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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 September 2019 (12.09.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/173293 A1

(51) International Patent Classification:
C07K 1/22 (2006.01)

(21) International Application Number:
PCT/US2019/020690

(22) International Filing Date:
05 March 2019 (05.03.2019)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/639,062 06 March 2018 (06.03.2018) US
62/695,543 09 July 2018 (09.07.2018) US

(71) Applicant: **SHIRE HUMAN GENETIC THERAPIES, INC.** [US/US]; 300 Shire Way, Lexington, MA 02421 (US).

(72) Inventors: **DWYER, Brian**; C/o Shire Human Genetic Therapies, Inc., 300 Shire Way, Lexington, MA 02421 (US). **ZHANG, Bohong**; C/o Shire Human Genetic Therapies, Inc., 300 Shire Way, Lexington, MA 02421 (US). **HU, Jun**; C/o Shire Human Genetic Therapies, Inc., 300 Shire Way, Lexington, MA 02421 (US). **MEIYAPPAN, Muthuraman**; C/o Shire Human Genetic Therapies, Inc., 300 Shire Way, Lexington, MA 02421 (US). **MILLER, Thomas**; C/o Shire Human Genetic Therapies, Inc., 300 Shire Way, Lexington, MA 02421 (US). **MCLEAN, Paul**; C/o Shire Human Genetic Therapies, Inc., 300 Shire Way, Lexington, MA 02421 (US). **PAN, Clark**; C/o Shire Human Genetic Therapies, Inc., 300 Shire Way, Lexington, MA 02421 (US).

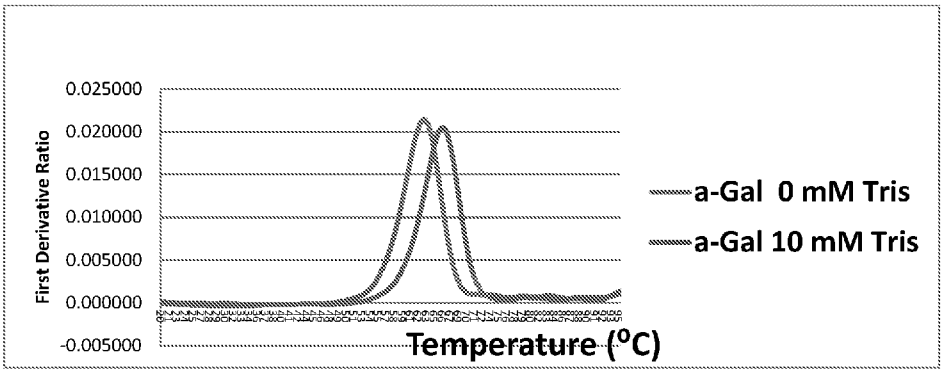
(74) Agent: **KHANKIN, Alina, K.**; Troutman Sanders LLP, 875 Third Avenue, New York, NY 10022 (US).

(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, JO, 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, TJ,

(54) Title: AFFINITY PURIFICATION OF GLYCOSIDE-CLEAVING ENZYMES

FIGURE 1



(57) Abstract: The invention relates to an affinity resin functionalized with small molecule inhibitors of glycoside-cleaving enzymes, *e.g.*, a-galactosidase A (α-Gal A), glucocerebrosidase (GCB), β- galactosidase, and acid alpha-glucosidase (GAA), and a method for purifying glycoside-cleaving enzymes produced in a cell line using the small molecule inhibitor-functionalized affinity resin.

WO 2019/173293 A1

[Continued on next page]

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))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

AFFINITY PURIFICATION OF GLYCOSIDE-CLEAVING ENZYMES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 62/639,062, filed March 6, 2018, and U.S. Provisional Application No. 62/695,543, filed July 9, 2018, the disclosures of which are herein incorporated by reference in their entirety.

FIELD OF INVENTION

[0002] The present invention relates to affinity resins functionalized with small molecule inhibitors of glycoside-cleaving enzymes, *e.g.*, α-galactosidase A (α-Gal A), glucocerebrosidase (GCB), β-galactosidase, and acid alpha-glucosidase (GAA), and methods for purifying glycoside-cleaving enzymes produced in a cell line using the small molecule inhibitor-functionalized affinity resins.

BACKGROUND OF THE INVENTION

[0003] Fabry disease is caused by a deficiency in the enzyme α-galactosidase A (α-Gal A): The pathophysiology of Fabry Disease is well established: due to a lack of the lysosomal enzyme α-galactosidase A (α-Gal A), there is accumulation of globotriaosylceramide (Gb3) throughout the body.

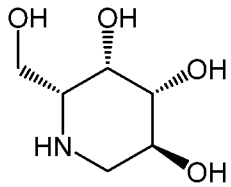
[0004] Enzyme replacement therapy ("ERT") is a currently used method of treatment for Fabry disease. Two α-Gal A products are commercially available for the treatment of Fabry disease: agalsidase alfa (Replagal®, Shire Human Genetic Therapies) and agalsidase beta (Fabrazyme®; Genzyme Corporation). These two forms of ERT effectively compensate for a patient's inadequate α-Gal A activity with a recombinant form of the enzyme, administered intravenously.

[0005] The cDNA and gene encoding human α-Gal A have been isolated and sequenced. Human α-Gal A is expressed as a 429-amino acid polypeptide, of which the N-terminal 31 amino acids are the signal peptide. The human enzyme has been expressed in Chinese Hamster Ovary (CHO) cells (Desnick et al., U.S. Patent 5,356,804; Ioannou et al., J Cell Biol. 119: 1137 (1992)); insect cells (Calhoun et al., WO 90/11353); and human cells (Selden et al., U.S. Patents 6,083,725 and 6,458,574).

SUBSTITUTE SHEET (RULE 26)

[0006] Recombinant α-Gal A is produced in a mammalian cell line by genetic engineering technology. The mature enzyme that is secreted in the cell culture media is a homodimeric glycoprotein, with each subunit consisting of 398 amino acids after removal of a 31-residue signal sequence. The protein undergoes post-translational modification by and the addition of 3 N-linked carbohydrate chains.

[0007] 1-deoxygalactonojirimycin ("DGJ") and its salt, 1-deoxygalactonojirimycin hydrochloride (also known by its United States Adopted Name (USAN), migalastat hydrochloride) acts as a pharmacological chaperone for mutant α-Gal A by selectively binding to the enzyme, thereby increasing its stability and helping the enzyme fold into its correct three-dimensional shape. This stabilization of α-Gal A allows the cell's quality control mechanisms to recognize the enzyme as properly folded so that trafficking of the enzyme to the lysosome is increased, allowing it to carry out its intended biological function, the metabolism of Globotriaosylceramide (GL-3). The chemical structure of DGJ is shown below:



[0008] Purification of α-Gal A by affinity column chromatography using Concanavalin A (Con A)-Sepharose and immobilized thio-α-galactoside (thio-Gal) has been disclosed (see Yasuda et al. Prot. Expr. & Purif. 2004, 37, 499-506). However, this and other reported methodology for purification of α-Gal A has a number of serious drawbacks, such as requiring multi-step manipulation of raw material, requiring costly elution solutions, and/or not being easily scalable.

[0009] Gaucher disease is an autosomal recessive lysosomal storage disorder characterized by a deficiency in the lysosomal enzyme, glucocerebrosidase (GCB). GCB hydrolyzes the glycolipid glucocerebroside that is formed after degradation of glycosphingolipids in the membranes of white blood cells and red blood cells. The deficiency in this enzyme causes glucocerebroside to accumulate in large quantities in the lysosomes of phagocytic cells located in the liver, spleen and bone marrow of Gaucher patients. Accumulation of these molecules causes a range of clinical manifestations including splenomegaly, hepatomegaly, skeletal disorder, thrombocytopenia and anemia. (Beutler et al. Gaucher disease;

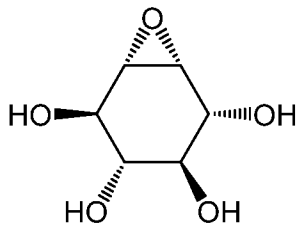
In: The Metabolic and Molecular Bases of Inherited Disease (McGraw-Hill, Inc, New York, 1995) pp. 2625-2639)

[00010] Treatments for patients suffering from this disease include administration of analgesics for relief of bone pain, blood and platelet transfusions and, in some cases, splenectomy. Joint replacement is sometimes necessary for patients who experience bone erosion.

[00011] Enzyme replacement therapy with GCB has been used as a treatment for Gaucher disease. Current treatment of patients with Gaucher disease includes administration of Velaglucerase, available under the name VPRIV® from Shire Human Genetic Therapies.

[00012] Velaglucerase is human beta-glucocerebrosidase produced by gene-activation in a human cell line. Gene activation refers to targeted recombination with a promoter that activates the endogenous beta-glucocerebrosidase gene in the selected human cell line. Velaglucerase is secreted as a monomeric glycoprotein of approximately 63 kDa and is composed of 497 amino acids with a sequence identical to the natural human protein. The amino acid sequence of velaglucerase is described in Zimran et al. (2007) Blood Cells Mol Dis, 39: 115-118. As with α-Gal A, purification of GCB has a number of serious drawbacks, such as requiring multi-step manipulation of raw material, requiring costly elution solutions, and/or not being easily scalable.

[00013] Conduritol B epoxide is an inhibitor of GCB. The chemical structure of Conduritol B is shown below:



[00014] The epoxide of Conduritol B reacts covalently with the enzyme active site. A series of competitive (reversible) inhibitors referred to as amino myo-inositols have been described, which have close similarity to Conduritol, except that they do not react chemically with the enzyme (see Trapero et al. J. Med. Chem. 2012, 55, 4479−4488).

[00015] Glycogen storage disease type II, also called Pompe disease, is an autosomal recessive metabolic disorder which damages muscle and nerve cells throughout the body. It is caused by an accumulation of glycogen in the lysosome due to deficiency of the lysosomal acid alpha-glucosidase enzyme (GAA). Pompe disease can be treated by enzyme replacement therapy

SUBSTITUTE SHEET (RULE 26)

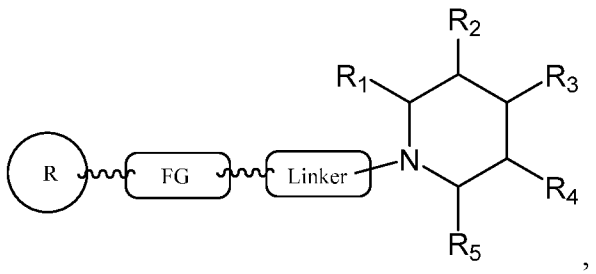
using biologically active recombinant human acid alpha-glucosidase commercially available as Myozyme® or Lumizyme® (Genzyme Corporation).

[00016] Thus, there exists an unmet need for novel methods for purifying glycoside-cleaving enzymes, such as α-Gal A, GCB, and GAA, that are easily scalable, require no manipulation of raw material, and minimize production costs.

SUMMARY OF THE INVENTION

[00017] Various non-limiting aspects and embodiments of the invention are described below.

[00018] In one aspect of the present invention, an affinity resin is provided, comprising a small molecule ligand having the formula:



,

wherein R is a resin matrix;

FG is absent or is a functional group selected from -O-, -NH-, -C(O)N-, -C(O)O-, CH_2, and -S-;

Linker is absent or is selected from a straight chained, branched or cyclic aliphatic C_2-C_{20} hydrocarbon; a heteroaliphatic C_2-C_{20} hydrocarbon; an aromatic C_6-C_{20} hydrocarbon; a heteroaromatic C_2-C_{20} hydrocarbon; an aryl C_6-C_{20} hydrocarbon; a heteroaryl C_2-C_{20} hydrocarbon, a C_2-C_{12} perfluorocarbon, or a combination thereof, each of which optionally contains 1-8 heteroatoms selected from halogen, O, N, and S and combinations thereof;

R_1, R_2, R_3, R_4, and R_5 are each independently selected from H, OH, CH_2OH and -NH-C(O)-CH_3, and

wherein the affinity resin is capable of binding a glycoside-cleaving enzyme.

[00019] In some embodiments, the affinity resin is capable of binding a glycoside-cleaving enzyme from conditioned medium of a cell line.

[00020] In one embodiment, the affinity resin is capable of binding a glycoside-cleaving enzyme directly from conditioned medium of a cell line without manipulation of the conditioned medium.

[00021] In one embodiment, R_1 is CH_2OH, R_2, R_3, and R_4 are each OH, and R_5 is H or -OH.

[00022] In another embodiment, R_1 is CH_2OH, R_2 and R_3 are each OH, R_4 is -NH-C(O)-CH_3, and R_5 is H.

[00023] In yet another embodiment, R_1 is H, R_2 is CH_2OH, R_3 and R_4 are each OH, and R_5 is H.

[00024] In one embodiment, the functional group is -C(O)N-.

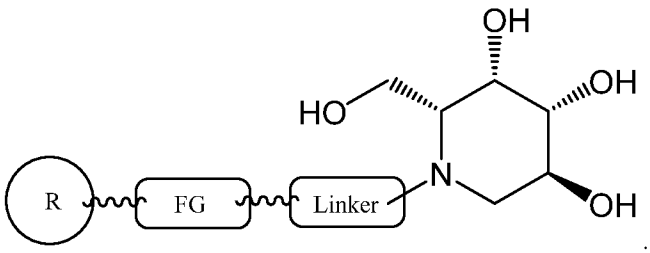
[00025] In one embodiment, the functional group is -O-.

[00026] In one embodiment, the linker is a straight chained, branched or cyclic aliphatic C_2-C_{20} hydrocarbon optionally containing 1-8 heteroatoms selected from halogen, O, N, and S and combinations thereof.

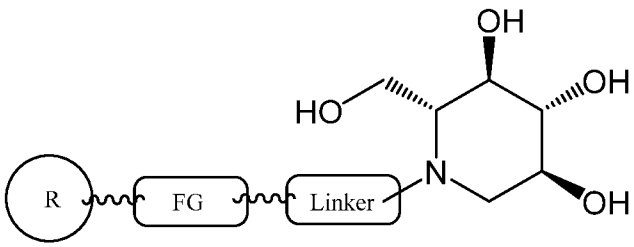
[00027] In one embodiment, the linker is a straight chained aliphatic C_2-C_{10} hydrocarbon.

[00028] In one embodiment, the linker is a straight chained aliphatic C_5 hydrocarbon.

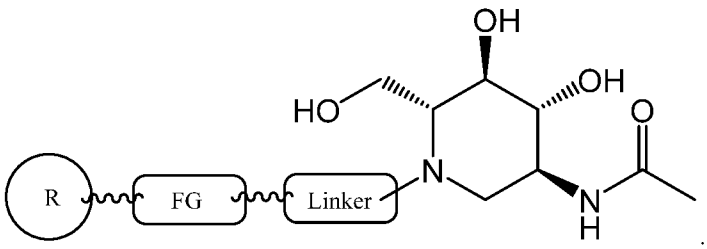
[00029] In one embodiment, the affinity resin of the invention has the formula:



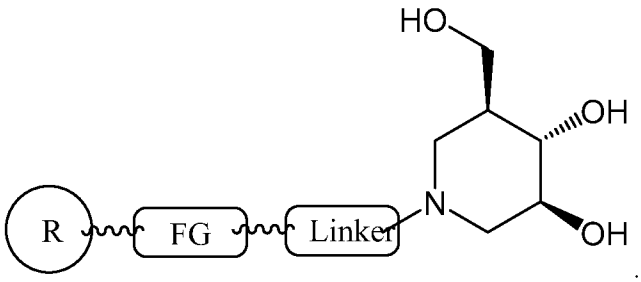
[00030] In one embodiment, the affinity resin has the formula:



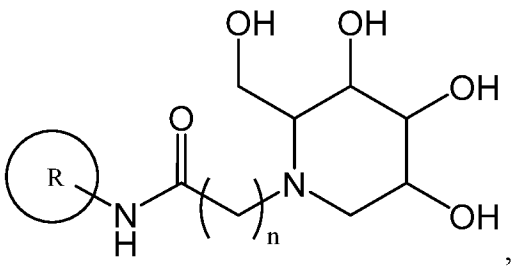
[00031] In one embodiment, the affinity resin has the formula:



[00032] In one embodiment, the affinity resin has the formula:



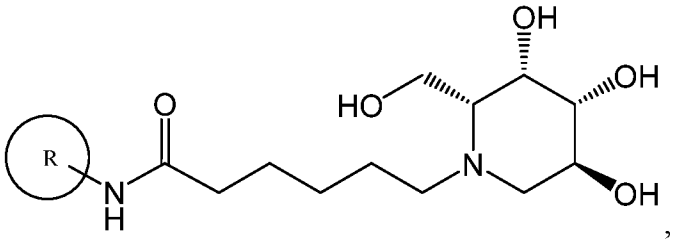
[00033] In one embodiment, the affinity resin has the formula:



wherein R is a resin matrix and n is an integer from 2 to 20.

[00034] In one embodiment, n is 5.

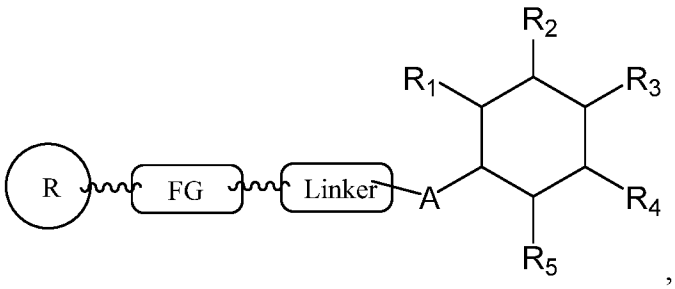
[00035] In one aspect, an affinity resin is provided, comprising a small molecule ligand having the formula:



,

wherein R is a resin matrix.

[00036] In one aspect, an affinity resin is provided, comprising a small molecule ligand having the formula:



,

wherein R is a resin matrix;

FG is absent or is a functional group selected from -O-, -NH-, -C(O)N-, -C(O)O-, CH_2, and -S-;

Linker is absent or is selected from a straight chained, branched or cyclic aliphatic C_2-C_{20} hydrocarbon; a heteroaliphatic C_2-C_{20} hydrocarbon; an aromatic C_6-C_{20} hydrocarbon; a heteroaromatic C_2-C_{20} hydrocarbon; an aryl C_6-C_{20} hydrocarbon; a heteroaryl C_2-C_{20} hydrocarbon, a C_2-C_{12} perfluorocarbon, or a combination thereof, each of which optionally contains 1-8 heteroatoms selected from halogen, O, N, and S and combinations thereof;

A is selected from -NH- and -S-;

R_1, R_2, R_3, R_4, and R_5 are each independently selected from H, OH, CH_2OH, and -NH-C(O)-CH_3, and

wherein the affinity resin is capable of binding a glycoside-cleaving enzyme.

[00037] In some embodiments, the affinity resin is capable of binding a glycoside-cleaving enzyme from conditioned medium of a cell line.

[00038] In one embodiment, the affinity resin is capable of binding a glycoside-cleaving enzyme directly from conditioned medium of a cell line without manipulation of the conditioned medium.

[00039] In one embodiment, R_1, R_2, R_3, R_4, and R_5 are each OH.

[00040] In one embodiment, R_1 is CH_2OH, R_2 and R_3 are each OH, R_4 is -NH-C(O)-CH_3, and R_5 is H.

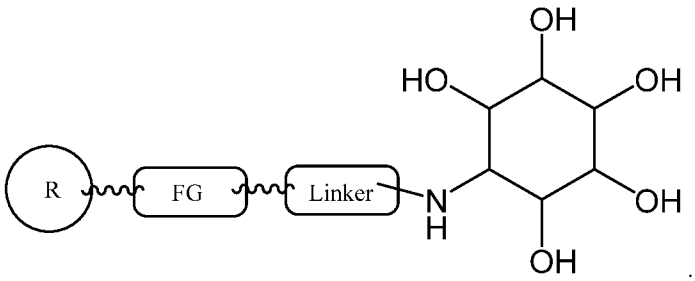
[00041] In one embodiment, the linker is a straight chained, branched or cyclic aliphatic C_2-C_{20} hydrocarbon optionally containing 1-8 heteroatoms selected from halogen, O, N, and S and combinations thereof.

[00042] In one embodiment, the linker is a straight chained aliphatic C_2-C_{10} hydrocarbon.

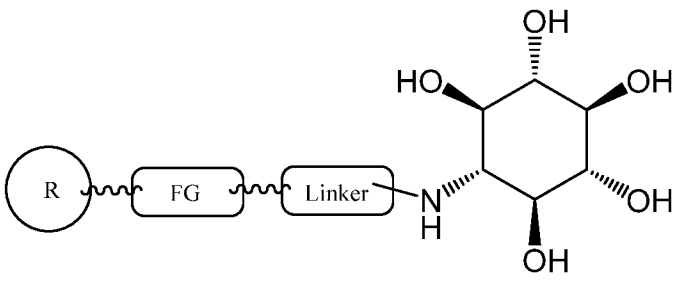
[00043] In one embodiment, A is -NH-.

[00044] In one embodiment, A is -S-.

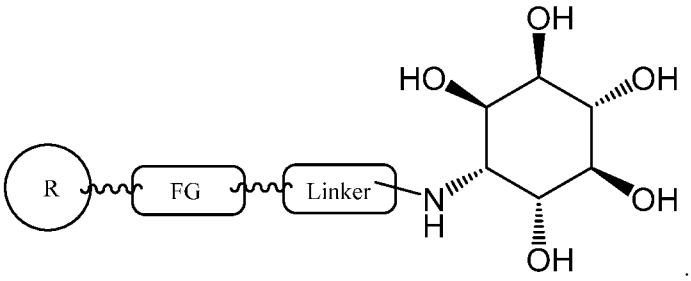
[00045] In one embodiment, the affinity resin has the formula:



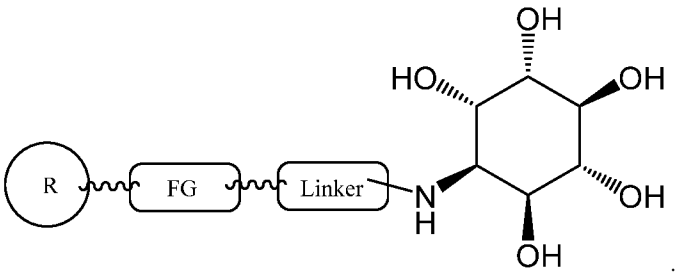
[00046] In one embodiment, the affinity resin has the formula:



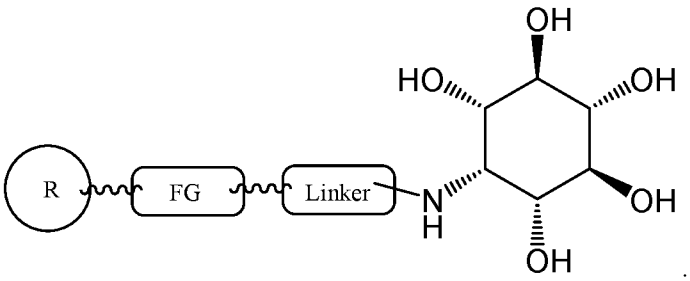
[00047] In another embodiment, the affinity resin has the formula:



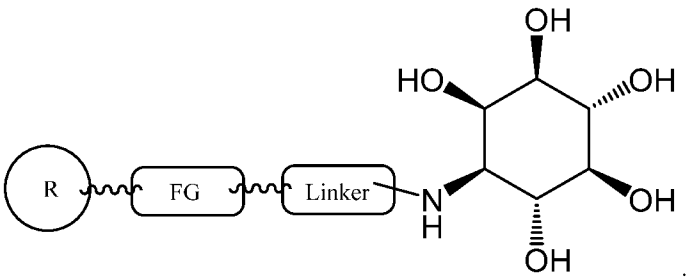
[00048] In another embodiment, the affinity resin has the formula:



[00049] In yet another embodiment, the affinity resin has the formula:



[00050] In yet another embodiment, the affinity resin has the formula:



[00051] In one embodiment, the affinity resin according to any of the above embodiments has a binding capacity of at least 10 mg of glycoside-cleaving enzyme per 1 mL of the affinity resin.

[00052] In one embodiment, the affinity resin according to any of the above embodiments has a binding capacity of about 50 mL to about 100 mL of the conditioned medium per 1 mL of the affinity resin. In one embodiment, the affinity resin according to any of the above embodiments has a binding capacity of about 80 mL of the conditioned medium per 1 mL of the affinity resin.

[00053] In one embodiment, the affinity resin according to any of the above embodiments maintains purification performance after exposure to NaOH.

[00054] In one aspect of the present invention, a method of purifying a glycoside-cleaving enzyme produced in a cell line is provided, said method comprising the steps of binding the glycoside-cleaving enzyme to the affinity resin of any of the above embodiments, and eluting the bound glycoside-cleaving enzyme.

[00055] In one embodiment, the glycoside-cleaving enzyme is purified from conditioned medium.

[00056] In one embodiment, the glycoside-cleaving enzyme is purified directly from conditioned medium without manipulation of the conditioned medium.

[00057] In one aspect of the present invention, a method of purifying a glycoside-cleaving enzyme produced in a cell line, *e.g.*, directly from conditioned medium of the cell line, *e.g.*, without manipulation of the conditioned medium is provided, said method comprising the steps of binding the glycoside-cleaving enzyme to the affinity resin of any of the above embodiments, and eluting the bound glycoside-cleaving enzyme.

[00058] In one embodiment of the above method, the cell line is a recombinant CHO cell line.

[00059] In one embodiment of the above method, the cell line is a human cell line.

[00060] In one embodiment of the above method, the purity of the eluted glycoside-cleaving enzyme is at least 95%.

[00061] In one embodiment of the above method, the binding capacity of the affinity resin is at least 10 mg of glycoside-cleaving enzyme per 1 mL of the affinity resin.

[00062] In one embodiment of the above method, the glycoside-cleaving enzyme is selected from α-galactosidase A (α-Gal A), glucocerebrosidase (GCB), β-galactosidase, and acid alpha-glucosidase (GAA).

[00063] In one embodiment of the above method, the glycoside-cleaving enzyme is α-Gal A.

[00064] In one embodiment of the above method, the glycoside-cleaving enzyme is GCB.

[00065] In one embodiment of the above method, the glycoside-cleaving enzyme is GAA.In one embodiment, the step of eluting the bound glycoside-cleaving enzyme is performed with an elution buffer having a pH between about pH 5 and about pH 9. In one embodiment, the step of eluting the bound glycoside-cleaving enzyme is performed with an elution buffer having a pH between about pH 7 and about pH 8.5.

[00066] In one embodiment, the step of eluting the bound glycoside-cleaving enzyme is performed with an elution buffer comprising a salt. In one embodiment, the salt is NaCl. In one embodiment, the step of eluting the bound glycoside-cleaving enzyme is performed with an elution buffer comprising a different concentration of salt than the concentration found in the medium or buffer in which the enzyme bound to the affinity resin.

[00067] In one embodiment, the step of eluting the bound glycoside-cleaving enzyme is performed with an elution buffer comprising a small molecule ligand, which small molecule ligand is a competitive inhibitor of the bound glycoside-cleaving enzyme. In one embodiment, the small molecule ligand present in the elution buffer is the same small molecule ligand as immobilized on the affinity matrix. In another embodiment, the small molecule ligand present in the elution buffer is a different small molecule ligand from the small molecule ligand immobilized on the affinity matrix.

[00068] In one embodiment of the above method, the step of eluting the bound glycoside-cleaving enzyme is performed with an elution buffer comprising tris(hydroxymethyl) aminomethane (Tris).

[00069] In one aspect of the present invention, a method of purifying α-Gal A is provided, said method comprising the steps of binding the α-Gal A to the affinity resin of any of the above embodiments, and eluting the bound α-Gal A.

[00070] In one embodiment, a method of purifying α-Gal A directly from conditioned medium of a cell line is provided, said method comprising the steps of binding the α-Gal A to the affinity resin of any of the above embodiments, and eluting the bound α-Gal A.

[00071] In one embodiment, the α-Gal A is purified directly from conditioned medium of a cell line without manipulation of the conditioned medium.

[00072] In one embodiment, a method of purifying GCB directly from conditioned medium of a cell line is provided, said method comprising the steps of binding the GCB to the affinity resin of any of the above embodiments, and eluting the bound GCB.

[00073] In one embodiment, the GCB is purified directly from conditioned medium of a cell line without manipulation of the conditioned medium.

[00074] In one embodiment, a method of purifying GAA directly from conditioned medium of a cell line is provided, said method comprising the steps of binding the GAA to the affinity resin of any of the above embodiments, and eluting the bound GAA.

[00075] In one embodiment, the GAA is purified directly from conditioned medium of a cell line without manipulation of the conditioned medium.

[00076] In one embodiment, the step of eluting the bound glycoside-cleaving enzyme is performed with an elution buffer comprising tris(hydroxymethyl) aminomethane (Tris).

[00077] These and other aspects of the present invention will become apparent to those skilled in the art after a reading of the following detailed description of the invention, including the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[00078] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[00079] **Figure 1** depicts Thermal Stability curves of α-Gal A at 0 mM (blue) and 10 mM (red) Tris buffer.

[00080] **Figure 2** shows an SDS-PAGE gel of α-Gal A affinity elution with Tris buffers.

[00081] **Figure 3** shows results of initial α-Gal A affinity purification testing. **Figure 3A** shows an SDS-PAGE gel of initial α-Gal A affinity purification testing, and **Figure 3B** shows anti- α-Gal A Western blot.

[00082] **Figure 4** shows results of the affinity resin binding capacity testing. **Figure 4A** shows a chromatogram of α-Gal A affinity resin binding capacity testing; **Figure 4B** shows an SDS-PAGE gel of α-Gal A affinity resin binding capacity testing, and **Figure 4C** shows an anti-α-Gal A Western blot of α-Gal A affinity resin binding capacity testing.

[00083] **Figure 5** shows results of the affinity resin stability against sanitization testing. **Figure 5A** is a chromatogram of the affinity resin stability against sanitization testing with five sanitization cycles, and **Figure 5B** is an SDS-PAGE gel of eluted α-Gal A.

DETAILED DESCRIPTION

[00084] Detailed embodiments of the present invention are disclosed herein; however, it is to be understood that the disclosed embodiments are merely illustrative of the invention that may be embodied in various forms. In addition, each of the examples given in connection with the various embodiments of the invention is intended to be illustrative, and not restrictive. Therefore, specific structural and functional details disclosed herein are not to be interpreted as limiting, but merely as a representative basis for teaching one skilled in the art to variously employ the present invention.

[00085] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[00086] As used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural references unless the context clearly dictates otherwise. Thus, for example, a reference to "a method" includes one or more methods, and/or steps of the type described herein and/or which will become apparent to those persons skilled in the art upon reading this disclosure.

[00087] ***Glycoside-cleaving enzyme production***

[00088] As described above, individuals with glycoside-cleaving enzyme deficiency, *e.g.*, α-Gal A deficiency, GCB deficiency, or GAA deficiency, can be treated with purified glycoside-cleaving enzyme, *e.g.*, α-Gal A, GCB, or GAA (i.e., enzyme replacement therapy). Cell lines, *e.g.*, mammalian cell lines, *e.g.*, CHO or human cell lines, or plant cell lines, genetically modified to overexpress the human glycoside-cleaving enzyme, *e.g.*, α-Gal A, GCB, or GAA, may be used for in vitro protein production, to produce the glycoside-cleaving enzyme, *e.g.*, α-Gal A, GCB, or GAA, which may be purified for enzyme replacement therapy. Secondary or immortalized human cells may be used and may be genetically modified by the transfection or transduction methods described elsewhere (see, *e.g.*, PCT/US00/06118, U.S. Pat. No. 6,458,574, PCT/US97/16603, USSN 2011/0280856, U.S. Pat. No. 7,833,742). After genetic modification,

the cells are cultured under conditions permitting overexpression and secretion of the human glycoside-cleaving enzyme, *e.g.*, α-Gal A, GCB, or GAA.

[00089] The protein is isolated from the cultured cells, *e.g.*, by collecting the medium in which the cells are grown, and/or lysing the cells to release their contents, concentrating the medium (*e.g.*, by diafiltration/ultrafiltration), and then applying standard protein purification techniques, such as passing the protein through, *e.g.*, a hydrophobic interaction resin, an ion exchange resin, *e.g.*, an anion exchange or a cation exchange resin, or a mixed mode resin, and the like. Passing the sample over a resin may constitute the first chromatography step. Typically further purification is required, such as passing the glycoside-cleaving enzyme-containing material eluted from the hydrophobic interaction resin over a column containing a second resin, such as an immobilized heparin resin such as Heparin Sepharose®, hydroxyapatite, an anion exchange resin such as Q Sepharose®, or a size exclusion resin such as Superdex® 200.

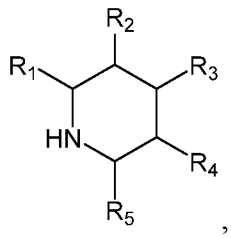
[00090] As such, the present purification methodology has a number of serious drawbacks, such as requiring multi-step manipulation of raw material, requiring costly elution solutions, and/or not being easily scalable.

[00091] ***Affinity resin functionalized with small molecule inhibitors***

[00092] As disclosed in the present invention, it has been unexpectedly discovered that a variety of small molecules that bind to and/or inhibit glycoside-cleaving enzymes may be immobilized on solid support, i.e., conjugated to the solid matrix resin, and used effectively and efficiently for affinity column chromatography purification of glycoside-cleaving enzymes.

[00093] In one aspect, a small molecule capable of being immobilized may be an azasugar, i.e., a carbohydrate derivative in which a piperidine ring replaces a tetrahydrofuran ring, *e.g.*, an iminosugar.

[00094] In one embodiment, the azasugar capable of being immobilized may have the following structural formula:

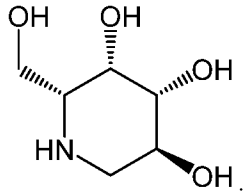


,

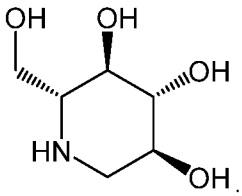
wherein R_1, R_2, R_3, R_4, and R_5 may be independently selected from H, OH, CH_2OH, and -NH-C(O)-CH_3.

SUBSTITUTE SHEET (RULE 26)

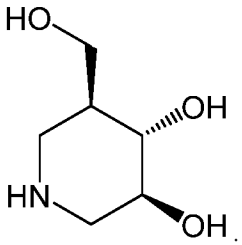
[00095] In one particular embodiment, the azasugar capable of being immobilized may be an analog of DGJ, where DGJ has the following structural formula:



[00096] In another embodiment, the azasugar capable of being immobilized may have the following structural formula:

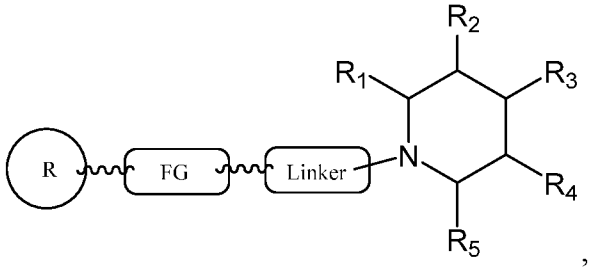


[00097] In another embodiment, the azasugar capable of being immobilized may be an analog of isofagomine, where isofagomine has the following structural formula:



[00098] In one aspect of the present invention, an azasugar may be immobilized onto a solid support directly or via a linker or spacer, which linker or spacer may be covalently attached to the azasugar via the nitrogen atom of the carbohydrate ring, and further attached to a resin matrix directly or via a suitable functional group. In one embodiment, the azasugar immobilized onto a solid support may be represented by the following formula:

[00099]



,

[000100] wherein R is a resin matrix;

[000101] FG is absent or is a functional group selected from -O-, -NH-, -C(O)N-, -C(O)O-, CH_2, and -S-; and

[000102] Linker is absent or is selected from a straight chained, branched or cyclic aliphatic C_2-C_{20} hydrocarbon; a heteroaliphatic C_2-C_{20} hydrocarbon; an aromatic C_6-C_{20} hydrocarbon; a heteroaromatic C_2-C_{20} hydrocarbon; an aryl C_6-C_{20} hydrocarbon; a heteroaryl C_2-C_{20} hydrocarbon, a C_2-C_{12} perfluorocarbon, or a combination thereof, each of which optionally contains 1-8 heteroatoms selected from halogen, O, N, and S and combinations thereof, and

[000103] wherein R_1, R_2, R_3, R_4, and R_5 may be independently selected from H, OH, CH_2OH, and -NH-C(O)-CH_3.

[000104] In one embodiment, R_1 is CH_2OH, R_2, R_3, and R_4 are each OH, and R_5 is H.

[000105] In another embodiment, R_1 is CH_2OH, R_2 and R_3 are each OH, R_4 is -NH-C(O)-CH_3, and R_5 is H.

[000106] In another embodiment, R_1 is H, R_2 is CH_2OH, R_3 and R_4 are each OH, and R_5 is H.

[000107] In one embodiment, the functional group is -C(O)N-.

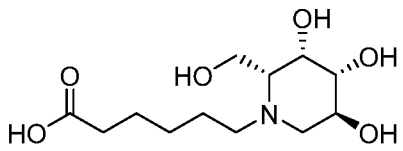
[000108] In one embodiment, the functional group is -O-.

[000109] In one embodiment, the linker is a straight chained, branched or cyclic aliphatic C_2-C_{20} hydrocarbon optionally containing 1-8 heteroatoms selected from halogen, O, N, and S and combinations thereof.

[000110] In one embodiment, the linker is a straight chained aliphatic C_2-C_{10} hydrocarbon.

[000111] In one embodiment, the linker is a straight chained aliphatic C_5 hydrocarbon.

[000112] In one embodiment, a DGJ analog may be N-5-Carboxypentyl-1-deoxygalactonojirimycin as shown in the following structure:



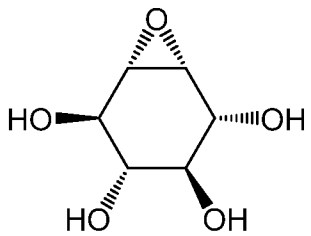
[000113] N-5-Carboxypentyl-1-deoxygalactonojirimycin,

[000114] conjugated to an affinity resin to generate a small molecule-based affinity purification chromatography resin.

[000115] In one embodiment, N-5-Carboxypentyl-1-deoxygalactonojirimycin may be immobilized onto a solid support and used for purification of glycoside-cleaving enzymes, *e.g.*, α-Gal A, GCB, and GAA, by affinity chromatography. In one particular embodiment, N-5-Carboxypentyl-1-deoxygalactonojirimycin may be conjugated to an affinity resin and used for purification of α-Gal A by affinity chromatography, *e.g.*, directly from the conditioned medium.

[000116] In another aspect, a small molecule capable of being immobilized may be a derivative of inositol, *e.g.*, a derivative of Conduritol B epoxide, where Conduritol B epoxide has the following structural formula:

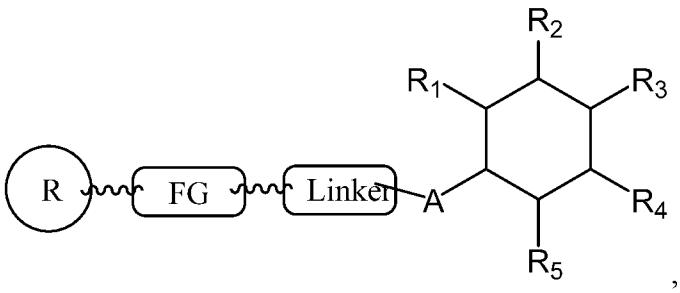
[000117]



[000118] In one embodiment, an inositol derivative, *e.g.*, Conduritol B epoxide, may be immobilized on solid support by chemically reacting with an amino group on a resin matrix, giving a structure with an exo-cyclic amino group.

[000119] In another embodiment, an inositol derivative, *e.g.*, Conduritol B epoxide, may be immobilized on solid support by chemically reacting with a sulfhydryl group on a resin matrix, giving a structure with an exo-cyclic thioether group.

[000120] In one aspect of the present invention, an inositol derivative may be immobilized onto a solid support directly or via a linker or spacer, which linker or spacer may be covalently attached to the inositol via, *e.g.*, an amino group or a sulfhydryl group, and further attached to a resin matrix directly or via a suitable functional group. In one embodiment, the inositol derivative immobilized onto a solid support may be represented by the following formula:



,

[000121] wherein R is a resin matrix;

[000122] FG is absent or is a functional group selected from -O-, -NH-, -C(O)N-, -C(O)O-, CH_2, and -S-;

[000123] Linker is absent or is selected from a straight chained, branched or cyclic aliphatic C_2-C_{20} hydrocarbon; a heteroaliphatic C_2-C_{20} hydrocarbon; an aromatic C_6-C_{20} hydrocarbon; a heteroaromatic C_2-C_{20} hydrocarbon; an aryl C_6-C_{20} hydrocarbon; a heteroaryl C_2-C_{20} hydrocarbon, a C_2-C_{12} perfluorocarbon, or a combination thereof, each of which optionally contains 1-8 heteroatoms selected from halogen, O, N, and S and combinations thereof;

[000124] A is selected from -NH- and -S-, and

[000125] wherein R_1, R_2, R_3, R_4, and R_5 may be independently selected from H, OH, CH_2OH, and -NH-C(O)-CH_3.

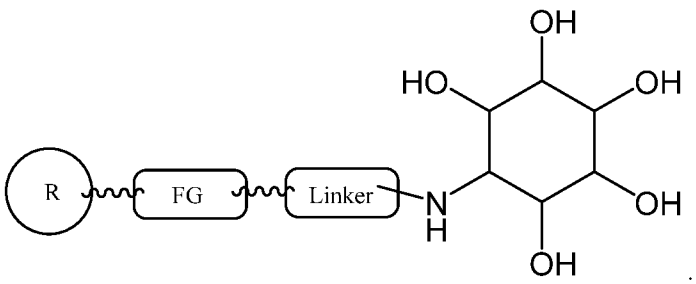
[000126] In one embodiment, R_1, R_2, R_3, R_4, and R_5 are each OH.

[000127] In one embodiment, R_1 is CH_2OH, R_2 and R_3 are each OH, R_4 is -NH-C(O)-CH_3, and R_5 is H.

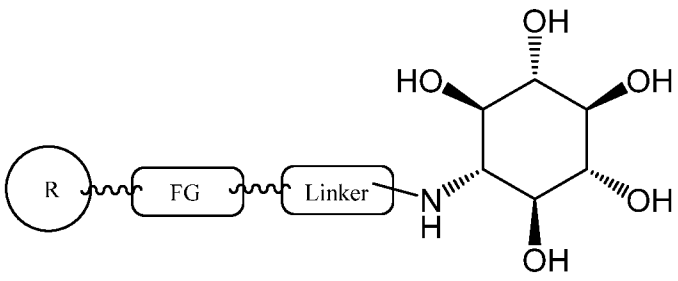
[000128] In one embodiment, the linker is a straight chained, branched or cyclic aliphatic C_2-C_{20} hydrocarbon optionally containing 1-8 heteroatoms selected from halogen, O, N, and S and combinations thereof.

[000129] In one embodiment, the linker is a straight chained aliphatic C_2-C_{10} hydrocarbon.

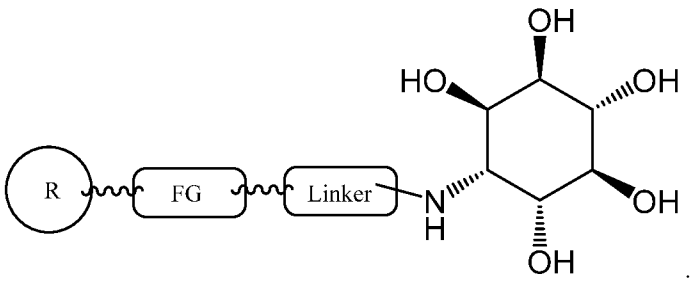
[000130] In one embodiment, the affinity resin has the formula:



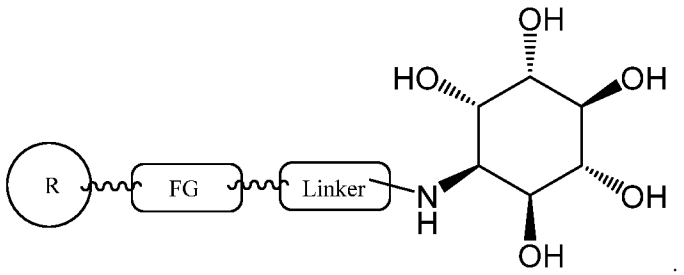
[000131] In one embodiment, the affinity resin has the formula:



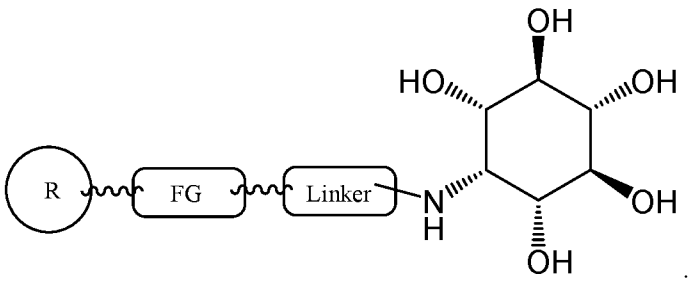
[000132] In another embodiment, the affinity resin has the formula:



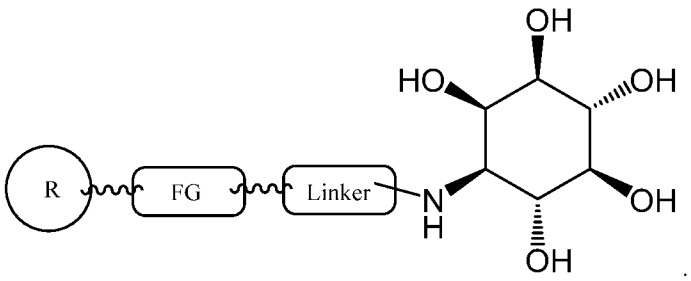
[000133] In another embodiment, the affinity resin has the formula:



[000134] In yet another embodiment, the affinity resin has the formula:



[000135] In yet another embodiment, the affinity resin has the formula:



[000136] In one embodiment, the affinity resin according to any of the above embodiments has a binding capacity of at least 1 mg, or at least 2 mg, or at least 3 mg, or at least 4 mg, or at

least 5 mg, or at least 6 mg, or at least 7 mg, or at least 8 mg, or at least 9 mg, or at least 10 mg, or at least 11 mg, or at least 12 mg, or at least 14 mg, or at least 16 mg of glycoside-cleaving enzyme per 1 mL of the affinity resin. In one embodiment, the affinity resin according to any of the above embodiments has a binding capacity of at least 10 mg of glycoside-cleaving enzyme per 1 mL of the affinity resin.

[000137] In one embodiment, the affinity resin according to any of the above embodiments has a binding capacity of at least 10 mL, or at least 20 mL, or at least 30 mL, or at least 40 mL, or at least 50 mL, or at least 60 mL, or at least 70 mL, or at least 80 mL, or at least 90 mL, or at least 100 mL of the conditioned medium per 1 mL of the affinity resin.

[000138] In one embodiment, the affinity resin according to any of the above embodiments has a binding capacity of about 10 mL to about 20 mL, or about 20 mL to about 40 mL, or about 40 mL to about 60 mL, or about 50 mL to about 100 mL, or about 60 mL to about 90 mL of the conditioned medium per 1 mL of the affinity resin.

[000139] In one embodiment, the affinity resin according to any of the above embodiments has a binding capacity of about 80 mL of the conditioned medium per 1 mL of the affinity resin.

[000140] In one embodiment, the affinity resin according to any of the above embodiments maintains purification performance after exposure to NaOH. In one embodiment, the affinity resin according to any of the above embodiments maintains purification performance after 30 min, or after 60 min, or after 90 min, or after 120 min, or after 150 min of exposure to NaOH.

[000141] In one embodiment, the affinity resin conjugated to a small molecule is capable of binding glycoside-cleaving enzymes, *e.g.*, α-Gal A, GCB, and GAA, directly from conditioned medium of a cell line without any manipulation of the conditioned medium.

[000142] ***Elution Conditions***

[000143] Various elution conditions including, without limitation, varying pH, addition of an ionic modifier, *e.g.*, a salt, at a varying concentration, and addition of a small molecule competitive inhibitor at a varying concentration, may be employed for the step of eluting the bound glycoside-cleaving enzymes from the affinity column matrix according to the invention.

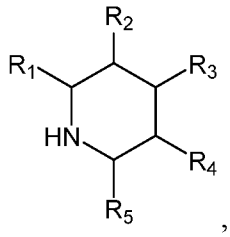
[000144] In some embodiments, varying pH conditions may be employed, including, without limitation, a buffer having a constant pH, a buffered pH gradient, and sequential elution buffers, each having a different pH. In one embodiment, the pH of the elution buffers may be

between about pH 5 and about pH 9. In one embodiment, the pH of the elution buffer may be in the physiological pH range of about pH 7 to about pH 8.5.

[000145] In some embodiments, an ionic modifier, *e.g.*, a salt, may be added to the elution solution, *e.g.*, an elution buffer. In some embodiments, a suitable salt may have a cation selected from sodium, lithium, magnesium, calcium, and guanidinium, and an anion selected from chloride, nitrate, bromide, iodide, perchlorate, and chlorate. In one embodiment, the salt may be NaCl. The salt may be present at any concentration as determined optimal by one of skill in the art, for example from 0 mM to 1 M.

[000146] In some embodiments, small molecule competitive inhibitor ligands may be added to the elution solution, *e.g.*, an elution buffer, to assist with elution of the bound glycoside-cleaving enzyme. By way of a non-limiting example, in some embodiments the small molecules that bind to and/or inhibit glycoside-cleaving enzymes, *e.g.*, aza-sugar derivatives and/or inositol derivatives, may be added to the elution buffer.

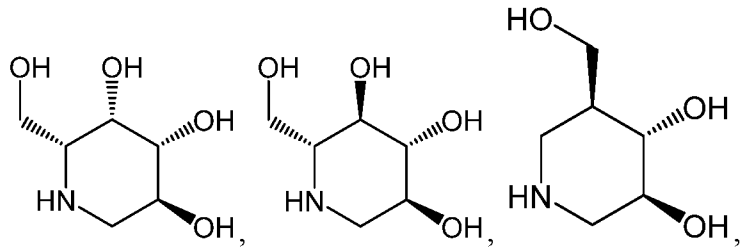
[000147] In some embodiments, the small molecules that may be added to the elution buffer may be the same small molecule ligands as the ligands immobilized on the affinity matrix as described above. In some embodiments, the small molecules that may be added to the elution buffer may have the following structural formula:



,

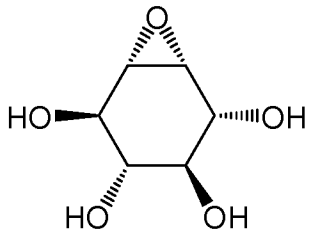
wherein R_1, R_2, R_3, R_4, and R_5 may be independently selected from H, OH, CH_2OH, and -NH-C(O)-CH_3.

[000148] In some embodiments, the small molecules that may be added to the elution buffer may be selected from:



or pharmaceutically acceptable salts, esters, and derivatives thereof.

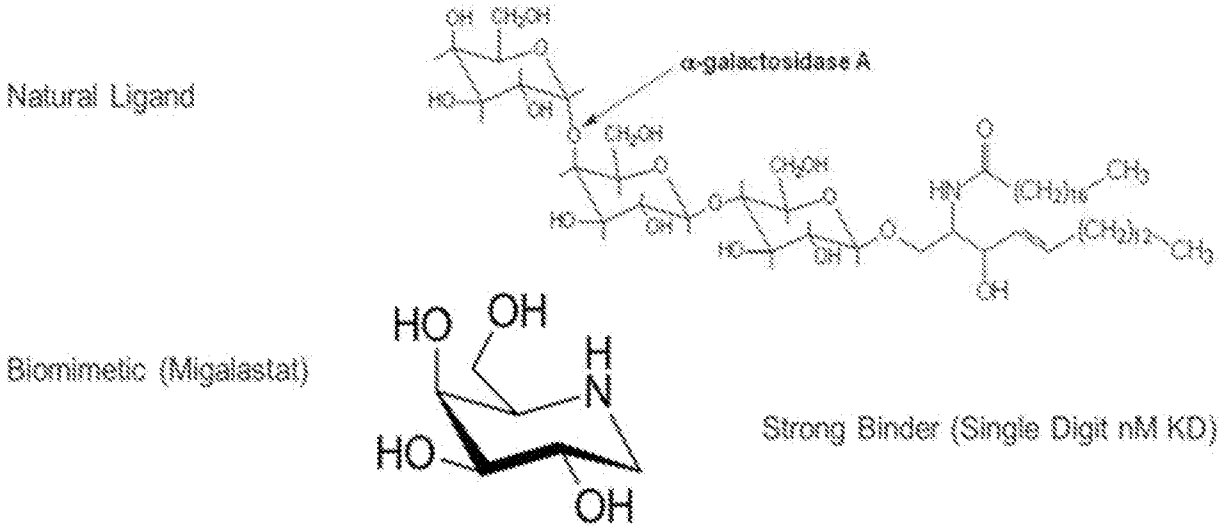
[000149] In some embodiments, the small molecules that may be added to the elution buffer may be a derivative of inositol, *e.g.*, a derivative of Conduritol B epoxide, where Conduritol B epoxide has the following structural formula:



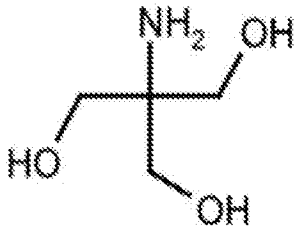
[000150] As stated above, in one embodiment, the step of eluting the bound glycoside-cleaving enzyme is performed with an elution buffer comprising a small molecule ligand, which small molecule ligand is a competitive inhibitor of the bound glycoside-cleaving enzyme. In one embodiment, the small molecule ligand present in the elution buffer is the same small molecule ligand as immobilized on the affinity matrix. In another embodiment, the small molecule ligand present in the elution buffer is a different small molecule ligand from the small molecule ligand immobilized on the affinity matrix.

[000151] ***Tris Elution Buffer***

[000152] It has been noted by the authors of the present invention that the structure of tris(hydroxymethyl)aminomethane, also known as tromethamine or Tris, bears some similarities to the structure of the natural ligand of α-Gal A and the structure of the azasugar biomimetic DGJ, as shown below:



Biomimetic (Tris)



Weak Binder (Triple Digit mM KD)

[000153] It has been discovered that α-Gal A thermal stability increases by 3 °C at 10mM Tris (**Figure 1**) and is dose concentration-dependent. Therefore, it has been hypothesized that Tris, a weak active site binder of α-Gal A, may be utilized as a competitive ligand to assist with the affinity chromatography purification of α-Gal A.

[000154] It has been discovered that using a buffer comprising Tris at 150 mM α-Gal A is effectively eluted from the affinity resin (**Figure 2**).

[000155] ***Purification methods***

[000156] In one aspect of the present invention, a method of purifying a glycoside-cleaving enzyme produced in a cell line is provided, said method comprising the steps of binding the glycoside-cleaving enzyme to the affinity resin as described above and eluting the bound glycoside-cleaving enzyme.

[000157] In one embodiment, a glycoside-cleaving enzyme is purified directly from conditioned medium of the cell line.

[000158] In one embodiment, a glycoside-cleaving enzyme is purified directly from conditioned medium of the cell line without manipulation of the conditioned medium.

[000159] For example, in some embodiments, a glycoside-cleaving enzyme is purified from conditioned medium which has not been subjected to one or more of: clarification, concentration (*e.g.*, UF/DF), and pH alteration, following harvest.

[000160] As stated above, in some embodiments the method of the invention allows for the purification of a glycoside-cleaving enzyme, *e.g.*, directly from conditioned medium of the cell line, *e.g.*, without manipulation of the conditioned medium.

[000161] In one embodiment, the cell line may be a mammalian cell line. In one embodiment the cell line is a recombinant CHO cell line. In another embodiment, the cell line is a human cell line. In another embodiment, the cell line may be a plant cell line.

[000162] In one embodiment, the purity of the eluted glycoside-cleaving enzyme is at least 90%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99%, or at least

99.5%, or at least 99.8%, or 99.9%. In one embodiment, the purity of the eluted glycoside-cleaving enzyme is at least 95%.

[000163] In one embodiment, the binding capacity of the affinity resin is at least 1 mg, or at least 2 mg, or at least 3 mg, or at least 4 mg, or at least 5 mg, or at least 6 mg, or at least 7 mg, or at least 8 mg, or at least 9 mg, or at least 10 mg, or at least 11 mg, or at least 12 mg, or at least 14 mg, or at least 16 mg of glycoside-cleaving enzyme per 1 mL of the affinity resin. In one embodiment, the binding capacity of the affinity resin is at least 10 mg of glycoside-cleaving enzyme per 1 mL of the affinity resin.

[000164] In one embodiment, the affinity resin according to any of the above embodiments has a binding capacity of at least 10 mL, or at least 20 mL, or at least 30 mL, or at least 40 mL, or at least 50 mL, or at least 60 mL, or at least 70 mL, or at least 80 mL, or at least 90 mL, or at least 100 mL of the conditioned medium per 1 mL of the affinity resin.

[000165] In one embodiment, the affinity resin according to any of the above embodiments has a binding capacity of about 10 mL to about 20 mL, or about 20 mL to about 40 mL, or about 40 mL to about 60 mL, or about 50 mL to about 100 mL, or about 60 mL to about 90 mL of the conditioned medium per 1 mL of the affinity resin.

[000166] In one embodiment, the affinity resin according to any of the above embodiments has a binding capacity of about 80 mL of the conditioned medium per 1 mL of the affinity resin.

[000167] In one embodiment, the glycoside-cleaving enzyme capable of being purified by the method of the invention is selected from α-galactosidase A (α-Gal A), glucocerebrosidase (GCB), β-galactosidase, or GAA.

[000168] In one embodiment, the glycoside-cleaving enzyme is α-Gal A.

[000169] In another embodiment, the glycoside-cleaving enzyme is GCB.

[000170] In one embodiment, the step of eluting the bound glycoside-cleaving enzyme is performed with an elution buffer comprising tris(hydroxymethyl) aminomethane (Tris). In one embodiment, the step of eluting the bound glycoside-cleaving enzyme is performed with an elution buffer comprising 100 mM Tris, or 150 mM Tris, or 200 mM Tris, or 250 mM Tris, or 300 mM Tris, or 350 mM Tris, or 400 mM Tris, or 450 mM Tris, or 500 mM Tris.

[000171] In one aspect of the invention, a method of purifying α-Gal A is provided, said method comprising the steps of binding the α-Gal A to the affinity resin as described above and eluting the bound α-Gal A. In one embodiment, α-Gal A is purified directly from conditioned

medium of a cell line. In one embodiment, α-Gal A is purified directly from conditioned medium of a cell line without manipulation of the conditioned medium.

[000172] In another aspect of the invention, a method of purifying GCB is provided, said method comprising the steps of binding GCB to the affinity resin as described above and eluting the bound GCB. In one embodiment, GCB is purified directly from conditioned medium of a cell line. In one embodiment, GCB is purified directly from conditioned medium of a cell line without manipulation of the conditioned medium.

[000173] In yet another aspect of the invention, a method of purifying GAA is provided, said method comprising the steps of binding GAA to the affinity resin as described above and eluting the bound GAA. In one embodiment, GAA is purified directly from conditioned medium of a cell line. In one embodiment, GAA is purified directly from conditioned medium of a cell line without manipulation of the conditioned medium.

[000174] ***Affinity Resin***

[000175] In principle, any porous, beaded resin suitable for protein purification may be functionalized with small molecule inhibitors according to embodiments of the present invention using methods known in the art. Some examples of resins suitable for functionalization with small molecule inhibitors include, but are not limited to, CarboxyLink Resin (ThermoFisher), AminoLink Resin (ThermoFisher), SulfoLink Resin (ThermoFisher), Carbohydrate Coupling Resin (G-Biosciences), and the like.

[000176] One non-limiting example of a suitable resin is CarboxyLink Resin, available from Thermo Scientific. CarboxyLink Coupling Resin may be used for covalent immobilization of peptides or other carboxyl-containing (—COOH) molecules to a porous, beaded resin for use in affinity purification procedures.

[000177] CarboxyLink Resin is crosslinked beaded agarose that has been activated with diamino-dipropylamine (DADPA) to contain long spacer arms, each with a primary amine at the end. When incubated with the resin and the carbodiimide crosslinker EDC (included in the CarboxyLink Immobilization Kit), carboxyl-containing molecules become permanently attached to the support by stable amide bonds. Once a molecule is coupled, the prepared affinity column can be used multiple times in typical protein affinity purification procedures. CarboxyLink Coupling Resins can also be used to immobilize other kinds of molecules using alternative amine-reactive crosslinking chemistries.

EXAMPLES

[000178] The following examples illustrate specific aspects of the instant description. The examples should not be construed as limiting, as the examples merely provide specific understanding and practice of the embodiments and their various aspects.

[000179] EXAMPLE 1: DGJ-resin conjugation

[000180] 30 mg of DGJ (Santa Cruz) was dissolved into 2.0 mL of 2-(N-morpholino) ethanesulfonic acid (MES)-based coupling buffer (0.1M MES, 0.9% NaCl, at pH = 4). The mixture was added to 2 mL of Carboxylink Resin (ThermoFisher), which was pre-equilibrated with the coupling buffer, at 54 µmol/mL concentration of resin. The mixture was allowed to react for 3 hours with end-over-end rotation at room temperature, then placed at 4.0 °C overnight. The column was then drained of the post-coupling DGJ analog solution and washed with 5.0 C.V. of wash buffer. The column was then stored in PBS or equilibrated in relevant equilibration buffer for purification. Post-coupling DGJ Analog Solution was analyzed by 1D 1H NMR analysis to determine percent coupling of the DGJ Analog to the Carboxylink Resin, which was 84%. The small molecule-loaded resin was equilibrated with buffer prior to sample loading.

[000181] EXAMPLE 2: Initial testing of α-Gal A purification directly from conditioned medium using DGJ-conjugated resin

[000182] 2.0 mL DGJ-conjugated resin (54 µmol/mL DGJ) was equilibrated in 1X PBS (-/-) buffer. A sample of 40.0 mL of CHO α-Gal A conditioned medium (CM) was loaded onto the resin by gravity flow at concentration of 0.28 mg/mL. Total α-Gal A in crude CM was 11.2 mg. The column was washed with 10 C.V. of 1X PBS (-/-) buffer. α-Gal A was eluted with 5 C.V. of 150 mM tris(hydroxymethyl)aminomethane (Tris) elution buffer (pH = 7.5), followed by 100 mM NaCl. Samples were evaluated by SDS-PAGE (**Figure 3A**) and Western Blot (**Figure 3B**). Total α-Gal A purified was 6.83 mg. Purity was 99% by HPLC, >90% by SDS-PAGE.

[000183] Initial testing demonstrated that utilizing a small molecule biomimetic based affinity column, and Tris based elution buffer, α-Gal A could be affinity purified directly from conditioned medium of a cell line without any manipulation of the conditioned medium. SDS-PAGE and Western Blot data demonstrated all of the α-Gal A in the 40.0 mL of the conditioned medium was captured by the DGJ analog affinity resin, resulting in 6-7 mg of affinity purified protein.

[000184] EXAMPLE 3: Capacity testing of α-Gal A purification directly from conditioned medium using DGJ-conjugated resin

[000185] To determine the binding capacity of the affinity resin, the load was increased from 40 mL to 200 mL of neat conditioned medium with the goal to overload the column and to determine the approximate binding capacity of the column. Based on UV (280 nm) absorbance (**Figure 4A**), SDS-PAGE (**Figure 4B**), and Western Blot (**Figure 4C**) analysis, α-Gal A broke through around 100 mL of *conditioned* medium, resulting in an approximate binding capacity of 16-17 mg of α-Gal A per mL of resin. As the resin was overloaded by design, it was expected to have some α-Gal A wash off, and α-Gal A was indeed found in the wash of the affinity column.

[000186] EXAMPLE 4: Stability against sanitization

[000187] To determine whether the DGJ analog would be able to maintain integrity over a significant amount of sodium hydroxide-based sanitization, which is a common sanitization method for conventional chromatography resin such as ion-exchange and hydrophobic resin, a study of resin performance after repeated NaOH sanitization was carried out. The method exposes the resin to 5 X 30-minute washes of 0.3 N NaOH, with neutralization between sanitization. This results in 2.5 hours of sanitization time per purification cycle. A total of 4 purification cycles were conducted to evaluate column performance over time. The chromatogram trace (**Figure 5A**) and SDS-PAGE gel (**Figure 5B**) analysis were very similar for each purification cycle. No loss of protein recovery was observed. Table 1, below, summarizes the total eluted protein quantitation over 4 purification cycles:

[000188] **Table 1: Total eluted Protein Quantification**

	Total Protein (mg)
Purification Cycle #1	4
Purification Cycle #2	5.2
Purification Cycle #3	3
Purification Cycle #4	5.5
Average over four cycles	**4.4**

[000189] As Table 1, above, demonstrates, the average protein recovery was 4.4 mg/purification cycle. Overall, the data support the conclusion that the DGJ analog retains structural integrity over the course of the sanitization testing.

[000190] All of the α-Gal A protein purified from the DGJ-analog resin had the expected enzyme activity and biochemical and biophysical properties.

[000191] It has been demonstrated that DGJ analog affinity resin of the invention can bind α-Gal A protein directly from the conditioned medium without any manipulation of the conditioned medium. The binding capacity of the affinity resin has been shown to be about 80 mL of the conditioned medium per 1 mL of the resin. The protein binding capacity of the affinity resin has been shown to be about 16-17 mg of protein per 1 mL of the resin. The DGJ analog affinity resin of the invention maintains purification performance after a total of about 10 hours of exposure to NaOH.

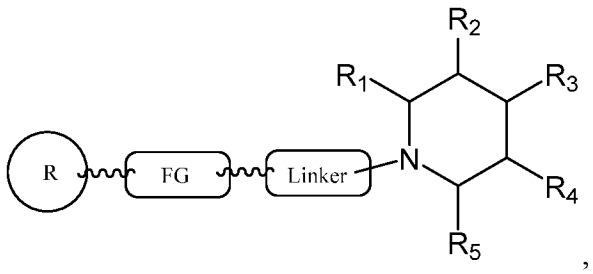
* * *

[000192] As various changes can be made in the above-described subject matter without departing from the scope and spirit of the present invention, it is intended that all subject matter contained in the above description, or defined in the appended claims, be interpreted as descriptive and illustrative of the present invention. Many modifications and variations of the present invention are possible in light of the above teachings. Accordingly, the present description is intended to embrace all such alternatives, modifications, and variances which fall within the scope of the appended claims.

[000193] All patents, applications, publications, test methods, literature, and other materials cited herein are hereby incorporated by reference in their entirety as if physically present in this specification.

WHAT IS CLAIMED IS:

1. An affinity resin comprising a small molecule ligand having the formula:



,

wherein R is a resin matrix;

FG is absent or is a functional group selected from -O-, -NH-, -C(O)N-, -C(O)O-, CH_2, and -S-;

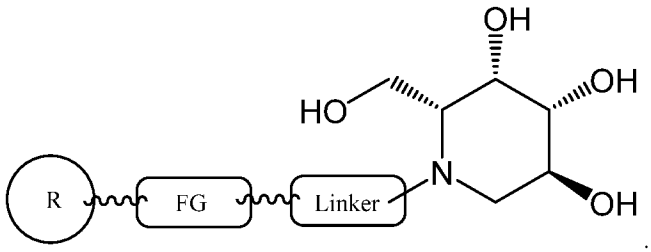
Linker is absent or is selected from a straight chained, branched or cyclic aliphatic C_2-C_{20} hydrocarbon; a heteroaliphatic C_2-C_{20} hydrocarbon; an aromatic C_6-C_{20} hydrocarbon; a heteroaromatic C_2-C_{20} hydrocarbon; an aryl C_6-C_{20} hydrocarbon; a heteroaryl C_2-C_{20} hydrocarbon, a C_2-C_{12} perfluorocarbon, or a combination thereof, each of which optionally contains 1-8 heteroatoms selected from halogen, O, N, and S and combinations thereof;

R_1, R_2, R_3, R_4, and R_5 are each independently selected from H, OH, CH_2OH, and -NH-C(O)-CH_3, and

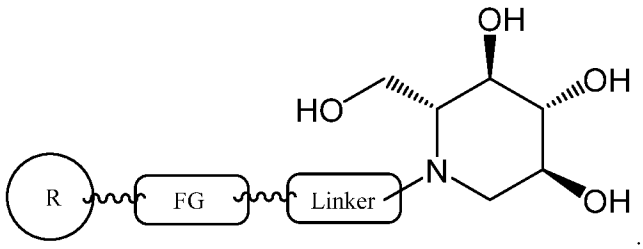
wherein the affinity resin is capable of binding a glycoside-cleaving enzyme.

2. The affinity resin of claim 1, wherein R_1 is CH_2OH, R_2, R_3, and R_4 are each OH, and R_5 is H.
3. The affinity resin of claim 1, wherein R_1 is CH_2OH, R_2 and R_3 are each OH, R_4 is -NH-C(O)-CH_3, and R_5 is H.
4. The affinity resin of claim 1, wherein R_1 is H, R_2 is CH_2OH, R_3 and R_4 are each OH, and R_5 is H.
5. The affinity resin of claim 1, wherein the functional group is -C(O)N-.
6. The affinity resin of claim 1, wherein the functional group is -O-.

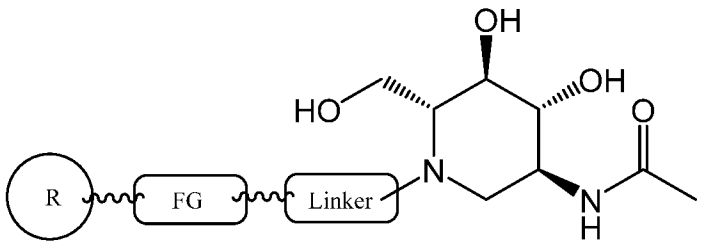
7. The affinity resin of claim 1, wherein the linker is a straight chained, branched or cyclic aliphatic C_2-C_{20} hydrocarbon optionally containing 1-8 heteroatoms selected from halogen, O, N, and S and combinations thereof.
8. The affinity resin of claim 1, wherein the linker is a straight chained aliphatic C_2-C_{10} hydrocarbon.
9. The affinity resin of claim 1, wherein the linker is a straight chained aliphatic C_5 hydrocarbon.
10. The affinity resin of claim 1, having the formula:



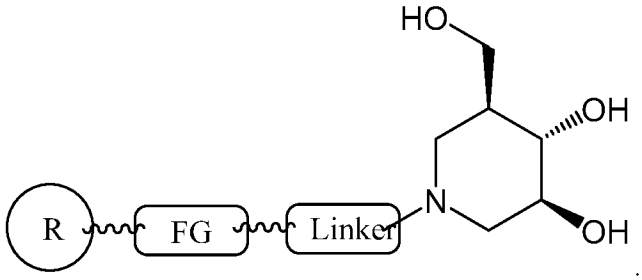
11. The affinity resin of claim 1, having the formula:



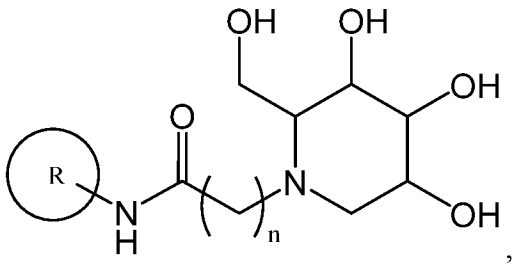
12. The affinity resin of claim 1, having the formula:



13. The affinity resin of claim 1, having the formula:



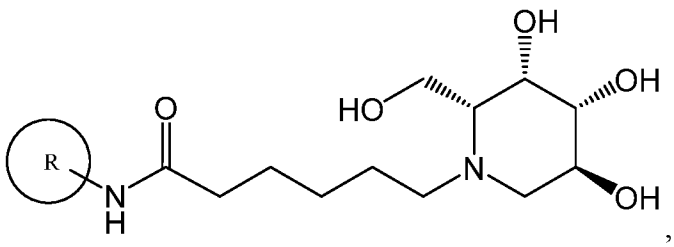
14. The affinity resin of claim 1, having the formula:



wherein R is a resin matrix and n is an integer from 2 to 20.

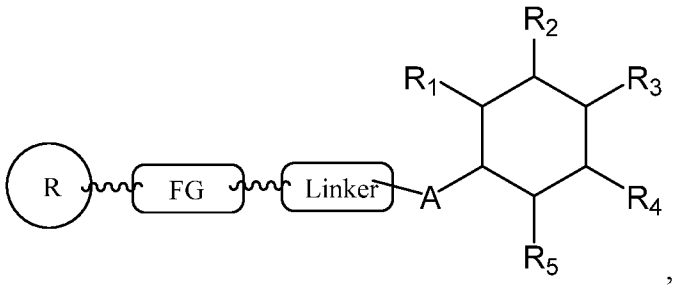
15. The affinity resin of claim 14, wherein n is 5.

16. An affinity resin comprising a small molecule ligand having the formula:



wherein R is a resin matrix.

17. An affinity resin comprising a small molecule ligand having the formula:



wherein R is a resin matrix;

FG is absent or is a functional group selected from -O-, -NH-, -C(O)N-, -C(O)O-, CH_2, and -S-;

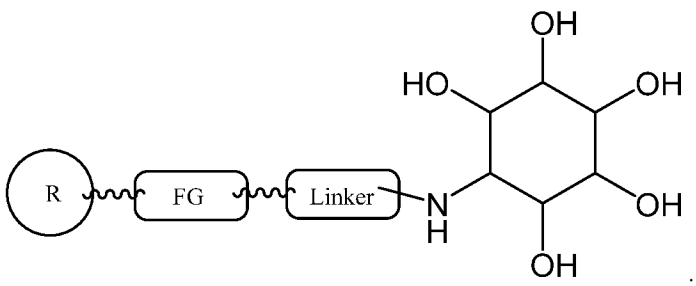
Linker is absent or is selected from a straight chained, branched or cyclic aliphatic C_2-C_{20} hydrocarbon; a heteroaliphatic C_2-C_{20} hydrocarbon; an aromatic C_6-C_{20} hydrocarbon; a heteroaromatic C_2-C_{20} hydrocarbon; an aryl C_6-C_{20} hydrocarbon; a heteroaryl C_2-C_{20} hydrocarbon, a C_2-C_{12} perfluorocarbon, or a combination thereof, each of which optionally contains 1-8 heteroatoms selected from halogen, O, N, and S and combinations thereof;

A is selected from -NH- and -S-;

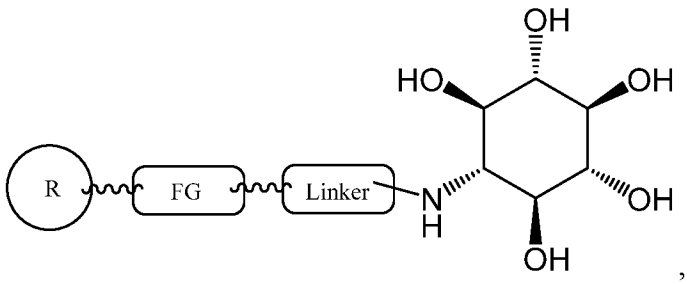
R_1, R_2, R_3, R_4, and R_5 are each independently selected from H, OH, and -NH-C(O)-CH_3, and

wherein the affinity resin is capable of binding a glycoside-cleaving enzyme.

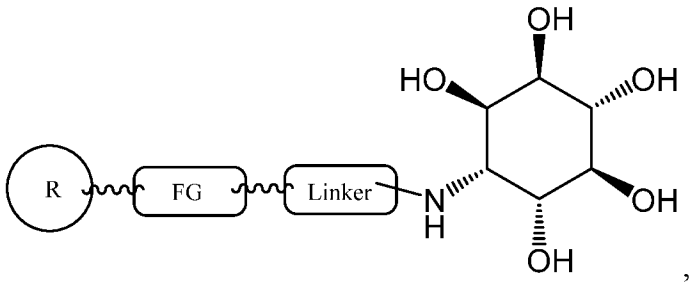
18. The affinity resin of claim 17, wherein R_1, R_2, R_3, R_4, and R_5 are each OH.
19. The affinity resin of claim 17, wherein R_1 is CH_2OH, R_2 and R_3 are each OH, R_4 is -NH-C(O)-CH_3, and R_5 is H.
20. The affinity resin of claim 17, wherein A is -NH-.
21. The affinity resin of claim 17, wherein A is -S-.
22. The affinity resin of claim 17, wherein the linker is a straight chained, branched or cyclic aliphatic C_2-C_{20} hydrocarbon optionally containing 1-8 heteroatoms selected from halogen, O, N, and S and combinations thereof.
23. The affinity resin of claim 17, wherein the linker is a straight chained aliphatic C_2-C_{10} hydrocarbon.
24. The affinity resin of claim 17, having the formula:



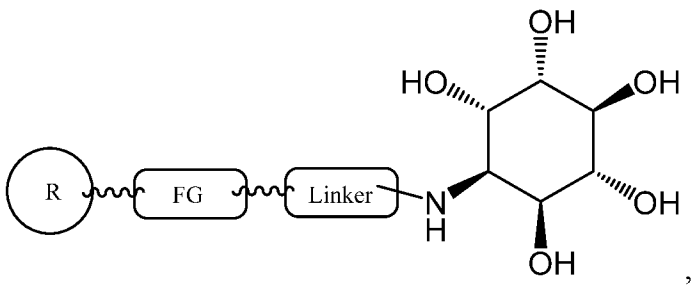
25. The affinity resin of claim 17, having the formula selected from:



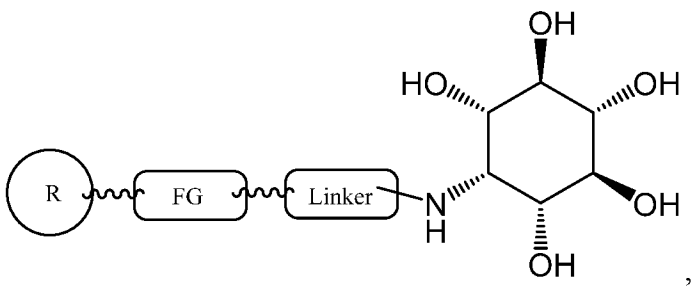
,



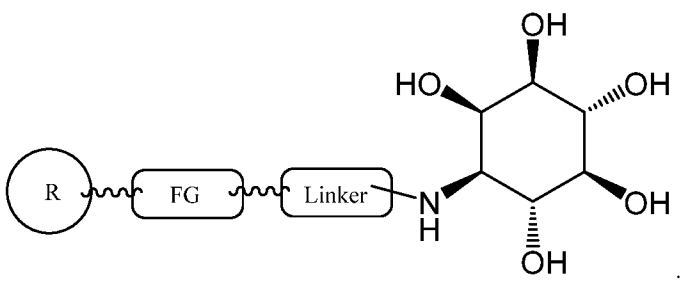
,



,



, and



.

26. The affinity resin of any of claims 1-25, wherein the affinity resin is capable of purifying the glycoside-cleaving enzyme directly from conditioned medium of a cell line.
27. The affinity resin of any of claims 1-25, wherein the affinity resin is capable of purifying the glycoside-cleaving enzyme directly from conditioned medium of a cell line without manipulation of the conditioned medium.
28. The affinity resin of any of claims 1-27 having the binding capacity of at least 10 mg of glycoside-cleaving enzyme per 1 mL of the affinity resin.
29. The affinity resin of any of claims 1-27 having the binding capacity of about 50 mL to about 100 mL of the conditioned medium per 1 mL of the affinity resin.
30. The affinity resin of any of claims 1-27, wherein the affinity resin maintains purification performance after exposure to NaOH.
31. A method of purifying a glycoside-cleaving enzyme produced in a cell line, said method comprising the steps of binding the glycoside-cleaving enzyme to the affinity resin of any of claims 1-30 and eluting the bound glycoside-cleaving enzyme.
32. The method of claim 31, wherein the glycoside-cleaving enzyme is purified from conditioned medium.
33. The method of claim 31, wherein the glycoside-cleaving enzyme is purified directly from conditioned medium without manipulation of the conditioned medium.
34. A method of purifying a glycoside-cleaving enzyme produced in a cell line directly from conditioned medium of the cell line without manipulation of the conditioned medium, said method comprising the steps of binding the glycoside-cleaving enzyme to the affinity resin of any of claims 1-30 and eluting the bound glycoside-cleaving enzyme.
35. The method of any of claims 31-34, wherein the cell line is a recombinant CHO cell line.
36. The method of any of claims 31-34, wherein the cell line is a human cell line.
37. The method of any of claims 31-34, wherein the purity of the eluted glycoside-cleaving enzyme is at least 95%.
38. The method of any of claims 31-34, wherein the binding capacity of the affinity resin is at least 10 mg of glycoside-cleaving enzyme per 1 mL of the affinity resin.
39. The method of any of claims 31-34, wherein the glycoside-cleaving enzyme is selected from α-galactosidase A (α-Gal A), glucocerebrosidase (GCB), β-galactosidase, and acid alpha-glucosidase (GAA).

40. The method of any of claims 31-34, wherein the glycoside-cleaving enzyme is α-Gal A.
41. The method of any of claims 31-34, wherein the glycoside-cleaving enzyme is GCB.
42. The method of any of claims 31-34, wherein the glycoside-cleaving enzyme is GAA.
43. The method of any of claims 31-42, wherein the step of eluting the bound glycoside-cleaving enzyme is performed with an elution buffer comprising tris(hydroxymethyl) aminomethane (Tris).
44. The method of any of claims 31-42, wherein the step of eluting the bound glycoside-cleaving enzyme is performed with an elution buffer comprising the same small molecule ligand as the small molecule ligand immobilized on the affinity matrix.
45. The method of any of claims 31-42, wherein the step of eluting the bound glycoside-cleaving enzyme is performed with an elution buffer having a pH between about pH 5 and about pH 9.
46. The method of any of claims 31-42, wherein the step of eluting the bound glycoside-cleaving enzyme is performed with an elution buffer having a pH between about pH 7 and about pH 8.5.
47. The method of any of claims 31-42, wherein the step of eluting the bound glycoside-cleaving enzyme is performed with an elution buffer comprising a salt.
48. The method of claim 47, wherein the salt is NaCl.
49. A method of purifying α-Gal A, said method comprising the steps of binding the α-Gal A to the affinity resin of any of claims 1-30 and eluting the bound α-Gal A.
50. A method of purifying α-Gal A directly from conditioned medium of a cell line, said method comprising the steps of binding the α-Gal A to the affinity resin of any of claims 1-30 and eluting the bound α-Gal A.
51. The method of claim 50, wherein the α-Gal A is purified directly from conditioned medium of a cell line without manipulation of the conditioned medium.
52. The method of claim 50, wherein the step of eluting the bound glycoside-cleaving enzyme is performed with an elution buffer comprising tris(hydroxymethyl) aminomethane (Tris).
53. A method of purifying GCB, said method comprising the steps of binding GCB to the affinity resin of any of claims 17-27 and eluting the bound GCB.
54. A method of purifying GAA, said method comprising the steps of binding GAA to the affinity resin of any of claims 1-30 and eluting the bound GAA.

FIGURE 1

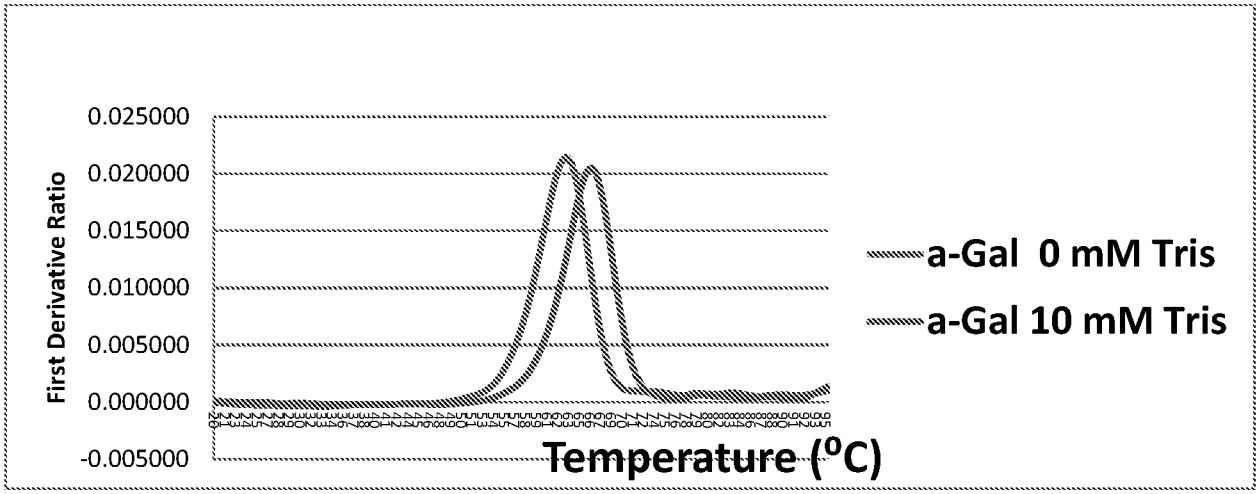

0.025000
0.020000
0.015000
0.010000
0.005000
0.000000
-0.005000
First Derivative Ratio
Temperature (°C)
a-Gal 0 mM Tris
a-Gal 10 mM Tris

FIGURE 2

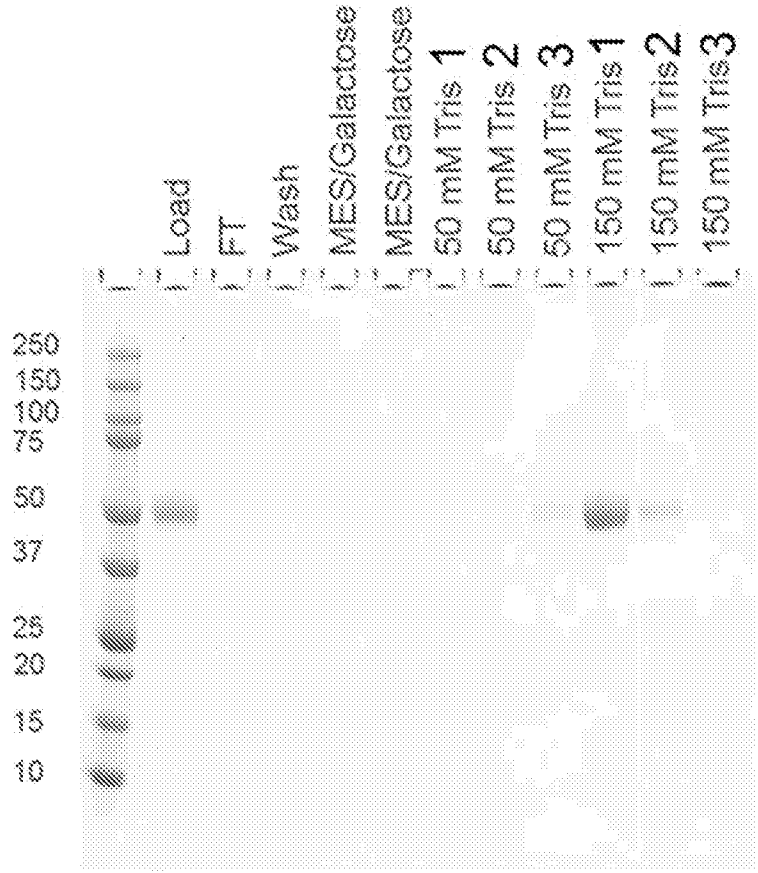

Load
FT
Wash
MES/Galactose
MES/Galactose
50 mM Tris 1
50 mM Tris 2
50 mM Tris 3
150 mM Tris 1
150 mM Tris 2
150 mM Tris 3
250
150
100
75
50
37
25
20
15
10

FIGURE 3

FIG 3A

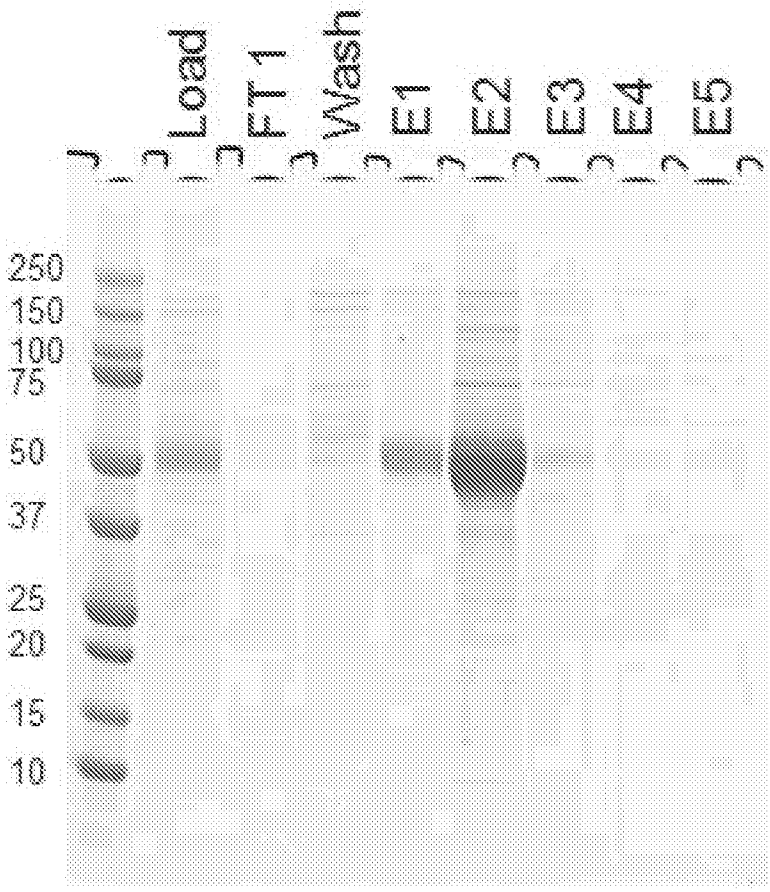


FIG 3B

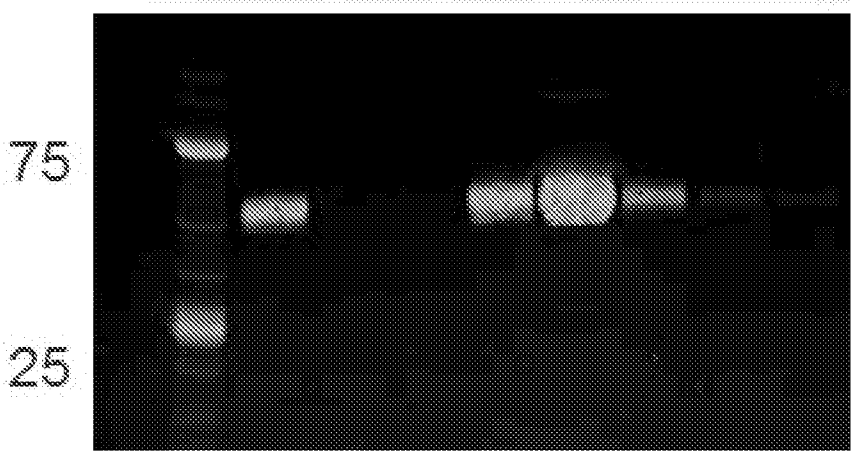


FIGURE 4A

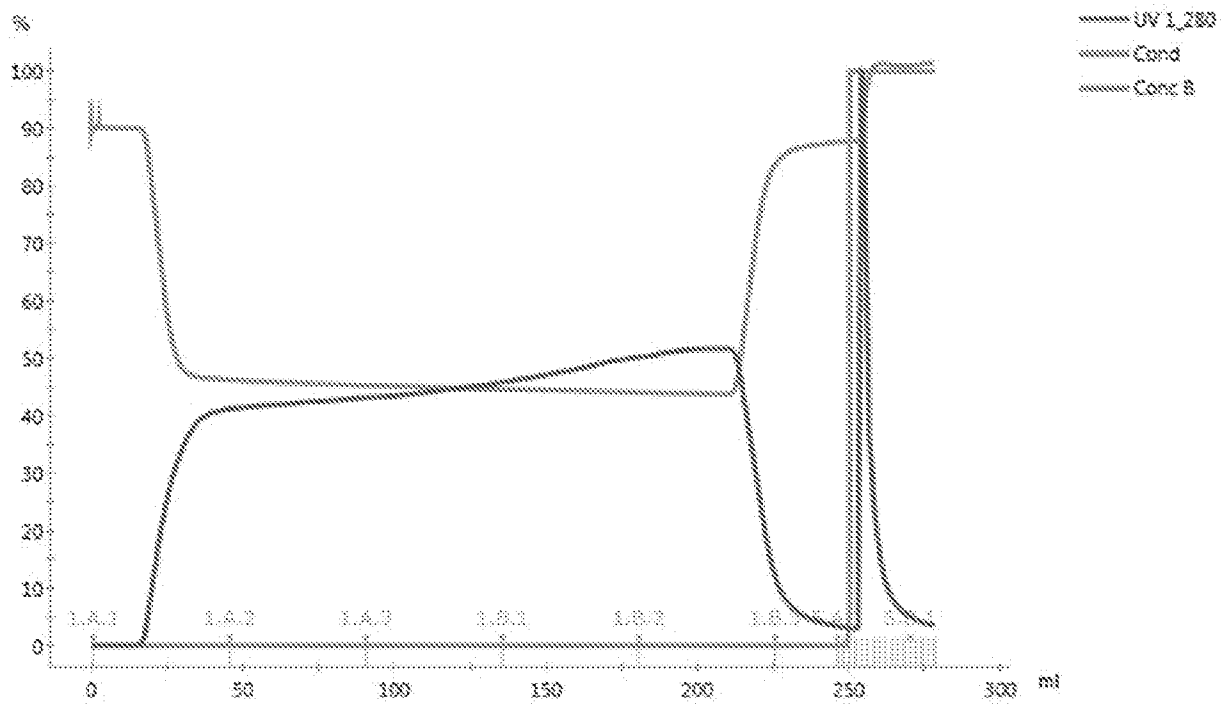

%
100
90
80
70
60
50
40
30
20
10
0
0
50
100
150
200
250
300
ml
UV 1_280
Cond
Conc B

FIGURE 4B

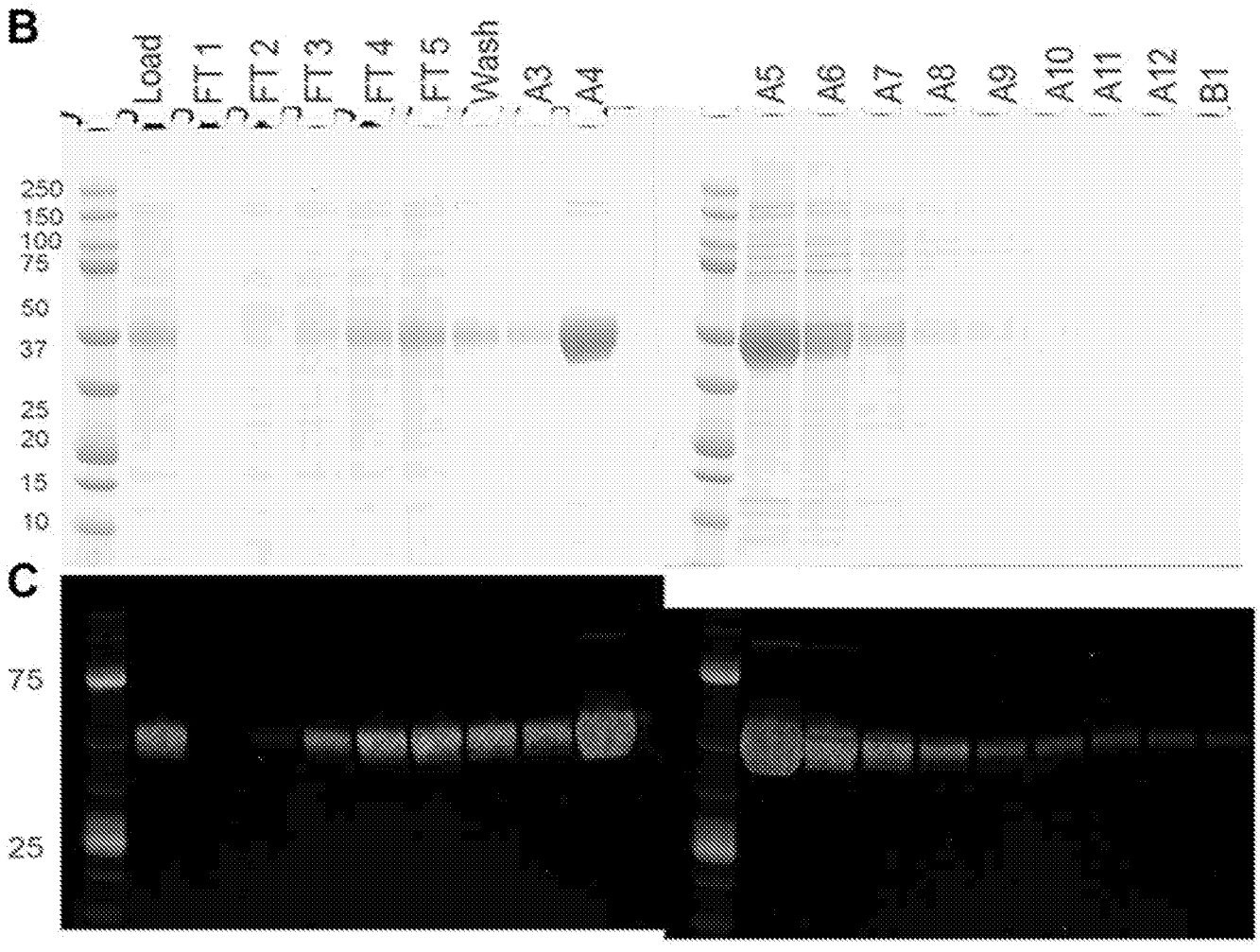


FIGURE 4C

FIGURE 5A

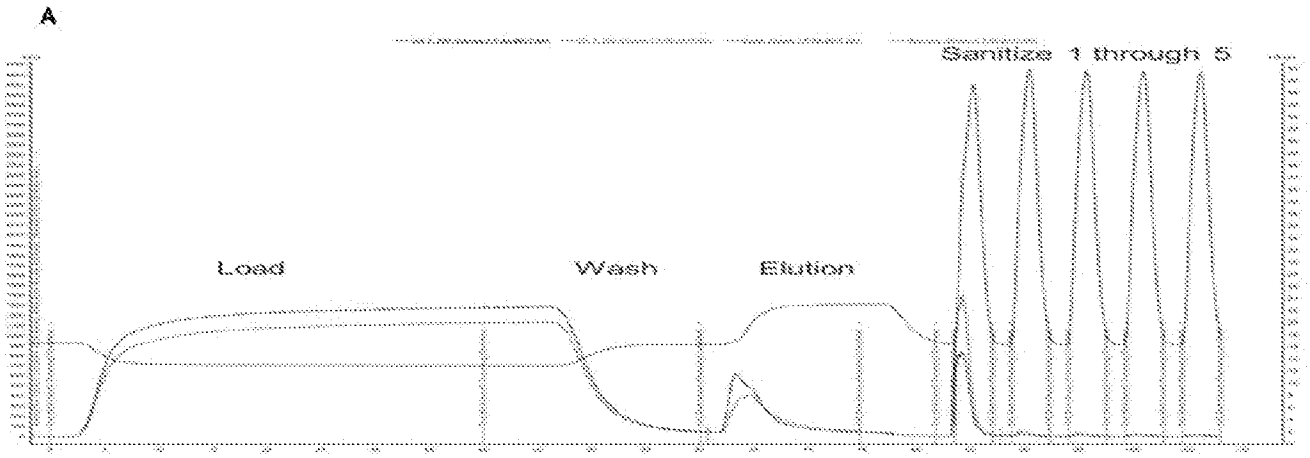
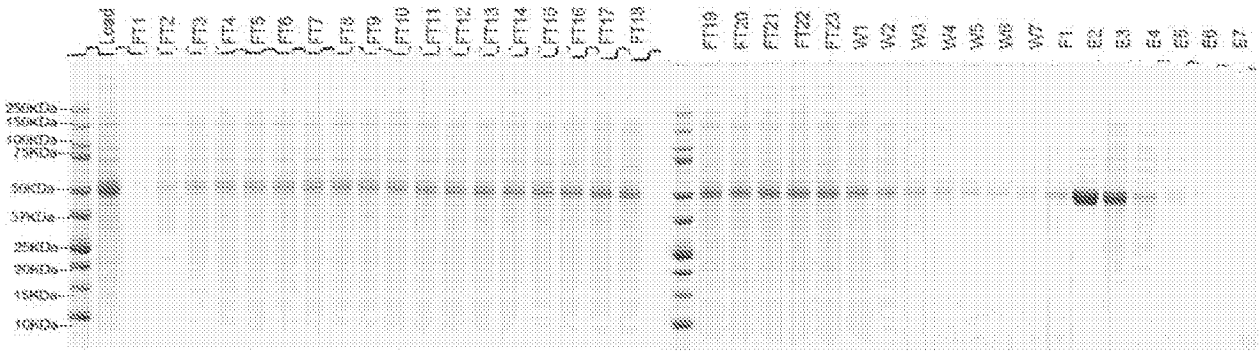


FIGURE 5B


Load
LF1
LF2
LF3
LF4
LF5
LF6
LF7
LF8
LF9
LF10
LF11
LF12
LF13
LF14
LF15
LF16
LF17
LF18
FT19
FT20
FT21
FT22
FT23
W1
W2
W3
W4
W5
W6
W7
E1
E2
E3
E4
E5
E6
E7
250KDa
150KDa
100KDa
75KDa
50KDa
37KDa
25KDa
20KDa
15KDa
10KDa

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/20690

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A23K 1/165; A23L 1/30; C07K 16/40 (2019.01)
CPC - A21D 8/042; A23K 10/14; A23K 10/30

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)
See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Baindur et al. 'Solution-Phase Synthesis of a Library of 3,5,7-Trisubstituted 3H-[1,2,3]triazolo[4,5-d]pyrimidines', Journal of Combinatorial Chemistry, 18 July 2003 (18.07.2003), Vol.5, pages653-659; Abstract	1, 26-27
A	Pubmed Substance Record for SID 329758054, 'StratoSpheres(TM) PL-PIP (Piperidine) resin, 50-100 mesh, extent of labeling: 3.0-4.0 mmol/g loading, 1 % cross-linked', U.S. National Library of Medicine, 03 March 2017 (03.03.2017), (https://pubchem.ncbi.nlm.nih.gov/substance/329758054); p1	1, 26-27
A	Trapero et al. 'Adamantane substituted aminocyclitols as pharmacological chaperones for Gaucher disease', MedChemComm, 01 October 2013 (01.10.2013), Vol.4, pages1584-1589; p1585	17, 18, 20, 22-27
A	Harrak et al. 'Aminocyclitol-Substituted Phytoceramides and their Effects on iNKT Cell Stimulation', MedChemComm, 28 September 2009 (28.09.2009), Vol.4, pages1608-1613; p1608	17, 18, 20, 22-27
A	Bause et al. 'N-Methyl-N-(5-carboxypentyl)-1-deoxynojirimycin, a new affinity ligand for the purification of trimming glucosidase I', FEBS Letters, 28 January 1991 (28.01.1991), Vol.278, pages167-170; p167, p169	1, 17, 18, 20, 22-27

☐ 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
09 July 2019	05 AUG 2019
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/20690

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

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

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

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

3. ☒ Claims Nos.: 28-54
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:

See Supplemental Box

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

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

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1st invention: Claims 1 (in part), and 26-27
2nf Invention: Claims 17 (in part), 18, 20, 22 (in part) and 23-27

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

International application No.
PCT/US 19/20690

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I+: Claims 1-27 directed to an affinity resin comprising a small molecule ligand having the formula in claims 1 or 17. The ligand having the formula in claims 1 or 17 will be searched to the extent that it encompasses a ligand having the formula in claim 1, wherein R is a resin matrix; FG is absent; Linker is absent; R1, R2, R3, R4, and R5 are H; and wherein the affinity resin is capable of binding a glycoside-cleaving enzyme. It is believed that claims 1(in part), and 26-27 read on this first named invention, and thus these claims will be searched without fee. Applicant is invited to elect additional ligands of claims 1 or 17, wherein each additional compound elected will require one additional invention fee. Applicants must specify the claims that encompass any additionally elected compound. Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the '+' group(s) will result in only the first claimed invention to be searched. Additionally, an exemplary election wherein different actual variables are selected is suggested. An exemplary election would be a ligand having the formula in claim 17: wherein R is a resin matrix; FG is -C(O)N-; Linker is a straight chained aliphatic C2-C20 hydrocarbon; A is -NH-; R1, R2, R3, R4, and R5 are each OH; and wherein the affinity resin is capable of binding a glycoside-cleaving enzyme (i.e., claims 17(in part), 18, 20, 22(in part), and 23-27).

The group of inventions listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:

Group I+ includes the technical feature of an affinity resin comprising a small molecule ligand having the formula in claims 1 or 17 containing the same, which is not required by any other invention of Group I+.

Common technical features:

The inventions of Group I+ share the technical feature of an affinity resin comprising a small molecule ligand having the formula in claims 1 or 17 containing the same.

These shared technical features, however, do not provide a contribution over the prior art, as being anticipated by the journal article entitled 'N-Methyl-N-(5-carboxypentyl)-1-deoxynojirimycin, a new affinity ligand for the purification of trimming glucosidase I' by Bause et al. (hereinafter 'Bause'). Bause discloses an affinity resin comprising a small molecule ligand having the formula in claim 1: wherein R is a resin matrix; FG is -C(O)O-; Linker is a straight chained C5 hydrocarbon; R1 is CH2OH; R2, R3, and R4 are OH; and R5 is H; and wherein the affinity resin is capable of binding a glycoside-cleaving enzyme (p167, left col 1st para "a new type of affinity resin containing N-methyl-N-(5-carboxypentyl)-1-deoxynojirimycin as the ligand. It will be shown that this affinity resin binds glucostdase I activtty specifically", p169, Fig. 1 "1st compound").

As said compound and compositions were known in the art at the time of the invention, these cannot be considered special technical features that would otherwise unify the inventions of Groups I+. The inventions of Group I+ thus lack unity under PCT Rule 13.

Note:
claims 28-54 determined unsearchable because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).