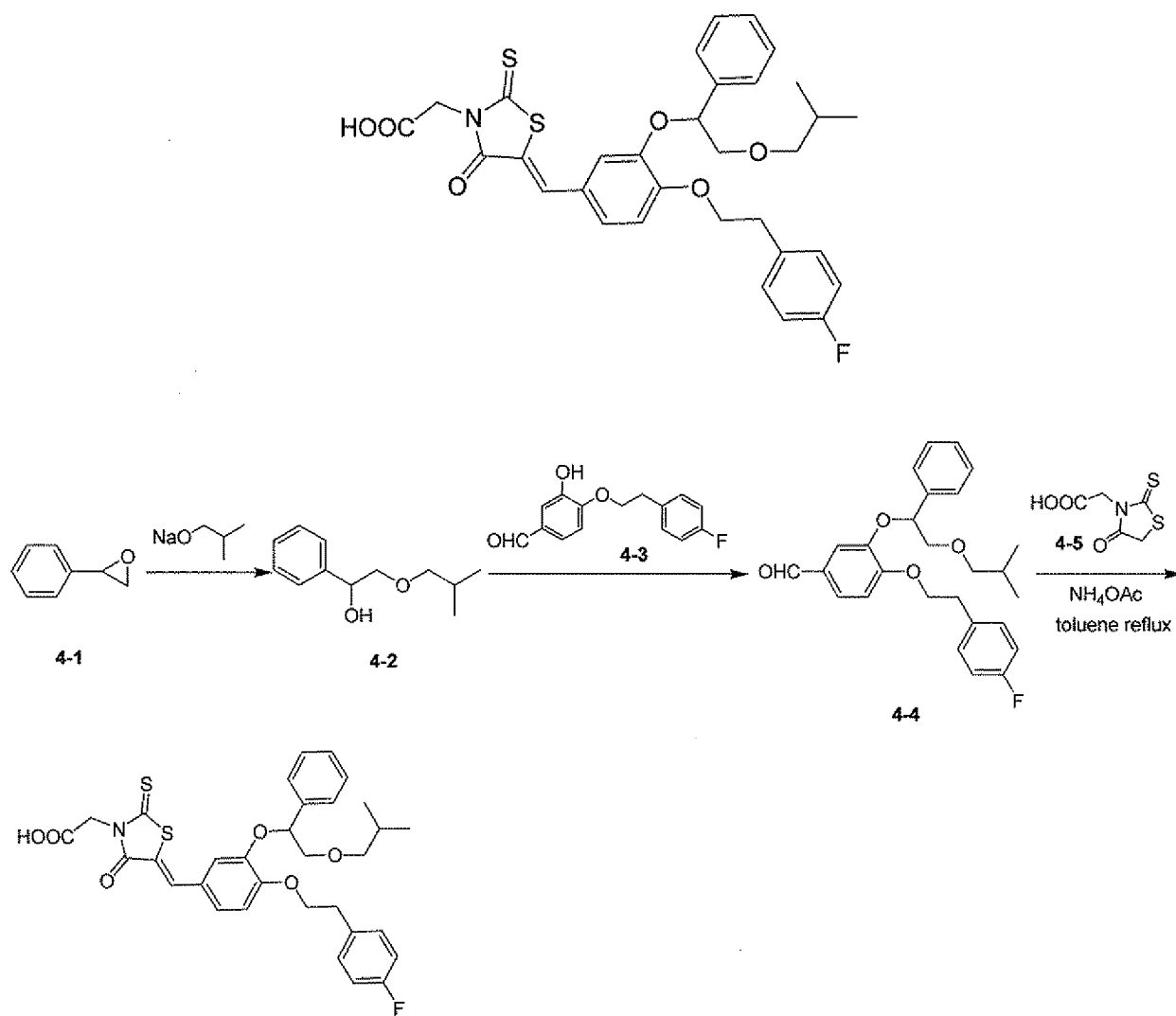
15 The mixture was diluted with H_2O and extracted with EtOAc. The organic layers were combined and dried over Na_2SO_4 , concentrated. The residue was purified by TLC to afford **3-5** (130 mg) as oil, which was used for next step directly.

Step D



To a mixture of **3-5** (130 mg, 0.3 mmol) and **3-6** (60 mg, 0.31 mmol) in toluene (15 mL) was added NH₄OAc (180 mg, 2.34 mmol) in one portion and then the mixture was heated to reflux for 3.5 h. Then the mixture was cooled to room temperature and aqueous 10% HCl was added to pH=5. The mixture was extracted with EtOAc. The organic layers were combined and dried over Na₂SO₄, concentrated to give yellow oil. The residue was purified by prep-TLC to afford **EXAMPLE 3** (150 mg, yield 82.4%). ¹H-NMR (300 MHz, MeOD) δ 7.60 (s, 1H), 7.19~7.49 (m, 11H), 6.88 (s, 1H), 6.81 (s, 1H), 5.05 (s, 1H), 4.65 (s, 2H), 4.25~4.39 (m, 2H), 3.15 (m, 2H), 1.60~1.98 (m, 5H), 1.00~1.46 (m, 6H). MS m/z: 607 (M+1)⁺.

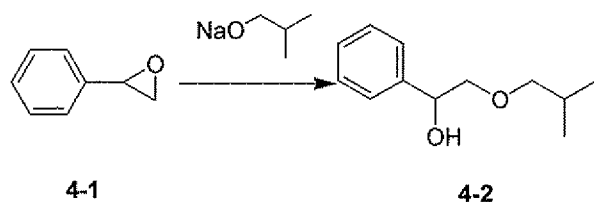
EXAMPLE 4



EXAMPLE 4

5

Step A

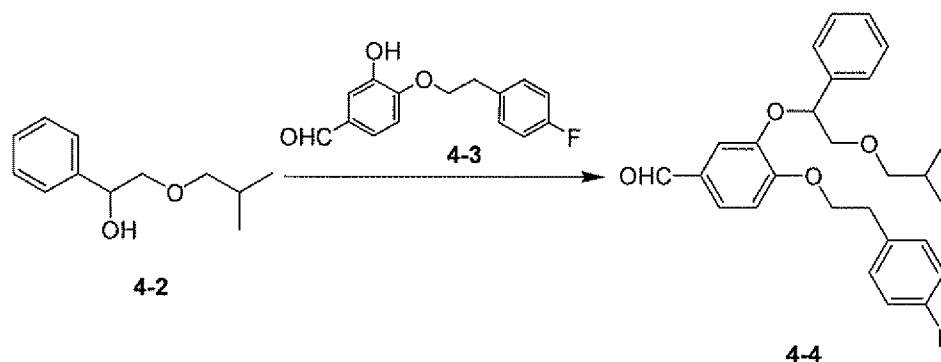


Sodium (8.5 mg, 0.37 mmol) was dissolved at 40 °C in 2-methyl-propan-1-ol (1.3 g, 17.5 mmol) and compound 4-1 (2 g, 16.7 mmol) were subsequently added dropwise at 50 °C over 30 min. Then the reaction mixture was heated to 70 °C and stirred overnight. The resulting mixture was partitioned between H₂O and EtOAc. The organic layer was washed with brine, dried over

sodium sulfate and concentrated to give **4-2** (1.0 g, crude), which is used for the next step directly.

Step B

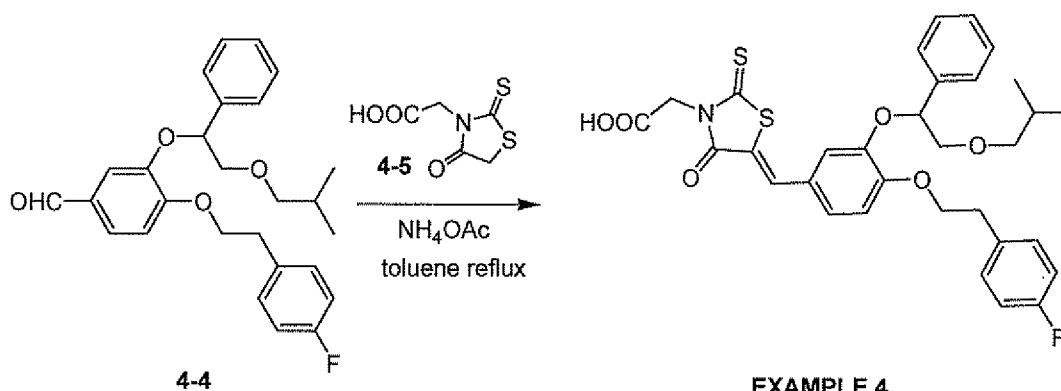
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To a solution of **4-2** (1.0 g, crude), **4-3** (400 mg, 1.64 mmol, 1.0 eq) and PPh₃ (516 mg, 1.96 mmol, 1.2 eq) in THF (20 mL) was added DIAD (406 mg, 1.96 mmol, 1.2 eq) dropwise at 0 °C. The resulting mixture was stirred at room temperature overnight and then partitioned between H₂O and EtOAc. The organic layer was washed by brine, dried over Na₂SO₄, concentrated and purified by prep-HPLC to give **4** (90 mg, yield 12.0 %). ¹H-NMR (400 MHz, MeOD) δ 9.72 (s, 1H), 7.28-7.40 (m, 9H), 6.98-7.05 (t, *J* = 8.78 Hz, 2H), 6.89-6.92 (d, *J* = 8.28 Hz, 1H), 5.42-5.46 (m, 1H), 4.22-4.32 (m, 2H), 3.86-3.92 (m, 1H), 3.68-3.74 (m, 1H), 3.24-3.34 (m, 2H), 3.12-3.18 (m, 2H), 1.82-1.90 (m, 1H), 0.84-0.88 (m, 6H).

15

Step C



The preparation of **EXAMPLE 4** is the same as that for **EXAMPLE 3**. ¹H-NMR (400 MHz, MeOD) δ 7.49 (s, 1H), 7.24-7.40 (m, 7H), 6.98-7.05 (m, 4H), 6.90-6.91 (m, 1H), 5.37-5.40 (m, 1H), 4.75 (s, 2H),

20

4.21-4.32 (m, 2H), 3.83-3.88 (m, 1H), 3.69-3.72 (m, 1H), 3.33-3.35 (m, 2H), 3.09-3.13 (t, $J = 6.57$ Hz, 2H), 1.79-1.87 (m, 1H), 0.86-0.88(m, 6H).

EXAMPLE 5

This example shows that *Pichia pastoris* transformed with an expression vector encoding the heavy chain (Hc) and light chain (Lc) of the human anti-Her2 antibody and treated with the novel Pmt inhibitors described herein produced a glycoprotein having reduced O-glycosylation.

Expression/integration plasmid vector pGLY2988 contains expression cassettes under control of the methanol-inducible *Pichia pastoris* AOX1 promoter that encode the heavy (Hc) and light (Lc) chains of anti-Her2. Anti-Her2 Hc and Lc fused at the N-terminus to α -MAT pre signal peptide (SEQ ID Nos:1 and 2) were synthesized by GeneArt AG. Each was synthesized with unique 5' *Eco*R1 and 3' *Fse*1 sites. The nucleotide and amino acid sequences of the anti-Her2 Hc are shown in SEQ ID Nos:3 and 4, respectively. The nucleotide and amino acid sequences of the anti-Her2 Lc are shown in SEQ ID Nos:5 and 6, respectively. Both nucleic acid fragments encoding the Hc and Lc proteins fused to the α -MAT pre signal peptide were separately subcloned using 5' *Eco*R1 and 3' *Fse*1 unique sites into an expression plasmid vector pGLY2198, which contains the *Pichia pastoris* *TRP2* targeting nucleic acid and the Zeocin-resistance marker and generates expression cassettes under the control of the AOX1 promoter and *Saccharomyces cerevisiae* *CYC* terminator, to form plasmid vectors pGLY2987 and pGLY2338, respectively. The Lc expression cassette was then removed from plasmid vector pGLY2338 by digesting with *Bam*HI and *Not*I and subcloned into plasmid vector pGLY2987 digested with *Bam*HI and *Not*I, thus generating the final expression plasmid vector pGLY2988.

Anti-Her2 expression strain yGLY4280 was constructed as follows: Five micrograms of pGLY2988 digested with restriction enzyme *Spe*I which cuts in the *TRP2* targeting region were used to transform strain yGLY22-1. Strain yGLY22-1 (*och1* Δ ::*lacZ* *bmt2* Δ ::*lacZ*/KIMNN2-2 / *mnn4L1* Δ ::*lacZ*/ *MmSLC35A3* *pno1* Δ *mnn4* Δ ::*lacZ* *met16* Δ ::*lacZ*), was constructed using methods described earlier (Nett and Gerngross, Yeast 20:1279 (2003); Choi *et al.*, PNAS USA 100:5022 (2003); Hamilton *et al.*, Science 301:1244 (2003)).

Transformation of yGLY22-1 performed essentially as follows: YGLY22-1 was grown in 50 mL YPD media (yeast extract (1%), peptone (2%), dextrose (2%)) overnight to an OD of between about 0.2 to 6. After incubation on ice for 30 minutes, cells were pelleted by

centrifugation at 2500-3000 rpm for 5 minutes. Media was removed and the cells washed three times with ice cold sterile 1M sorbitol before resuspension in 0.5 ml ice cold sterile 1M sorbitol. Ten μ L of linearized DNA (10 ug) and 100 μ L cell suspension was combined in an electroporation cuvette and incubated for 5 minutes on ice. Electroporation was in a Bio-Rad GenePulser Xcell following the preset *Pichia pastoris* protocol (2 kV, 25 μ F, 200 Ω), immediately followed by the addition of 1 mL YPDS recovery media (YPD media plus 1 M sorbitol). The transformed cells were allowed to recover for four hours to overnight at room temperature (26°C) before plating the cells on the selective media. Following selection on media containing zeocin, transformants were screened by small scale expression analysis to detect anti-Her2 expression. Strain yGLY4280 was selected based on high level anti-Her2 expression.

Anti-Her2 protein expression for strain yGLY4280 was carried out in shake flasks at 24°C with buffered glycerol-complex medium (BMGY) consisting of 1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer pH 6.0, 1.34% yeast nitrogen base, 4 x 10⁻⁵ % biotin, and 1% glycerol. The induction medium for protein expression was buffered methanol-complex medium (BMMY) consisting of 1% methanol instead of glycerol in BMGY. Pmt inhibitors in 100% methanol were added to the growth medium to a final concentration of 0.15 ug/mL at the time the induction medium was added. This is an intermediate dose that is sufficient to reduce O-glycosylation to roughly 50% of that observed without Pmt inhibitor treatment, and thus allows a comparison of potency for the different inhibitors. Following 24 hr further growth in the induction media, cultures were harvested and centrifuged at 2,000 rpm for five minutes to remove cells from the supernatant.

O-glycan determination was performed using a Dionex-HPLC (HPAEC-PAD) as follows. To measure O-glycosylation reduction, protein was purified from the growth medium using protein A chromatography (Li et al. Nat. Biotechnol. 24(2):210-5 (2006)) and the O-glycans released from and separated from protein by alkaline elimination (beta -elimination) (Harvey, Mass Spectrometry Reviews 18: 349- 451 (1999)). This process also reduces the newly formed reducing terminus of the released O-glycan (either oligomannose or mannose) to mannitol. The mannitol group thus serves as a unique indicator of each O- glycan. 0.5 nmole or more of protein, contained within a volume of 100 μ L PBS buffer, was required for beta elimination. The sample was treated with 25 μ L alkaline borohydride reagent and incubated at 50°C for 16 hours. About 20 uL arabitol internal standard was added, followed by 10 μ L glacial acetic acid. The sample was then centrifuged through a Millipore filter containing both SEPABEADS and AG 50W-X8 resin and washed with water. The samples, including wash, were transferred to plastic

autosampler vials and evaporated to dryness in a centrifugal evaporator. 150 μ L 1% AcOH/MeOH was added to the samples and the samples evaporated to dryness in a centrifugal evaporator. This last step was repeated five more times. 200 μ L of water was added and 100 μ L of the sample was analyzed by high pH anion-exchange chromatography coupled with pulsed electrochemical detection-Dionex HPLC (HPAEC-PAD). Average O-glycan occupancy was determined based upon the amount of mannitol recovered.

The results are summarized in Table 1, which shows that the four novel Pmt inhibitors, Examples 1 to 4, reduce O-glycosylation to levels lower than that observed with Pmt inhibitor Pmti-3 as described in Orchard *et al.* (Bioorgan & Med Chem Letters (2004) 14:3975-3978) and patent publications by the same authors, including EP 1313471 B1 and Bobrowicz *et al.*, U.S. Published Application No. 2007061631. Pmti-3 has the following chemical structure:

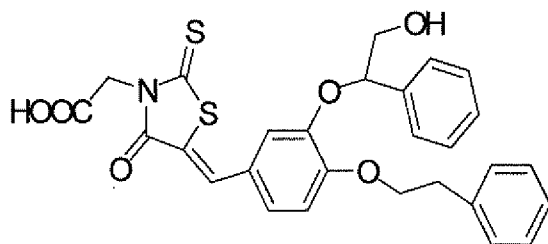


Table 1.

Inhibitor (0.15 μ g/mL)	O-glycan occupancy (moles O-mannose/moles Ab)
none	> 14.0
Pmti-3	8.1
Example 1	4.1
Example 2	4.3
Example 3	5.5
Example 4	5.2

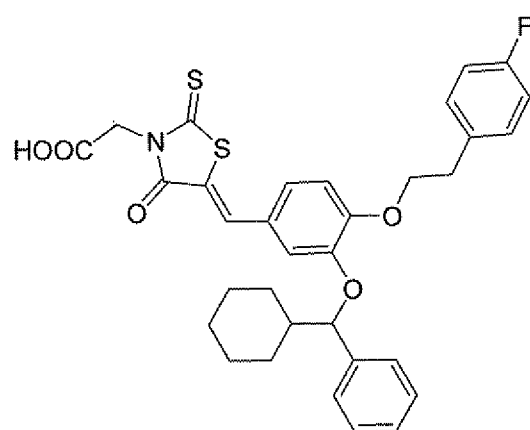
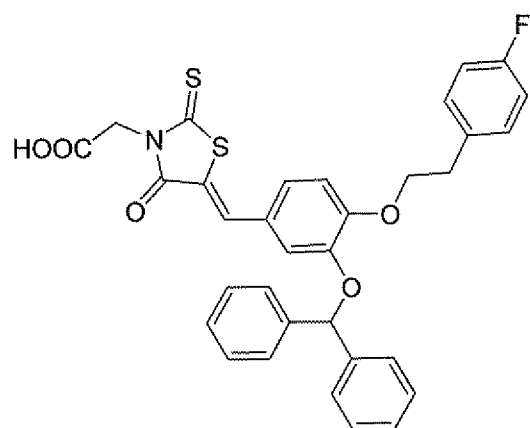
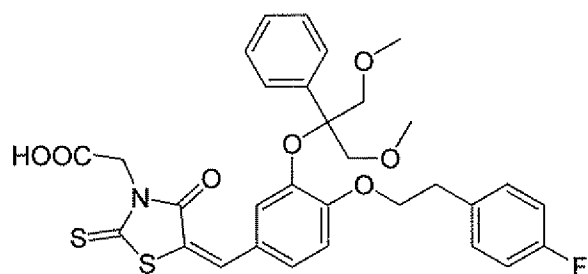
Visual proof that the novel Pmt inhibitors effectively reduce O-glycosylation of *Pichia*-produced recombinant protein is provided by Figure 1, which shows the effects of increasing amounts of Pmt inhibitors on O-glycosylation of anti-Her2 heavy chain (Hc). Strain

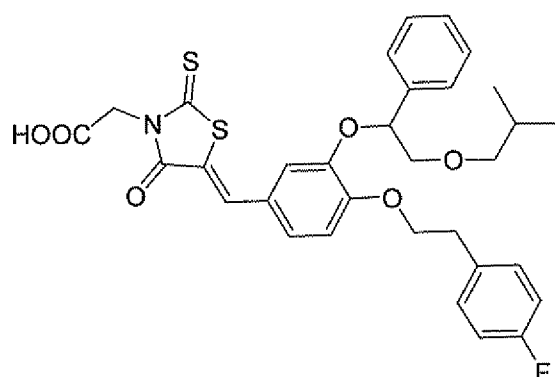
yGLY4280 was inoculated into 96-well deep well plates (Qiagen, Valencia, CA) containing 0.5ml of BMGY media per well. After 24 hours growth with vigorous shaking, the 96-well plate was centrifuged at 2,000 rpm for five minutes to pellet cells. The media was removed and, following a wash step with 0.5mL of BMMY media, the cells resuspended in 0.2 mL BMMY media in which Pmt inhibitors were diluted 2-fold across the rows (11 wells). Well #1 contained 5ug/mL of inhibitor, well # 2 contained 2.5 ug/mL and so on until well # 10 contained 0.009 ug/mL; well #11 contained no inhibitor. After an additional 24 hours growth with vigorous shaking, the plate was centrifuged at 2,000 rpm for five minutes to pellet cells, and the cleared supernatant subjected to Western blot analysis to detect anti-Her2 expression. The Western blotting was performed as follows: seven μ L of the supernatants were separated by reducing polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli, U. K. (1970) Nature 227, 680–685 and then electroblotted onto nitrocellulose membranes (Schleicher & Schuell, now Whatman, Inc., Florham Park, NJ). Anti-Her2 antibody chains were detected on the Western blots using a peroxidase-conjugated anti-human Hc and Lc antibody (Calbiochem/EMD Biosciences, La Jolla, CA) and developed using the ImmunoPure Metal Enhanced DAB Substrate Kit (Pierce Biotechnology, Rockford, IL). Figure 1 shows the results of one such analysis in which the novel Pmt inhibitor from Example 4 (EX. 4, panel A) was tested against Pmti-3 from Orchard *et al.* (panel B) and also an inactive compound as a control (panel C). As shown in panels A and B, Example 4 and Pmti-3 effectively reduced O-glycosylation of anti-Her2 Hc at concentrations as low as 0.018 ug/mL. In contrast, the inactive control compound (panel C) showed no reduction in Hc O-glycosylation. Similar results were obtained with inhibitors shown in Examples 1, 2, and 3. Taken together, these results indicate that the novel Pmt inhibitors shown in Examples 1 to 4 are effective inhibitors of fungal O-glycosylation.

While the present invention is described herein with reference to illustrated embodiments, it should be understood that the invention is not limited hereto. Those having ordinary skill in the art and access to the teachings herein will recognize additional modifications and embodiments within the scope thereof. Therefore, the present invention is limited only by the claims attached herein.

WHAT IS CLAIMED:

1. A compound selected from the following group:





or a salt thereof.

2. A method of producing a protein having reduced O-linked glycosylation
5 comprising:

- (a) growing a cell in a culture that produces the protein;
- (b) contacting the culture with one or more compounds of Claim 1, which
inhibit Pmt-mediated O-linked glycosylation; and
- (c) isolating the protein produced by the host cell.

3. The method of Claim 2 wherein the culture of the cell is provided by:

- (a) providing a nucleic acid encoding a protein; and
- (b) introducing the nucleic acid into the cell.

4. The method of Claim 3 wherein the culture is grown for a time sufficient
to provide a multiplicity of the cells having the nucleic acid before contacting the culture with
any of the one or more compounds which inhibit Pmt-mediated O-linked glycosylation.

5. The method of Claim 3 wherein the culture is grown in the presence of any
of the one or more compounds which inhibit Pmt-mediated O-linked glycosylation.

6. The method of Claim 3 wherein the nucleic acid is operably linked to an
inducible promoter.

7. The method of Claim 6 wherein the culture is grown for a time sufficient
to provide a multiplicity of the cells having the nucleic acid before contacting the culture with the

one or more compounds which inhibit Pmt-mediated O-linked glycosylation and an inducer of the promoter to induce expression of the protein and isolating the protein produced by the cell in the presence of the one or more inhibitors and the inducer to produce the protein having reduced O-linked glycosylation.

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8. The method of Claim 6 wherein the culture is contacted with an inducer of the promoter to induce expression of the protein for a time before contacting the culture with the one or more compounds which inhibit Pmt-mediated O-linked glycosylation and isolating the protein produced by the cell in the presence of the one or more compounds which inhibit Pmt-mediated O-linked glycosylation and the inducer to produce the protein having reduced O-linked glycosylation.

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9. The method of claim 3, wherein the cell is a fungal cell.

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10. The method of claim 3, wherein the cell is a yeast cell.

11. The method of claim 3, wherein the cell is selected from the group consisting of *K. lactis*, *Pichia pastoris*, *Pichia methanolica*, and *Hansenula*.

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12. The method of claim 3, wherein the cell is *Pichia pastoris*.

13. The method of claim 3, wherein the cell is a yeast or filamentous fungal cell that has been genetically modified to produce glycoproteins with a predominant N-glycan glycoform.

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14. The method of claim 3 wherein the cells have been genetically modified to produce glycoproteins in which the N-glycosylation pattern is human-like or humanized.

15. The method of claim 3 wherein the protein is produced at a yield of at least 100 mg/liter of culture medium.

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