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Fibroblaszt növekedési faktor (FGF) átmodellezése és glikopegilezése

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(54) **Remodeling and glycopegylation of fibroblast growth factor (FGF)**

Umformung und GlycoPEGylierung des Fibroblastenwachstumsfaktors (FGF)

Remodelage et glycopégylation de facteur de croissance de fibroblaste (FGF)

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Description**CROSS-REFERENCE TO RELATED APPLICATIONS**

5 [0001] The present application is related to U.S. Provisional Patent Application 60/623,342, filed October 29, 2004.

BACKGROUND OF THE INVENTION

10 [0002] Fibroblast Growth Factors (FGFs) promote growth, proliferation, survival and differentiation of a wide variety of cells and tissue types. The prototypic fibroblast growth factors (FGFs), FGF-1 and FGF-2, were originally isolated from brain and pituitary as mitogens for fibroblasts. However, FGF-1 and FGF-2, and fibroblast growth factors generally, are widely expressed in developing and adult tissues, and have multiple biological activities including angiogenesis, mitogenesis, cellular differentiation and repair of tissue injury (see e.g., Baird, A. et al., Cancer Cells 3:239-243 (1991) and Burgess, W. H. et al., Annu. Rev. Biochem. 58:575-606 (1989)).

15 [0003] According to the published literature, the FGF family now consists of at least twenty five members, FGF-1 to FGF-25. The 25 members of the FGF family range in molecular mass from 17 to 34 kDa and share 13-71% amino acid identity. Between vertebrate species, FGFs are highly conserved in both gene structure and amino-acid sequence.

20 [0004] The 25 members of the mammalian FGF family are differentially expressed in many tissues. The members are divided into subfamilies that have similar, though individually unique, patterns of expression. Some FGFs are expressed exclusively during embryonic development (for example, *Fgf3*, 4, 8, 15, 17 and 19), whereas others are expressed in embryonic and adult tissues. For example, *FGF-16* mRNA is predominantly expressed in the rat heart in adult tissues. However, in rat embryos, *FGF-16* mRNA is predominantly expressed in the brown adipose tissue (see e.g., Miyake A, et al. Biochem Biophys Res Commun 1998, 243:148-152).

25 [0005] Although most FGFs (FGFs 3-8, 10, 15, 17-19, and 21-25) have amino-terminal signal peptides and are readily secreted from cells, FGFs 9, 16 and 20 lack an obvious amino-terminal signal peptide but are nevertheless secreted (see e.g., Miyamoto M, et al. Mol Cell Biol 1993, 13:4251-4259). A third subset of FGFs (FGF 11-14) lack signal sequences and are thought to remain intracellular.

30 [0006] As noted above, the sub-family of FGF proteins comprising FGF-9, FGF-16, and FGF-20 lack a classical signal sequence, although they contain nuclear localization signals, and are secreted. These FGFs are expressed in the developing and adult nervous systems, suggesting a role in nervous system development and function (see e.g., Smallwood P.M., et al. Proc Natl Acad Sci USA (1996) 93:9850-9857). Indeed, a cDNA encoding FGF-20 was isolated from rat brain (see e.g., U.S. Patent 6,797,695). Among FGF family members, FGF-20 is most similar to FGF-9 and FGF-16 (70 and 62% amino acid identity, respectively).

35 [0007] Numerous studies of human disorders as well as gene knock-out studies in mice indicate that FGFs are neurotrophic for cells of both the peripheral and central nervous system, and are important in the development of the skeletal system in mammals. A role in nervous system development and function is supported by *in situ* hybridization studies that show that FGF-20 mRNA is preferentially expressed in the substantia nigra pars compacta of the brain. Further support for a nervous system function is found in studies showing that *in vitro*, recombinant rat FGF-20 enhanced the survival of midbrain dopaminergic neurons in culture (see e.g., Ohmachi S. Biochem Biophys Res Commun 2000, 277:355-360).

40 [0008] In other studies, high levels of FGF-21 mRNA expression has been shown to occur in the liver, and human FGF-21 may play a role in the development of and recovery from liver disease. FGF-21 is also expressed in testis and thymus, and therefore may play a role in the development or recovery from disorders of testicular function or function of cells derived from the thymus (see e.g., U.S. Patent No. 6,716,626).

45 [0009] Because of their wide ranging and potent activities, FGFs are pursued as therapeutic agents for a number of different indications, including wound healing, such as musculoskeletal conditions, bone fractures, ligament and tissue repair, tendonitis, bursitis, etc.; skin conditions, for example, burns, cuts, lacerations, bed sores, slow healing ulcers, etc.; tissue protection, repair, and the induction of angiogenesis during myocardial infarction and ischemia, inflammatory conditions and diseases (e.g., intestinal inflammation, including inflammatory bowel disease see e.g., Jeffers et al. Gastroenterology 2002, 123:1151-1162), in the treatment of neurological conditions such as neuro-degenerative diseases (e.g., Parkinson's disease), and stroke, in the treatment of eye disease, including macular degeneration, the pathology and treatment of cancer (see e.g., Jeffers, M., et al. Cancer Research 61, 3131-3138, April 1, (2001) and Jeffers et al. Expert Opinion on Therapeutic Targets (2002) 6(4):469-482) and for the treatment of diabetes. Unfortunately, the administration of therapeutic proteins such as FGF-9, FGF-18, FGF-20, and FGF-21 for the treatment of diseases and conditions can be complicated by, for example, short half life and mutagenic properties.

50 [0010] Poly(ethylene glycol) ("PEG") is an exemplary polymer that has been conjugated to polypeptides. The use of PEG to derivatize peptide therapeutics has been demonstrated to reduce the immunogenicity of the peptides and improve pharmacodynamics including half-life. For example, U.S. Pat. No. 4,179,337 (Davis et al.) concerns non-immunogenic

polypeptides, such as enzymes and peptide hormones coupled to polyethylene glycol (PEG) or polypropylene glycol. Between 10 and 100 moles of polymer are used per mole polypeptide and at least 15% of the physiological activity is maintained. In addition, the clearance time in circulation is prolonged due to the increased size of the PEG-conjugate of the polypeptides in question. The methods disclosed by Davis et al. are chemical PEGylation methods.

[0011] The chemical modification of peptides, frequently results in an undesirable loss of peptide activity, which is attributable to the non-selective nature of the chemistries utilized to modify the peptide. For example, when the modifying group is a water-soluble peptide, e.g., PEG, the principal mode of attachment of PEG, and its derivatives, to peptides is a non-specific bonding through a peptide amino acid residue. Studies of conjugates of water-soluble polymers and interleukin-2 (Fisher et al., Br. J. Haematol., 82: 654 (1992)), granulocyte colony stimulating factor (Satake-Ishikawa et al., Cell Struct. Funct., 17: 157 (1992)), tumor necrosis factor (Tsutsumi et al., Br. J. Cancer, 71: 963 (1996)) and Fibroblast Growth Factor (Clark, et al., J. Biol. Chem., 271:21969 (1996)) have revealed that chemical PEGylation of these proteins decreases the in vivo receptor binding activity of the peptides.

[0012] In many chemical PEGylation methods, poly(ethylene glycol) is added in an essentially random, non-specific manner to reactive residues on a peptide backbone. For the production of therapeutic peptides, it is clearly desirable to utilize a derivitization strategy that results in the formation of a specifically labeled, readily characterizable, essentially homogeneous product. A promising route to preparing specifically labeled peptides is through the use of enzymes, such as glycosyltransferases to append a modified sugar moiety onto a peptide.

[0013] Enzyme-based syntheses have the advantages of regioselectivity and stereoselectivity. Moreover, enzymatic syntheses are performed using unprotected substrates. Two principal classes of enzymes are used in the synthesis of carbohydrates, glycosyltransferases (e.g., sialyltransferases, oligosaccharyltransferases, N-acetylglucosaminyltransferases), and glycosidases. The glycosidases are further classified as exoglycosidases (e.g., β -mannosidase, β -glucosidase), and endoglycosidases (e.g., Endo-A, Endo-M). Each of these classes of enzymes has been successfully used synthetically to prepare carbohydrates. For a general review, see, Crout et al., Curr. Opin. Chem. Biol. 2: 98-111 (1998).

[0014] Glycosyltransferases modify the oligosaccharide structures on glycopeptides, producing specific products with good stereochemical and regiochemical control. Glycosyltransferases are used to prepare oligosaccharides and to modify terminal N- and O-linked carbohydrate structures, particularly on glycopeptides produced in mammalian cells. For example, the terminal oligosaccharides of glycopeptides have been completely sialylated and/or fucosylated to provide more consistent sugar structures, which improves glycopeptide pharmacodynamics and a variety of other biological properties. For example, β -1,4-galactosyltransferase was used to synthesize lactosamine, an illustration of the utility of glycosyltransferases in the synthesis of carbohydrates (see, e.g., Wong et al., J. Org. Chem. 47: 5416-5418 (1982)). Moreover, numerous synthetic procedures have made use of α -sialyltransferases to transfer sialic acid from cytidine-5'-monophospho-N-acetylneuraminic acid to the 3-OH or 6-OH of galactose (see, e.g., Kevin et al., Chem. Eur. J. 2: 1359-1362 (1996)). Fucosyltransferases are used in synthetic pathways to transfer a fucose unit from guanosine-5'-diphosphofucose to a specific hydroxyl of a saccharide acceptor. For example, Ichikawa prepared sialyl Lewis-X by a method that involves the fucosylation of sialylated lactosamine with a cloned fucosyltransferase (Ichikawa et al., J. Am. Chem. Soc. 114: 9283-9298 (1992)). For a discussion of recent advances in glycoconjugate synthesis for therapeutic use see, Koeller et al., Nature Biotechnology 18: 835-841 (2000). See also, U.S. Patent No. 5,876,980; 6,030,815; 5,728,554; 5,922,577; and WO/9831826.

[0015] Glycosidases can also be used to prepare saccharides. Glycosidases normally catalyze the hydrolysis of a glycosidic bond. Under appropriate conditions, however, they can be used to form this linkage. Most glycosidases used for carbohydrate synthesis are exoglycosidases; the glycosyl transfer occurs at the non-reducing terminus of the substrate. The glycosidase takes up a glycosyl donor in a glycosyl-enzyme intermediate that is either intercepted by water to give the hydrolysis product, or by an acceptor, to give a new glycoside or oligosaccharide. An exemplary pathway using an exoglycosidase is the synthesis of the core trisaccharide of all N-linked glycopeptides, including the difficult β -mannoside linkage, which was formed by the action of β -mannosidase (Singh et al., Chem. Commun. 993-994 (1996)).

[0016] In another exemplary application of the use of a glycosidase to form a glycosidic linkage, a mutant glycosidase has been prepared in which the normal nucleophilic amino acid within the active site is changed to a non-nucleophilic amino acid. The mutant enzymes do not hydrolyze glycosidic linkages, but can still form them. The mutant glycosidases are used to prepare oligosaccharides using an α -glycosyl fluoride donor and a glycoside acceptor molecule (Withers et al., U.S. Patent No. 5,716,812). Although the mutant glycosidases are useful for forming free oligosaccharides, it has yet to be demonstrated that such enzymes are capable of appending glycosyl donors onto glycosylated or non-glycosylated peptides, nor have these enzymes been used with unactivated glycosyl donors.

[0017] Although their use is less common than that of the exoglycosidases, endoglycosidases are also utilized to prepare carbohydrates. Methods based on the use of endoglycosidases have the advantage that an oligosaccharide, rather than a monosaccharide, is transferred. Oligosaccharide fragments have been added to substrates using *endo*- β -N-acetylglucosamines such as *endo*-F, *endo*-M (Wang et al., Tetrahedron Lett. 37: 1975-1978); and Haneda et al., Carbohydr. Res. 292: 61-70 (1996)).

[0018] In addition to their use in preparing carbohydrates, the enzymes discussed above are applied to the synthesis

of glycopeptides as well. The synthesis of a homogenous glycoform of ribonuclease B has been published (Witte K. et al., J. Am. Chem. Soc. 119: 2114-2118 (1997)). The high mannose core of ribonuclease B was cleaved by treating the glycopeptide with endoglycosidase H. The cleavage occurred specifically between the two core GlcNAc residues. The tetrasaccharide sialyl Lewis X was then enzymatically rebuilt on the remaining GlcNAc anchor site on the now homogenous protein by the sequential use of β -1,4-galactosyltransferase, α -2,3-sialyltransferase and α -1,3-fucosyltransferase V. Each enzymatically catalyzed step proceeded in excellent yield.

[0019] Methods combining both chemical and enzymatic synthetic elements are also known. For example, Yamamoto and coworkers (Carbohydr. Res. 305: 415-422 (1998)) reported the chemoenzymatic synthesis of the glycopeptide, glycosylated Peptide T, using an endoglycosidase. The N-acetylglucosaminyl peptide was synthesized by purely chemical means. The peptide was subsequently enzymatically elaborated with the oligosaccharide of human transferrin glycopeptide. The saccharide portion was added to the peptide by treating it with an endo- β -N-acetylglucosaminidase. The resulting glycosylated peptide was highly stable and resistant to proteolysis when compared to the peptide T and N-acetylglucosaminyl peptide T.

[0020] The use of glycosyltransferases to modify peptide structure with reporter groups has been explored. For example, Brossmer et al. (U.S. Patent No. 5,405,753) discloses the formation of a fluorescent-labeled cytidine monophosphate ("CMP") derivative of sialic acid and the use of the fluorescent glycoside in an assay for sialyl transferase activity and for the fluorescent-labeling of cell surfaces, glycoproteins and gangliosides. Gross et al. (Analyt. Biochem. 186: 127 (1990)) describe a similar assay. Bean et al. (U.S. Patent No. 5,432,059) discloses an assay for glycosylation deficiency disorders utilizing reglycosylation of a deficiently glycosylated protein. The deficient protein is reglycosylated with a fluorescent-labeled CMP glycoside. Each of the fluorescent sialic acid derivatives is substituted with the fluorescent moiety at either the 9-position or at the amine that is normally acetylated in sialic acid. The methods using the fluorescent sialic acid derivatives are assays for the presence of glycosyltransferases or for non-glycosylated or improperly glycosylated glycoproteins. The assays are conducted on small amounts of enzyme or glycoprotein in a sample of biological origin. The enzymatic derivatization of a glycosylated or non-glycosylated peptide on a preparative or industrial scale using a modified sialic acid was not disclosed or suggested in either of these references.

[0021] Enzymatic methods have also been used to activate glycosyl residues on a glycopeptide towards subsequent chemical elaboration. The glycosyl residues are typically activated using galactose oxidase, which converts a terminal galactose residue to the corresponding aldehyde. The aldehyde is subsequently coupled to an amine-containing modifying group. For example, Casares et al. (Nature Biotech. 19: 142 (2001)) have attached doxorubicin to the oxidized galactose residues of a recombinant MHCII-peptide chimera.

[0022] Glycosyl residues have also been modified to bear ketone groups. For example, Mahal and co-workers (Science 276: 1125 (1997)) have prepared N-levulinoyl mannosamine ("ManLev"), which has a ketone functionality at the position normally occupied by the acetyl group in the natural substrate. Cells were treated with the ManLev, thereby incorporating a ketone group onto the cell surface. See, also Saxon et al., Science 287: 2007 (2000); Hang et al., J. Am. Chem. Soc. 123: 1242 (2001); Yarema et al., J. Biol. Chem. 273: 31168 (1998); and Charter et al., Glycobiology 10: 1049 (2000).

[0023] Carbohydrates are attached to glycopeptides in several ways of which N-linked to asparagine and mucin-type O-linked to serine and threonine are the most relevant for recombinant glycoprotein therapeutics. A determining factor for initiation of glycosylation of a protein is the primary sequence context, although clearly other factors including protein region and conformation play roles. N-linked glycosylation occurs at the consensus sequence NXS/T, where X can be any amino acid but proline.

[0024] The present invention answers these needs by providing FGF mutants that contain newly introduced N-linked or O-linked glycosylation sites, providing flexibility in glycosylation and/or glycopegylation of these recombinant FGF mutants. Moreover, the invention provides an industrially practical method for the modification of N- or O-linked mutant FGF peptides with modifying groups such as water-soluble polymers, therapeutic moieties, biomolecules, and the like. Of particular interest are methods in which the modified mutant FGF has improved properties, which enhance its use as a therapeutic or diagnostic agent.

BRIEF SUMMARY OF THE INVENTION

[0025] It has now been discovered that the controlled modification of Fibroblast Growth Factor with one or more modifying groups (e.g., non-glycosidic modifying groups) affords a novel FGF peptide conjugate with pharmacokinetic properties that are improved relative to the corresponding native (un-modified) FGF. Furthermore, cost effective methods for reliable and reproducible production of the FGF peptide conjugates of the invention have been discovered and developed.

[0026] In one aspect, the invention provides an FGF conjugate comprising a FGF peptide and a glycosyl linking group-poly(ethylene glycol) cassette attached to an amino acid residue of the FGF peptide.

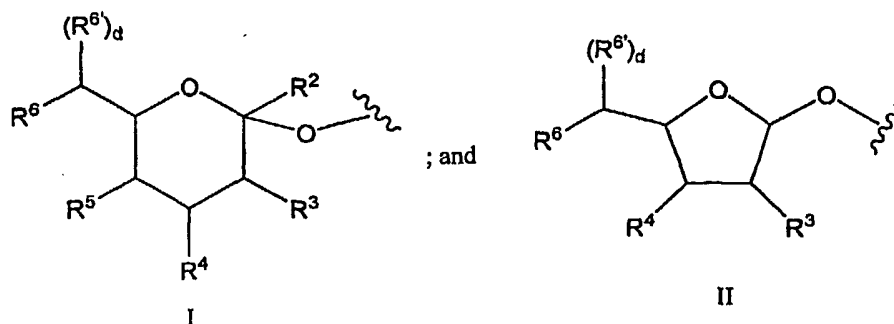
[0027] In an exemplary embodiment, glyconjugated FGF molecules of the invention are produced by the enzyme mediated formation of a conjugate between a glycosylated or non-glycosylated FGF peptide and an enzymatically

transferable saccharyl moiety that includes a modifying group, such as a polymeric modifying group, *e.g.*, poly(ethylene glycol), within its structure. The modifying group is attached to the saccharyl moiety directly (*i.e.*, through a single group formed by the reaction of two reactive groups) or through a linker moiety, *e.g.*, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, *etc.*

[0028] In one aspect, the present invention provides a conjugate between a PEG moiety, and a peptide that has an *in vivo* activity similar or otherwise analogous to art-recognized FGF. In the conjugate of the invention, the PEG moiety is covalently attached to the peptide via a glycosyl linking group or an intact glycosyl linking group. Exemplary intact glycosyl linking groups include sialic acid moieties that are derivatized with PEG.

[0029] The saccharyl moiety bearing the polymeric modifying group can be attached at any position of a glycosyl moiety of FGF. Moreover, the polymeric modifying group can be bound to a glycosyl residue at any position in the amino acid sequence of a wild type or mutant FGF peptide.

[0030] In an exemplary embodiment, the invention provides a FGF peptide that is conjugated through a glycosyl linking group to a polymeric modifying group. Exemplary FGF peptide conjugates include a glycosyl linking group having a formula selected from:



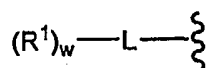
[0031] In Formulae I and II, R² is H, CH₂OR⁷, COOR⁷, COO-M⁺ or OR⁷, in which R⁷ represents H, substituted or unsubstituted alkyl or substituted or unsubstituted heteroalkyl. The symbols R³, R⁴, R⁵, R⁶ and R^{6'} independently represent H, substituted or unsubstituted alkyl, OR⁸, NHC(O)R⁹. M⁺ is a metal. The index d is 0 or 1. R⁸ and R⁹ are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl or sialic acid. At least one of R³, R⁴, R⁵, R⁶ or R^{6'} includes the polymeric modifying group e.g., PEG. In an exemplary embodiment, R⁶ and R^{6'}, together with the carbon to which they are attached are components of the side chain of a sialyl moiety. In a further exemplary embodiment, this side chain is functionalized with the polymeric modifying group.

[0032] As discussed herein, the PEG of use in the conjugates of the invention can be linear or branched. An exemplary precursor of use to form the branched PEG containing peptide conjugates according to this embodiment of the invention has the formula:



[0033] The branched polymer species according to this formula are essentially pure water-soluble polymers. X^3 is a moiety that includes an ionizable (e.g., OH, COOH, H_2PO_4 , HSO_3 , NH_2 , and salts thereof, etc.) or other reactive functional group, e.g., *infra*. C is carbon. X^5 , R^{16} and R^{17} are independently selected from non-reactive groups (e.g., H, unsubstituted alkyl, unsubstituted heteroalkyl) and polymeric arms (e.g., PEG). X^2 and X^4 are linkage fragments that are preferably essentially non-reactive under physiological conditions, which may be the same or different. An exemplary linker includes neither aromatic nor ester moieties. Alternatively, these linkages can include one or more moiety that is designed to degrade under physiologically relevant conditions, e.g., esters, disulfides, etc. X^2 and X^4 join polymeric arms R^{16} and R^{17} to C. When X^3 is reacted with a reactive functional group of complementary reactivity on a linker, sugar or linker-sugar cassette, X^3 is converted to a component of linkage fragment X^3 .

[0034] In an exemplary embodiment, the polymeric modifying group is bound to the glycosyl linking group, generally through a heteroatom on the glycosyl core (e.g., N, O), through a linker, L, as shown below:



[0035] R^1 is the polymeric modifying group and L is selected from a bond and a linking group. The index w represents an integer selected from 1-6, preferably 1-3 and more preferably 1-2. Exemplary linking groups include substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl moieties and sialic acid. An exemplary component of the linker is an acyl moiety. Another exemplary linking group is an amino acid residue (e.g., cysteine, serine, lysine, and short oligopeptides, e.g., Lys-Lys, Lys-Lys-Lys, Cys-Lys, Ser-Lys, etc.)

[0036] When L is a bond, it is formed by reaction of a reactive functional group on a precursor of R^1 and a reactive functional group of complementary reactivity on a precursor of the glycosyl linking group. When L is a non-zero order linking group, L can be in place on the glycosyl moiety prior to reaction with the R^1 precursor. Alternatively, the precursors of R^1 and L can be incorporated into a preformed cassette that is subsequently attached to the glycosyl moiety. As set forth herein, the selection and preparation of precursors with appropriate reactive functional groups is within the ability of those skilled in the art. Moreover, coupling of the precursors proceeds by chemistry that is well understood in the art.

[0037] In another aspect, the present invention provides an isolated nucleic acid comprising a polynucleotide sequence encoding a mutant Fibroblast Growth Factor. The mutant Fibroblast Growth Factor comprises one or more N-linked or O-linked glycosylation site that is not present in wild-type Fibroblast Growth Factor. In some embodiments, the nucleic acid encoding the mutant FGF-20 has a corresponding wild-type sequence that encodes a wild-type Fibroblast Growth Factor that has the amino acid sequence of SEQ ID NO:1. In some preferred embodiments, the mutant Fibroblast Growth Factor includes at least one amino acid sequence selected from SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, and 112-145. In some other embodiments, the nucleic acid encoding the mutant FGF-21 has a corresponding wild-type sequence that encodes a wild-type Fibroblast Growth Factor that has the amino acid sequence of SEQ ID NO:146. In some preferred embodiments, the mutant Fibroblast Growth Factor includes at least one amino acid sequence selected from SEQ ID NOs: 161-214, 220-320, and 323-360.

[0038] In another aspect, the present invention provides an expression cassette or a cell that comprises a nucleic acid, e.g., an isolated nucleic acid, including a polynucleotide sequence encoding a mutant Fibroblast Growth Factor. The mutant Fibroblast Growth Factor includes one or more N-linked or O-linked glycosylation site that is not present in the wild-type Fibroblast Growth Factor.

[0039] In another aspect, the present invention provides a mutant Fibroblast Growth Factor, that includes one or more N-linked or O-linked glycosylation site that is not present in the wild-type Fibroblast Growth Factor. In some embodiments, the wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO:1. In some preferred embodiments, the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, and 112-145. In some other embodiments, the wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO:146. In some preferred embodiments, the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from SEQ ID NOs: 161-214, 220-320, and 323-360.

[0040] In another aspect, the present invention provides a method for making a mutant Fibroblast Growth Factor that includes an N-linked or O-linked glycosylation site that is not present in the wild-type Fibroblast Growth Factor. This method includes the steps of recombinantly producing the mutant Fibroblast Growth Factor, and glycosylating the mutant Fibroblast Growth Factor at the new glycosylation site. In some embodiments, the wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO:1. In some preferred embodiments, the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, and 112-145. In some other embodiments, the wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO:146. In some preferred embodiments, the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from SEQ ID NOs: 161-214, 220-320, and 323-360.

[0041] In still a further aspect, the present invention provides a pharmaceutical composition having a therapeutically effective amount of a mutant Fibroblast Growth Factor that includes an N-linked or O-linked glycosylation site not present in the wild-type Fibroblast Growth Factor. In some embodiments, the wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO:1. In some preferred embodiments, the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, and 112-145. In some other embodiments, the wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO:146. In some preferred embodiments, the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from SEQ ID NOs: 161-214, 220-320, and 323-360.

[0042] In each of the aspects described above, the mutant Fibroblast Growth Factor is optionally conjugated to one or more modifying groups, preferably via glycoconjugation, giving rise to a glycosyl linking group between the glycosylation site and the modifying group. An exemplary modifying group is poly(ethylene glycol).

BRIEF DESCRIPTION OF THE DRAWINGS

[0043]

FIG. 1A displays results of an SDS-PAGE analysis of human FGF-20 induction at varied temperature, time, vector and E.Coli strains: lanes 1 and 14: molecular weight marker (sized in kDa), (induction temperature); lanes 2-9 and 15-18: 37°C, lanes 10-13 and 19-22: 20°C. Strain used: lanes 2-4 and 6-8 and 10-12, W3110; lanes 5, 9, and 13 BL21(DE3); lanes 15-17 and 19-21, E.Coli_(trxb, gor, supp); lanes 18 and 22, E.Coli_(trxb, gor, supp)(DE3). Vector used: lanes 2, 6, 10, 15, 19 use vector #1; lanes 3, 7, 11, 16, 20 use vector #2; lanes 4, 8, 12, 17, 21 use vector #3; lanes 5, 9, 13, 18 and 22 use vector #4.

FIG. 1B displays results of an SDS-PAGE analysis of human FGF-20 solubility at varied temperature and E.Coli strains, lane 1: molecular weight marker (sized in kDa). Even numbers represent pellet and odd numbers represent supernatant. Induction temperatures used: lanes 2-3: 20°C; lanes 4-5: 30°C; lanes 6-7: 37°C; lanes 8-9: 37°C. Strain used: lanes 6-7, BL21 (DE3); lanes 2-5 and 8-9, E.Coli_(trxb, gor, supp)(DE3). Vector #4 was employed.

FIG. 1C displays results of an SDS-PAGE analysis of human FGF-21 induction at varied temperature, time, vector and E.Coli strains: lane 1 and 15: molecular weight marker (sized in kDa), lane 2 no induction; (induction temperature); lanes 3-10 and 16-20: 37°C, lanes 11-14 and 21-23: 20°C. Strains used: lanes 3-5 and 7-9 and 11-13, W3110; lanes 6, 10, and 14 BL21(DE3); lanes 16-19 and 21-23, E.Coli_(trxb, gor, supp); lane 20, E.Coli_(trxb, gor, supp)(DE3). Vectors used: lanes 3, 7, 11, 17, 21 are vector #1; lanes 4, 8, 12, 18, 22 are vector #2; lanes 5, 9, 13, 19 and 23 are vector #3; lanes 6, 10, 14, and 20 are vector #4.

FIG. 1D displays results of an SDS-PAGE analysis of human FGF-21 solubility at varied temperature and E.Coli strains, lane 1+1b: molecular weight marker (sized in kDa). Even numbers represent pellet and odd numbers represent supernatant. Induction temperature used: lanes 2-3 and 6-7: 37°C; lanes 4-5 and 8-9: 20°C; lanes 11-12: 18°C. Strain used: lanes 2-5, W3110; lanes 6-12, E.Coli_(trxb, gor, supp). Vector #3 was employed.

FIG. 2 is a table providing exemplary sialyltransferases of use in forming the glycoconjugates of the invention, e.g., to glycoPEGylate peptides with a modified sialic acid.

DETAILED DESCRIPTION OF THE INVENTION AND THE PREFERRED EMBODIMENTS

Abbreviations

[0044] PEG, poly(ethyleneglycol); PPG, poly(propyleneglycol); Ara, arabinosyl; Fru, fructosyl; Fuc, fucosyl; Gal, galactosyl; GalNAc, N-acetylgalactosaminyl; Glc, glucosyl; GlcNAc, N-acetylglucosaminyl; Man, mannosyl; ManAc, mannosaminyl acetate; Xyl, xylosyl; NeuAc, sialyl or N-acetylneuraminyl; Sia, sialyl or N-acetylneuraminyl; and derivatives and analogues thereof.

Definitions

[0045] Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic chemistry and nucleic acid chemistry and hybridization are those well known and commonly employed in the art. Standard techniques are used for nucleic acid and peptide synthesis. The techniques and procedures are generally performed according to conventional methods in the art and various general references (see *generally*, Sambrook et al. MOLECULAR CLONING: A LABORATORY MANUAL, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which is incorporated herein by reference), which are provided throughout this document. The nomenclature used herein and the laboratory procedures in analytical chemistry, and organic synthetic described below are those well known and commonly employed in the art. Standard techniques, or modifications thereof, are used for chemical syntheses and chemical analyses.

[0046] The term "nucleic acid" or "polynucleotide" refers to deoxyribonucleic acids (DNA) or ribonucleic acids (RNA) and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated.

Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al., Nucleic Acid Res. 19:5081 (1991); Ohtsuka et al., J. Biol. Chem. 260:2605-2608 (1985); and Rossolini et al., Mol. Cell. Probes 8:91-98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, and mRNA encoded by a gene.

[0047] The term "gene" means the segment of DNA involved in producing a polypeptide chain. It may include regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

[0048] The term "isolated," when applied to a nucleic acid or protein, denotes that the nucleic acid or protein is essentially free of other cellular components with which it is associated in the natural state. It is preferably in a homogeneous state although it can be in either a dry or aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein that is the predominant species present in a preparation is substantially purified. In particular, an isolated gene is separated from open reading frames that flank the gene and encode a protein other than the gene of interest. The term "purified" denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. Particularly, it means that the nucleic acid or protein is at least 85% pure, more preferably at least 95% pure, and most preferably at least 99% pure.

[0049] The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, γ -carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, e.g., homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid analogs described in the following patent application can be incorporated into the FGF peptide conjugates and mutant FGF sequences of the invention: U.S. Pat. App. Nos. 11/094677 (filed March 29, 2005); 11/094676 (filed March 29, 2005); 11/093798 (filed March 29, 2005); 11/093797 (filed March 29, 2005); 11/093597 (filed March 29, 2005); 10/965218 (filed Oct. 13, 2004); 11/093797 (filed March 29, 2005); 11/009635 (filed Dec. 10, 2004); 11/016348 (filed Dec. 16, 2004); 10/825867 (filed Apr. 16, 2004); 10/826919 (filed Apr. 16, 2004); and 10/686944 (now U.S. Pat. No. 6,927,042, issued Aug. 9, 2005). The methods described in these applications can also be used to produce the the FGF peptide conjugates and mutant FGF sequences of the invention. "Amino acid mimetics" refers to chemical compounds having a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

[0050] There are various known methods in the art that permit the incorporation of an unnatural amino acid derivative or analog into a polypeptide chain in a site-specific manner, see, e.g., WO 02/086075.

[0051] Amino acids may be referred to herein by either the commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0052] "Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, "conservatively modified variants" refers to those nucleic acids that encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein that encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid that encodes a polypeptide is implicit in each described sequence.

[0053] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

[0054] The following eight groups each contain amino acids that are conservative substitutions for one another:

- 1) Alanine (A), Glycine (G);

- 2) Aspartic acid (D), Glutamic acid (E);
- 3) Asparagine (N), Glutamine (Q);
- 4) Arginine (R), Lysine (K);
- 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V);
- 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W);
- 7) Serine (S), Threonine (T); and
- 8) Cysteine (C), Methionine (M)

(see, e.g., Creighton, *Proteins* (1984)).

[0055] Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0056] In the present application, amino acid residues are numbered according to their relative positions from the most N-terminal residue, which is numbered 1, in an unmodified wild-type polypeptide sequence.

[0057] "Proximate to a proline residue," as used herein refers to an amino acid that is less than about 10 amino acids removed from a proline residue, preferably, less than about 9, 8, 7, 6 or 5 amino acids removed from a proline residue, more preferably, less than about 4, 3, 2 or 1 residues removed from a proline residue. The amino acid "proximate a proline residue" may be on the C- or N-terminal side of the proline residue.

[0058] "Polypeptide," "peptide," and "protein" are used interchangeably herein to refer to a polymer in which the monomers are amino acids and are joined together through amide bonds, alternatively referred to as a polypeptide. Additionally, unnatural amino acids, for example, β -alanine, phenylglycine and homoarginine are also included. Amino acids that are not gene-encoded may also be used in the present invention. Furthermore, amino acids that have been modified to include reactive groups, glycosylation sites, polymers, therapeutic moieties, biomolecules and the like may also be used in the invention. All of the amino acids used in the present invention may be either the D- or L-isomer. The L-isomer is generally preferred. In addition, other peptidomimetics are also useful in the present invention. As used herein, "peptide" refers to both glycosylated and unglycosylated peptides. Also included are peptides that are incompletely glycosylated by a system that expresses the peptide. For a general review, see, Spatola, A. F., in CHEMISTRY AND BIOCHEMISTRY OF AMINO ACIDS, PEPTIDES AND PROTEINS, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983).

[0059] The term "peptide conjugate," refers to species of the invention in which a peptide is conjugated with a modified sugar as set forth herein.

[0060] The term "FGF" or "Fibroblast Growth Factor" refers to any of the family of twenty-five known wild-type peptides. The term also refers to amino acid sequences with the same, fewer or additional amino acids as compared to the wild-type sequence. Additional amino acids, which can be natural or unnatural, can be inserted into the beginning, middle, or end of the amino acid sequence.

[0061] The term "mutating" or "mutation," as used in the context of introducing additional N- or O-linked glycosylation site(s) into a wild-type Fibroblast Growth Factor, refers to the deletion, insertion, or substitution of any nucleotide or amino acid residue, by chemical, enzymatic, or any other means, in a polynucleotide sequence encoding a wild-type Fibroblast Growth Factor or the amino acid sequence of a wild-type Fibroblast Growth Factor, respectively, such that the amino acid sequence of the resulting Fibroblast Growth Factor comprises at least one N- or O-linked glycosylation site that does not exist in the corresponding wild-type Fibroblast Growth Factor. In the case of amino acid substitution, both conservative and non-conservative substitutions may be used to create a FGF mutant that contains a new N- or O-linked glycosylation site.

[0062] The site for a mutation introducing a new N- or O-linked glycosylation site may be located anywhere in the polypeptide. Exemplary amino acid sequences for Fibroblast Growth Factor mutants are depicted in SEQ ID NOs: 9-14, 18-22, 23-45, 48-65, 69-109, 112-145, 161-214, 220-320, and 323-360. A "mutant Fibroblast Growth Factor" of this invention thus comprises at least one amino acid substitution, insertion, or mutated amino acid residue. On the other hand, the wild-type Fibroblast Growth Factor whose coding sequence is modified to generate a mutant Fibroblast Growth Factor can be referred to in this application as "the corresponding wild-type Fibroblast Growth Factor", or simply "wild-type peptide". For example, SEQ ID NO:1 is the amino acid sequence of the corresponding wild-type Fibroblast Growth Factor-20 for mutant Fibroblast Growth Factors having the amino acid sequences of SEQ ID NOs: 9-14, 18-22, 23-45, 48-65, 69-109, and 112-145. Likewise, SEQ ID NO:146 is the amino acid sequence of the corresponding wild-type Fibroblast Growth Factor-21 for mutant Fibroblast Growth Factors having the amino acid sequences of SEQ ID NOs: 161-214, 220-320, and 323-360.

[0063] The term "effective amount," or "an amount effective to" or a "therapeutically effective amount" or any grammatically equivalent term means the amount that produces therapeutic effects for which a substance is administered. The effects include the prevention, correction, or inhibition of progression of the symptoms of a disease/condition and related complications to any detectable extent. The exact amount will depend on the purpose of the treatment, and will

be ascertainable by one skilled in the art using known techniques (see, e.g., Lieberman, *Pharmaceutical Dosage Forms* (vols. 1-3, 1992); Lloyd, *The Art, Science and Technology of Pharmaceutical Compounding* (1999); and Pickar, *Dosage Calculations* (1999)).

[0064] As used herein, the term "modified sugar," refers to a naturally- or non-naturally-occurring carbohydrate that is enzymatically added onto an amino acid or a glycosyl residue of a peptide in a process of the invention. The modified sugar is selected from a number of enzyme substrates including, but not limited to sugar nucleotides (mono-, di-, and triphosphates), activated sugars (e.g., glycosyl halides, glycosyl mesylates) and sugars that are neither activated nor nucleotides. The "modified sugar" is covalently functionalized with a "modifying group." Useful modifying groups include, but are not limited to, water-soluble polymers (PEG moieties), therapeutic moieties, diagnostic moieties, biomolecules and the like. The modifying group is preferably not a naturally occurring, or an unmodified carbohydrate. The locus of functionalization with the modifying group is selected such that it does not prevent the "modified sugar" from being added enzymatically to a peptide.

[0065] The term "water-soluble" refers to moieties that have some detectable degree of solubility in water. Methods to detect and/or quantify water solubility are well known in the art. Exemplary water-soluble polymers include peptides, saccharides, poly(ethers), poly(amines), poly(carboxylic acids) and the like. Peptides can have mixed sequences of be composed of a single amino acid, e.g., poly(lysine). An exemplary polysaccharide is poly(sialic acid). An exemplary poly(ether) is poly(ethylene glycol), e.g., m-PEG. Poly(ethylene imine) is an exemplary polyamine, and poly(acrylic acid) is a representative poly(carboxylic acid).

[0066] The polymer backbone of the water-soluble polymer can be poly(ethylene glycol) (i.e. PEG). However, it should be understood that other related polymers are also suitable for use in the practice of this invention and that the use of the term PEG or poly(ethylene glycol) is intended to be inclusive and not exclusive in this respect. The term PEG includes poly(ethylene glycol) in any of its forms, including alkoxy PEG, difunctional PEG, multiarmed PEG, forked PEG, branched PEG, pendent PEG (i.e. PEG or related polymers having one or more functional groups pendent to the polymer backbone), or PEG with degradable linkages therein.

[0067] The polymer backbone can be linear or branched. Branched polymer backbones are generally known in the art. Typically, a branched polymer has a central branch core moiety and a plurality of linear polymer chains linked to the central branch core. PEG is commonly used in branched forms that can be prepared by addition of ethylene oxide to various polyols, such as glycerol, pentaerythritol and sorbitol. The central branch moiety can also be derived from several amino acids, such as lysine. The branched poly(ethylene glycol) can be represented in general form as $R(-\text{PEG}-\text{OX})_m$ in which R represents the core moiety, such as glycerol or pentaerythritol, X represents a capping group or an end group, and m represents the number of arms. Multi-armed PEG molecules, such as those described in U.S. Pat. No. 5,932,462, which is incorporated by reference herein in its entirety, can also be used as the polymer backbone.

[0068] Many other polymers are also suitable for the invention. Polymer backbones that are non-peptidic and water-soluble, with from 2 to about 300 termini, are particularly useful in the invention. Examples of suitable polymers include, but are not limited to, other poly(alkylene glycols), such as poly(propylene glycol) ("PPG"), copolymers of ethylene glycol and propylene glycol and the like, poly(oxyethylated polyol), poly(olefinic alcohol), poly(vinylpyrrolidone), poly(hydroxypropylmethacrylamide), poly(α -hydroxy acid), poly(vinyl alcohol), polyphosphazene, polyoxazoline, poly(N-acryloylmorpholine), such as described in U.S. Pat. No. 5,629,384, which is incorporated by reference herein in its entirety, and copolymers, terpolymers, and mixtures thereof. Although the molecular weight of each chain of the polymer backbone can vary, it is typically in the range of from about 100 Da to about 100,000 Da, often from about 6,000 Da to about 80,000 Da.

[0069] The term "sialic acid" or "sialyl" refers to any member of a family of nine-carbon carboxylated sugars. The most common member of the sialic acid family is N-acetylneuraminic acid (2-keto-5-acetamido-3,5-dideoxy-D-glycero-D-galactononulopyranos-1-onic acid (often abbreviated as Neu5Ac, NeuAc, or NANA). A second member of the family is N-glycolyl-neuraminic acid (Neu5Gc or NeuGc), in which the N-acetyl group of NeuAc is hydroxylated. A third sialic acid family member is 2-keto-3-deoxy-nonulosonic acid (KDN) (Nadano et al. (1986) *J. Biol. Chem.* 261: 11550-11557; Kanamori et al., *J. Biol. Chem.* 265: 21811-21819 (1990)). Also included are 9-substituted sialic acids such as a 9-O-C₁-C₆ acyl-Neu5Ac like 9-O-lactyl-Neu5Ac or 9-O-acetyl-Neu5Ac, 9-deoxy-9-fluoro-Neu5Ac and 9-azido-9-deoxy-Neu5Ac. For review of the sialic acid family, see, e.g., Varki, *Glycobiology* 2: 25-40 (1992); *Sialic Acids: Chemistry, Metabolism and Function*, R. Schauer, Ed. (Springer-Verlag, New York (1992)). The synthesis and use of sialic acid compounds in a sialylation procedure is disclosed in international application WO 92/16640, published October 1, 1992.

[0070] The "area under the curve" or "AUC", as used herein in the context of administering a peptide drug to a patient, is defined as total area under the curve that describes the concentration of drug in systemic circulation in the patient as a function of time from zero to infinity.

[0071] The term "half-life" or " $t_{1/2}$ ", as used herein in the context of administering a peptide drug to a patient, is defined as the time required for plasma concentration of a drug in a patient to be reduced by one half. There may be more than one half-life associated with the peptide drug depending on multiple clearance mechanisms, redistribution, and other mechanisms well known in the art. Usually, alpha and beta half-lives are defined such that the alpha phase is associated with redistribution, and the beta phase is associated with clearance. However, with protein drugs that are, for the most

part, confined to the bloodstream, there can be at least two clearance half-lives. For some glycosylated peptides, rapid beta phase clearance may be mediated via receptors on macrophages, or endothelial cells that recognize terminal galactose, N-acetylgalactosamine, N-acetylglucosamine, mannose, or fucose. Slower beta phase clearance may occur via renal glomerular filtration for molecules with an effective radius < 2 nm (approximately 68 kD) and/or specific or non-specific uptake and metabolism in tissues. GlycoPEGylation may cap terminal sugars (e.g., galactose or N-acetylgalactosamine) and thereby block rapid alpha phase clearance via receptors that recognize these sugars. It may also confer a larger effective radius and thereby decrease the volume of distribution and tissue uptake, thereby prolonging the late beta phase. Thus, the precise impact of glycoPEGylation on alpha phase and beta phase half-lives will vary depending upon the size, state of glycosylation, and other parameters, as is well known in the art. Further explanation of "half-life" is found in *Pharmaceutical Biotechnology* (1997, DFA Crommelin and RD Sindelar, eds., Harwood Publishers, Amsterdam, pp 101 - 120).

[0072] The term "glycoconjugation," as used herein, refers to the enzymatically mediated conjugation of a modified sugar species to an amino acid or glycosyl residue of a polypeptide, e.g., a mutant Fibroblast Growth Factor of the present invention. A subgenus of "glycoconjugation" is "glyco-PEGylation," in which the modifying group of the modified sugar is poly(ethylene glycol), an alkyl derivative of PEG (e.g., m-PEG) or a reactive derivative of PEG (e.g., H₂N-PEG, HOOC-PEG) thereof.

[0073] The terms "large-scale" and "industrial-scale" are used interchangeably and refer to a reaction cycle that produces at least about 250 mg, preferably at least about 500 mg, and more preferably at least about 1 gram of glycoconjugate at the completion of a single reaction cycle.

[0074] The term, "glycosyl linking group," as used herein refers to a glycosyl residue to which a modifying group (e.g., PEG moiety, therapeutic moiety, biomolecule) is covalently attached; the glycosyl linking group joins the modifying group to the remainder of the conjugate. In the methods of the invention, the "glycosyl linking group" becomes covalently attached to a glycosylated or unglycosylated peptide, thereby linking the agent to an amino acid and/or glycosyl residue on the peptide. A "glycosyl linking group" is generally derived from a "modified sugar" by the enzymatic attachment of the "modified sugar" to an amino acid and/or glycosyl residue of the peptide. The glycosyl linking group can be a saccharide-derived structure that is degraded during formation of modifying group-modified sugar cassette (e.g., oxidation→Schiff base formation→reduction), or the glycosyl linking group may be intact. An "intact glycosyl linking group" refers to a linking group that is derived from a glycosyl moiety in which the saccharide monomer that links the modifying group and to the remainder of the conjugate is not degraded, e.g., oxidized, e.g., by sodium metaperiodate. "Intact glycosyl linking groups" of the invention may be derived from a naturally occurring oligosaccharide by addition of glycosyl unit(s) or removal of one or more glycosyl unit from a parent saccharide structure.

[0075] The term, "non-glycosidic modifying group", as used herein, refers to modifying groups which do not include a naturally occurring sugar linked directly to the glycosyl linking group.

[0076] As used herein, "a radioactive agent" includes any radioisotope that is effective in diagnosing or destroying a tumor. Examples include, but are not limited to, indium-111, cobalt-60. Additionally, naturally occurring radioactive elements such as uranium, radium, and thorium, which typically represent mixtures of radioisotopes, are suitable examples of a radioactive agent. The metal ions are typically chelated with an organic chelating moiety.

[0077] Many useful chelating groups, crown ethers, cryptands and the like are known in the art and can be incorporated into the compounds of the invention (e.g., EDTA, DTPA, DOTA, NTA, HDTA, etc. and their phosphonate analogs such as DTPP, EDTP, HDTP, NTP, etc). See, for example, Pitt et al., "The Design of Chelating Agents for the Treatment of Iron Overload," in, *INORGANIC CHEMISTRY IN BIOLOGY AND MEDICINE*; Martell, Ed.; American Chemical Society, Washington, D.C., 1980, pp. 279-312; Lindoy, *THE CHEMISTRY OF MACROCYCLIC LIGAND COMPLEXES*; Cambridge University Press, Cambridge, 1989; Dugas, *BIOORGANIC CHEMISTRY*; Springer-Verlag, New York, 1989, and references contained therein.

[0078] Additionally, a manifold of routes allowing the attachment of chelating agents, crown ethers and cyclodextrins to other molecules is available to those of skill in the art. See, for example, Meares et al., "Properties of In Vivo Chelate-Tagged Proteins and Polypeptides." In, *MODIFICATION OF PROTEINS: FOOD, NUTRITIONAL, AND PHARMACOLOGICAL ASPECTS*; Feeney, et al., Eds., American Chemical Society, Washington, D.C., 1982, pp. 370-387; Kasina et al., *Bioconjugate Chem.*, 9: 108-117 (1998); Song et al., *Bioconjugate Chem.*, 8: 249-255 (1997).

[0079] As used herein, "pharmaceutically acceptable carrier" includes any material, which when combined with the conjugate retains the conjugates' activity and is non-reactive with the subject's immune systems. Examples include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting agents. Other carriers may also include sterile solutions, tablets including coated tablets and capsules. Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid or salts thereof, magnesium or calcium stearate, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients. Compositions comprising such carriers are formulated by well known conventional methods.

[0080] As used herein, "administering," means oral administration, inhalation, administration as a suppository, topical

contact, intravenous, intraperitoneal, intramuscular, intralesional, intranasal or subcutaneous administration, or the implantation of a slow-release device e.g., a mini-osmotic pump, to the subject. Administration is by any route including parenteral, and transmucosal (e.g., oral, nasal, vaginal, rectal, or transdermal). Parenteral administration includes, e.g., intravenous, intramuscular, intra-arteriole, intradermal, subcutaneous, intraperitoneal, intraventricular, and intracranial. Moreover, where injection is to treat a tumor, e.g., induce apoptosis, administration may be directly to the tumor and/or into tissues surrounding the tumor. Other modes of delivery include, but are not limited to, the use of liposomal formulations, intravenous infusion, transdermal patches, etc.

[0081] The term "ameliorating" or "ameliorate" refers to any indicia of success in the treatment of a pathology or condition, including any objective or subjective parameter such as abatement, remission or diminishing of symptoms or an improvement in a patient's physical or mental well-being. Amelioration of symptoms can be based on objective or subjective parameters; including the results of a physical examination and/or a psychiatric evaluation.

[0082] The term "therapy" refers to "treating" or "treatment" of a disease or condition including preventing the disease or condition from occurring in an animal that may be predisposed to the disease but does not yet experience or exhibit symptoms of the disease (prophylactic treatment), inhibiting the disease (slowing or arresting its development), providing relief from the symptoms or side-effects of the disease (including palliative treatment), and relieving the disease (causing regression of the disease).

[0083] The term "isolated" refers to a material that is substantially or essentially free from components, which are used to produce the material. For peptide conjugates of the invention, the term "isolated" refers to material that is substantially or essentially free from components, which normally accompany the material in the mixture used to prepare the peptide conjugate. "Isolated" and "pure" are used interchangeably. Typically, isolated peptide conjugates of the invention have a level of purity preferably expressed as a range. The lower end of the range of purity for the peptide conjugates is about 60%, about 70% or about 80% and the upper end of the range of purity is about 70%, about 80%, about 90% or more than about 90%.

[0084] When the peptide conjugates are more than about 90% pure, their purities are also preferably expressed as a range. The lower end of the range of purity is about 90%, about 92%, about 94%, about 96% or about 98%. The upper end of the range of purity is about 92%, about 94%, about 96%, about 98% or about 100% purity.

[0085] Purity is determined by any art-recognized method of analysis (e.g., band intensity on a silver stained gel, polyacrylamide gel electrophoresis, HPLC, or a similar means).

[0086] "Essentially each member of the population," as used herein, describes a characteristic of a population of peptide conjugates of the invention in which a selected percentage of the modified sugars added to a peptide are added to multiple, identical acceptor sites on the peptide. "Essentially each member of the population" speaks to the "homogeneity" of the sites on the peptide conjugated to a modified sugar and refers to conjugates of the invention, which are at least about 80%, preferably at least about 90% and more preferably at least about 95% homogenous.

[0087] "Homogeneity," refers to the structural consistency across a population of acceptor moieties to which the modified sugars are conjugated. Thus, in a peptide conjugate of the invention in which each modified sugar moiety is conjugated to an acceptor site having the same structure as the acceptor site to which every other modified sugar is conjugated, the peptide conjugate is said to be about 100% homogeneous. Homogeneity is typically expressed as a range. The lower end of the range of homogeneity for the peptide conjugates is about 60%, about 70% or about 80% and the upper end of the range of purity is about 70%, about 80%, about 90% or more than about 90%.

[0088] When the peptide conjugates are more than or equal to about 90% homogeneous, their homogeneity is also preferably expressed as a range. The lower end of the range of homogeneity is about 90%, about 92%, about 94%, about 96% or about 98%. The upper end of the range of purity is about 92%, about 94%, about 96%, about 98% or about 100% homogeneity. The purity of the peptide conjugates is typically determined by one or more methods known to those of skill in the art, e.g., liquid chromatography-mass spectrometry (LC-MS), matrix assisted laser desorption mass time of flight spectrometry (MALDITOF), capillary electrophoresis, and the like.

[0089] "Substantially uniform glycoform" or a "substantially uniform glycosylation pattern," when referring to a glycopeptide species, refers to the percentage of acceptor moieties that are glycosylated by the glycosyltransferase of interest (e.g., fucosyltransferase). For example, in the case of a α 1,2 fucosyltransferase, a substantially uniform fucosylation pattern exists if substantially all (as defined below) of the Gal β 1,4-GlcNAc-R and sialylated analogues thereof are fucosylated in a peptide conjugate of the invention. It will be understood by one of skill in the art, that the starting material may contain glycosylated acceptor moieties (e.g., fucosylated Gal β 1,4-GlcNAc-R moieties). Thus, the calculated percent glycosylation will include acceptor moieties that are glycosylated by the methods of the invention, as well as those acceptor moieties already glycosylated in the starting material.

[0090] The term "substantially" in the above definitions of "substantially uniform" generally means at least about 40%, at least about 70%, at least about 80%, or more preferably at least about 90%, and still more preferably at least about 95% of the acceptor moieties for a particular glycosyltransferase are glycosylated.

[0091] Where substituent groups are specified by their conventional chemical formulae, written from left to right, they equally encompass the chemically identical substituents, which would result from writing the structure from right to left,

e.g., $-\text{CH}_2\text{O}-$ is intended to also recite $-\text{OCH}_2-$.

[0092] The term "alkyl," by itself or as part of another substituent means, unless otherwise stated, a straight or branched chain, or cyclic hydrocarbon radical, or combination thereof, which may be fully saturated, mono- or polyunsaturated and can include di- and multivalent radicals, having the number of carbon atoms designated (*i.e.* $\text{C}_1\text{-C}_{10}$ means one to ten carbons). Examples of saturated hydrocarbon radicals include, but are not limited to, groups such as methyl, ethyl, n-propyl, isopropyl, n-butyl, t-butyl, isobutyl, sec-butyl, cyclohexyl, (cyclohexyl)methyl, cyclopropylmethyl, homologs and isomers of, for example, n-pentyl, n-hexyl, n-heptyl, n-octyl, and the like. An unsaturated alkyl group is one having one or more double bonds or triple bonds. Examples of unsaturated alkyl groups include, but are not limited to, vinyl, 2-propenyl, crotyl, 2-isopentenyl, 2-(butadienyl), 2,4-pentadienyl, 3-(1,4-pentadienyl), ethynyl, 1- and 3-propynyl, 3-butylnyl, and the higher homologs and isomers. The term "alkyl," unless otherwise noted, is also meant to include those derivatives of alkyl defined in more detail below, such as "heteroalkyl." Alkyl groups that are limited to hydrocarbon groups are termed "homoalkyl".

[0093] The term "alkylene" by itself or as part of another substituent means a divalent radical derived from an alkane, as exemplified, but not limited, by $-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-$, and further includes those groups described below as "heteroalkylene." Typically, an alkyl (or alkylene) group will have from 1 to 24 carbon atoms, with those groups having 10 or fewer carbon atoms being preferred in the present invention. A "lower alkyl" or "lower alkylene" is a shorter chain alkyl or alkylene group, generally having eight or fewer carbon atoms.

[0094] The terms "alkoxy," "alkylamino" and "alkylthio" (or thioalkoxy) are used in their conventional sense, and refer to those alkyl groups attached to the remainder of the molecule via an oxygen atom, an amino group, or a sulfur atom, respectively.

[0095] The term "heteroalkyl," by itself or in combination with another term, means, unless otherwise stated, a stable straight or branched chain, or cyclic hydrocarbon radical, or combinations thereof, consisting of the stated number of carbon atoms and at least one heteroatom selected from the group consisting of O, N, Si and S, and wherein the nitrogen and sulfur atoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized. The heteroatom(s) O, N and S and Si may be placed at any interior position of the heteroalkyl group or at the position at which the alkyl group is attached to the remainder of the molecule. Examples include, but are not limited to, $-\text{CH}_2\text{-CH}_2\text{-O-CH}_3$, $-\text{CH}_2\text{-CH}_2\text{-NH-CH}_3$, $-\text{CH}_2\text{-CH}_2\text{-N(CH}_3\text{)-CH}_3$, $-\text{CH}_2\text{-S-CH}_2\text{-CH}_3$, $-\text{CH}_2\text{-CH}_2\text{-S(O)-CH}_3$, $-\text{CH}_2\text{-CH}_2\text{-S(O)}_2\text{-CH}_3$, $-\text{CH=CH-O-CH}_3$, $-\text{Si(CH}_3\text{)}_3$, $-\text{CH}_2\text{-CH=N-OCH}_3$, and $-\text{CH=CH-N(CH}_3\text{)-CH}_3$. Up to two heteroatoms may be consecutive, such as, for example, $-\text{CH}_2\text{-NH-OCH}_3$ and $-\text{CH}_2\text{-O-Si(CH}_3\text{)}_3$. Similarly, the term "heteroalkylene" by itself or as part of another substituent means a divalent radical derived from heteroalkyl, as exemplified, but not limited by, $-\text{CH}_2\text{-CH}_2\text{-S-CH}_2\text{-CH}_2-$ and $-\text{CH}_2\text{-S-CH}_2\text{-CH}_2\text{-NH-CH}_2-$. For heteroalkylene groups, heteroatoms can also occupy either or both of the chain termini (e.g., alkyleneoxy, alkyleneedioxy, alkyleneamino, alkylene diamino, and the like). Still further, for alkylene and heteroalkylene linking groups, no orientation of the linking group is implied by the direction in which the formula of the linking group is written. For example, the formula $-\text{C(O)}_2\text{R}'$ represents both $-\text{C(O)}_2\text{R}'$ and $\text{R}'\text{-C(O)}_2-$.

[0096] The terms "cycloalkyl" and "heterocycloalkyl", by themselves or in combination with other terms, represent, unless otherwise stated, cyclic versions of "alkyl" and "heteroalkyl", respectively. Additionally, for heterocycloalkyl, a heteroatom can occupy the position at which the heterocycle is attached to the remainder of the molecule. Examples of cycloalkyl include, but are not limited to, cyclopentyl, cyclohexyl, 1-cyclohexenyl, 3-cyclohexenyl, cycloheptyl, and the like. Examples of heterocycloalkyl include, but are not limited to, 1-(1,2,5,6-tetrahydropyridyl), 1-piperidinyl, 2-piperidinyl, 3-piperidinyl, 4-morpholinyl, 3-morpholinyl, tetrahydrofuran-2-yl, tetrahydrofuran-3-yl, tetrahydrothien-2-yl, tetrahydrothien-3-yl, 1-piperazinyl, 2-piperazinyl, and the like.

[0097] The terms "halo" or "halogen," by themselves or as part of another substituent, mean, unless otherwise stated, a fluorine, chlorine, bromine, or iodine atom. Additionally, terms such as "haloalkyl," are meant to include monohaloalkyl and polyhaloalkyl. For example, the term "halo($\text{C}_1\text{-C}_4$)alkyl" is meant to include, but not be limited to, trifluoromethyl, 2,2,2-trifluoroethyl, 4-chlorobutyl, 3-bromopropyl, and the like.

[0098] The term "aryl" means, unless otherwise stated, a polyunsaturated, aromatic, substituent that can be a single ring or multiple rings (preferably from 1 to 3 rings), which are fused together or linked covalently. The term "heteroaryl" refers to aryl groups (or rings) that contain from one to four heteroatoms selected from N, O, and S, wherein the nitrogen and sulfur atoms are optionally oxidized, and the nitrogen atom(s) are optionally quaternized. A heteroaryl group can be attached to the remainder of the molecule through a heteroatom. Non-limiting examples of aryl and heteroaryl groups include phenyl, 1-naphthyl, 2-naphthyl, 4-biphenyl, 1-pyrrolyl, 2-pyrrolyl, 3-pyrrolyl, 3-pyrazolyl, 2-imidazolyl, 4-imidazolyl, pyrazinyl, 2-oxazolyl, 4-oxazolyl, 2-phenyl-4-oxazolyl, 5-oxazolyl, 3-isoxazolyl, 4-isoxazolyl, 5-isoxazolyl, 2-thiazolyl, 4-thiazolyl, 5-thiazolyl, 2-furyl, 3-furyl, 2-thienyl, 3-thienyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrimidyl, 4-pyrimidyl, 5-benzothiazolyl, purinyl, 2-benzimidazolyl, 5-indolyl, 1-isoquinolyl, 5-isoquinolyl, 2-quinoxalyl, 5-quinoxalyl, 3-quinolyl, tetrazolyl, benzo[b]furanyl, benzo[b]thienyl, 2,3-dihydrobenzo[1,4]dioxin-6-yl, benzo[1,3]dioxol-5-yl and 6-quinolyl. Substituents for each of the above noted aryl and heteroaryl ring systems are selected from the group of acceptable substituents described below.

[0099] For brevity, the term "aryl" when used in combination with other terms (e.g., aryloxy, arylthioxy, arylalkyl) includes

both aryl and heteroaryl rings as defined above. Thus, the term "arylalkyl" is meant to include those radicals in which an aryl group is attached to an alkyl group (e.g., benzyl, phenethyl, pyridylmethyl and the like) including those alkyl groups in which a carbon atom (e.g., a methylene group) has been replaced by, for example, an oxygen atom (e.g., phenoxyethyl, 2-pyridyloxymethyl, 3-(1-naphthyloxy)propyl, and the like).

[0100] Each of the above terms (e.g., "alkyl," "heteroalkyl," "aryl" and "heteroaryl") is meant to include both substituted and unsubstituted forms of the indicated radical. Preferred substituents for *each* type of radical are provided below.

[0101] Substituents for the alkyl and heteroalkyl radicals (including those groups often referred to as alkylene, alkenyl, heteroalkylene, heteroalkenyl, alkynyl, cycloalkyl, heterocycloalkyl, cycloalkenyl, and heterocycloalkenyl) are generically referred to as "alkyl group substituents," and they can be one or more of a variety of groups selected from, but not limited to: -OR', =O, =NR', =N-OR', -NR'R'', -SR', -halogen, -SiR'R''R''', -OC(O)R', -C(O)R', -CO₂R', -CONR'R'', -OC(O)NR'R'', -NR'C(O)R', -NR'-C(O)NR'R'', -NR'C(O)₂R', -NR-C(NR'R''R''')=NR''', -NR-C(NR'R'')=NR'', -S(O)R', -S(O)₂R', -S(O)₂NR'R'', NRSO₂R', -CN and -NO₂ in a number ranging from zero to (2m'+1), where m' is the total number of carbon atoms in such radical. R', R'', R''' and R'''' each preferably independently refer to hydrogen, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, e.g., aryl substituted with 1-3 halogens, substituted or unsubstituted alkyl, alkoxy or thioalkoxy groups, or arylalkyl groups. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R', R'', R''' and R'''' groups when more than one of these groups is present. When R' and R'' are attached to the same nitrogen atom, they can be combined with the nitrogen atom to form a 5-, 6-, or 7-membered ring. For example, -NR'R'' is meant to include, but not be limited to, 1-pyrrolidinyl and 4-morpholinyl. From the above discussion of substituents, one of skill in the art will understand that the term "alkyl" is meant to include groups including carbon atoms bound to groups other than hydrogen groups, such as haloalkyl (e.g., -CF₃ and -CH₂CF₃) and acyl (e.g., -C(O)CH₃, -C(O)CF₃, -C(O)CH₂OCH₃, and the like).

[0102] Similar to the substituents described for the alkyl radical, substituents for the aryl and heteroaryl groups are generically referred to as "aryl group substituents." The substituents are selected from, for example: halogen, -OR', =O, =NR', =N-OR', -NR'R'', -SR', -halogen, -SiR'R''R''', -OC(O)R', -C(O)R', -CO₂R', -CONR'R'', -OC(O)NR'R'', -NR'C(O)R', -NR'-C(O)NR'R'', -NR'C(O)₂R', -NR-C(NR'R''R''')=NR''', -NR-C(NR'R'')=NR'', -S(O)R', -S(O)₂R', -S(O)₂NR'R'', -NRSO₂R', -CN and -NO₂, -R', -N₃, -CH(Ph)₂, fluoro(C₁-C₄)alkoxy, and fluoro(C₁-C₄)alkyl, in a number ranging from zero to the total number of open valences on the aromatic ring system; and where R', R'', R''' and R'''' are preferably independently selected from hydrogen, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl and substituted or unsubstituted heteroaryl. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R', R'', R''' and R'''' groups when more than one of these groups is present. In the schemes that follow, the symbol X represents "R" as described above.

[0103] Two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula -T-C(O)-(CRR')_u-U-, wherein T and U are independently NR-, -O-, -CRR'- or a single bond, and u is an integer of from 0 to 3. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula A-(CH₂)_r-B-, wherein A and B are independently -CRR'-, -O-, -NR-, -S-, -S(O)-, -S(O)₂-, -S(O)₂NR'- or a single bond, and r is an integer of from 1 to 4. One of the single bonds of the new ring so formed may optionally be replaced with a double bond. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula -(CRR')_z-X-(CR''R''')_d-, where z and d are independently integers of from 0 to 3, and X is -O-, -NR'-, -S-, -S(O)-, -S(O)₂-, or -S(O)₂NR'-. The substituents R, R', R'' and R''' are preferably independently selected from hydrogen or substituted or unsubstituted (C₁-C₆)alkyl.

[0104] As used herein, the term "heteroatom" is meant to include oxygen (O), nitrogen (N), sulfur (S) and silicon (Si).

Introduction

[0105] FGF-9 is a fibroblast growth factor that is expressed as a secreted protein in the brain and the uterine endometrium. The 208 amino acid heparin binding protein is thought to be unglycosylated in its wild-type state. As an autocrine/paracrine growth factor, FGF-9 plays an important role in glial cell development and in the proliferation and activation of other cells expressing FGF receptors, such as those found in the motorneurons and prostate.

[0106] FGF-18 is another member of the family of the FGF family. It is involved in stimulating hepatic and intestinal proliferation and is an essential regulator of bone and cartilage differentiation. Like FGF-9, it too is thought to be unglycosylated in its wild-type state. This 207 amino acid protein is also involved in postnatal lung development through the stimulation of myofibroblast proliferation and differentiation. Induced by calcineurin, FGF-18 has the ability to repress noggin expression and act as an effective neuroprotective agent.

[0107] FGF-20 is a novel fibroblast growth factor that is expressed as a secreted protein in the brain (e.g., cerebellum and substantia nigra pars compacta) and expressed in *E. coli* as a monomer of apparent molecular weight of 23 kDa. This 211 amino acid heparin binding protein is thought to be un-glycosylated in its wild-type state. Its biological activities include neurogenesis, neuroprotection, CNS regeneration, anti-inflammatory effects (e.g., bowel anti-inflammatory

agent) and wound healing, making it a useful agent for treating diseases such as Parkinson's and Alzheimer's. FGF-20 can also be used as a prophylactic or mitigating agent against radiation toxicity to the GI and other parts of the body, *e.g.* arising from chemo- and radiation therapy, nuclear/radiological terrorism, radiation accidents, etc. In several studies, FGF-20 has also demonstrated its effectiveness in preventing and treating oral mucositis, a condition characterized by symptoms ranging from mild erythema to severe painful ulcerations.

[0108] FGF-21, another novel fibroblast growth factor, is expressed in liver, thymic and testicular tissue. The 209 amino acid protein is also thought to be unglycosylated in the wild-type state. In recent studies, FGF-21 was shown to regulate glucose uptake in human fat cells, suggesting its role as a metabolic regulator. Its effect on insulin activity and its regulation of lipidolysis make FGF-21 a useful treatment for type II diabetes and obesity. It has been implicated in various diseases characterized by complete or partial loss of cellular, tissue, or organ function as well as abnormalities in the function or number of cells and/or tissue. FGF-21 also has numerous other therapeutic applications, as will be described below.

[0109] One disease amenable to FGF-21 treatment is ischemic vascular disease. Treatment with the peptide may induce therapeutic angiogenesis or preserve function/survival of cells in patients suffering from diseases such as myocardial ischemia/infarction, peripheral vascular disease, renal artery disease, or stroke, etc.

[0110] Other diseases for which FGF-21 therapy can be useful include cardiomyopathies, which are characterized by loss of function or death of cardiac myocytes or supporting cells in the heart as occurs in, *e.g.* congestive heart failure, myocarditis, and musculoskeletal diseases, which are characterized by loss of function, inadequate function or death of skeletal muscle cells, bone cells or supporting cells, *e.g.* skeletal myopathies, bone disease, and arthritis. In addition, congenital defects in, *e.g.* liver, heart, lung, brain, limbs, kidney, etc., arising from the loss of FGF-21 or its function are treatable with FGF-21.

[0111] FGF-21 polypeptides and polynucleotides can also facilitate the healing of wounds originating from trauma, disease, medical or surgical treatment, and aid in cell and tissue regeneration necessitated by the above circumstances. For example, FGF-21 can effect liver regeneration, operative wound healing, re-endothelialization of injured blood vessels, healing of traumatic wounds, healing of ulcers due to vascular, metabolic disease, etc., bone fractures, loss of cells due to inflammatory disease, etc.

[0112] To improve the effectiveness of recombinant FGF used for therapeutic purposes, the present invention provides conjugates of FGF peptides with a modifying group. Some of the peptides in these FGF peptide conjugates have the same amino acid or nucleotide sequence as the wild-type FGF, while others are mutants.

[0113] The modifying groups can be selected from polymeric modifying groups such as, *e.g.*, PEG (m-PEG), PPG (m-PPG), etc., therapeutic moieties, diagnostic moieties, targeting moieties and the like. Creation of a FGF peptide conjugate, *e.g.*, adding a water-soluble polymeric modifying group, can improve the stability and retention time of FGF in a patient's circulation, and/or reduce the antigenicity of FGF.

[0114] The peptide conjugates of the invention can be formed by the enzymatic attachment of a modified sugar to a glycosylated or unglycosylated peptide. An amino acid glycosylation site and/or a glycosyl group provides a locus for conjugating a modified sugar bearing a modifying group to the peptide, *e.g.*, by glycoconjugation.

[0115] The present invention also provides genetically engineered mutants of Fibroblast Growth Factor that contain N-linked or O-linked glycosylation sites not present in naturally occurring Fibroblast Growth Factor. While these FGF mutants substantially retain the biological activity of the wild-type hormone, the newly introduced glycosylation sites allow the recombinantly produced FGF mutants to be glycosylated in a large variety of patterns.

[0116] The methods of the invention also make it possible to assemble peptide conjugates and glycopeptide conjugates that have a substantially homogeneous derivatization pattern. The enzymes used in the invention are generally selective for a particular amino acid residue, combination of amino acid residues, particular glycosyl residues, or combination of glycosyl residues of the peptide. The methods are also practical for large-scale production of peptide conjugates. Thus, the methods of the invention provide a practical means for large-scale preparation of peptide conjugates having preselected uniform derivatization patterns. The methods are particularly well suited for modification of therapeutic peptides, including but not limited to, glycopeptides that are incompletely glycosylated during production in cell culture cells (*e.g.*, mammalian cells, insect cells, plant cells, fungal cells, yeast cells, or prokaryotic cells) or transgenic plants or animals.

[0117] The FGF peptide conjugates can be included in pharmaceutical formulations comprising a FGF peptide conjugate as well as a pharmaceutically acceptable carrier.

[0118] The present invention also provides conjugates of FGF peptides with increased therapeutic half-life due to, for example, reduced clearance rate, or reduced rate of uptake by the immune or reticuloendothelial system (RES). Moreover, the methods of the invention provide a means for masking antigenic determinants on peptides, thus reducing or eliminating a host immune response against the peptide. Selective attachment of targeting agents can also be used to target a peptide to a particular tissue or cell surface receptor that is specific for the particular targeting agent.

The Mutants

[0119] The present invention provides mutants of FGF that include one or more O- or N-linked glycosylation sites that are not found in the wild type peptide. The mutants are substrates for enzymatic glycosylation at one or more sites that would not normally be glycosylated, or would be poorly glycosylated, in the wild type peptide. Thus, the mutants allow the position of a glycosyl residue or a glycosyl linking group to be engineered to obtain a peptide having selected desirable properties. In addition to the position and number of glycosyl residues or glycosyl linking groups, other properties that can be varied using the mutants and methods of the invention include pharmacokinetics, pharmacodynamics, resistance to proteolysis, immunogenicity, recognition by the reticuloendothelial system, tissue distribution and the like.

[0120] Accordingly, in one aspect, the present invention provides an isolated nucleic acid comprising a polynucleotide sequence encoding a mutant Fibroblast Growth Factor. The mutant Fibroblast Growth Factor comprises an N-linked or O-linked glycosylation site that does not exist in the corresponding wild-type Fibroblast Growth Factor. In some embodiments, the corresponding wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO:1. In some preferred embodiments, the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, and 112-145. In some other embodiments, the wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO:146. In some preferred embodiments, the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from SEQ ID NOs: 161-214, 220-320, and 323-360. In an exemplary embodiment, a peptide having Fibroblast Growth Factor activity has an amino acid sequence that is at least about 95% homologous to the amino acid sequences set forth herein. Preferably, the amino acid sequence is at least about 96%, 97%, 98% or 99% homologous to the amino acid sequences set forth herein.

[0121] In another aspect, the present invention provides an expression cassette or a cell that comprises a nucleic acid, e.g., an isolated nucleic acid, including a polynucleotide sequence encoding a mutant Fibroblast Growth Factor. The mutant Fibroblast Growth Factor includes one or more N-linked or O-linked glycosylation site that does not exist in the corresponding wild-type Fibroblast Growth Factor.

[0122] In another aspect, the present invention provides a mutant Fibroblast Growth Factor, that includes an N-linked or O-linked glycosylation site that does not exist in the corresponding wild-type Fibroblast Growth Factor. In some embodiments, the corresponding wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO:1. In some preferred embodiments, the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, and 112-145. In some other embodiments, the wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO:146. In some preferred embodiments, the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from SEQ ID NOs: 161-214, 220-320, and 323-360. In an exemplary embodiment, a peptide having Fibroblast Growth Factor activity has an amino acid sequence that is at least about 95% homologous to the amino acid sequences set forth herein. Preferably, the amino acid sequence is at least about 96%, 97%, 98% or 99% homologous to the amino acid sequences set forth herein.

[0123] In another aspect, the present invention provides a method for making a mutant Fibroblast Growth Factor that includes an N-linked or O-linked glycosylation site that does not exist in the corresponding wild-type Fibroblast Growth Factor. This method comprises the steps of recombinantly producing the mutant Fibroblast Growth Factor, and glycosylating the mutant Fibroblast Growth Factor at the new glycosylation site. In some embodiments, the corresponding wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO:1. In some preferred embodiments, the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, and 112-145. In some other embodiments, the wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO:146. In some preferred embodiments, the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from SEQ ID NOs: 161-214, 220-320, and 323-360. In an exemplary embodiment, a peptide having Fibroblast Growth Factor activity has an amino acid sequence that is at least about 95% homologous to the amino acid sequences set forth herein. Preferably, the amino acid sequence is at least about 96%, 97%, 98% or 99% homologous to the amino acid sequences set forth herein.

Acquisition of FGF Coding Sequences

General Recombinant Technology

[0124] This invention relies on routine techniques in the field of recombinant genetics. Basic texts disclosing the general methods of use in this invention include Sambrook and Russell, *Molecular Cloning, A Laboratory Manual* (3rd ed. 2001); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and Ausubel et al., eds., *Current Protocols in Molecular Biology* (1994).

[0125] For nucleic acids, sizes are given in either kilobases (kb) or base pairs (bp). These are estimates derived from agarose or acrylamide gel electrophoresis, from sequenced nucleic acids, or from published DNA sequences. For proteins, sizes are given in kilodaltons (kDa) or amino acid residue numbers. Proteins sizes are estimated from gel

electrophoresis, from sequenced proteins, from derived amino acid sequences, or from published protein sequences.

[0126] Oligonucleotides that are not commercially available can be chemically synthesized, *e.g.*, according to the solid phase phosphoramidite triester method first described by Beaucage & Caruthers, *Tetrahedron Lett.* 22: 1859-1862 (1981), using an automated synthesizer, as described in Van Devanter *et al.*, *Nucleic Acids Res.* 12: 6159-6168 (1984).

Purification of oligonucleotides is performed using any art-recognized strategy, *e.g.*, native acrylamide gel electrophoresis or anion-exchange HPLC as described in Pearson & Reanier, *J. Chrom.* 255: 137-149 (1983).

[0127] The sequence of the cloned wild-type Fibroblast Growth Factor genes, polynucleotide encoding mutant Fibroblast Growth Factors, and synthetic oligonucleotides can be verified after cloning using, *e.g.*, the chain termination method for sequencing double-stranded templates of Wallace *et al.*, *Gene* 16: 21-26 (1981).

Cloning and Subcloning of a Wild-Type FGF Coding Sequence

[0128] A number of polynucleotide sequences encoding a wild-type Fibroblast Growth Factor-20, *e.g.*, GenBank Accession No. NM_019851, NM_019113, have been determined and can be obtained from a commercial supplier.

[0129] The rapid progress in the studies of human genome has made possible a cloning approach where a human DNA sequence database can be searched for any gene segment that has a certain percentage of sequence homology to a known nucleotide sequence, such as one encoding a previously identified Fibroblast Growth Factor. Any DNA sequence so identified can be subsequently obtained by chemical synthesis and/or a polymerase chain reaction (PCR) technique such as overlap extension method. For a short sequence, completely *de novo* synthesis may be sufficient; whereas further isolation of full length coding sequence from a human cDNA or genomic library using a synthetic probe may be necessary to obtain a larger gene.

[0130] Alternatively, a nucleic acid sequence encoding a Fibroblast Growth Factor can be isolated from a human cDNA or genomic DNA library using standard cloning techniques such as polymerase chain reaction (PCR), where homology-based primers can often be derived from a known nucleic acid sequence encoding a Fibroblast Growth Factor. Most commonly used techniques for this purpose are described in standard texts, *e.g.*, Sambrook and Russell, *supra*.

[0131] cDNA libraries suitable for obtaining a coding sequence for a wild-type Fibroblast Growth Factor may be commercially available or can be constructed. The general methods of isolating mRNA, making cDNA by reverse transcription, ligating cDNA into a recombinant vector, transfecting into a recombinant host for propagation, screening, and cloning are well known (*see, e.g.*, Gubler and Hoffman, *Gene*, 25: 263-269 (1983); Ausubel *et al.*, *supra*). Upon obtaining an amplified segment of nucleotide sequence by PCR, the segment can be further used as a probe to isolate the full length polynucleotide sequence encoding the wild-type Fibroblast Growth Factor from the cDNA library. A general description of appropriate procedures can be found in Sambrook and Russell, *supra*.

[0132] A similar procedure can be followed to obtain a full length sequence encoding a wild-type Fibroblast Growth Factor, *e.g.*, any one of the GenBank Accession Nos. mentioned above, from a human genomic library. Human genomic libraries are commercially available or can be constructed according to various art-recognized methods. In general, to construct a genomic library, the DNA is first extracted from an tissue where a Fibroblast Growth Factor is likely found. The DNA is then either mechanically sheared or enzymatically digested to yield fragments of about 12-20 kb in length. The fragments are subsequently separated by gradient centrifugation from polynucleotide fragments of undesired sizes and are inserted in bacteriophage λ vectors. These vectors and phages are packaged *in vitro*. Recombinant phages are analyzed by plaque hybridization as described in Benton and Davis, *Science*, 196: 180-182 (1977). Colony hybridization is carried out as described by Grunstein *et al.*, *Proc. Natl. Acad. Sci. USA*, 72: 3961-3965 (1975).

[0133] Based on sequence homology, degenerate oligonucleotides can be designed as primer sets and PCR can be performed under suitable conditions (*see, e.g.*, White *et al.*, *PCR Protocols: Current Methods and Applications*, 1993; Griffin and Griffin, *PCR Technology*, CRC Press Inc. 1994) to amplify a segment of nucleotide sequence from a cDNA or genomic library. Using the amplified segment as a probe, the full length nucleic acid encoding a wild-type Fibroblast Growth Factor is obtained.

[0134] Upon acquiring a nucleic acid sequence encoding a wild-type Fibroblast Growth Factor, the coding sequence can be subcloned into a vector, for instance, an expression vector, so that a recombinant wild-type Fibroblast Growth Factor can be produced from the resulting construct. Further modifications to the wild-type Fibroblast Growth Factor coding sequence, *e.g.*, nucleotide substitutions, may be subsequently made to alter the characteristics of the molecule.

Introducing Mutations into an FGF Sequence

[0135] From an encoding polynucleotide sequence, the amino acid sequence of a wild-type Fibroblast Growth Factor, *e.g.*, SEQ ID NO:1, SEQ ID NO:146, can be determined. Subsequently, this amino acid sequence may be modified to alter the protein's glycosylation pattern, by introducing additional glycosylation site(s) at various locations in the amino acid sequence.

[0136] Several types of protein glycosylation sites are well known in the art. For instance, in eukaryotes, N-linked

glycosylation occurs on the asparagine of the consensus sequence Asn-X_{aa}-Ser/Thr, in which X_{aa} is any amino acid except proline (Kornfeld et al., Ann Rev Biochem 54:631-664 (1985); Kukuruzinska et al., Proc. Natl. Acad. Sci. USA 84:2145-2149 (1987); Herscovics et al., FASEB J 7:540-550 (1993); and Orlean, Saccharomyces Vol. 3 (1996)). O-linked glycosylation takes place at serine or threonine residues (Tanner et al., Biochim. Biophys. Acta. 906:81-91 (1987); and Hounsell et al., Glycoconj. J. 13:19-26 (1996)). Other glycosylation patterns are formed by linking glycosylphosphatidylinositol to the carboxyl-terminal carboxyl group of the protein (Takeda et al., Trends Biochem. Sci. 20:367-371 (1995); and Udenfriend et al., Ann. Rev. Biochem. 64:593-591 (1995). Based on this knowledge, suitable mutations can thus be introduced into a wild-type Fibroblast Growth Factor sequence to form new glycosylation sites.

[0137] Although direct modification of an amino acid residue within a Fibroblast Growth Factor polypeptide sequence may be suitable to introduce a new N-linked or O-linked glycosylation site, more frequently, introduction of a new glycosylation site is accomplished by mutating the polynucleotide sequence encoding a Fibroblast Growth Factor. This can be achieved by using any of known mutagenesis methods, some of which are discussed below. Exemplary modifications to Fibroblast Growth Factor include those illustrated in SEQ ID NO:9 or SEQ ID NO:87.

[0138] A variety of mutation-generating protocols are established and described in the art. See, e.g., Zhang et al., Proc. Natl. Acad. Sci. USA, 94: 4504-4509 (1997); and Stemmer, Nature, 370: 389-391 (1994). The procedures can be used separately or in combination to produce variants of a set of nucleic acids, and hence variants of encoded polypeptides. Kits for mutagenesis, library construction, and other diversity-generating methods are commercially available.

[0139] Mutational methods of generating diversity include, for example, site-directed mutagenesis (Botstein and Shurtle, Science, 229: 1193-1201 (1985)), mutagenesis using uracil-containing templates (Kunkel, Proc. Natl. Acad. Sci. USA, 82: 488-492 (1985)), oligonucleotide-directed mutagenesis (Zoller and Smith, Nucl. Acids Res., 10: 6487-6500 (1982)), phosphorothioate-modified DNA mutagenesis (Taylor et al., Nucl. Acids Res., 13: 8749-8764 and 8765-8787 (1985)), and mutagenesis using gapped duplex DNA (Kramer et al., Nucl. Acids Res., 12: 9441-9456 (1984)).

[0140] Other possible methods for generating mutations include point mismatch repair (Kramer et al., Cell, 38: 879-887 (1984)), mutagenesis using repair-deficient host strains (Carter et al., Nucl. Acids Res., 13: 4431-4443 (1985)), deletion mutagenesis (Eghtedarzadeh and Henikoff, Nucl. Acids Res., 14: 5115 (1986)), restriction-selection and restriction-purification (Wells et al., Phil. Trans. R. Soc. Lond. A, 317: 415-423 (1986)), mutagenesis by total gene synthesis (Nambiar et al., Science, 223: 1299-1301 (1984)), double-strand break repair (Mandecki, Proc. Natl. Acad. Sci. USA, 83: 7177-7181 (1986)), mutagenesis by polynucleotide chain termination methods (U.S. Patent No. 5,965,408), and error-prone PCR (Leung et al., Biotechniques, 1: 11-15 (1989)).

Modification of Nucleic Acids for Preferred Codon Usage in a Host Organism

[0141] The polynucleotide sequence encoding a mutant Fibroblast Growth Factor can be further altered to coincide with the preferred codon usage of a particular host. For example, the preferred codon usage of one strain of bacterial cells can be used to derive a polynucleotide that encodes a mutant Fibroblast Growth Factor of the invention and includes the codons favored by this strain. The frequency of preferred codon usage exhibited by a host cell can be calculated by averaging frequency of preferred codon usage in a large number of genes expressed by the host cell (e.g., calculation service is available from web site of the Kazusa DNA Research Institute, Japan). This analysis is preferably limited to genes that are highly expressed by the host cell. U.S. Patent No. 5,824,864, for example, provides the frequency of codon usage by highly expressed genes exhibited by dicotyledonous plants and monocotyledonous plants.

[0142] At the completion of modification, the mutant Fibroblast Growth Factor coding sequences are verified by sequencing and are then subcloned into an appropriate expression vector for recombinant production in the same manner as the wild-type Fibroblast Growth Factors.

Expression and Purification of the Mutant FGF

[0143] Following sequence verification, the mutant Fibroblast Growth Factor of the present invention can be produced using routine techniques in the field of recombinant genetics, relying on the polynucleotide sequences encoding the polypeptide disclosed herein.

Expression Systems

[0144] To obtain high level expression of a nucleic acid encoding a mutant Fibroblast Growth Factor of the present invention, one typically subclones a polynucleotide encoding the mutant Fibroblast Growth Factor into an expression vector that contains a strong promoter to direct transcription, a transcription/translation terminator and a ribosome binding site for translational initiation. Suitable bacterial promoters are well known in the art and described, e.g., in Sambrook and Russell, *supra*, and Ausubel *et al.*, *supra*. Bacterial expression systems for expressing the wild-type or mutant Fibroblast Growth Factor are available in, e.g., *E. coli*, *Bacillus sp.*, *Salmonella*, and *Caulobacter*. Kits for such expression

systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known in the art and are also commercially available. In one embodiment, the eukaryotic expression vector is an adenoviral vector, an adeno-associated vector, or a retroviral vector.

[0145] The promoter used to direct expression of a heterologous nucleic acid depends on the particular application. The promoter is optionally positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

[0146] In addition to the promoter, the expression vector typically includes a transcription unit or expression cassette that contains all the additional elements required for the expression of the mutant Fibroblast Growth Factor in host cells.

A typical expression cassette thus contains a promoter operably linked to the nucleic acid sequence encoding the mutant Fibroblast Growth Factor and signals required for efficient polyadenylation of the transcript, ribosome binding sites, and translation termination. The nucleic acid sequence encoding the Fibroblast Growth Factor is typically linked to a cleavable signal peptide sequence to promote secretion of the Fibroblast Growth Factor by the transformed cell. Such signal peptides include, among others, the signal peptides from tissue plasminogen activator, insulin, and neuron growth factor, and juvenile hormone esterase of *Heliothis virescens*. Additional elements of the cassette may include enhancers and, if genomic DNA is used as the structural gene, introns with functional splice donor and acceptor sites.

[0147] In addition to a promoter sequence, the expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes.

[0148] The particular expression vector used to transport the genetic information into the cell is not particularly critical. Any of the conventional vectors used for expression in eukaryotic or prokaryotic cells may be used. Standard bacterial expression vectors include plasmids such as pBR322 based plasmids, pSKF, pET23D, and fusion expression systems such as GST and LacZ. Epitope tags can also be added to recombinant proteins to provide convenient methods of isolation, e.g., c-myc.

[0149] Expression vectors containing regulatory elements from eukaryotic viruses are typically used in eukaryotic expression vectors, e.g., SV40 vectors, papilloma virus vectors, and vectors derived from Epstein-Barr virus. Other exemplary eukaryotic vectors include pMSG, pAV009/A⁺, pMTO10/A⁺, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV40 early promoter, SV40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

[0150] Some expression systems have markers that provide gene amplification such as thymidine kinase, hygromycin B phosphotransferase, and dihydrofolate reductase. Alternatively, high yield expression systems not involving gene amplification are also suitable, such as a baculovirus vector in insect cells, with a polynucleotide sequence encoding the mutant Fibroblast Growth Factor under the direction of the polyhedrin promoter or other strong baculovirus promoters.

[0151] The elements that are typically included in expression vectors also include a replicon that functions in *E. coli*, a gene encoding antibiotic resistance to permit selection of bacteria that harbor recombinant plasmids, and unique restriction sites in nonessential regions of the plasmid to allow insertion of eukaryotic sequences. The particular antibiotic resistance gene chosen is not critical, any of the many resistance genes known in the art are suitable. The prokaryotic sequences are optionally chosen such that they do not interfere with the replication of the DNA in eukaryotic cells, if necessary. Similar to antibiotic resistance selection markers, metabolic selection markers based on known metabolic pathways may also be used as a means for selecting transformed host cells.

[0152] When periplasmic expression of a recombinant protein (e.g., a FGF mutant of the present invention) is desired, the expression vector further comprises a sequence encoding a secretion signal, such as the *E. coli* OppA (Periplasmic Oligopeptide Binding Protein) secretion signal or a modified version thereof, which is directly connected to 5' of the coding sequence of the protein to be expressed. This signal sequence directs the recombinant protein produced in cytoplasm through the cell membrane into the periplasmic space. The expression vector may further comprise a coding sequence for signal peptidase 1, which is capable of enzymatically cleaving the signal sequence when the recombinant protein is entering the periplasmic space. More detailed description for periplasmic production of a recombinant protein can be found in, e.g., Gray et al., Gene 39: 247-254 (1985), U.S. Patent Nos. 6,160,089 and 6,436,674.

[0153] As discussed above, a person skilled in the art will recognize that various conservative substitutions can be made to any wild-type or mutant Fibroblast Growth Factor or its coding sequence while still retaining the biological activity of the Fibroblast Growth Factor. Moreover, modifications of a polynucleotide coding sequence may also be made to accommodate preferred codon usage in a particular expression host without altering the resulting amino acid sequence.

Transfection Methods

[0154] Standard transfection methods are used to produce bacterial, mammalian, yeast, insect, or plant cell lines that express large quantities of the mutant Fibroblast Growth Factor, which are then purified using standard techniques (see,

e.g., Colley et al., J. Biol. Chem. 264: 17619-17622 (1989); Guide to Protein Purification, in Methods in Enzymology, vol. 182 (Deutscher, ed., 1990)). Transformation of eukaryotic and prokaryotic cells are performed according to standard techniques (see, e.g., Morrison, J. Bact. 132: 349-351 (1977); Clark-Curtiss & Curtiss, Methods in Enzymology 101: 347-362 (Wu et al., eds, 1983).

5 **[0155]** Any of the well known procedures for introducing foreign nucleotide sequences into host cells may be used. These include the use of calcium phosphate transfection, polybrene, protoplast fusion, electroporation, liposomes, microinjection, plasma vectors, viral vectors and any of the other well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA, or other foreign genetic material into a host cell (see, e.g., Sambrook and Russell, *supra*). It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing at least 10 one gene into the host cell capable of expressing the mutant Fibroblast Growth Factor.

Detection of Expression of Mutant FGF in Host Cells

15 **[0156]** After the expression vector is introduced into appropriate host cells, the transfected cells are cultured under conditions favoring expression of the mutant Fibroblast Growth Factor. The cells are then screened for the expression of the recombinant polypeptide, which is subsequently recovered from the culture using standard techniques (see, e.g., Scopes, Protein Purification: Principles and Practice (1982); U.S. Patent No. 4,673,641; Ausubel *et al.*, *supra*; and Sambrook and Russell, *supra*).

20 **[0157]** Several general methods for screening gene expression are well known among those skilled in the art. First, gene expression can be detected at the nucleic acid level. A variety of methods of specific DNA and RNA measurement using nucleic acid hybridization techniques are commonly used (e.g., Sambrook and Russell, *supra*). Some methods involve an electrophoretic separation (e.g., Southern blot for detecting DNA and Northern blot for detecting RNA), but detection of DNA or RNA can be carried out without electrophoresis as well (such as by dot blot). The presence of nucleic acid encoding a mutant Fibroblast Growth Factor in transfected cells can also be detected by PCR or RT-PCR using 25 sequence-specific primers.

30 **[0158]** Second, gene expression can be detected at the polypeptide level. Various immunological assays are routinely used by those skilled in the art to measure the level of a gene product, particularly using polyclonal or monoclonal antibodies that react specifically with a mutant Fibroblast Growth Factor of the present invention, such as a polypeptide having the amino acid sequence of SEQ ID NO:3, 4, or 5, (e.g., Harlow and Lane, Antibodies, A Laboratory Manual, Chapter 14, Cold Spring Harbor, 1988; Kohler and Milstein, Nature, 256: 495-497 (1975)). Such techniques require antibody preparation by selecting antibodies with high specificity against the mutant Fibroblast Growth Factor or an antigenic portion thereof. The methods of raising polyclonal and monoclonal antibodies are well established and their descriptions can be found in the literature, see, e.g., Harlow and Lane, *supra*; Kohler and Milstein, Eur. J. Immunol., 6: 511-519 (1976). More detailed descriptions of preparing antibody against the mutant Fibroblast Growth Factor of the 35 present invention and conducting immunological assays detecting the mutant Fibroblast Growth Factor are provided in a later section.

Purification of Recombinantly Produced Mutant FGF

40 **[0159]** Once the expression of a recombinant mutant Fibroblast Growth Factor in transfected host cells is confirmed, the host cells are then cultured in an appropriate scale for the purpose of purifying the recombinant polypeptide.

Purification of Recombinantly Produced Mutant FGF from Bacteria

45 **[0160]** When the mutant Fibroblast Growth Factors of the present invention are produced recombinantly by transformed bacteria in large amounts, typically after promoter induction, although expression can be constitutive, the proteins may form insoluble aggregates. There are several protocols that are suitable for purification of protein inclusion bodies. For example, purification of aggregate proteins (hereinafter referred to as inclusion bodies) typically involves the extraction, separation and/or purification of inclusion bodies by disruption of bacterial cells, e.g., by incubation in a buffer of about 50 100-150 µg/ml lysozyme and 0.1 % Nonidet P40, a non-ionic detergent. The cell suspension can be ground using a Polytron grinder (Brinkman Instruments, Westbury, NY). Alternatively, the cells can be sonicated on ice. Alternate methods of lysing bacteria are described in Ausubel *et al.* and Sambrook and Russell, both *supra*, and will be apparent to those of skill in the art.

55 **[0161]** The cell suspension is generally centrifuged and the pellet containing the inclusion bodies resuspended in buffer which does not dissolve but washes the inclusion bodies, e.g., 20 mM Tris-HCl (pH 7.2), 1 mM EDTA, 150 mM NaCl and 2% Triton-X 100, a non-ionic detergent. It may be necessary to repeat the wash step to remove as much cellular debris as possible. The remaining pellet of inclusion bodies may be resuspended in an appropriate buffer (e.g., 20 mM sodium phosphate, pH 6.8, 150 mM NaCl). Other appropriate buffers will be apparent to those of skill in the art.

[0162] Following the washing step, the inclusion bodies are solubilized by the addition of a solvent that is both a strong hydrogen acceptor and a strong hydrogen donor (or a combination of solvents each having one of these properties). The proteins that formed the inclusion bodies may then be renatured by dilution or dialysis with a compatible buffer. Suitable solvents include, but are not limited to, urea (from about 4 M to about 8 M), formamide (at least about 80%,
 5 volume/volume basis), and guanidine hydrochloride (from about 4 M to about 8 M). Some solvents that are capable of solubilizing aggregate-forming proteins, such as SDS (sodium dodecyl sulfate) and 70% formic acid, may be inappropriate for use in this procedure due to the possibility of irreversible denaturation of the proteins, accompanied by a lack of immunogenicity and/or activity. Although guanidine hydrochloride and similar agents are denaturants, this denaturation is not irreversible and renaturation may occur upon removal (by dialysis, for example) or dilution of the denaturant,
 10 allowing re-formation of the immunologically and/or biologically active protein of interest. After solubilization, the protein can be separated from other bacterial proteins by standard separation techniques. For further description of purifying recombinant Fibroblast Growth Factor from bacterial inclusion body, see, e.g., Patra et al., Protein Expression and Purification 18: 182-190 (2000).

[0163] Alternatively, it is possible to purify recombinant polypeptides, e.g., a mutant Fibroblast Growth Factor, from bacterial periplasm. Where the recombinant protein is exported into the periplasm of the bacteria, the periplasmic fraction of the bacteria can be isolated by cold osmotic shock in addition to other methods known to those of skill in the art (see e.g., Ausubel *et al.*, *supra*). To isolate recombinant proteins from the periplasm, the bacterial cells are centrifuged to form a pellet. The pellet is resuspended in a buffer containing 20% sucrose. To lyse the cells, the bacteria are centrifuged and the pellet is resuspended in ice-cold 5 mM MgSO₄ and kept in an ice bath for approximately 10 minutes. The cell
 20 suspension is centrifuged and the supernatant decanted and saved. The recombinant proteins present in the supernatant can be separated from the host proteins by standard separation techniques well known to those of skill in the art.

Standard Protein Separation Techniques for Purification

[0164] When a recombinant polypeptide, e.g., the mutant Fibroblast Growth Factor of the present invention, is expressed in host cells in a soluble form, its purification can follow the standard protein purification procedure described below.

Solubility Fractionation

[0165] Often as an initial step, and if the protein mixture is complex, an initial salt fractionation can separate many of the unwanted host cell proteins (or proteins derived from the cell culture media) from the recombinant protein of interest, e.g., a mutant Fibroblast Growth Factor of the present invention. The preferred salt is ammonium sulfate. Ammonium sulfate precipitates proteins by effectively reducing the amount of water in the protein mixture. Proteins then precipitate
 35 on the basis of their solubility. The more hydrophobic a protein is, the more likely it is to precipitate at lower ammonium sulfate concentrations. A typical protocol is to add saturated ammonium sulfate to a protein solution so that the resultant ammonium sulfate concentration is between 20-30%. This will precipitate the most hydrophobic proteins. The precipitate is discarded (unless the protein of interest is hydrophobic) and ammonium sulfate is added to the supernatant to a concentration known to precipitate the protein of interest. The precipitate is then solubilized in buffer and the excess salt removed if necessary, through either dialysis or diafiltration. Other methods that rely on solubility of proteins, such as cold ethanol precipitation, are well known to those of skill in the art and can be used to fractionate complex protein mixtures.

Size Differential Filtration

[0166] Based on a calculated molecular weight, a protein of greater and lesser size can be isolated using ultrafiltration through membranes of different pore sizes (for example, Amicon or Millipore membranes). As a first step, the protein mixture is ultrafiltered through a membrane with a pore size that has a lower molecular weight cut-off than the molecular weight of a protein of interest, e.g., a mutant Fibroblast Growth Factor. The retentate of the ultrafiltration is then ultrafiltered against a membrane with a molecular cut off greater than the molecular weight of the protein of interest. The recombinant
 50 protein will pass through the membrane into the filtrate. The filtrate can then be chromatographed as described below.

Column Chromatography

[0167] The proteins of interest (such as the mutant Fibroblast Growth Factor of the present invention) can also be separated from other proteins on the basis of their size, net surface charge, hydrophobicity, or affinity for ligands. In addition, antibodies raised against Fibroblast Growth Factor can be conjugated to column matrices and the Fibroblast Growth Factor immunopurified. All of these methods are well known in the art.

[0168] It will be apparent to one of skill that chromatographic techniques can be performed at any scale and using

equipment from many different manufacturers (e.g., Pharmacia Biotech).

Immunoassays for Detection of Mutant FGF Expression

[0169] To confirm the production of a recombinant mutant Fibroblast Growth Factor, immunological assays may be useful to detect in a sample the expression of the polypeptide. Immunological assays are also useful for quantifying the expression level of the recombinant hormone. Antibodies against a mutant Fibroblast Growth Factor are necessary for carrying out these immunological assays.

Production of Antibodies against Mutant FGF

[0170] Methods for producing polyclonal and monoclonal antibodies that react specifically with an immunogen of interest are known to those of skill in the art (see, e.g., Coligan, Current Protocols in Immunology Wiley/Greene, NY, 1991; Harlow and Lane, Antibodies: A Laboratory Manual Cold Spring Harbor Press, NY, 1989; Stites et al. (eds.) Basic and Clinical Immunology (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Goding, Monoclonal Antibodies: Principles and Practice (2d ed.) Academic Press, New York, NY, 1986; and Kohler and Milstein Nature 256: 495-497, 1975). Such techniques include antibody preparation by selection of antibodies from libraries of recombinant antibodies in phage or similar vectors (see, Huse et al., Science 246: 1275-1281, 1989; and Ward et al., Nature 341: 544-546, 1989).

[0171] In order to produce antisera containing antibodies with desired specificity, the polypeptide of interest (e.g., a mutant Fibroblast Growth Factor of the present invention) or an antigenic fragment thereof can be used to immunize suitable animals, e.g., mice, rabbits, or primates. A standard adjuvant, such as Freund's adjuvant, can be used in accordance with a standard immunization protocol. Alternatively, a synthetic antigenic peptide derived from that particular polypeptide can be conjugated to a carrier protein and subsequently used as an immunogen.

[0172] The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the antigen of interest. When appropriately high titers of antibody to the antigen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich antibodies specifically reactive to the antigen and purification of the antibodies can be performed subsequently, see, Harlow and Lane, *supra*, and the general descriptions of protein purification provided above.

[0173] Monoclonal antibodies are obtained using various techniques familiar to those of skill in the art. Typically, spleen cells from an animal immunized with a desired antigen are immortalized, commonly by fusion with a myeloma cell (see, Kohler and Milstein, Eur. J. Immunol. 6:511-519, 1976). Alternative methods of immortalization include, e.g., transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods well known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and the yield of the monoclonal antibodies produced by such cells may be enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate host.

[0174] Additionally, monoclonal antibodies may also be recombinantly produced upon identification of nucleic acid sequences encoding an antibody with desired specificity or a binding fragment of such antibody by screening a human B cell cDNA library according to the general protocol outlined by Huse *et al.*, *supra*. The general principles and methods of recombinant polypeptide production discussed above are applicable for antibody production by recombinant methods.

[0175] When desired, antibodies capable of specifically recognizing a mutant Fibroblast Growth Factor of the present invention can be tested for their cross-reactivity against the wild-type Fibroblast Growth Factor and thus distinguished from the antibodies against the wild-type protein. For instance, antisera obtained from an animal immunized with a mutant Fibroblast Growth Factor can be run through a column on which a wild-type Fibroblast Growth Factor is immobilized. The portion of the antisera that passes through the column recognizes only the mutant Fibroblast Growth Factor and not the wild-type Fibroblast Growth Factor. Similarly, monoclonal antibodies against a mutant Fibroblast Growth Factor can also be screened for their exclusivity in recognizing only the mutant but not the wild-type Fibroblast Growth Factor.

[0176] Polyclonal or monoclonal antibodies that specifically recognize only the mutant Fibroblast Growth Factor of the present invention but not the wild-type Fibroblast Growth Factor are useful for isolating the mutant protein from the wild-type protein, for example, by incubating a sample with a mutant Fibroblast Growth Factor-specific polyclonal or monoclonal antibody immobilized on a solid support.

Immunoassays for Detecting Mutant FGF Expression

[0177] Once antibodies specific for a mutant Fibroblast Growth Factor of the present invention are available, the amount of the polypeptide in a sample, e.g., a cell lysate, can be measured by a variety of immunoassay methods providing qualitative and quantitative results to a skilled artisan. For a review of immunological and immunoassay pro-

cedures in general see, *e.g.*, Stites, *supra*; U.S. Patent Nos. 4,366,241; 4,376,110; 4,517,288; and 4,837,168.

Glycosylation and Glycoconjugation of the Mutant FGF

Glycosylation and Glycoconjugation by Enzymatic Methods

[0178] Post-expression *in vitro* modification of peptides is an attractive strategy to remedy the deficiencies of methods that rely on controlling glycosylation by engineering expression systems; including both modification of glycan structures or introduction of glycans at novel sites. A comprehensive arsenal of enzymes that transfer saccharide donor moieties is becoming available, making *in vitro* enzymatic synthesis of glycoconjugates with custom designed glycosylation patterns and glycosyl structures possible. See, for example, U.S. Patent Nos. 5,876,980; 6,030,815; 5,728,554; 5,922,577; and published patent applications WO 98/31826; WO 01/88117; WO 03/031464; WO 03/046150; WO 03/045980; WO 03/093448; WO 04/009838; US2002/142370; US2003/040037; US2003/180835; US2004/063911; US2003/207406; and US2003/124645, each of which is incorporated herein by reference.

[0179] The invention provides methods for preparing conjugates of glycosylated and unglycosylated mutant Fibroblast Growth Factors, which have glycosylation sites that do not exist in the corresponding wild-type FGF. Such conjugation may take place directly on the appropriate sugar units of a glycosylated mutant FGF, or following the removal (*i.e.*, "trimming back") of any undesired sugar units. The conjugates are formed between peptides and diverse species such as water-soluble polymers, therapeutic moieties, diagnostic moieties, targeting moieties and the like. Also provided are conjugates that include two or more peptides linked together through a linker arm, *i.e.*, multifunctional conjugates. The multi-functional conjugates of the invention can include two or more copies of the same peptide or a collection of diverse peptides with different structures, and/or properties.

[0180] The conjugates of the invention are formed by the enzymatic attachment of a modified sugar to the glycosylated or unglycosylated peptide. The modified sugar, when interposed between the peptide and the modifying group on the sugar becomes what is referred to herein as "an glycosyl linking group." Using the exquisite selectivity of enzymes, such as glycosyltransferases, the present method provides peptides that bear a desired group at one or more specific locations. Thus, according to the present invention, a modified sugar is attached directly to a selected locus on the peptide chain or, alternatively, the modified sugar is appended onto a carbohydrate moiety of a glycopeptide. Peptides in which modified sugars are bound to both a glycopeptide carbohydrate and directly to an amino acid residue of the peptide backbone are also within the scope of the present invention.

[0181] In contrast to known chemical and enzymatic peptide elaboration strategies, the methods of the invention, make it possible to assemble peptides and glycopeptides that have a substantially homogeneous derivatization pattern; the enzymes used in the invention are generally selective for a particular amino acid residue or combination of amino acid residues of the peptide. The methods are also practical for large-scale production of modified peptides and glycopeptides. Thus, the methods of the invention provide a practical means for large-scale preparation of glycopeptides having preselected uniform derivatization patterns. The methods are particularly well suited for modification of therapeutic peptides, including but not limited to, glycopeptides that are incompletely glycosylated during production in cell culture cells (*e.g.*, mammalian cells, insect cells, plant cells, fungal cells, yeast cells, or prokaryotic cells) or transgenic plants or animals.

[0182] The methods of the invention also provide conjugates of glycosylated and unglycosylated peptides with increased therapeutic half-life due to, for example, reduced clearance rate, or reduced rate of uptake by the immune or reticuloendothelial system (RES). Moreover, the methods of the invention provide a means for masking antigenic determinants on peptides, thus reducing or eliminating a host immune response against the peptide. Selective attachment of targeting agents can also be used to target a peptide to a particular tissue or cell surface receptor that is specific for the particular targeting agent.

Peptide Conjugates

[0183] In another aspect, the present invention provides a conjugate between a modified sugar and a FGF peptide. The FGF peptide in these cases may have the same sequence as a wild-type peptide, or it may be a mutant peptide. A peptide conjugate can have one of several forms. In an exemplary embodiment, a peptide conjugate can comprise a FGF peptide and a modifying group linked to an amino acid of the peptide through a glycosyl linking group.

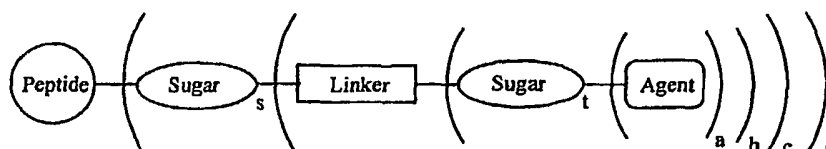
[0184] In another exemplary embodiment, a Fibroblast Growth Factor (FGF) peptide conjugate can comprise a FGF peptide and a glycosyl group attached to an amino acid residue of the FGF peptide. In another exemplary embodiment, the FGF peptide is a member selected from FGF-1, FGF-2, FGF-9, FGF-18, FGF-20 and FGF-21. In another exemplary embodiment, the FGF peptide comprises at least one amino acid sequence which is a member selected from SEQ IDNOs: 1, 9-14, 18-45, 48-65, 69-109, 112-145 and 146.

[0185] In an exemplary embodiment, the glycosyl group is an intact glycosyl linking group. In another exemplary

embodiment, the glycosyl group further comprises a modifying group. In another exemplary embodiment, the modifying group is a non-glycosidic modifying group. In another exemplary embodiment, the modifying group does not include a naturally occurring saccharide moiety.

[0186] In another exemplary embodiment, the peptide conjugate can comprise a FGF peptide and a glycosyl linking group which is bound to both a glycopeptide carbohydrate and directly to an amino acid residue of the peptide backbone. In yet another exemplary embodiment, a peptide conjugate can comprise a FGF peptide and a modifying group linked directly to an amino acid residue of the peptide. In this embodiment, the peptide conjugate may not comprise a glycosyl group. In any of these embodiments, the FGF peptide may or not be glycosylated. The present invention also encompasses a method for the modification of the glycan structure on FGF, providing a conjugate between FGF and a modifying group.

[0187] The conjugates of the invention will typically correspond to the general structure:



in which the symbols a, b, c, d and s represent a positive, non-zero integer; and t is either 0 or a positive integer. The "agent" is a therapeutic agent, a bioactive agent, a detectable label, water-soluble moiety (e.g., PEG, m-PEG, PPG, and m-PPG) or the like. The "agent" can be a peptide, e.g., enzyme, antibody, antigen, etc. The linker can be any of a wide array of linking groups, *infra*. Alternatively, the linker may be a single bond or a "zero order linker."

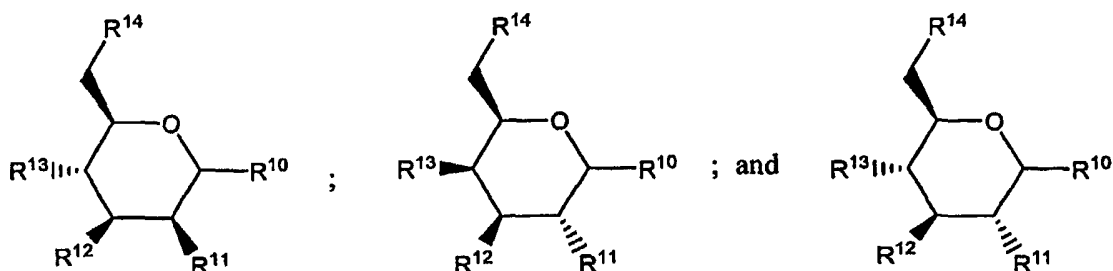
[0188] In the discussion that follows, the invention is illustrated by reference to the use of selected FGF peptides, such as FGF-20 and FGF-21. Those of skill in the art will recognize that the focus of the discussion is for clarity of illustration and that any FGF peptide, either wild-type or mutant, can be used to form these conjugates.

Modified Sugar

[0189] In an exemplary embodiment, the peptides of the invention are reacted with a modified sugar, thus forming a peptide conjugate. A modified sugar comprises a "sugar donor moiety" as well as a "sugar transfer moiety". The sugar donor moiety is any portion of the modified sugar that will be attached to the peptide, either through a glycosyl moiety or amino acid moiety, as a conjugate of the invention. The sugar donor moiety includes those atoms that are chemically altered during their conversion from the modified sugar to the glycosyl linking group of the peptide conjugate. The sugar transfer moiety is any portion of the modified sugar that will not be attached to the peptide as a conjugate of the invention. For example, a modified sugar of the invention is the PEGylated sugar nucleotide, CMP-SA-PEG. For CMP-SA-PEG, the sugar donor moiety, or PEG-sialyl donor moiety, comprises PEG-sialic acid while the sugar transfer moiety, or sialyl transfer moiety, comprises CMP.

[0190] In modified sugars of use in the invention, the saccharyl moiety is preferably a saccharide, a deoxy-saccharide, an amino-saccharide, or an N-acyl saccharide. The term "saccharide" and its equivalents, "saccharyl," "sugar," and "glycosyl" refer to monomers, dimers, oligomers and polymers. The sugar moiety is also functionalized with a modifying group. The modifying group is conjugated to the saccharyl moiety, typically, through conjugation with an amine, sulfhydryl or hydroxyl, e.g., primary hydroxyl, moiety on the sugar. In an exemplary embodiment, the modifying group is attached through an amine moiety on the sugar, e.g., through an amide, a urethane or a urea that is formed through the reaction of the amine with a reactive derivative of the modifying group.

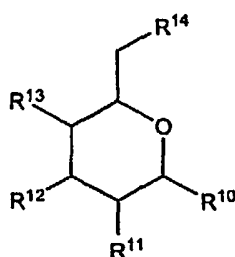
[0191] Any saccharyl moiety can be utilized as the sugar donor moiety of the modified sugar. The saccharyl moiety can be a known sugar, such as mannose, galactose or glucose, or a species having the stereochemistry of a known sugar. The general formulae of these modified sugars are:



[0192] Other saccharyl moieties that are useful in forming the compositions of the invention include, but are not limited to fucose and sialic acid, as well as amino sugars such as glucosamine, galactosamine, mannosamine, the 5-amine analogue of sialic acid and the like. The saccharyl moiety can be a structure found in nature or it can be modified to provide a site for conjugating the modifying group. For example, in one embodiment, the modified sugar provides a sialic acid derivative in which the 9-hydroxy moiety is replaced with an amine. The amine is readily derivatized with an activated analogue of a selected modifying group.

[0193] Examples of modified sugars of use in the invention are described in PCT Patent Application No. PCT/US05/002522, which is herein incorporated by reference.

[0194] In a further exemplary embodiment, the invention utilizes modified sugars in which the 6-hydroxyl position is converted to the corresponding amine moiety, which bears a linker-modifying group cassette such as those set forth above. Exemplary glycosyl groups that can be used as the core of these modified sugars include Gal, GalNAc, Glc, GlcNAc, Fuc, Xyl, Man, and the like. A representative modified sugar according to this embodiment has the formula:



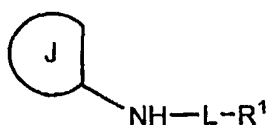
in which R^{11} - R^{14} are members independently selected from H, OH, $C(O)CH_3$, NH, and $NH\ C(O)CH_3$. R^{10} is a link to another glycosyl residue (-O-glycosyl) or to an amino acid of the Factor VII and/or Factor VIIa peptide (-NH-(Factor VII and/or Factor VIIa)). R^{14} is OR^1 , NHR^1 or $NH-L-R^1$. R^1 and $NH-L-R^1$ are as described above.

Glycosyl Linking Groups

[0195] In an exemplary embodiment, the invention provides a peptide conjugate formed between a modified sugar of the invention and a FGF peptide. In this embodiment, the sugar donor moiety (such as the saccharyl moiety and the modifying group) of the modified sugar becomes a "glycosyl linking group". The "glycosyl linking group" can alternatively refer to the glycosyl moiety which is interposed between the peptide and the modifying group.

[0196] Due to the versatility of the methods available for adding and/or modifying glycosyl residues on a peptide, the glycosyl linking groups can have substantially any structure. In the discussion that follows, the invention is illustrated by reference to the use of selected derivatives of furanose and pyranose. Those of skill in the art will recognize that the focus of the discussion is for clarity of illustration and that the structures and compositions set forth are generally applicable across the genus of glycosyl linking groups and modified sugars. The glycosyl linking group can comprise virtually any mono- or oligo-saccharide. The glycosyl linking groups can be attached to an amino acid either through the side chain or through the peptide backbone. Alternatively the glycosyl linking groups can be attached to the peptide through a saccharyl moiety. This saccharyl moiety can be a portion of an O-linked or N-linked glycan structure on the peptide.

[0197] In an exemplary embodiment, the invention utilizes a glycosyl linking group that has the formula:



in which J is a glycosyl moiety, L is a bond or a linker and R^1 is a modifying group, e.g., a polymeric modifying group. Exemplary bonds are those that are formed between an NH_2 moiety on the glycosyl moiety and a group of complementary reactivity on the modifying group. For example, when R^1 includes a carboxylic acid moiety, this moiety may be activated and coupled with the NH_2 moiety on the glycosyl residue affording a bond having the structure $NHC(O)R^1$. J is preferably a glycosyl moiety that is "intact", not having been degraded by exposure to conditions that cleave the pyranose or furanose structure, e.g. oxidative conditions, e.g., sodium periodate.

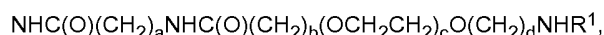
[0198] Exemplary linkers include alkyl and heteroalkyl moieties. The linkers include linking groups, for example acyl-based linking groups, e.g., $-C(O)NH-$, $-OC(O)NH-$, and the like. The linking groups are bonds formed between components of the species of the invention, e.g., between the glycosyl moiety and the linker (L), or between the linker and the modifying

group (R^1). Other exemplary linking groups are ethers, thioethers and amines. For example, in one embodiment, the linker is an amino acid residue, such as a glycine residue. The carboxylic acid moiety of the glycine is converted to the corresponding amide by reaction with an amine on the glycosyl residue, and the amine of the glycine is converted to the corresponding amide or urethane by reaction with an activated carboxylic acid or carbonate of the modifying group.

[0199] An exemplary species of $NH-L-R^1$ has the formula:

$-NH\{C(O)(CH_2)_aNH\}_s\{C(O)(CH_2)_b(OCH_2CH_2)_cO(CH_2)_dNH\}_tR^1$, in which the indices s and t are independently 0 or 1. The indices a , b and d are independently integers from 0 to 20, and c is an integer from 1 to 2500. Other similar linkers are based on species in which an $-NH$ moiety is replaced by another group, for example, $-S$, $-O$ or $-CH_2$. As those of skill will appreciate one or more of the bracketed moieties corresponding to indices s and t can be replaced with a substituted or unsubstituted alkyl or heteroalkyl moiety.

[0200] More particularly, the invention utilizes compounds in which $NH-L-R^1$ is:

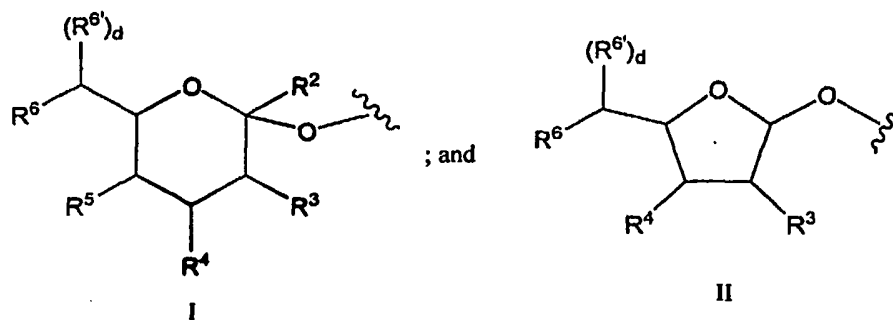


$NHC(O)(CH_2)_b(OCH_2CH_2)_cO(CH_2)_dNHR^1$, $NHC(O)O(CH_2)_b(OCH_2CH_2)_cO(CH_2)_dNHR^1$, $NH(CH_2)_aNHC(O)(CH_2)_b(OCH_2CH_2)_cO(CH_2)_dNHR^1$, $NHC(O)(CH_2)_aNHR^1$, $NH(CH_2)_aNHR^1$, and NHR^1 . In these formulae, the indices a , b and d are independently selected from the integers from 0 to 20, preferably from 1 to 5. The index c is an integer from 1 to about 2500.

[0201] In an exemplary embodiment, c is selected such that the PEG moiety is approximately 1 kD, 5 kD, 10 kD, 15 kD, 20 kD, 25 kD, 30 kD, 35 kD, 40 kD, 45 kD, 50 kD, 55 kD, 60 kD or 65 kD.

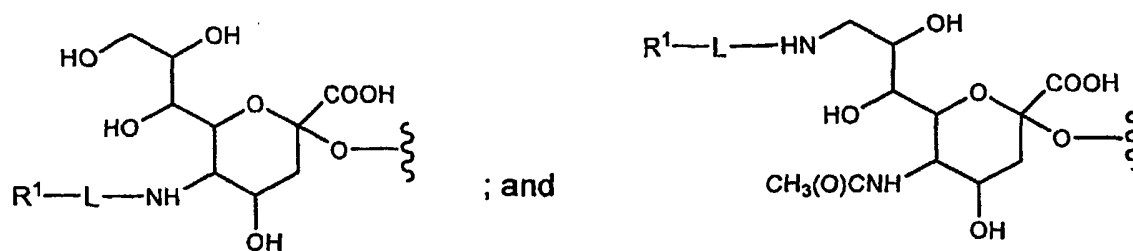
[0202] For the purposes of convenience, the glycosyl linking groups in the remainder of this section will be based on a sialyl moiety. However, one of skill in the art will recognize that another glycosyl moiety, such as mannosyl, galactosyl, glucosyl, or fucosyl, could be used in place of the sialyl moiety.

[0203] In an exemplary embodiment, the glycosyl linking group is an intact glycosyl linking group, in which the glycosyl moiety or moieties forming the linking group are not degraded by chemical (e.g., sodium metaperiodate) or enzymatic (e.g., oxidase) processes. Selected conjugates of the invention include a modifying group that is attached to the amine moiety of an amino-saccharide, e.g., mannosamine, glucosamine, galactosamine, sialic acid etc. In an exemplary embodiment, the invention provides a peptide conjugate comprising an intact glycosyl linking group having a formula that is selected from:



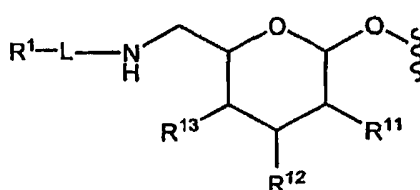
[0204] In Formulae I R^2 is H , CH_2OR^7 , $COOR^7$ or OR^7 , in which R^7 represents H , substituted or unsubstituted alkyl or substituted or unsubstituted heteroalkyl. When $COOR^7$ is a carboxylic acid or carboxylate, both forms are represented by the designation of the single structure COO^- or $COOH$. In Formulae I and II, the symbols R^3 , R^4 , R^5 , R^6 and R^6' independently represent H , substituted or unsubstituted alkyl, OR^8 , $NHC(O)R^9$. The index d is 0 or 1. R^8 and R^9 are independently selected from H , substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, sialic acid or polysialic acid. At least one of R^3 , R^4 , R^5 , R^6 or R^6' includes a modifying group. This modifying group can be a polymeric modifying moiety e.g., PEG, linked through a bond or a linking group. In an exemplary embodiment, R^6 and R^6' , together with the carbon to which they are attached are components of the pyruvyl side chain of sialic acid. In a further exemplary embodiment, the pyruvyl side chain is functionalized with the polymeric modifying group. In another exemplary embodiment, R^6 and R^6' , together with the carbon to which they are attached are components of the side chain of sialic acid and the polymeric modifying group is a component of R^5 .

[0205] Exemplary modifying group-intact glycosyl linking group cassettes according to this motif are based on a sialic acid structure, such as those having the formulae:



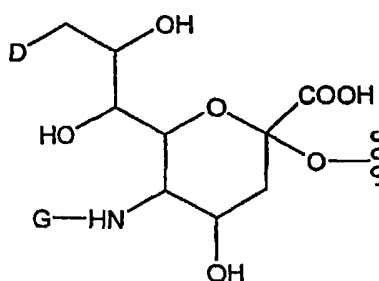
[0206] In the formulae above, R^1 and L are as described above. Further detail about the structure of exemplary R^1 groups is provided below.

[0207] In still a further exemplary embodiment, the conjugate is formed between a peptide and a modified sugar in which the modifying group is attached through a linker at the 6-carbon position of the modified sugar. Thus, illustrative glycosyl linking groups according to this embodiment have the formula:



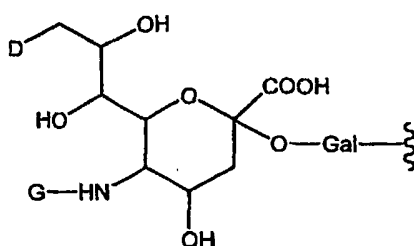
in which the radicals are as discussed above. Glycosyl linking groups include, without limitation, glucose, glucosamine, N-acetyl-glucosamine, galactose, galactosamine, N-acetylgalactosamine, mannose, mannosamine, N-acetyl-mannosamine, and the like.

[0208] In one embodiment, the present invention provides a peptide conjugate comprising the following glycosyl linking group:

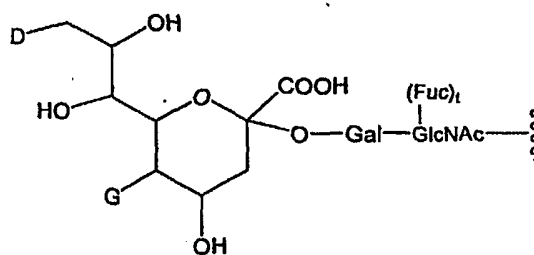


wherein D is a member selected from -OH and R^1 -L-NH-; G is a member selected from H and R^1 -L- and $-C'(O)(C_1-C_6)$ alkyl; R^1 is a moiety comprising a straight-chain or branched poly(ethylene glycol) residue; and L is a linker, e.g., a bond ("zero order"), substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl. In exemplary embodiments, when D is OH, G is R^1 -L-, and when G is $-C(O)(C_1-C_6)$ alkyl, D is R^1 -L-NH-.

[0209] The invention provides a peptide conjugate that includes a glycosyl linking group having the formula:

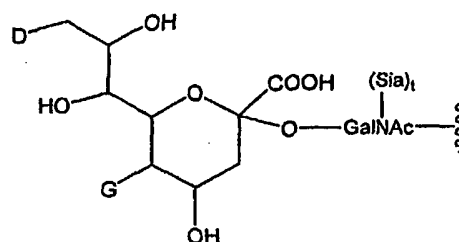


[0210] In other embodiments, the glycosyl linking group has the formula:



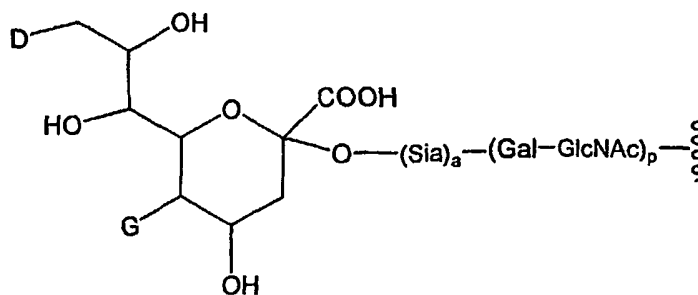
in which the index t is 0 or 1.

[0211] In a still further exemplary embodiment, the glycosyl linking group has the formula:



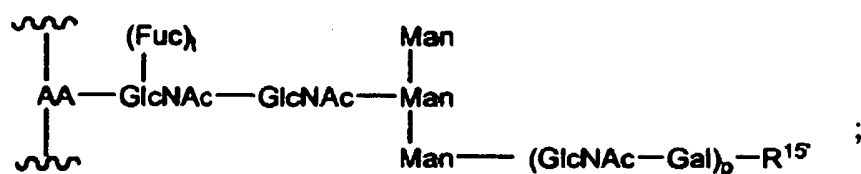
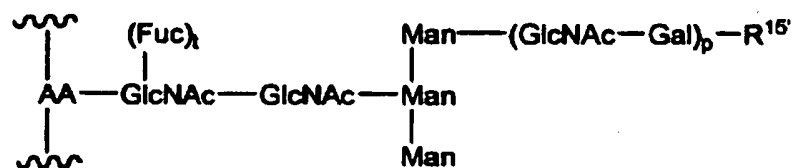
in which the index t is 0 or 1.

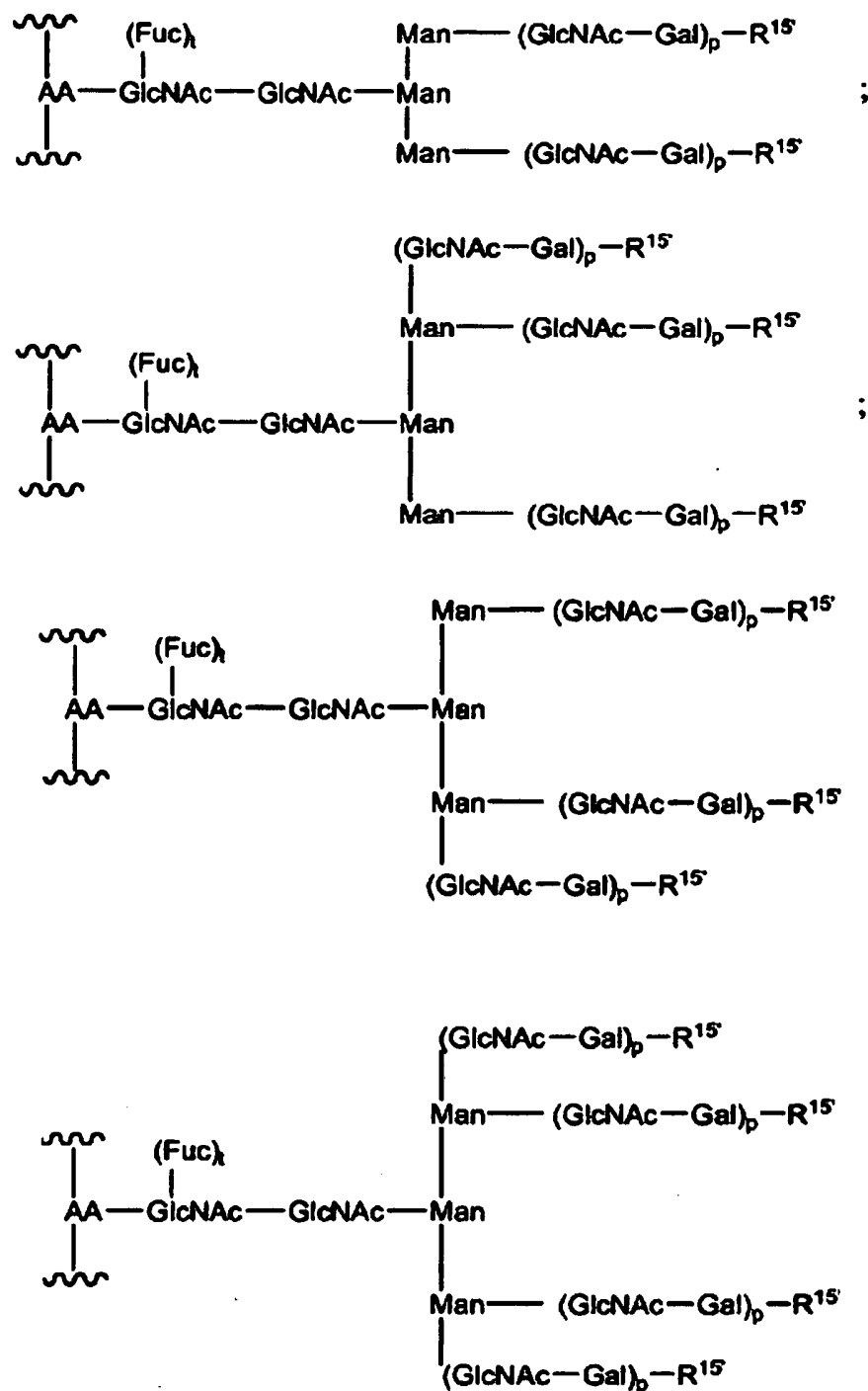
[0212] In yet another embodiment, the glycosyl linking group has the formula:



in which the index p represents an integer from 1 to 10; and a is either 0 or 1.

[0213] In an exemplary embodiment, a glycoPEGylated peptide conjugate of the invention selected from the formulae set forth below:

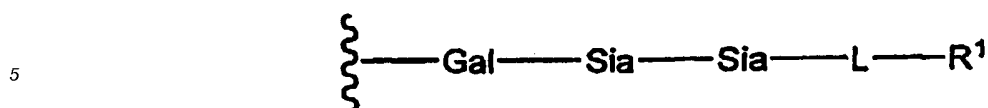




[0214] In the formulae above, the index t is an integer from 0 to 1 and the index p is an integer from 1 to 10. The symbol R^{15} represents H, OH (e.g., Gal-OH), a sialyl moiety, a sialyl linking group (i.e., sialyl linking group-polymeric modifying group (Sia-L- R^1), or a sialyl moiety to which is bound a polymer modified sialyl moiety (e.g., Sia-Sia-L- R^1) ("Sia-Sia P "). Exemplary polymer modified saccharyl moieties have a structure according to Formulae I and II. An exemplary peptide conjugate of the invention will include at least one glycan having a R^{15} that includes a structure according to Formulae I or II. The oxygen, with the open valence, of Formulae I and II is preferably attached through a glycosidic linkage to a carbon of a Gal or GalNAc moiety. In a further exemplary embodiment, the oxygen is attached to the carbon at position 3 of a galactose residue. In an exemplary embodiment, the modified sialic acid is linked $\alpha 2,3$ -to the galactose residue. In another exemplary embodiment, the sialic acid is linked $\alpha 2,6$ -to the galactose residue.

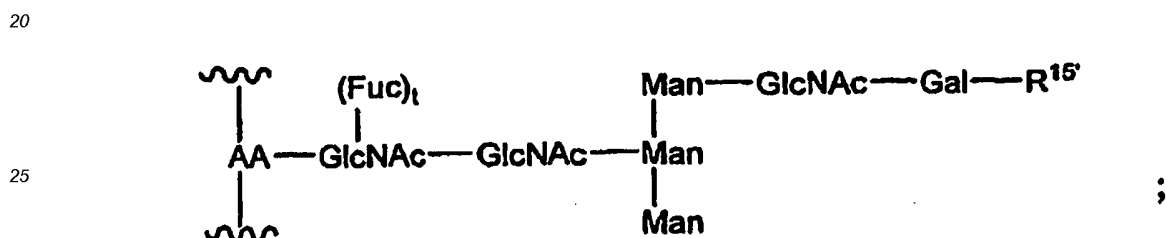
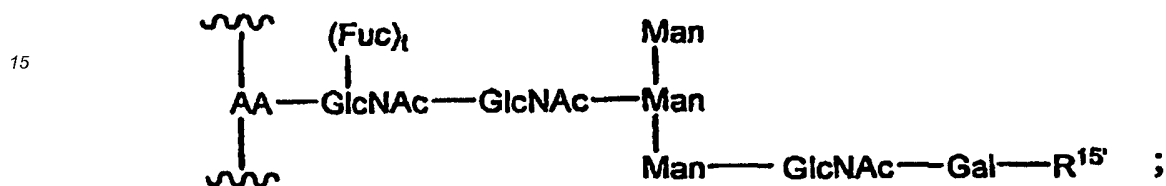
[0215] In an exemplary embodiment, the sialyl linking group is a sialyl moiety to which is bound a polymer modified sialyl moiety (e.g., Sia-Sia-L- R^1) ("Sia-Sia P "). Here, the glycosyl linking group is linked to a galactosyl moiety through a

sialyl moiety:

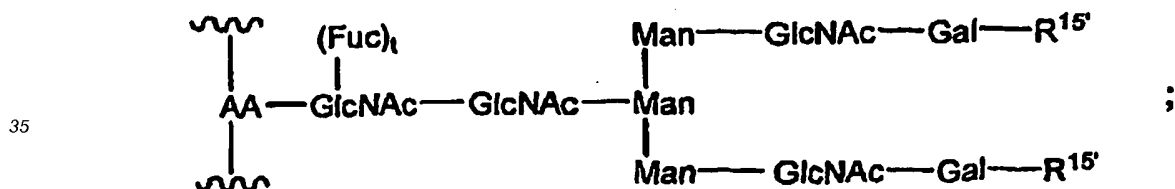


[0216] An exemplary species according to this motif is prepared by conjugating Sia-L-R¹ to a terminal sialic acid of a glycan using an enzyme that forms Sia-Sia bonds, e.g., CST-II, ST8Sia-II, ST8Sia-III and ST8Sia-IV.

10 [0217] In another exemplary embodiment, the glycans on the peptide conjugates have a formula that is selected from the group:



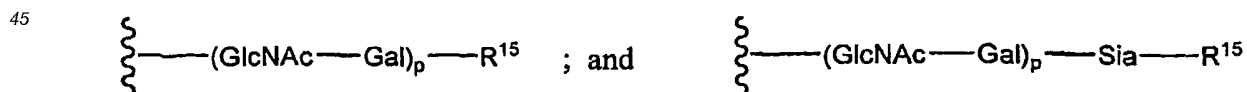
and



and combinations thereof.

40 [0218] In each of the formulae above, R^{15'} is as discussed above. Moreover, an exemplary peptide conjugate of the invention will include at least one glycan with an R¹⁵ moiety having a structure according to Formulae I or II.

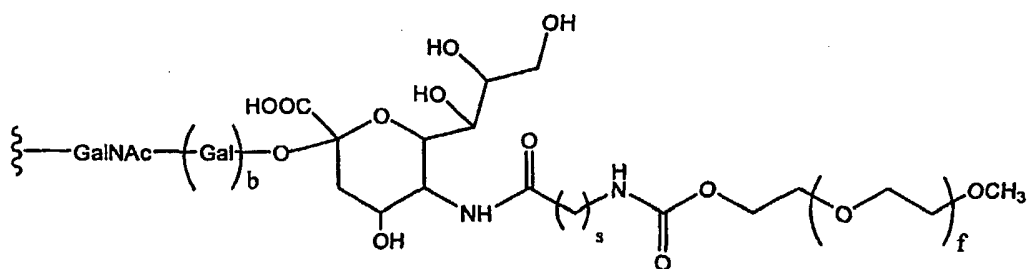
[0219] In another exemplary embodiment, the glycosyl linking group comprises at least one glycosyl linking group having the formula:



wherein R¹⁵ is said sialyl linking group; and the index p is an integer selected from 1 to 10.

50 [0220] In an exemplary embodiment, the glycosyl linking moiety has the formula:

55



in which b is an integer from 0 to 1. The index s represents an integer from 1 to 10; and the index f represents an integer from 1 to 2500.

[0221] In an exemplary embodiment, the polymeric modifying group is PEG. In another exemplary embodiment, the PEG moiety has a molecular weight of about 20 kDa. In another exemplary embodiment, the PEG moiety has a molecular weight of about 5 kDa. In another exemplary embodiment, the PEG moiety has a molecular weight of about 10 kDa. In another exemplary embodiment, the PEG moiety has a molecular weight of about 40 kDa.

[0222] In an exemplary embodiment, the glycosyl linking group is a linear 10 kDa-PEG-sialyl, and one or two of these glycosyl linking groups are covalently attached to the peptide. In an exemplary embodiment, the glycosyl linking group is a linear 20 kDa-PEG-sialyl, and one or two of these glycosyl linking groups are covalently attached to the peptide. In an exemplary embodiment, the glycosyl linking group is a linear 5 kDa-PEG-sialyl, and one, two or three of these glycosyl linking groups are covalently attached to the peptide. In an exemplary embodiment, the glycosyl linking group is a linear 40 kDa-PEG-sialyl, and one or two of these glycosyl linking groups are covalently attached to the peptide.

Modifying Groups

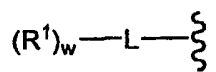
[0223] The peptide conjugates of the invention comprise a modifying group. This group can be covalently attached to a FGF peptide through an amino acid or a glycosyl linking group. "Modifying groups" can encompass a variety of structures including targeting moieties, therapeutic moieties, biomolecules. Additionally, "modifying groups" include polymeric modifying groups, which are polymers which can alter a property of the peptide such as its bioavailability or its half-life in the body.

[0224] In an exemplary embodiment, the modifying group is a targeting agent that localizes selectively in a particular tissue due to the presence of a targeting agent as a component of the conjugate. In an exemplary embodiment, the targeting agent is a protein. Exemplary proteins include transferrin (brain, blood pool), HS-glycoprotein (bone, brain, blood pool), antibodies (brain, tissue with antibody-specific antigen, blood pool), coagulation factors V-XII (damaged tissue, clots, cancer, blood pool), serum proteins, e.g., α -acid glycoprotein, fetuin, α -fetal protein (brain, blood pool), β 2-glycoprotein (liver, atherosclerosis plaques, brain, blood pool), G-CSF, GM-CSF, M-CSF, and EPO (immune stimulation, cancers, blood pool, red blood cell overproduction, neuroprotection), albumin (increase in half-life), and lipoprotein E.

[0225] For the purposes of convenience, the modifying groups in the remainder of this section will be largely based on polymeric modifying groups such as water soluble and water insoluble polymers. However, one of skill in the art will recognize that other modifying groups, such as targeting moieties, therapeutic moieties and biomolecules, could be used in place of the polymeric modifying groups.

Linkers of the Modifying Groups

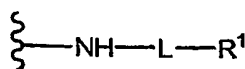
[0226] The linkers of the modifying group serve to attach the modifying group (ie polymeric modifying groups, targeting moieties, therapeutic moieties and biomolecules) to the peptide. In an exemplary embodiment, the polymeric modifying group is bound to a glycosyl linking group, generally through a heteroatom, e.g, nitrogen, on the core through a linker, L, as shown below:



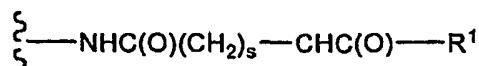
[0227] R^1 is the polymeric moiety and L is selected from a bond and a linking group. The index w represents an integer selected from 1-6, preferably 1-3 and more preferably 1-2. Exemplary linking groups include substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl moieties and sialic acid. An exemplary component of the linker is an acyl moiety.

[0228] An exemplary compound according to the invention has a structure according to Formulae I or II above, in

which at least one of R^2 , R^3 , R^4 , R^5 , R^6 or $R^{6'}$ has the formula:

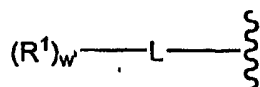


[0229] In another example according to this embodiment at least one of R^2 , R^3 , R^4 , R^5 , R^6 or $R^{6'}$ has the formula:



in which s is an integer from 0 to 20 and R^1 is a linear polymeric modifying moiety.

[0230] In an exemplary embodiment, the polymeric modifying group-linker construct is a branched structure that includes two or more polymeric chains attached to central moiety. In this embodiment, the construct has the formula:



in which R^1 and L are as discussed above and w is an integer from 2 to 6, preferably from 2 to 4 and more preferably from 2 to 3.

[0231] When L is a bond it is formed between a reactive functional group on a precursor of R^1 and a reactive functional group of complementary reactivity on the saccharyl core. When L is a non-zero order linker, a precursor of L can be in place on the glycosyl moiety prior to reaction with the R^1 precursor. Alternatively, the precursors of R^1 and L can be incorporated into a preformed cassette that is subsequently attached to the glycosyl moiety. As set forth herein, the selection and preparation of precursors with appropriate reactive functional groups is within the ability of those skilled in the art. Moreover, coupling the precursors proceeds by chemistry that is well understood in the art.

[0232] In an exemplary embodiment, L is a linking group that is formed from an amino acid, or small peptide (e.g., 1-4 amino acid residues) providing a modified sugar in which the polymeric modifying group is attached through a substituted alkyl linker. Exemplary linkers include glycine, lysine, serine and cysteine. The PEG moiety can be attached to the amine moiety of the linker through an amide or urethane bond. The PEG is linked to the sulfur or oxygen atoms of cysteine and serine through thioether or ether bonds, respectively.

[0233] In an exemplary embodiment, R^5 includes the polymeric modifying group. In another exemplary embodiment, R^5 includes both the polymeric modifying group and a linker, L , joining the modifying group to the remainder of the molecule. As discussed above, L can be a linear or branched structure. Similarly, the polymeric modifying group can be branched or linear.

Water-Soluble Polymers

[0234] Many water-soluble polymers are known to those of skill in the art and are useful in practicing the present invention. The term water-soluble polymer encompasses species such as saccharides (e.g., dextran, amylose, hyalouronic acid, poly(sialic acid), heparans, heparins, etc.); poly (amino acids), e.g., poly(aspartic acid) and poly(glutamic acid); nucleic acids; synthetic polymers (e.g., poly(acrylic acid), poly(ethers), e.g., poly(ethylene glycol); peptides, proteins, and the like. The present invention may be practiced with any water-soluble polymer with the sole limitation that the polymer must include a point at which the remainder of the conjugate can be attached.

[0235] Methods for activation of polymers can also be found in WO 94/17039, U.S. Pat. No. 5,324,844, WO 94/18247, WO 94/04193, U.S. Pat. No. 5,219,564, U.S. Pat. No. 5,122,614, WO 90/13540, U.S. Pat. No. 5,281,698, and more WO 93/15189, and for conjugation between activated polymers and peptides, e.g. Coagulation Factor VIII (WO 94/15625), hemoglobin (WO 94/09027), oxygen carrying molecule (U.S. Pat. No. 4,412,989), ribonuclease and superoxide dismutase (Veronese et al., App. Biochem. Biotech. 11: 141-45 (1985)).

[0236] Exemplary water-soluble polymers are those in which a substantial proportion of the polymer molecules in a sample of the polymer are of approximately the same molecular weight; such polymers are "homodisperse."

[0237] The present invention is further illustrated by reference to a poly(ethylene glycol) conjugate. Several reviews and monographs on the functionalization and conjugation of PEG are available. See, for example, Harris, Macromol. Chem. Phys. C25: 325-373 (1985); Scouten, Methods in Enzymology 135: 30-65 (1987); Wong et al., Enzyme Microb. Technol. 14: 866-874 (1992); Delgado et al., Critical Reviews in Therapeutic Drug Carrier Systems 9: 249-304 (1992); Zalipsky, Bioconjugate Chem. 6: 150-165 (1995); and Bhadra, et al., Pharmazie, 57:5-29 (2002). Routes for preparing

reactive PEG molecules and forming conjugates using the reactive molecules are known in the art. For example, U.S. Patent No. 5,672,662 discloses a water soluble and isolatable conjugate of an active ester of a polymer acid selected from linear or branched poly(alkylene oxides), poly(oxyethylated polyols), poly(olefinic alcohols), and poly(acrylomorpholine).

[0238] U.S. Patent No. 6,376,604 sets forth a method for preparing a water-soluble 1-benzotriazolylcarbonate ester of a water-soluble and non-peptidic polymer by reacting a terminal hydroxyl of the polymer with di(1-benzotriazolyl)carbonate in an organic solvent. The active ester is used to form conjugates with a biologically active agent such as a protein or peptide.

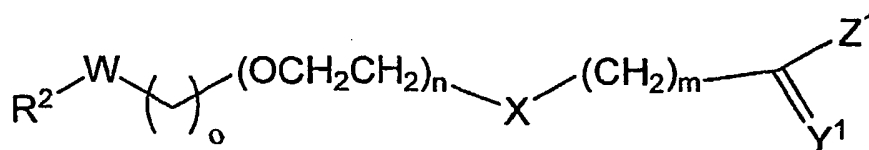
[0239] WO 99/45964 describes a conjugate comprising a biologically active agent and an activated water soluble polymer comprising a polymer backbone having at least one terminus linked to the polymer backbone through a stable linkage, wherein at least one terminus comprises a branching moiety having proximal reactive groups linked to the branching moiety, in which the biologically active agent is linked to at least one of the proximal reactive groups. Other branched poly(ethylene glycols) are described in WO 96/21469, U.S. Patent No. 5,932,462 describes a conjugate formed with a branched PEG molecule that includes a branched terminus that includes reactive functional groups. The free reactive groups are available to react with a biologically active species, such as a protein or peptide, forming conjugates between the poly(ethylene glycol) and the biologically active species. U.S. Patent No. 5,446,090 describes a bifunctional PEG linker and its use in forming conjugates having a peptide at each of the PEG linker termini.

[0240] Conjugates that include degradable PEG linkages are described in WO 99/34833; and WO 99/14259, as well as in U.S. Patent No. 6,348,558. Such degradable linkages are applicable in the present invention.

[0241] The art-recognized methods of polymer activation set forth above are of use in the context of the present invention in the formation of the branched polymers set forth herein and also for the conjugation of these branched polymers to other species, e.g., sugars, sugar nucleotides and the like.

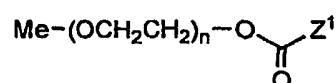
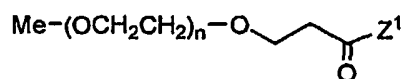
[0242] An exemplary water-soluble polymer is poly(ethylene glycol), e.g., methoxy-poly(ethylene glycol). The poly(ethylene glycol) used in the present invention is not restricted to any particular form or molecular weight range. For unbranched poly(ethylene glycol) molecules the molecular weight is preferably between 500 and 100,000. A molecular weight of 2000-60,000 is preferably used and preferably of from about 5,000 to about 40,000.

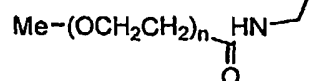
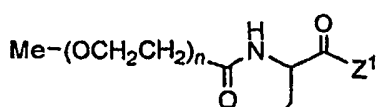
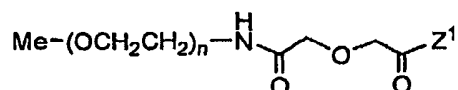
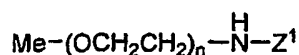
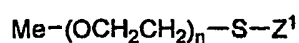
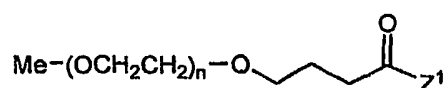
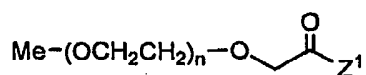
[0243] In an exemplary embodiment, poly(ethylene glycol) molecules of the invention include, but are not limited to, those species set forth below.



in which R^2 is H, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted heteroalkyl, e.g., acetal, $OHC-$, $H_2N-CH_2CH_2-$, $HS-CH_2CH_2-$, and $-(CH_2)_qC(Y^1)Z^2$; -sugar-nucleotide, or protein. The index "n" represents an integer from 1 to 2500. The indices m, o, and q independently represent integers from 0 to 20. The symbol Z represents OH, NH_2 , halogen, $S-R^3$, the alcohol portion of activated esters, $-(CH_2)_pC(Y^2)V$, $-(CH_2)_pU(CH_2)_sC(Y^2)_v$, sugar-nucleotide, protein, and leaving groups, e.g., imidazole, p-nitrophenyl, HOBT, tetrazole, halide. The symbols X, Y^1 , Y^2 , W, U independently represent the moieties O, S, N- R^4 . The symbol V represents OH, NH_2 , halogen, $S-R^5$, the alcohol component of activated esters, the amine component of activated amides, sugar-nucleotides, and proteins. The indices p, s and v are members independently selected from the integers from 0 to 20. The symbols R^3 , R^4 and R^5 independently represent H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heterocycloalkyl and substituted or unsubstituted heteroaryl.

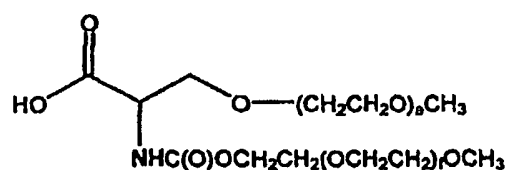
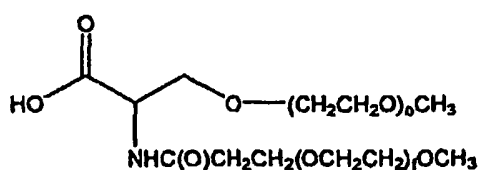
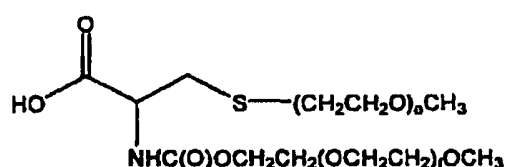
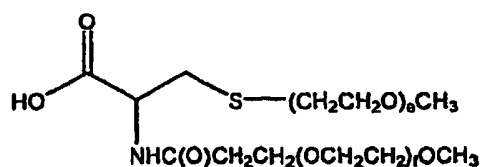
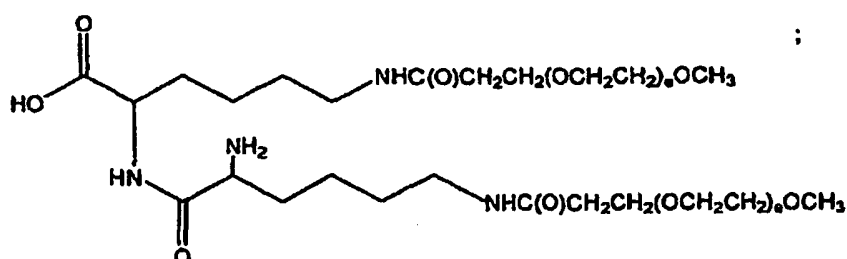
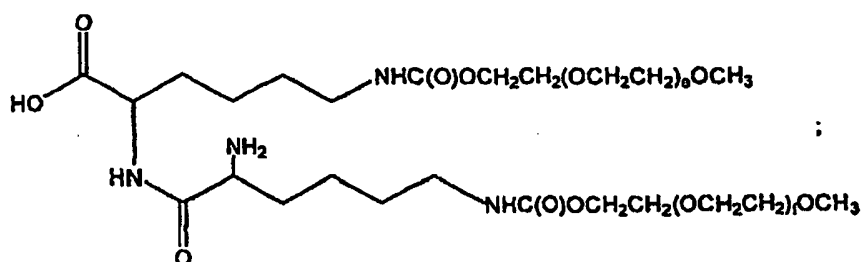
[0244] In other exemplary embodiments, the poly(ethylene glycol) molecule is selected from the following:

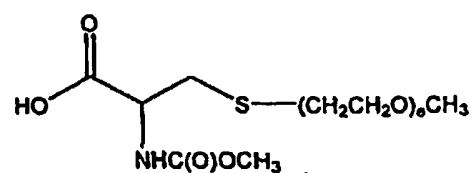
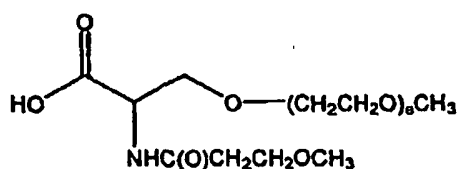




[0245] In another embodiment the poly(ethylene glycol) is a branched PEG having more than one PEG moiety attached. Examples of branched PEGs are described in U.S. Pat. No. 5,932,462; U.S. Pat. No. 5,342,940; U.S. Pat. No. 5,643,575; U.S. Pat. No. 5,919,455; U.S. Pat. No. 6,113,906; U.S. Pat. No. 5,183,660; WO 02/09766; Kodera Y., Bioconjugate Chemistry 5: 283-288 (1994); and Yamasaki et al., Agric. Biol. Chem., 52: 2125-2127, 1998. In a preferred embodiment the molecular weight of each poly(ethylene glycol) of the branched PEG is less than or equal to 40,000 daltons.

[0246] Representative polymeric modifying moieties include structures that are based on side chain-containing amino acids, e.g., serine, cysteine, lysine, and small peptides, e.g., lys-lys. Exemplary structures include:

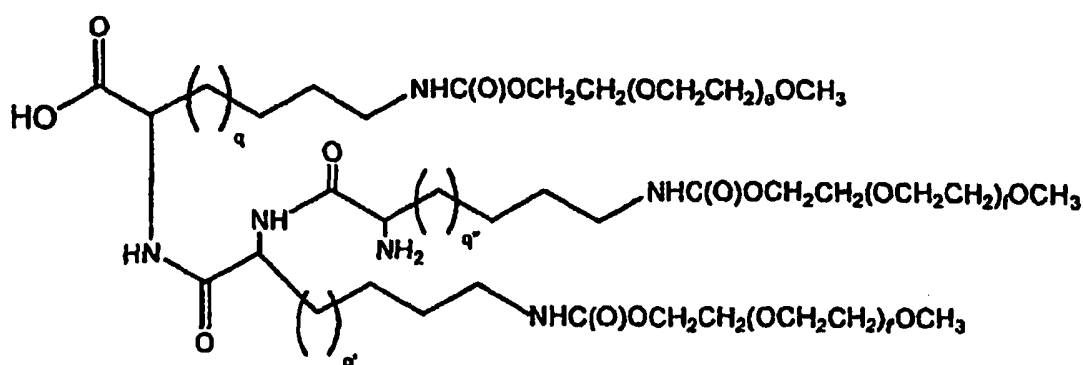




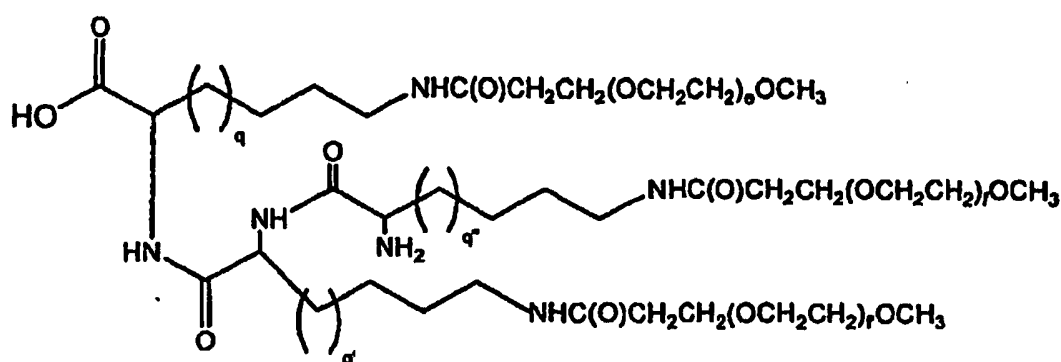
; and

[0247] Those of skill will appreciate that the free amine in the di-lysine structures can also be pegylated through an amide or urethane bond with a PEG moiety.

[0248] In yet another embodiment, the polymeric modifying moiety is a branched PEG moiety that is based upon a tri-lysine peptide. The tri-lysine can be mono-, di-, tri-, or tetra-PEG-ylated. Exemplary species according to this embodiment have the formulae:



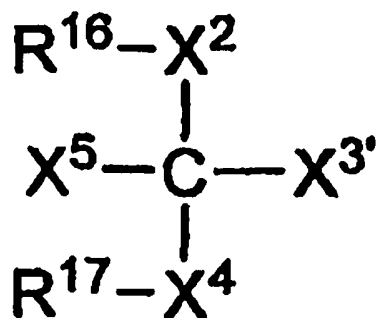
and



in which the indices e, f and f are independently selected integers from 1 to 2500; and the indices q, q' and q'' are independently selected integers from 1 to 20.

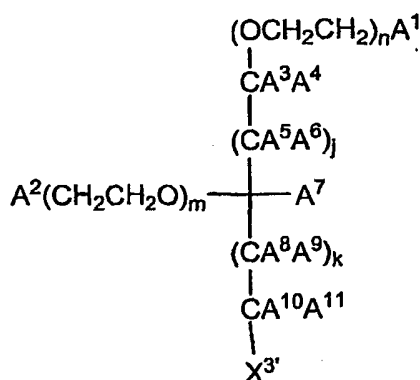
[0249] As will be apparent to those of skill, the branched polymers of use in the invention include variations on the themes set forth above. For example the di-lysine-PEG conjugate shown above can include three polymeric subunits, the third bonded to the α -amine shown as unmodified in the structure above. Similarly, the use of a tri-lysine functionalized with three or four polymeric subunits labeled with the polymeric modifying moiety in a desired manner is within the scope of the invention.

[0250] As discussed herein, the PEG of use in the conjugates of the invention can be linear or branched. An exemplary precursor of use to form the branched PEG containing peptide conjugates according to this embodiment of the invention has the formula:



(III).

[0251] Another exemplary precursor of use to form the branched PEG containing peptide conjugates according to this embodiment of the invention has the formula:



in which the indices m and n are integers independently selected from 0 to 5000. A¹, A², A³, A⁴, A⁵, A⁶, A⁷, A⁸, A⁹, A¹⁰ and A¹¹ are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, -NA¹²A¹³, -OA¹² and -SiA¹²A¹³. A¹² and A¹³ are members independently selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, and substituted or unsubstituted heteroaryl.

[0252] The branched polymer species according to this formula are essentially pure water-soluble polymers. X^{3'} is a moiety that includes an ionizable (e.g., OH, COOH, H₂PO₄, HSO₃, NH₂, and salts thereof, etc.) or other reactive functional group, e.g., *infra*. C is carbon. X⁵, R¹⁶ and R¹⁷ are independently selected from non-reactive groups (e.g., H, unsubstituted alkyl, unsubstituted heteroalkyl) and polymeric arms (e.g., PEG). X² and X⁴ are linkage fragments that are preferably essentially non-reactive under physiological conditions, which may be the same or different. An exemplary linker includes neither aromatic nor ester moieties. Alternatively, these linkages can include one or more moiety that is designed to degrade under physiologically relevant conditions, e.g., esters, disulfides, etc. X² and X⁴ join polymeric arms R¹⁶ and R¹⁷ to C. When X^{3'} is reacted with a reactive functional group of complementary reactivity on a linker, sugar or linker-sugar cassette, X^{3'} is converted to a component of linkage fragment X³.

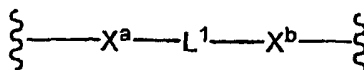
[0253] Exemplary linkage fragments for X², X³ and X⁴ are independently selected and include S, SC(O)NH, HNC(O)S, SC(O)O, O, NH, NHC(O), (O)CNH and NHC(O)O, and OC(O)NH, CH₂S, CH₂O, CH₂CH₂O, CH₂CH₂S, (CH₂)_oO, (CH₂)_oS or (CH₂)_oY'-PEG wherein, Y' is S, NH, NHC(O), C(O)NH, NHC(O)O, OC(O)NH, or O and o is an integer from 1 to 50. In an exemplary embodiment, the linkage fragments X² and X⁴ are different linkage fragments.

[0254] In an exemplary embodiment, the precursor (Formula III), or an activated derivative thereof, is reacted with, and thereby bound to a sugar, an activated sugar or a sugar nucleotide through a reaction between X^{3'} and a group of complementary reactivity on the sugar moiety, e.g., an amine. Alternatively, X^{3'} reacts with a reactive functional group on a precursor to linker, L. One or more of R², R³, R⁴, R⁵, R⁶ or R^{6'} of Formulae I and II can include the branched polymeric modifying moiety, or this moiety bound through L.

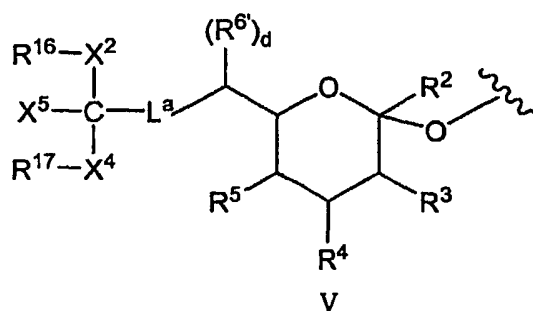
[0255] In an exemplary embodiment, the moiety:

$$\begin{array}{c} \text{O} \\ \parallel \\ \text{R}^{16}-\text{X}^2-\text{C}-\text{C}-\text{X}^a- \\ | \\ \text{X}^4 \\ | \\ \text{R}^{17} \end{array}$$

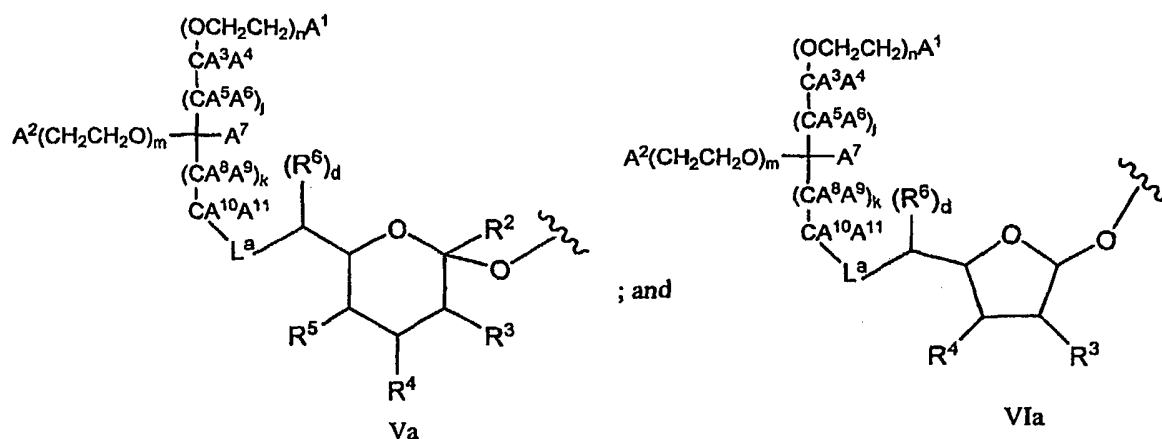
[0257] In another exemplary embodiment, X^a is a linking moiety formed with another linker:



[0260] In a further exemplary embodiment, the peptide conjugates of the invention include a moiety, e.g., an R^{15} moiety that has a formula that is selected from:

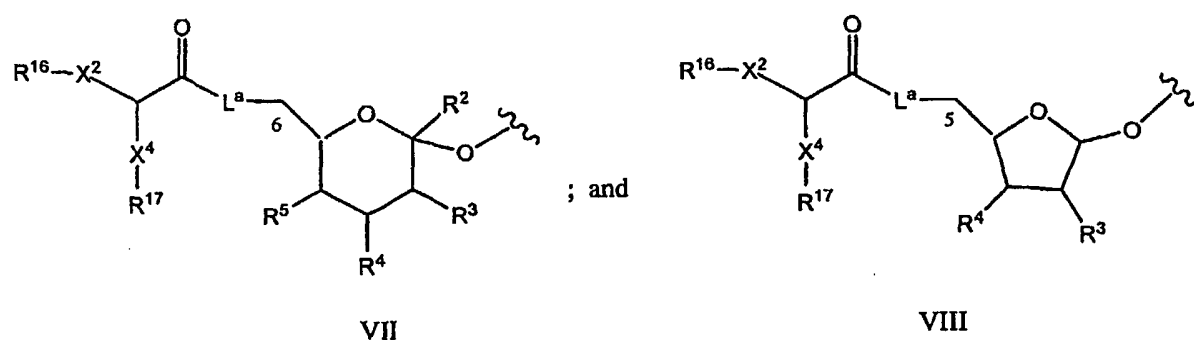


VI



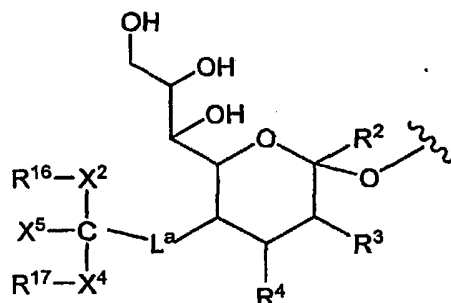
in which the identity of the radicals represented by the various symbols is the same as that discussed hereinabove. L^a is a bond or a linker as discussed above for L and L^1 , e.g., substituted or unsubstituted alkyl or substituted or unsubstituted heteroalkyl moiety. In an exemplary embodiment, L^a is a moiety of the side chain of sialic acid that is functionalized with the polymeric modifying moiety as shown. Exemplary L^a moieties include substituted or unsubstituted alkyl chains that include one or more OH or NH_2 .

[0261] In yet another exemplary embodiment, the invention provides peptide conjugates having a moiety, e.g., an R^{15} moiety with formula:

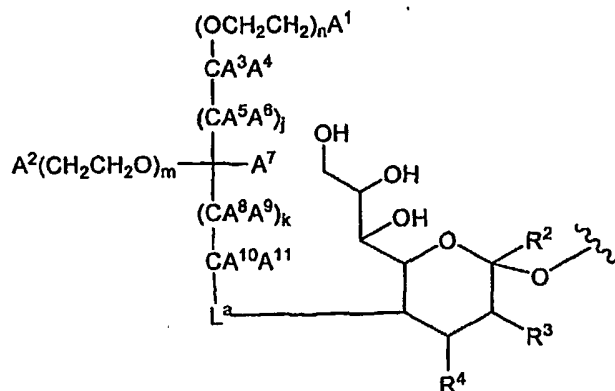


[0262] The identity of the radicals represented by the various symbols is the same as that discussed hereinabove. As those of skill will appreciate, the linker arm in Formulae VII and VIII is equally applicable to other modified sugars set forth herein. In exemplary embodiment, the species of Formulae VII and VIII are the R^{15} moieties attached to the glycan structures set forth herein.

[0263] In yet another exemplary embodiment, the Factor VII or Factor VIIa peptide conjugate includes a R^{15} moiety with a formula which is a member selected from:

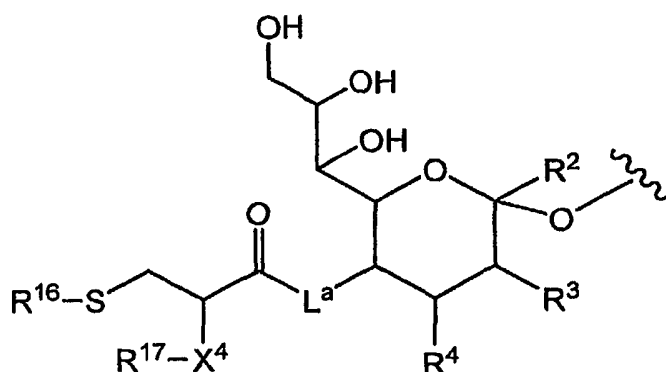


and



in which the identities of the radicals are as discussed above. An exemplary species for L^a is $-(CH_2)_jC(O)NH(CH_2)_hC(O)NH-$, in which the indices h and j are independently selected integers from 0 to 10. A further exemplary species is $-C(O)NH-$. The indices m and n are integers independently selected from 0 to 5000. A^1 , A^2 , A^3 , A^4 , A^5 , A^6 , A^7 , A^8 , A^9 , A^{10} and A^{11} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, $-NA^{12}A^{13}$, $-OA^{12}$ and $-SiA^{12}A^{13}$. A^{12} and A^{13} are members independently selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, and substituted or unsubstituted heteroaryl.

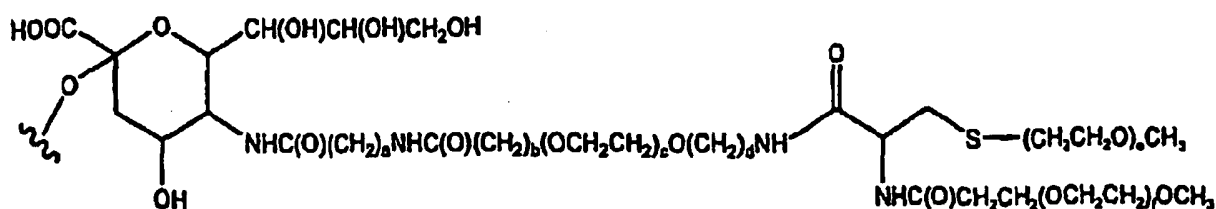
[0264] In an exemplary embodiment, the glycosyl linking group has a structure according to the following formula:

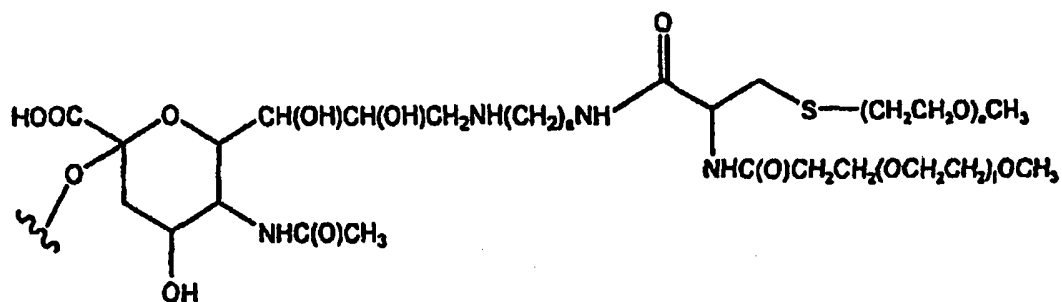
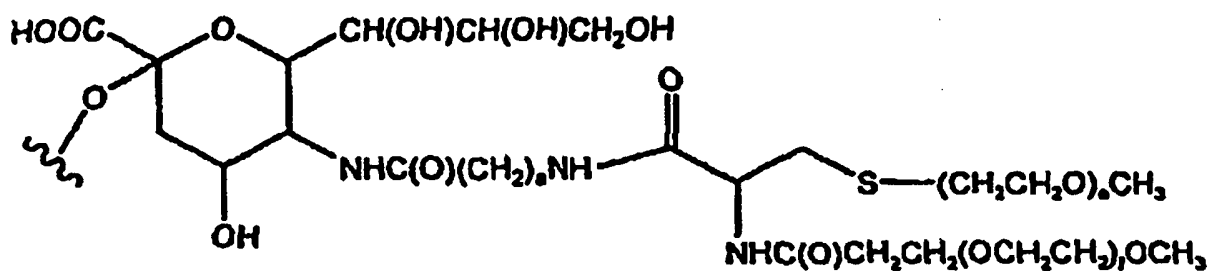


[0265] The embodiments of the invention set forth above are further exemplified by reference to species in which the polymer is a water-soluble polymer, particularly poly(ethylene glycol) ("PEG"), e.g., methoxy-poly(ethylene glycol). Those of skill will appreciate that the focus in the sections that follow is for clarity of illustration and the various motifs set forth using PEG as an exemplary polymer are equally applicable to species in which a polymer other than PEG is utilized.

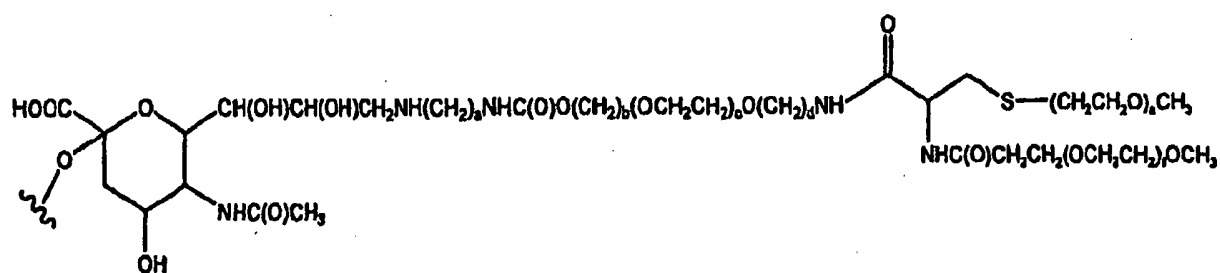
[0266] PEG of any molecular weight, e.g., 1 kDa, 2 kDa, 5 kDa, 10 kDa, 15 kDa, 20 kDa, 25 kDa, 30 kDa, 35 kDa, 40 kDa and 45 kDa is of use in the present invention.

[0267] In an exemplary embodiment, the R^{15} moiety has a formula that is a member selected from the group:



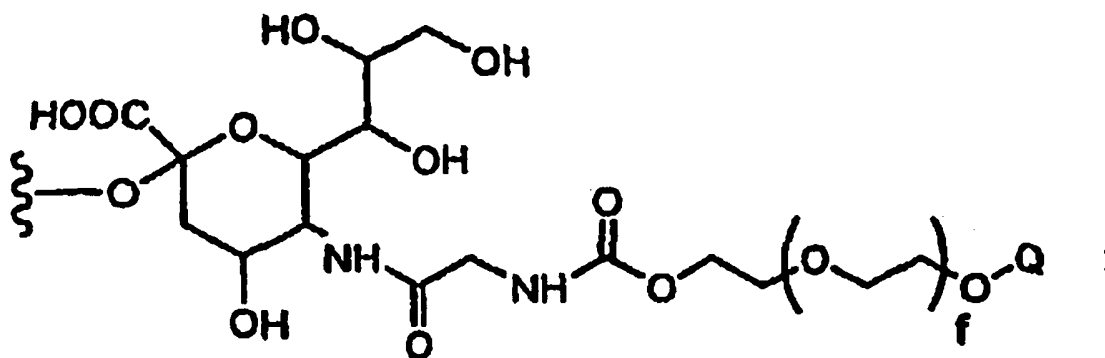


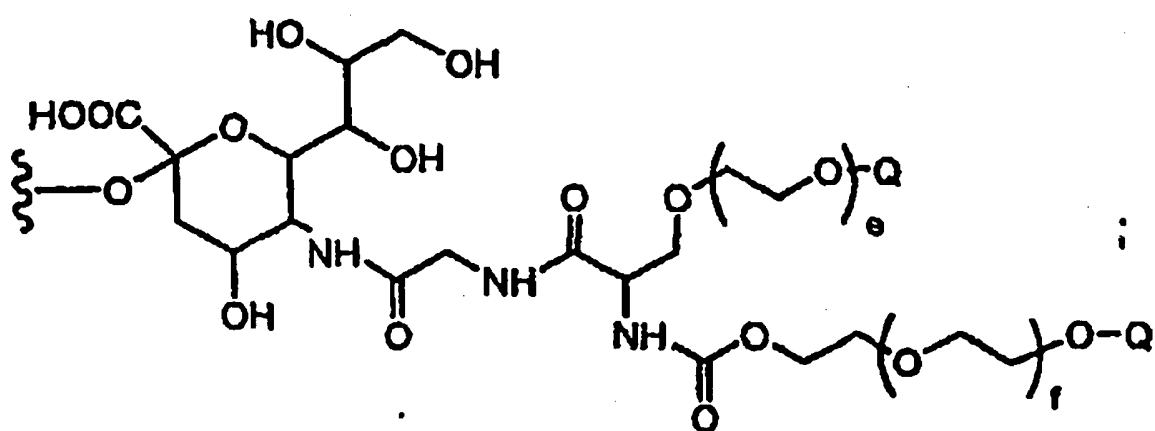
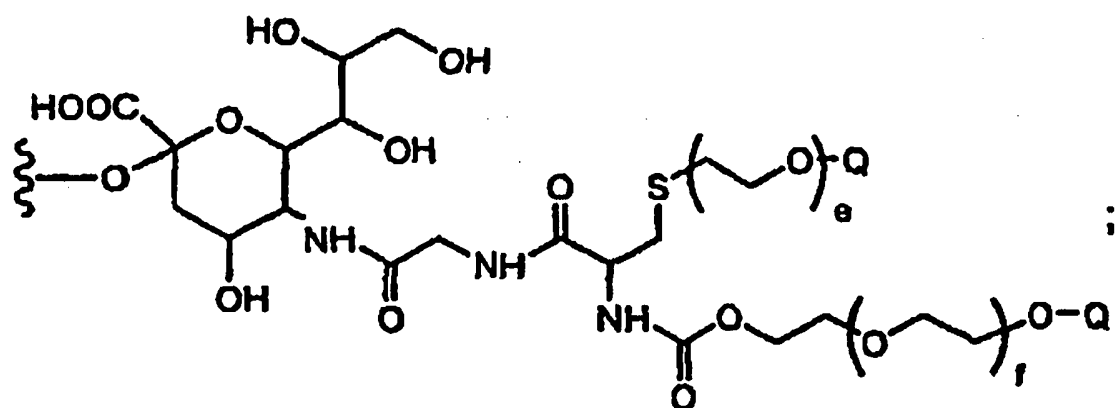
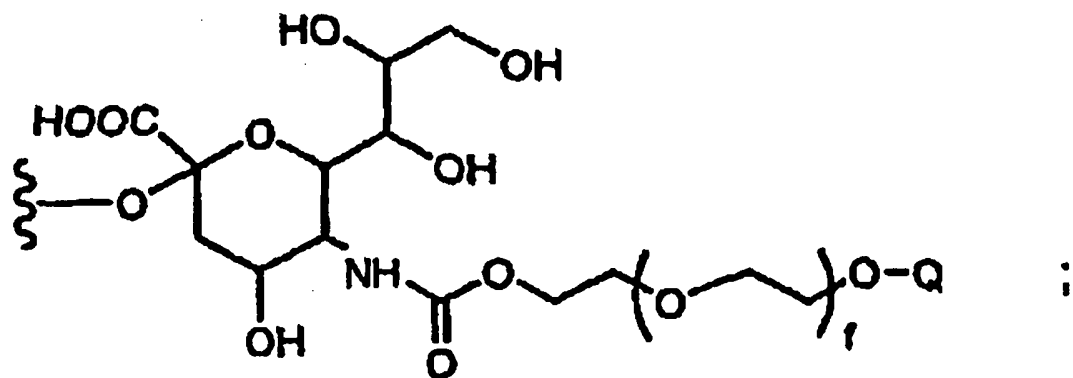
and



35 In each of the structures above, the linker fragment $-\text{NH}(\text{CH}_2)_a-$ can be present or absent.

[0268] In other exemplary embodiments, the peptide conjugate includes an R^{15} moiety selected from the group:





45 and



15

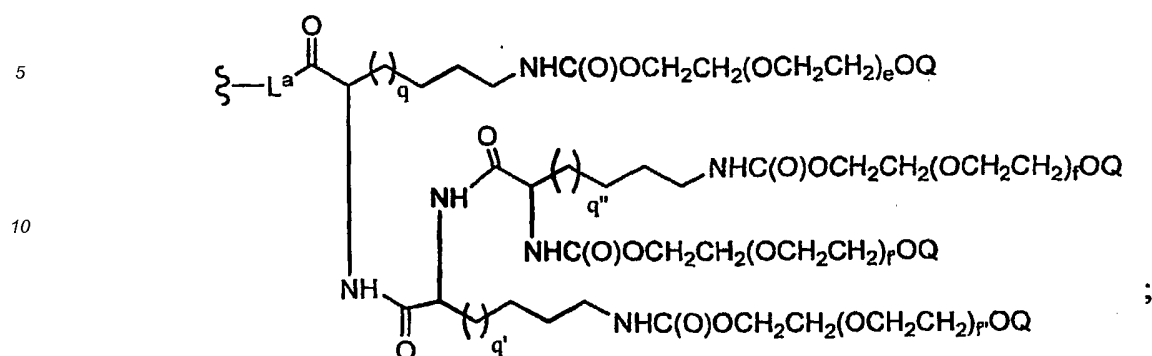
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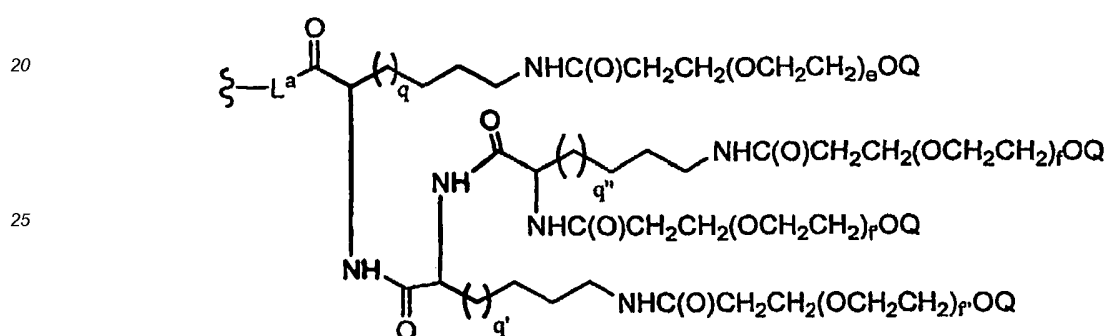
50



and tri-lysine peptides (Lys-Lys-Lys), e.g.:



and

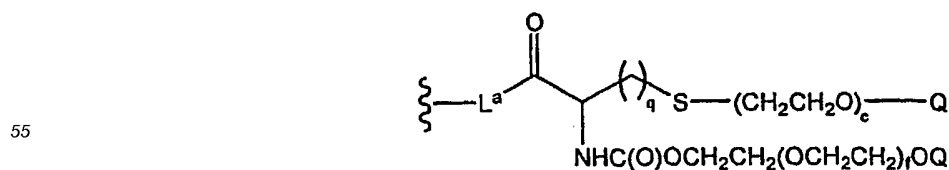
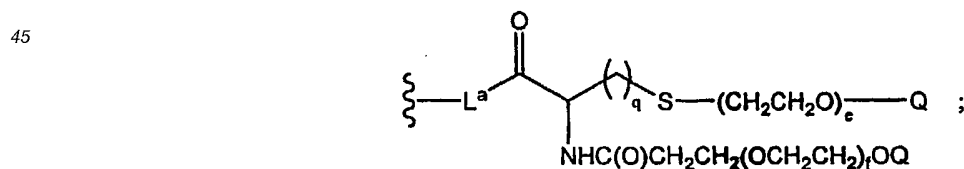


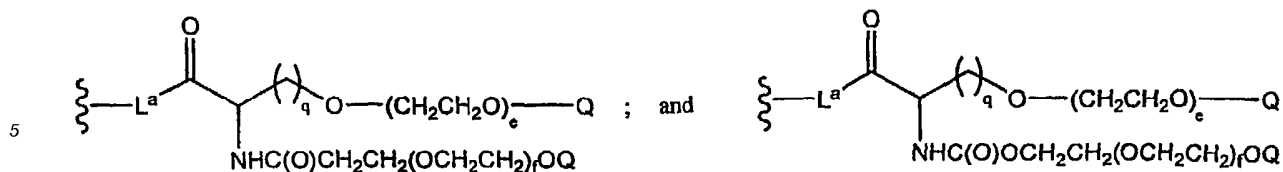
In each of the figures above, the indices e, f, f' and f'' represent integers independently selected from 1 to 2500. The indices q, q' and q'' represent integers independently selected from 1 to 20.

[0271] In another exemplary embodiment, the modifying group:



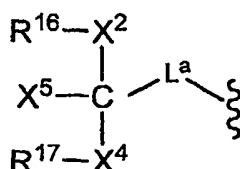
has a formula that is a member selected from:



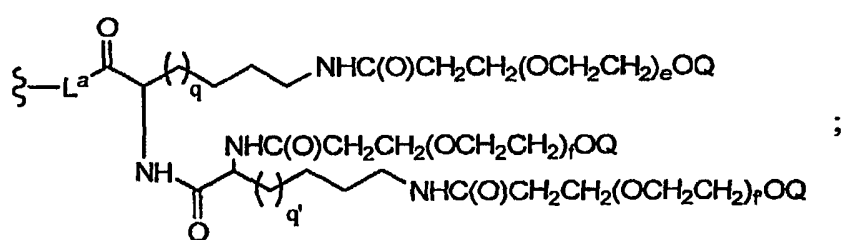
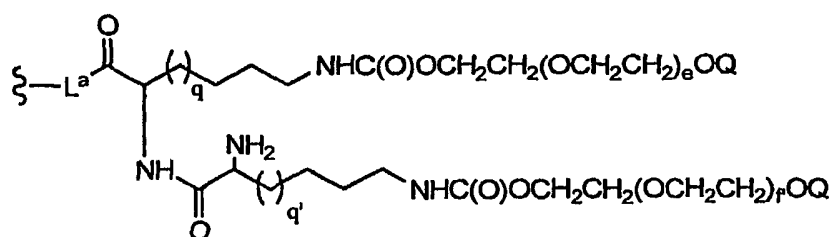
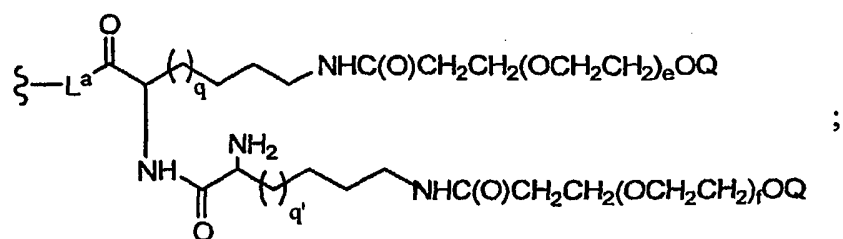


wherein Q is a member selected from H and substituted or unsubstituted C₁-C₆ alkyl. The indices e and f are integers independently selected from 1 to 2500, and the index q is an integer selected from 0 to 20.

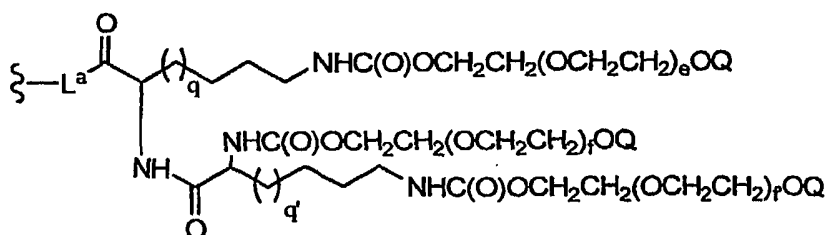
[0272] In another exemplary embodiment, the modifying group:



has a formula that is a member selected from:

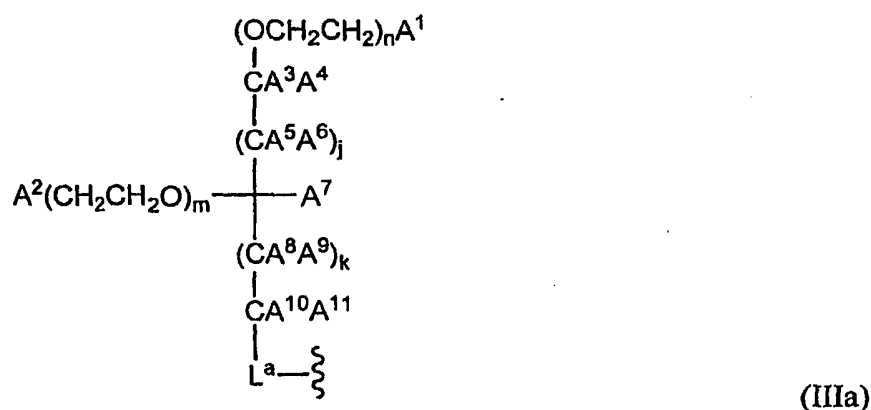


and



wherein Q is a member selected from H and substituted or unsubstituted C₁-C₆ alkyl. The indices e, f and f' are integers independently selected from 1 to 2500, and q and q' are integers independently selected from 1 to 20.

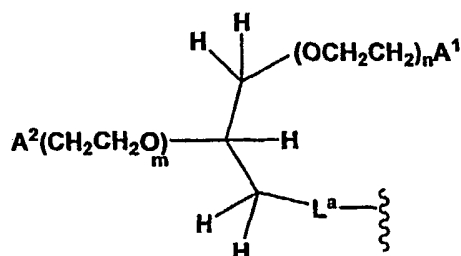
[0273] In another exemplary embodiment, the branched polymer has a structure according to the following formula:



in which the indices m and n are integers independently selected from 0 to 5000. A¹, A², A³, A⁴, A⁵, A⁶, A⁷, A⁸, A⁹, A¹⁰ and A¹¹ are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, -NA¹²A¹³, -OA¹² and -SiA¹²A¹³. A¹² and A¹³ are members independently selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, and substituted or unsubstituted heteroaryl.

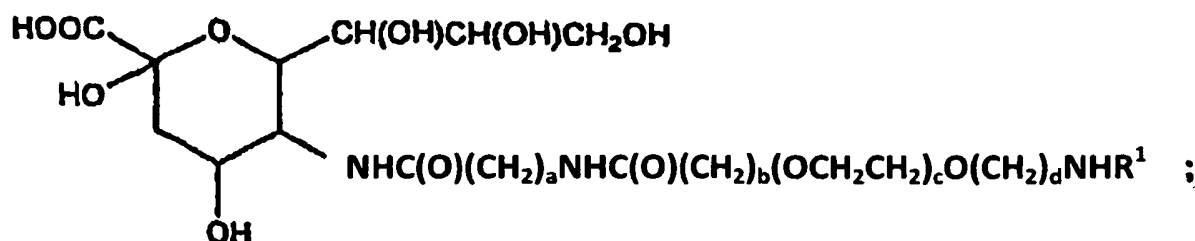
[0274] Formula IIIa is a subset of Formula III. The structures described by Formula IIIa are also encompassed by Formula III.

[0275] In another exemplary embodiment according to the formula above, the branched polymer has a structure according to the following formula:

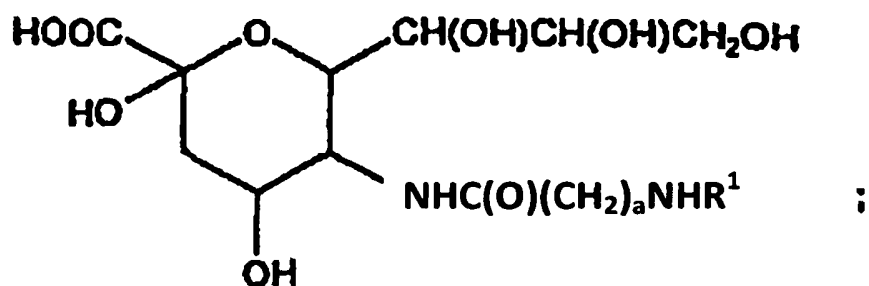


In an exemplary embodiment, A¹ and A² are each -OCH₃ or H.

[0276] In an illustrative embodiment, the modified sugar is sialic acid and selected modified sugar compounds of use in the invention have the formulae:

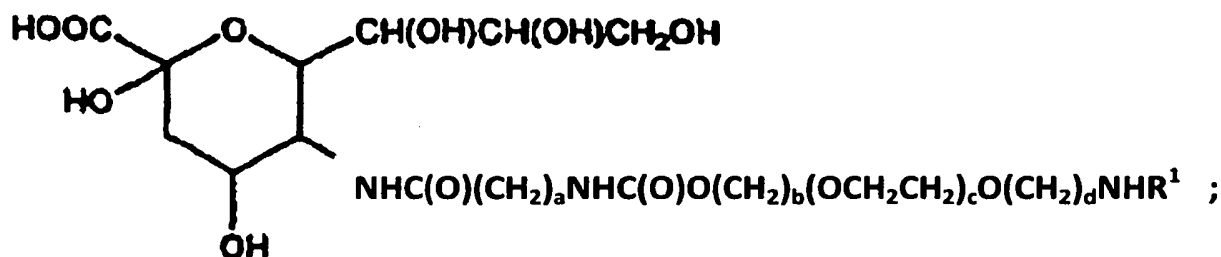


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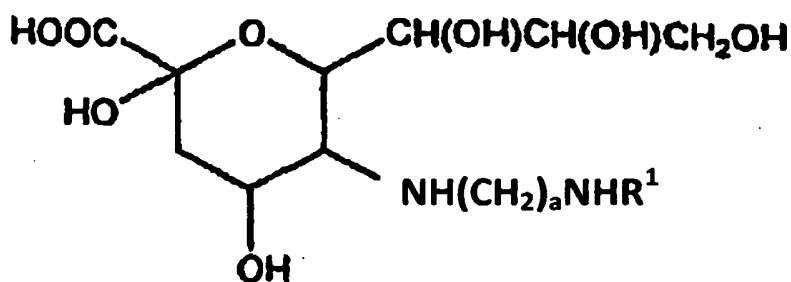
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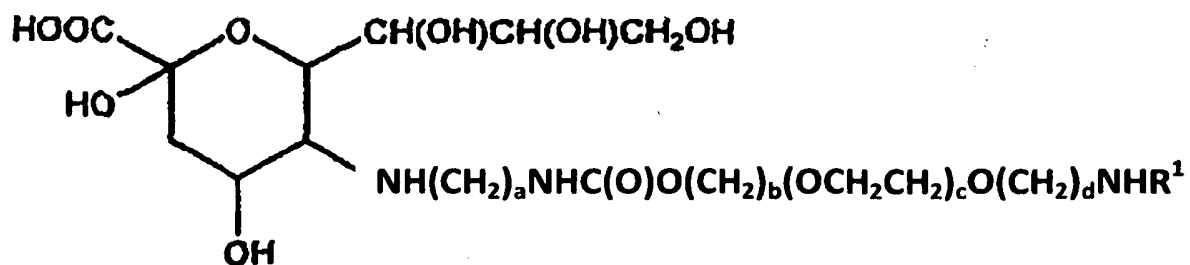
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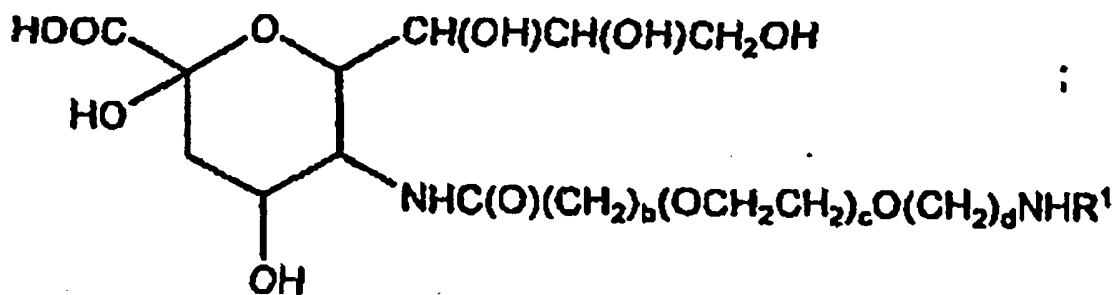
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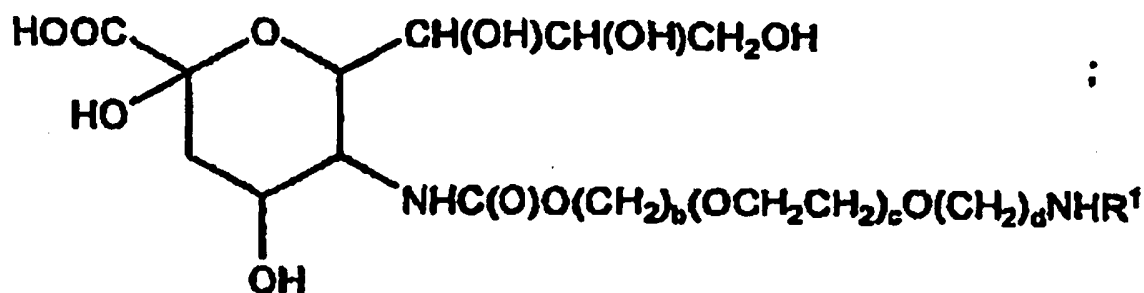
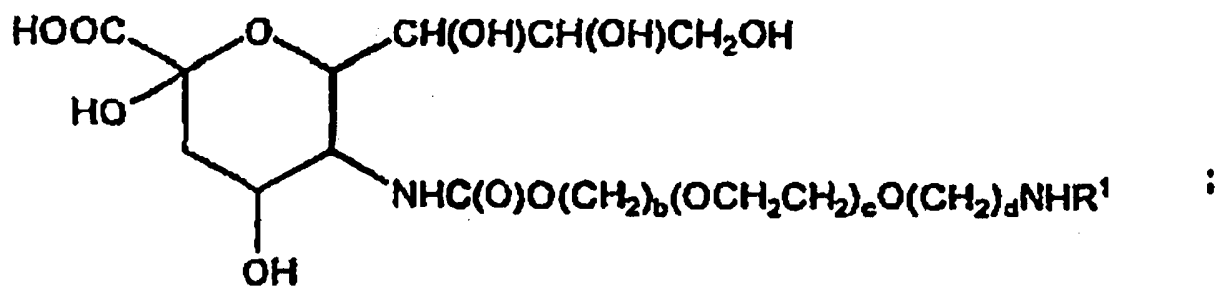
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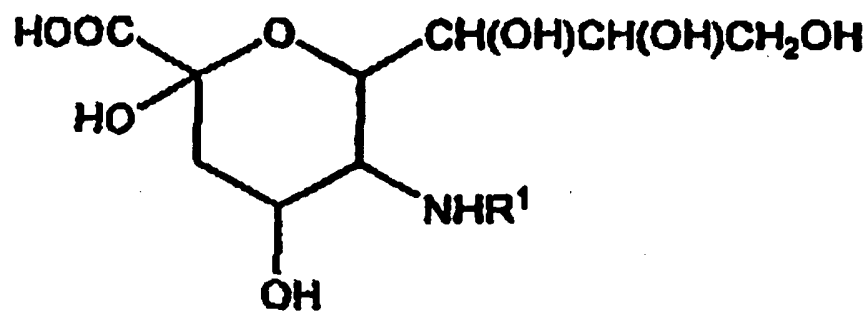


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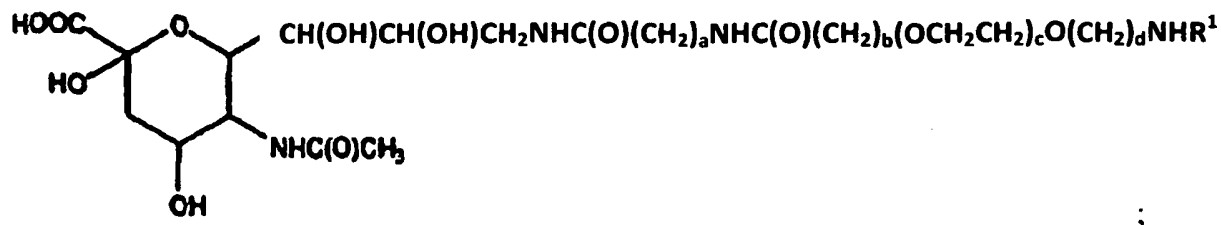


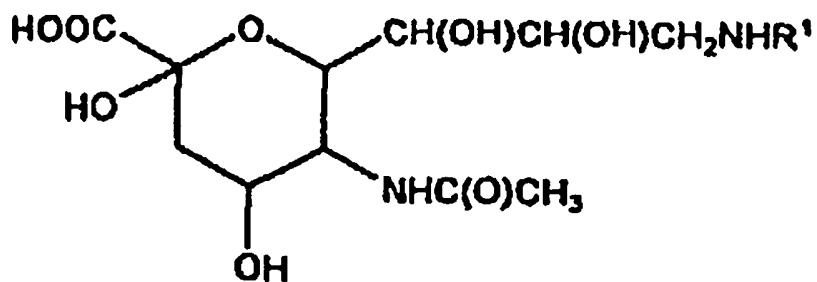
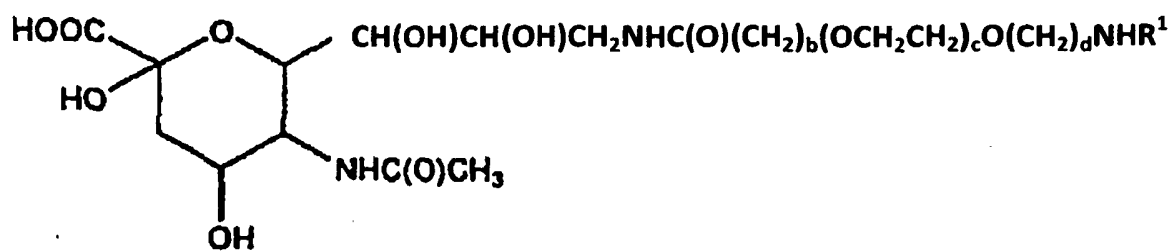
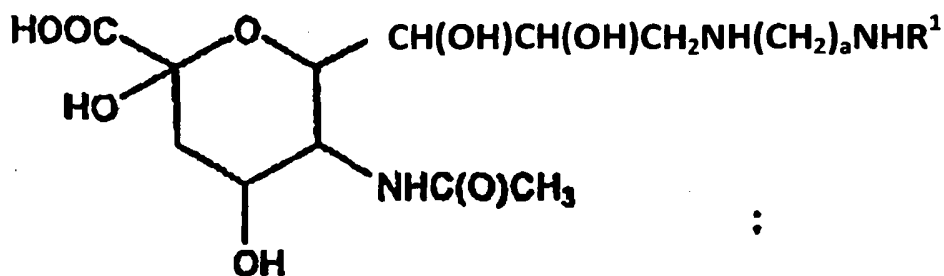
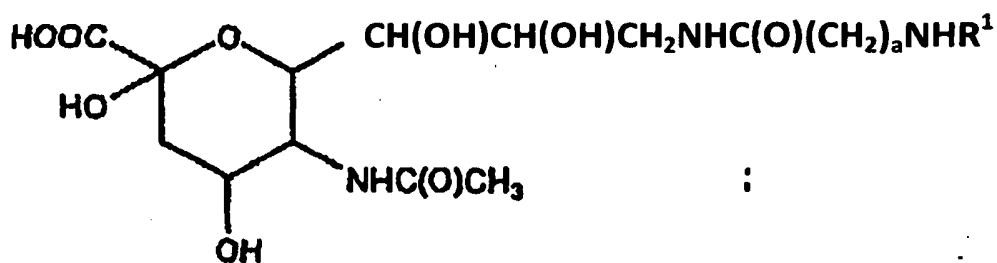
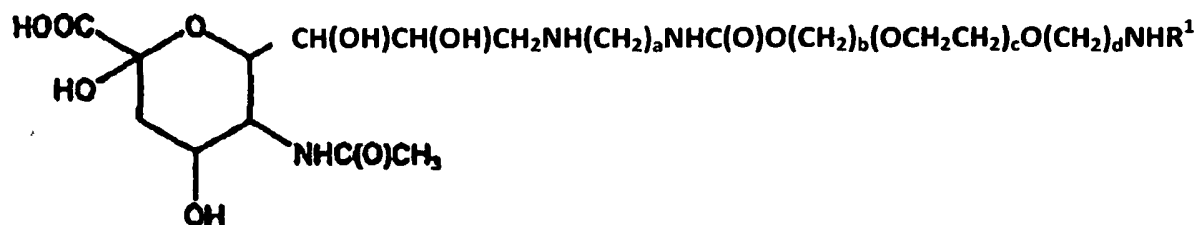
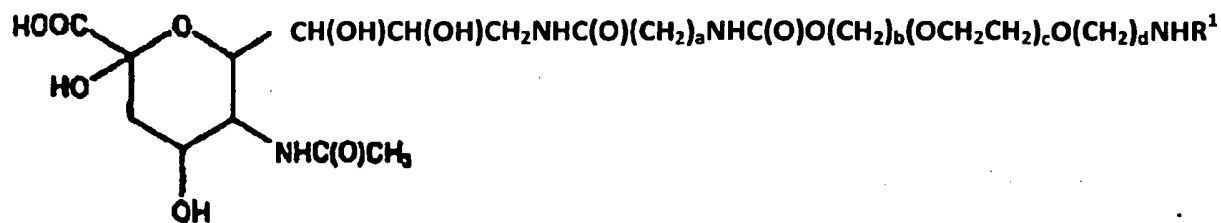
and

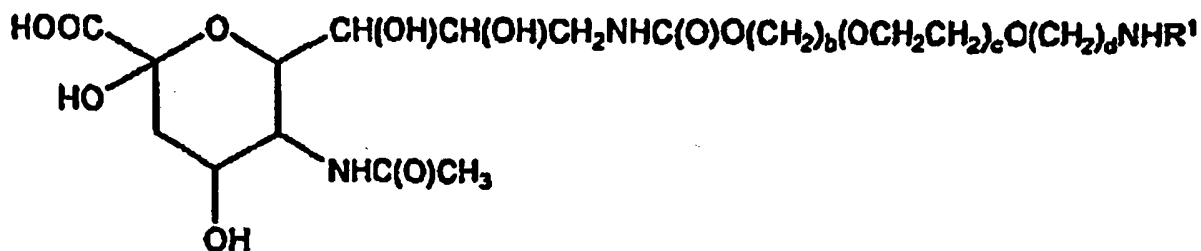


[0277] The indices a, b and d are integers from 0 to 20. The index c is an integer from 1 to 2500. The structures set forth above can be components of R^{15} .

[0278] In another illustrative embodiment, a primary hydroxyl moiety of the sugar is functionalized with the modifying group. For example, the 9-hydroxyl of sialic acid can be converted to the corresponding amine and functionalized to provide a compound according to the invention. Formulae according to this embodiment include:



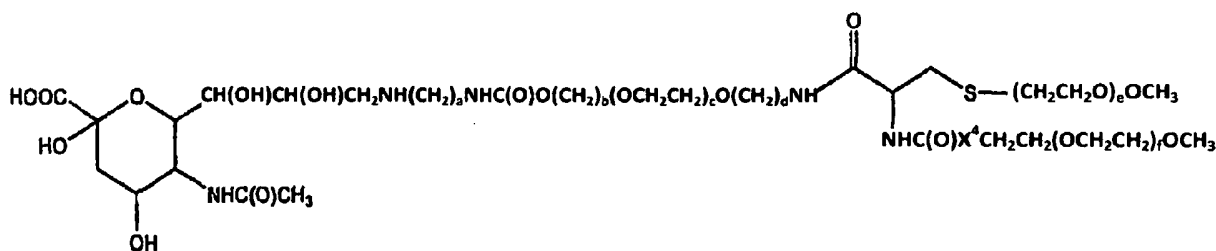
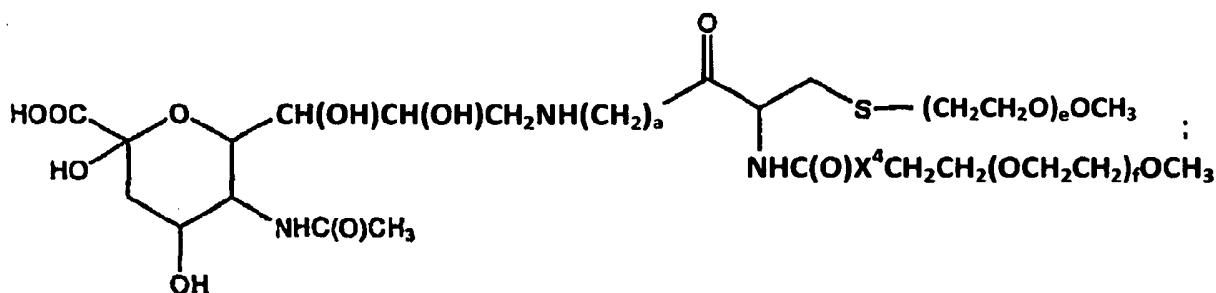
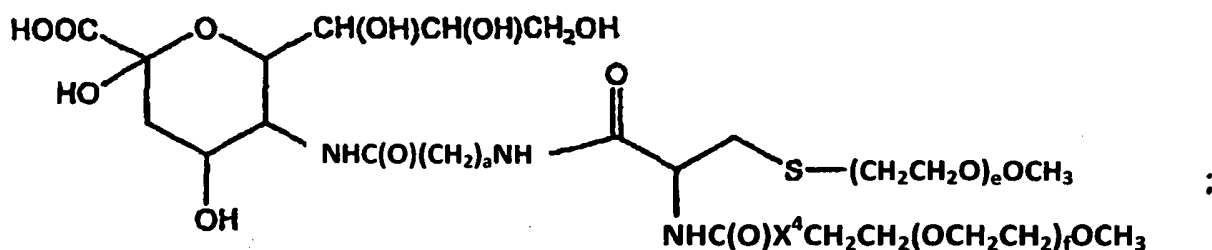
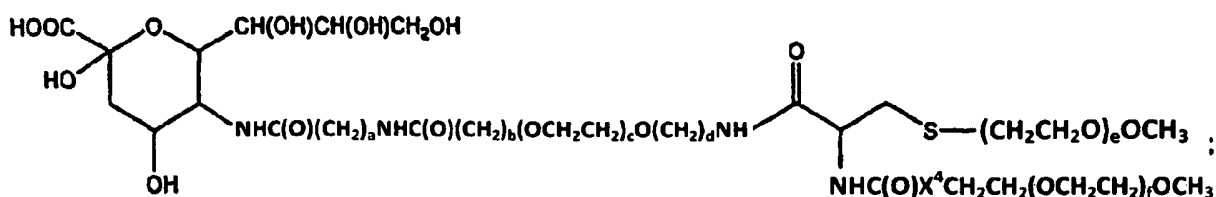




[0279] The structures set forth above can be components of R¹⁵.

[0280] Although the present invention is exemplified in the preceding sections by reference to PEG, as those of skill will appreciate, an array of polymeric modifying moieties is of use in the compounds and methods set forth herein.

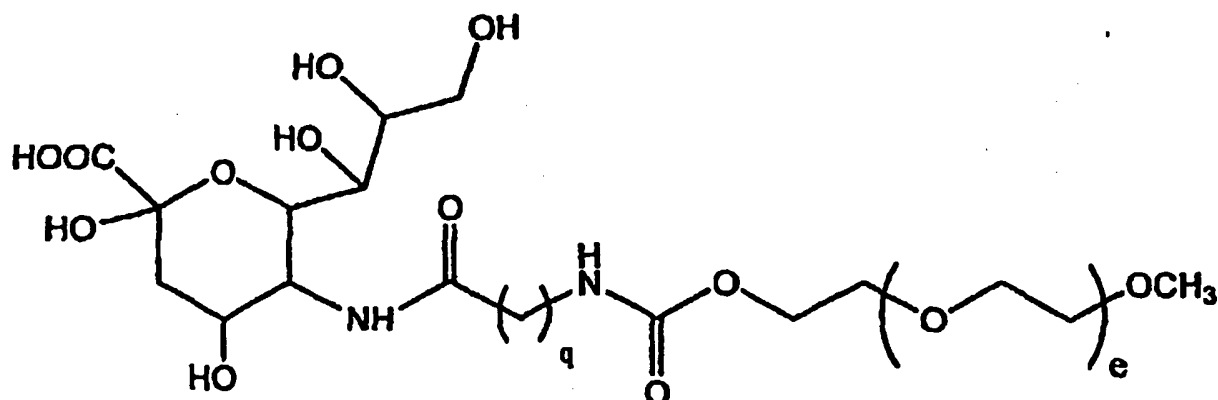
[0281] In selected embodiments, R¹ or L-R¹ is a branched PEG5 for example, one of the species set forth above. In an exemplary embodiment, the branched PEG structure is based on a cysteine peptide. Illustrative modified sugars according to this embodiment include:



in which X⁴ is a bond or O. In each of the structures above, the alkylamine linker -(CH₂)_aNH- can be present or absent.

The structures set forth above can be components of $R^{15}/R^{15'}$.

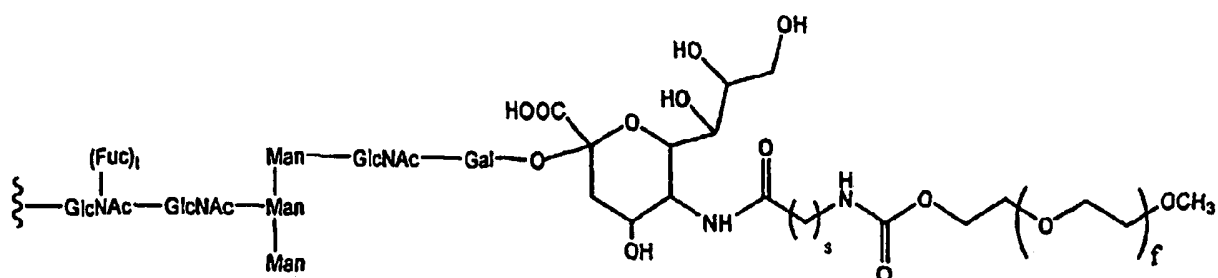
[0282] As discussed herein, the polymer-modified sialic acids of use in the invention may also be linear structures. Thus, the invention provides for conjugates that include a sialic acid moiety derived from a structure such as:



in which the indices q and e are as discussed above.

[0283] Exemplary modified sugars are modified with water-soluble or water-insoluble polymers. Examples of useful polymer are further exemplified below.

[0284] In another exemplary embodiment, the peptide is derived from insect cells, remodeled by adding GlcNAc and Gal to the mannose core and glycopegylated using a sialic acid bearing a linear PEG moiety, affording a Factor VII or Factor VIIa peptide that comprises at least one moiety having the formula:



in which the index t is an integer from 0 to 1; the index s represents an integer from 1 to 10; and the index f represents an integer from 1 to 2500.

Water-Insoluble Polymers

[0285] In another embodiment, analogous to those discussed above, the modified sugars include a water-insoluble polymer, rather than a water-soluble polymer. The conjugates of the invention may also include one or more water-insoluble polymers. This embodiment of the invention is illustrated by the use of the conjugate as a vehicle with which to deliver a therapeutic peptide in a controlled manner. Polymeric drug delivery systems are known in the art. See, for example, Dunn et al., Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991. Those of skill in the art will appreciate that substantially any known drug delivery system is applicable to the conjugates of the present invention.

[0286] The motifs forth above for R^1 , $L-R^1$, R^{15} , $R^{15'}$ and other radicals are equally applicable to water-insoluble polymers, which may be incorporated into the linear and branched structures without limitation utilizing chemistry readily accessible to those of skill in the art. Similarly, the incorporation of these species into any of the modified sugars discussed herein is within the scope of the present invention. Accordingly, the invention provides conjugates containing, and for the use of to prepare such conjugates, sialic acid and other sugar moieties modified with a linear or branched water-insoluble polymers, and activated analogues of the modified sialic acid species (e.g., CMP-Sia-(water insoluble polymer)).

[0287] Representative water-insoluble polymers include, but are not limited to, polyphosphazines, poly(vinyl alcohols), polyamides, polycarbonates, polyalkylenes, polyacrylamides, polyalkylene glycols, polyalkylene oxides, polyalkylene terephthalates, polyvinyl ethers, polyvinyl esters, polyvinyl halides, polyvinylpyrrolidone, polyglycolides, polysiloxanes,

polyurethanes, poly(methyl methacrylate), poly(ethyl methacrylate), poly(butyl methacrylate), poly(isobutyl methacrylate), poly(hexyl methacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate) polyethylene, polypropylene, poly(ethylene glycol), poly(ethylene oxide), poly(ethylene terephthalate), poly(vinyl acetate), polyvinyl chloride, polystyrene, polyvinyl pyrrolidone, pluronics and polyvinylphenol and copolymers thereof.

[0288] Synthetically modified natural polymers of use in conjugates of the invention include, but are not limited to, alkyl celluloses, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, and nitrocelluloses. Particularly preferred members of the broad classes of synthetically modified natural polymers include, but are not limited to, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, hydroxybutyl methyl cellulose, cellulose acetate, cellulose propionate, cellulose acetate butyrate, cellulose acetate phthalate, carboxymethyl cellulose, cellulose triacetate, cellulose sulfate sodium salt, and polymers of acrylic and methacrylic esters and alginic acid.

[0289] These and the other polymers discussed herein can be readily obtained from commercial sources such as Sigma Chemical Co. (St. Louis, MO.), Polysciences (Warrenton, PA.), Aldrich (Milwaukee, WI.), Fluka (Ronkonkoma, NY), and BioRad (Richmond, CA), or else synthesized from monomers obtained from these suppliers using standard techniques.

[0290] Representative biodegradable polymers of use in the conjugates of the invention include, but are not limited to, polylactides, polyglycolides and copolymers thereof, poly(ethylene terephthalate), poly(butyric acid), poly(valeric acid), poly(lactide-co-caprolactone), poly(lactide-co-glycolide), polyanhydrides, polyorthoesters, blends and copolymers thereof. Of particular use are compositions that form gels, such as those including collagen, pluronics and the like.

[0291] The polymers of use in the invention include "hybrid" polymers that include water-insoluble materials having within at least a portion of their structure, a bioresorbable molecule. An example of such a polymer is one that includes a water-insoluble copolymer, which has a bioresorbable region, a hydrophilic region and a plurality of crosslinkable functional groups per polymer chain.

[0292] For purposes of the present invention, "water-insoluble materials" includes materials that are substantially insoluble in water or water-containing environments. Thus, although certain regions or segments of the copolymer may be hydrophilic or even water-soluble, the polymer molecule, as a whole, does not to any substantial measure dissolve in water.

[0293] For purposes of the present invention, the term "bioresorbable molecule" includes a region that is capable of being metabolized or broken down and resorbed and/or eliminated through normal excretory routes by the body. Such metabolites or break down products are preferably substantially non-toxic to the body.

[0294] The bioresorbable region may be either hydrophobic or hydrophilic, so long as the copolymer composition as a whole is not rendered water-soluble. Thus, the bioresorbable region is selected based on the preference that the polymer, as a whole, remains water-insoluble. Accordingly, the relative properties, *i.e.*, the kinds of functional groups contained by, and the relative proportions of the bioresorbable region, and the hydrophilic region are selected to ensure that useful bioresorbable compositions remain water-insoluble.

[0295] Exemplary resorbable polymers include, for example, synthetically produced resorbable block copolymers of poly(α -hydroxy-carboxylic acid)/poly(oxyalkylene), (see, Cohn *et al.*, U.S. Patent No. 4,826,945). These copolymers are not crosslinked and are water-soluble so that the body can excrete the degraded block copolymer compositions. See, Younes *et al.*, J Biomed. Mater. Res. 21: 1301-1316 (1987); and Cohn *et al.*, J Biomed. Mater. Res. 22: 993-1009 (1988).

[0296] Presently preferred bioresorbable polymers include one or more components selected from poly(esters), poly(hydroxy acids), poly(lactones), poly(amides), poly(ester-amides), poly(amino acids), poly(anhydrides), poly(orthoesters), poly(carbonates), poly(phosphazines), poly(phosphoesters), poly(thioesters), polysaccharides and mixtures thereof. More preferably still, the bioresorbable polymer includes a poly(hydroxy) acid component. Of the poly(hydroxy) acids, polylactic acid, polyglycolic acid, polycaproic acid, polybutyric acid, polyvaleric acid and copolymers and mixtures thereof are preferred.

[0297] In addition to forming fragments that are absorbed *in vivo* ("bioresorbed"), preferred polymeric coatings for use in the methods of the invention can also form an excretable and/or metabolizable fragment.

[0298] Higher order copolymers can also be used in the present invention. For example, Casey *et al.*, U.S. Patent No. 4,438,253, which issued on March 20, 1984, discloses tri-block copolymers produced from the transesterification of poly(glycolic acid) and an hydroxyl-ended poly(alkylene glycol). Such compositions are disclosed for use as resorbable monofilament sutures. The flexibility of such compositions is controlled by the incorporation of an aromatic orthocarbonate, such as tetra-*p*-tolyl orthocarbonate into the copolymer structure.

[0299] Other polymers based on lactic and/or glycolic acids can also be utilized. For example, Spinu, U.S. Patent No. 5,202,413, which issued on April 13, 1993, discloses biodegradable multi-block copolymers having sequentially ordered blocks of polylactide and/or polyglycolide produced by ring-opening polymerization of lactide and/or glycolide onto either an oligomeric diol or a diamine residue followed by chain extension with a difunctional compound, such as, a diisocyanate, diacylchloride or dichlorosilane.

[0300] Bioresorbable regions of coatings useful in the present invention can be designed to be hydrolytically and/or

enzymatically cleavable. For purposes of the present invention, "hydrolytically cleavable" refers to the susceptibility of the copolymer, especially the bioresorbable region, to hydrolysis in water or a water-containing environment. Similarly, "enzymatically cleavable" as used herein refers to the susceptibility of the copolymer, especially the bioresorbable region, to cleavage by endogenous or exogenous enzymes.

[0301] When placed within the body, the hydrophilic region can be processed into excretable and/or metabolizable fragments. Thus, the hydrophilic region can include, for example, polyethers, polyalkylene oxides, polyols, poly(vinyl pyrrolidine), poly(vinyl alcohol), poly(alkyl oxazolines), polysaccharides, carbohydrates, peptides, proteins and copolymers and mixtures thereof. Furthermore, the hydrophilic region can also be, for example, a poly(alkylene) oxide. Such poly(alkylene) oxides can include, for example, poly(ethylene) oxide, poly(propylene) oxide and mixtures and copolymers thereof.

[0302] Polymers that are components of hydrogels are also useful in the present invention. Hydrogels are polymeric materials that are capable of absorbing relatively large quantities of water. Examples of hydrogel forming compounds include, but are not limited to, polyacrylic acids, sodium carboxymethylcellulose, polyvinyl alcohol, polyvinyl pyrrolidine, gelatin, carrageenan and other polysaccharides, hydroxyethylenemethacrylic acid (HEMA), as well as derivatives thereof, and the like. Hydrogels can be produced that are stable, biodegradable and bioresorbable. Moreover, hydrogel compositions can include subunits that exhibit one or more of these properties.

[0303] Bio-compatible hydrogel compositions whose integrity can be controlled through crosslinking are known and are presently preferred for use in the methods of the invention. For example, Hubbell et al., U.S. Patent Nos. 5,410,016, which issued on April 25, 1995 and 5,529,914, which issued on June 25, 1996, disclose water-soluble systems, which are crosslinked block copolymers having a water-soluble central block segment sandwiched between two hydrolytically labile extensions. Such copolymers are further end-capped with photopolymerizable acrylate functionalities. When crosslinked, these systems become hydrogels. The water soluble central block of such copolymers can include poly(ethylene glycol); whereas, the hydrolytically labile extensions can be a poly(α -hydroxy acid), such as polyglycolic acid or polylactic acid. See, Sawhney et al., *Macromolecules* 26: 581-587 (1993).

[0304] In another preferred embodiment, the gel is a thermoreversible gel. Thermoreversible gels including components, such as pluronics, collagen, gelatin, hyaluronic acid, polysaccharides, polyurethane hydrogel, polyurethane-urea hydrogel and combinations thereof are presently preferred.

[0305] In yet another exemplary embodiment, the conjugate of the invention includes a component of a liposome. Liposomes can be prepared according to methods known to those skilled in the art, for example, as described in Epstein et al., U.S. Patent No. 4,522,811. For example, liposome formulations may be prepared by dissolving appropriate lipid(s) (such as stearyl phosphatidyl ethanolamine, stearyl phosphatidyl choline, arachidoyl phosphatidyl choline, and cholesterol) in an inorganic solvent that is then evaporated, leaving behind a thin film of dried lipid on the surface of the container. An aqueous solution of the active compound or its pharmaceutically acceptable salt is then introduced into the container. The container is then swirled by hand to free lipid material from the sides of the container and to disperse lipid aggregates, thereby forming the liposomal suspension.

[0306] The above-recited microparticles and methods of preparing the microparticles are offered by way of example and they are not intended to define the scope of microparticles of use in the present invention. It will be apparent to those of skill in the art that an array of microparticles, fabricated by different methods, is of use in the present invention.

[0307] The structural formats discussed above in the context of the water-soluble polymers, both straight-chain and branched are generally applicable with respect to the water-insoluble polymers as well. Thus, for example, the cysteine, serine, dilysine, and trylsine branching cores can be functionalized with two water-insoluble polymer moieties. The methods used to produce these species are generally closely analogous to those used to produce the water-soluble polymers.

Biomolecules

[0308] In another preferred embodiment, the modified sugar bears a biomolecule. In still further preferred embodiments, the biomolecule is a functional protein, enzyme, antigen, antibody, peptide, nucleic acid (e.g., single nucleotides or nucleosides, oligonucleotides, polynucleotides and single- and higher-stranded nucleic acids), lectin, receptor or a combination thereof.

[0309] Preferred biomolecules are essentially non-fluorescent, or emit such a minimal amount of fluorescence that they are inappropriate for use as a fluorescent marker in an assay. Moreover, it is generally preferred to use biomolecules that are not sugars. An exception to this preference is the use of an otherwise naturally occurring sugar that is modified by covalent attachment of another entity (e.g., PEG, biomolecule, therapeutic moiety, diagnostic moiety, etc.). In an exemplary embodiment, a sugar moiety, which is a biomolecule, is conjugated to a linker arm and the sugar-linker arm cassette is subsequently conjugated to a peptide via a method of the invention.

[0310] Biomolecules useful in practicing the present invention can be derived from any source. The biomolecules can be isolated from natural sources or they can be produced by synthetic methods. Peptides can be natural peptides or

mutated peptides. Mutations can be effected by chemical mutagenesis, site-directed mutagenesis or other means of inducing mutations known to those of skill in the art. Peptides useful in practicing the instant invention include, for example, enzymes, antigens, antibodies and receptors. Antibodies can be either polyclonal or monoclonal; either intact or fragments. The peptides are optionally the products of a program of directed evolution.

5 **[0311]** Both naturally derived and synthetic peptides and nucleic acids are of use in conjunction with the present invention; these molecules can be attached to a sugar residue component or a crosslinking agent by any available reactive group. For example, peptides can be attached through a reactive amine, carboxyl, sulfhydryl, or hydroxyl group. The reactive group can reside at a peptide terminus or at a site internal to the peptide chain. Nucleic acids can be attached through a reactive group on a base (e.g., exocyclic amine) or an available hydroxyl group on a sugar moiety
10 (e.g., 3'- or 5'-hydroxy). The peptide and nucleic acid chains can be further derivatized at one or more sites to allow for the attachment of appropriate reactive groups onto the chain. See, Chrisey et al. Nucleic Acids Res. 24: 3031-3039 (1996).

[0312] In a further preferred embodiment, the biomolecule is selected to direct the peptide modified by the methods of the invention to a specific tissue, thereby enhancing the delivery of the peptide to that tissue relative to the amount of underivatized peptide that is delivered to the tissue. In a still further preferred embodiment, the amount of derivatized
15 peptide delivered to a specific tissue within a selected time period is enhanced by derivatization by at least about 20%, more preferably, at least about 40%, and more preferably still, at least about 100%. Presently, preferred biomolecules for targeting applications include antibodies, hormones and ligands for cell-surface receptors.

[0313] In still a further exemplary embodiment, there is provided as conjugate with biotin. Thus, for example, a selectively biotinylated peptide is elaborated by the attachment of an avidin or streptavidin moiety bearing one or more
20 modifying groups.

The Methods

[0314] In addition to the conjugates discussed above, the present invention provides methods for preparing these and other conjugates. Thus, in a further aspect, the invention provides a method of forming a covalent conjugate between
25 a selected moiety and a peptide. Additionally, the invention provides methods for targeting conjugates of the invention to a particular tissue or region of the body. Furthermore, the present invention provides a method for preventing, curing, or ameliorating a disease state by administering a conjugate of the invention to a subject at risk of developing the disease or a subject that has the disease.

30 **[0315]** In exemplary embodiments, the conjugate is formed between a water-soluble polymer, a therapeutic moiety, targeting moiety or a biomolecule, and a glycosylated or non-glycosylated peptide. The polymer, therapeutic moiety or biomolecule is conjugated to the peptide via an intact glycosyl linking group, which is interposed between, and covalently linked to both the peptide and the modifying group (e.g., water-soluble polymer).

[0316] In an exemplary embodiment, the conjugate is formed through a chemical process sometimes referred to as chemoPEGylation. Further discussion of the synthesis chemoPEGylated peptide conjugates is provided in
35 PCT/US02/3226, filed October 9, 2002 and U.S. Pat. App. No. 10/287,994, filed November 5, 2002, each of which are herein incorporated by reference in their entirety.

[0317] The method includes contacting the peptide with a mixture containing a modified sugar and a glycosyltransferase for which the modified sugar is a substrate. The reaction is conducted under conditions sufficient to form a covalent
40 bond between the modified sugar and the peptide. The sugar moiety of the modified sugar is preferably selected from nucleotide sugars, activated sugars, and sugars that are neither nucleotides nor activated.

[0318] The acceptor peptide (glycosylated or non-glycosylated) is typically synthesized *de novo*, or recombinantly expressed in a prokaryotic cell (e.g., bacterial cell, such as *E. coli*) or in a eukaryotic cell such as a mammalian cell (e.g., CHO cells), yeast (e.g., *Saccharomyces*), insect, or plant cell. The peptide can be either a full-length protein or a fragment.
45 Moreover, the peptide can be a wild type or mutated peptide. In an exemplary embodiment, the peptide includes a mutation that adds one or more consensus glycosylation sites to the peptide sequence.

[0319] The method of the invention also provides for modification of incompletely glycosylated peptides that are produced recombinantly. Many recombinantly produced glycoproteins are incompletely glycosylated, exposing carbohydrate residues that may have undesirable properties, e.g., immunogenicity, recognition by the RES. Employing a modified
50 sugar in a method of the invention, the peptide can be simultaneously further glycosylated and derivatized with, e.g., a water-soluble polymer, therapeutic agent, or the like. The sugar moiety of the modified sugar can be the residue that would properly be conjugated to the acceptor in a fully glycosylated peptide, or another sugar moiety with desirable properties.

[0320] Peptides modified by the methods of the invention can be synthetic or wild-type peptides or they can be mutated
55 peptides, produced by methods known in the art, such as site-directed mutagenesis. Glycosylation of peptides is typically either N-linked or O-linked. An exemplary N-linkage is the attachment of the modified sugar to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of a carbohydrate moiety to the asparagine

side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one sugar (e.g., N-acetylgalactosamine, galactose, mannose, GlcNAc, glucose, fucose or xylose) to the hydroxy side chain of a hydroxyamino acid, preferably serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

[0321] Addition of glycosylation sites to a peptide or other structure is conveniently accomplished by altering the amino acid sequence such that it contains one or more glycosylation sites. The addition may also be made by the incorporation of one or more species presenting an -OH group, preferably serine or threonine residues, within the sequence of the peptide (for O-linked glycosylation sites). The addition may be made by mutation or by full chemical synthesis of the peptide. The peptide amino acid sequence is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding the peptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) are preferably made using methods known in the art.

[0322] In an exemplary embodiment, the glycosylation site is added by shuffling polynucleotides. Polynucleotides encoding a candidate peptide can be modulated with DNA shuffling protocols. DNA shuffling is a process of recursive recombination and mutation, performed by random fragmentation of a pool of related genes, followed by reassembly of the fragments by a polymerase chain reaction-like process. See, e.g., Stemmer, Proc. Natl. Acad. Sci. USA 91:10747-10751 (1994); Stemmer, Nature 370:389-391 (1994); and U.S. Patent Nos. 5,605,793, 5,837,458, 5,830,721 and 5,811,238.

[0323] The present invention also provides means of adding (or removing) one or more selected glycosyl residues to a peptide, after which a modified sugar is conjugated to at least one of the selected glycosyl residues of the peptide. The present embodiment is useful, for example, when it is desired to conjugate the modified sugar to a selected glycosyl residue that is either not present on a peptide or is not present in a desired amount. Thus, prior to coupling a modified sugar to a peptide, the selected glycosyl residue is conjugated to the peptide by enzymatic or chemical coupling. In another embodiment, the glycosylation pattern of a glycopeptide is altered prior to the conjugation of the modified sugar by the removal of a carbohydrate residue from the glycopeptide. See, for example WO 98/31826.

[0324] Addition or removal of any carbohydrate moieties present on the glycopeptide is accomplished either chemically or enzymatically. Chemical deglycosylation is preferably brought about by exposure of the polypeptide variant to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the peptide intact. Chemical deglycosylation is described by Hakimuddin et al., Arch. Biochem. Biophys. 259: 52 (1987) and by Edge et al., Anal. Biochem. 118: 131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptide variants can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., Meth. Enzymol. 138: 350 (1987).

[0325] Chemical addition of glycosyl moieties is carried out by any art-recognized method. Enzymatic addition of sugar moieties is preferably achieved using a modification of the methods set forth herein, substituting native glycosyl units for the modified sugars used in the invention. Other methods of adding sugar moieties are disclosed in U.S. Patent Nos. 5,876,980, 6,030,815, 5,728,554, and 5,922,577.

[0326] Exemplary attachment points for selected glycosyl residue include, but are not limited to: (a) consensus sites for N-linked glycosylation and O-linked glycosylation; (b) terminal glycosyl moieties that are acceptors for a glycosyl-transferase; (c) arginine, asparagine and histidine; (d) free carboxyl groups; (e) free sulfhydryl groups such as those of cysteine; (f) free hydroxyl groups such as those of serine, threonine, or hydroxyproline; (g) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan; or (h) the amide group of glutamine. Exemplary methods of use in the present invention are described in WO 87/05330 published Sep. 11, 1987, and in Aplin and Wriston, CRC CRIT. REV. BIOCHEM., pp. 259-306(1981).

[0327] In one embodiment, the invention provides a method for linking FGF-21 and one or more peptide through a linking group. The linking group is of any useful structure and may be selected from straight-chain and branched chain structures. Preferably, each terminus of the linker, which is attached to a peptide, includes a modified sugar (i.e., a nascent intact glycosyl linking group).

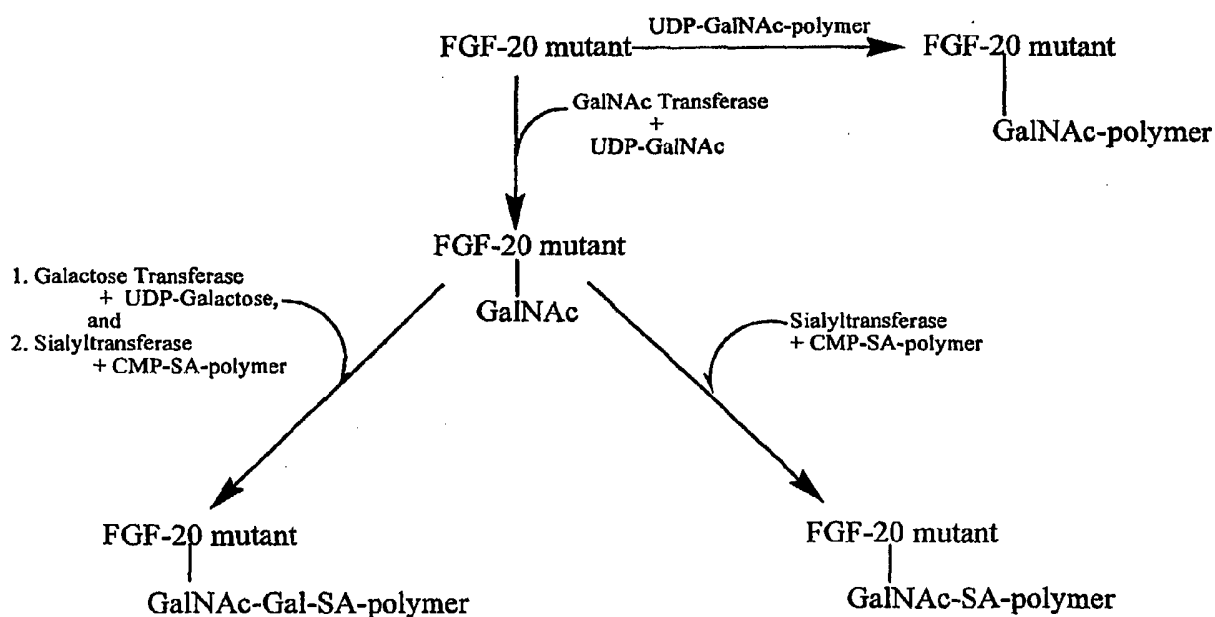
[0328] In an exemplary method of the invention, two peptides are linked together via a linker moiety that includes a PEG linker. The construct conforms to the general structure set forth in the cartoon above. As described herein, the construct of the invention includes two intact glycosyl linking groups (i.e., $s + t = 1$). The focus on a PEG linker that includes two glycosyl groups is for purposes of clarity and should not be interpreted as limiting the identity of linker arms of use in this embodiment of the invention.

[0329] Thus, a PEG moiety is functionalized at a first terminus with a first glycosyl unit and at a second terminus with a second glycosyl unit. The first and second glycosyl units are preferably substrates for different transferases, allowing orthogonal attachment of the first and second peptides to the first and second glycosyl units, respectively. In practice, the (glycosyl)¹-PEG-(glycosyl)² linker is contacted with the first peptide and a first transferase for which the first glycosyl unit is a substrate, thereby forming (peptide)¹-(glycosyl)¹-PEG-(glycosyl)². Glycosyltransferase and/or unreacted peptide is then optionally removed from the reaction mixture. The second peptide and a second transferase for which the second

glycosyl unit is a substrate are added to the (peptide)¹-(glycosyl)¹-PEG-(glycosyl)² conjugate, forming (peptide)¹-(glycosyl)¹-PEG-(glycosyl)²-(peptide)². Those of skill in the art will appreciate that the method outlined above is also applicable to forming conjugates between more than two peptides by, for example, the use of a branched PEG, dendrimer, poly(amino acid), polysaccharide or the like.

[0330] Another exemplary embodiment is set forth in Scheme 3. Scheme 3 shows a method of preparing a conjugate comprising a polymer. The polymer increases the circulatory half-life of the FGF protein.

Scheme 3



in which SA is sialic acid, and polymer is PEG, mPEG, poly sialic acid, a water soluble or water insoluble polymer. Though the method is exemplified by reference to FGF-20 and FGF-21, those of skill will appreciate it is equally applicable to other FGF peptides, e.g., FGF-9 and FGF-18.

[0331] The use of reactive derivatives of PEG (or other linkers) to attach one or more peptide moieties to the linker is within the scope of the present invention. The invention is not limited by the identity of the reactive PEG analogue. Many activated derivatives of poly(ethyleneglycol) are available commercially and in the literature. It is well within the abilities of one of skill to choose, and synthesize if necessary, an appropriate activated PEG derivative with which to prepare a substrate useful in the present invention. See, Abuchowski et al. *Cancer Biochem. Biophys.*, 7: 175-186 (1984); Abuchowski et al., *J. Biol. Chem.*, 252: 3582-3586 (1977); Jackson et al., *Anal. Biochem.*, 165: 114-127 (1987); Koide et al., *Biochem Biophys. Res. Commun.*, 111: 659-667 (1983)), tresylate (Nilsson et al., *Methods Enzymol.*, 104: 56-69 (1984); Delgado et al., *Biotechnol. Appl. Biochem.*, 12: 119-128 (1990)); N-hydroxysuccinimide derived active esters (Buckmann et al., *Makromol. Chem.*, 182: 1379-1384(1981); Joppich et al., *Makromol. Chem.*, 180: 1381-1384(1979); Abuchowski et al., *Cancer Biochem. Biophys.*, 7: 175-186 (1984); Katre et al. *Proc. Natl. Acad. Sci. U.S.A.*, 84: 1487-1491 (1987); Kitamura et al., *Cancer Res.*, 51: 4310-4315 (1991); Boccu et al., *Z. Naturforsch.*, 38C: 94-99 (1983), carbonates (Zalipsky et al., *POLY(ETHYLENE GLYCOL) CHEMISTRY: BIOTECHNICAL AND BIOMEDICAL APPLICATIONS*, Harris, Ed., Plenum Press, New York, 1992, pp. 347-370; Zalipsky et al., *Biotechnol. Appl. Biochem.*, 15: 100-114 (1992); Veronese et al., *Appl. Biochem. Biotech.*, 11: 141-152 (1985)), imidazolyl formates (Beauchamp et al., *Anal. Biochem.*, 131: 25-33 (1983); Berger et al., *Blood*, 71: 1641-1647 (1988)), 4-dithiopyridines (Woghiren et al., *Bioconjugate Chem.*, 4: 314-318 (1993)), isocyanates (Byun et al., *ASAIO Journal*, M649-M-653 (1992)) and epoxides (U.S. Pat. No. 4,806,595, issued to Noishiki et al., (1989). Other linking groups include the urethane linkage between amino groups and activated PEG. See, Veronese, et al., *Appl. Biochem. Biotechnol.*, 11: 141-152 (1985).

Preparation of Modified Sugars

[0332] In general, the sugar moiety and the modifying group are linked together through the use of reactive groups, which are typically transformed by the linking process into a new organic functional group or species that is unreactive

under physiologically relevant conditions. The sugar reactive functional group(s), is located at any position on the sugar moiety. Reactive groups and classes of reactions useful in practicing the present invention are generally those that are well known in the art of bioconjugate chemistry. Currently favored classes of reactions available with reactive sugar moieties are those, which proceed under relatively mild conditions. These include, but are not limited to nucleophilic substitutions (*e.g.*, reactions of amines and alcohols with acyl halides, active esters), electrophilic substitutions (*e.g.*, enamine reactions) and additions to carbon-carbon and carbon-heteroatom multiple bonds (*e.g.*, Michael reaction, Diels-Alder addition). These and other useful reactions are discussed in, for example, March, *ADVANCED ORGANIC CHEMISTRY*, 3rd Ed., John Wiley & Sons, New York, 1985; Hermanson, *BIOCONJUGATE TECHNIQUES*, Academic Press, San Diego, 1996; and Feeney et al., *MODIFICATION OF PROTEINS*; *Advances in Chemistry Series*, Vol. 198, American Chemical Society, Washington, D.C., 1982.

[0333] Useful reactive functional groups pendent from a sugar nucleus or modifying group include, but are not limited to:

(a) carboxyl groups and various derivatives thereof including, but not limited to, N-hydroxysuccinimide esters, N-hydroxybenztriazole esters, acid halides, acyl imidazoles, thioesters, p-nitrophenyl esters, alkyl, alkenyl, alkynyl and aromatic esters;

(b) hydroxyl groups, which can be converted to, *e.g.*, esters, ethers, aldehydes, *etc.*

(c) haloalkyl groups, wherein the halide can be later displaced with a nucleophilic group such as, for example, an amine, a carboxylate anion, thiol anion, carbanion, or an alkoxide ion, thereby resulting in the covalent attachment of a new group at the functional group of the halogen atom;

(d) dienophile groups, which are capable of participating in Diels-Alder reactions such as, for example, maleimido groups;

(e) aldehyde or ketone groups, such that subsequent derivatization is possible via formation of carbonyl derivatives such as, for example, imines, hydrazones, semicarbazones or oximes, or via such mechanisms as Grignard addition or alkyllithium addition;

(f) sulfonyl halide groups for subsequent reaction with amines, for example, to form sulfonamides;

(g) thiol groups, which can be, for example, converted to disulfides or reacted with acyl halides;

(h) amine or sulfhydryl groups, which can be, for example, acylated, alkylated or oxidized;

(i) alkenes, which can undergo, for example, cycloadditions, acylation, Michael addition, *etc.*; and

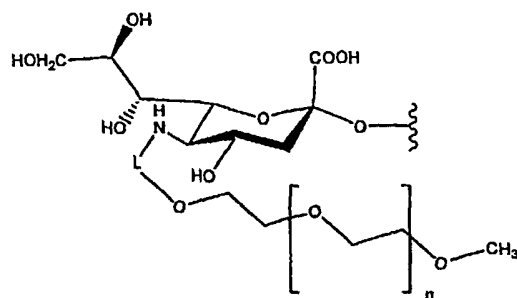
(j) epoxides, which can react with, for example, amines and hydroxyl compounds.

[0334] The reactive functional groups can be chosen such that they do not participate in, or interfere with, the reactions necessary to assemble the reactive sugar nucleus or modifying group. Alternatively, a reactive functional group can be protected from participating in the reaction by the presence of a protecting group. Those of skill in the art understand how to protect a particular functional group such that it does not interfere with a chosen set of reaction conditions. For examples of useful protecting groups, see, for example, Greene et al., *PROTECTIVE GROUPS IN ORGANIC SYNTHESIS*, John Wiley & Sons, New York, 1991.

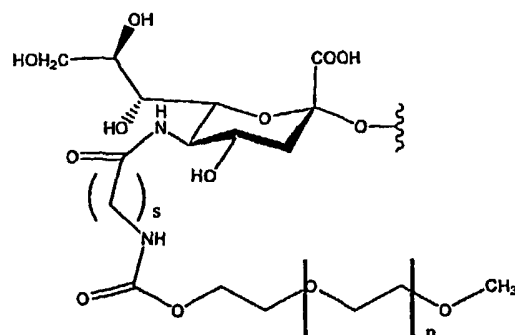
[0335] In the discussion that follows, a number of specific examples of modified sugars that are useful in practicing the present invention are set forth. In the exemplary embodiments, a sialic acid derivative is utilized as the sugar nucleus to which the modifying group is attached. The focus of the discussion on sialic acid derivatives is for clarity of illustration only and should not be construed to limit the scope of the invention. Those of skill in the art will appreciate that a variety of other sugar moieties can be activated and derivatized in a manner analogous to that set forth using sialic acid as an example. For example, numerous methods are available for modifying galactose, glucose, N-acetylgalactosamine and fucose to name a few sugar substrates, which are readily modified by art recognized methods. See, for example, Elhalabi et al., *Curr. Med. Chem.* 6: 93 (1999); and Schafer et al., *J. Org. Chem.* 65: 24 (2000)).

[0336] In an exemplary embodiment, the peptide that is modified by a method of the invention is a glycopeptide that is produced in prokaryotic cells (*e.g.*, *E.coli*), eukaryotic cells including yeast and mammalian cells (*e.g.*, CHO cells), or in a transgenic animal and thus, contains N- and/or O-linked oligosaccharide chains, which are incompletely sialylated. The oligosaccharide chains of the glycopeptide lacking a sialic acid and containing a terminal galactose residue can be PEG-ylated, PPG-ylated or otherwise modified with a modified sialic acid.

[0337] Exemplary PEG-sialic acid derivative include:



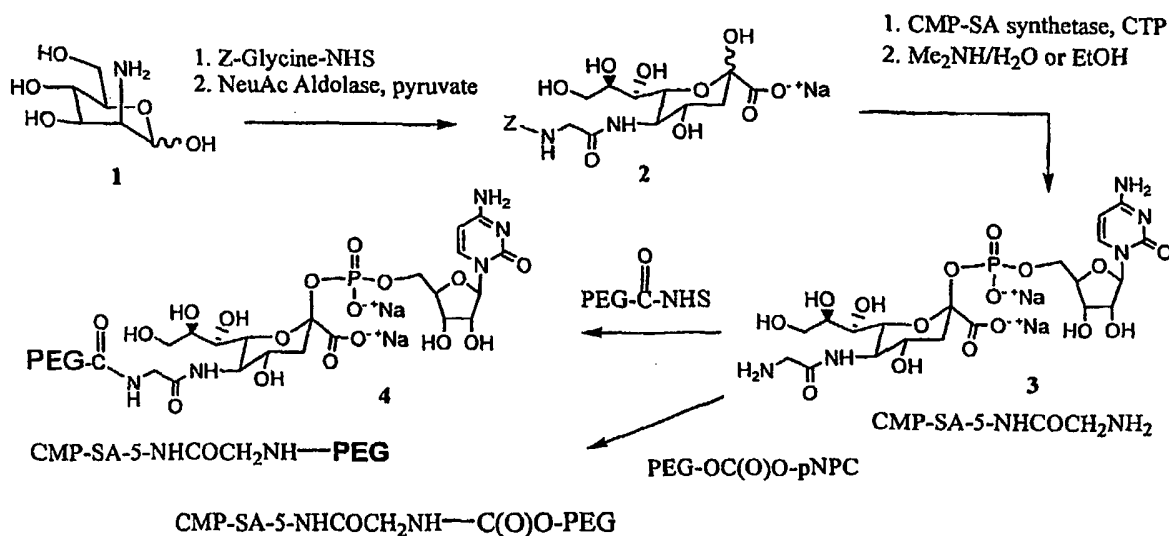
in which L is a substituted or unsubstituted alkyl or substituted or unsubstituted heteroalkyl linker moiety joining the sialic acid moiety and the PEG moiety, and "n" is 1 or greater; and



in which the index "s" represents an integer from 0 to 20, and "n" is 1 or greater.

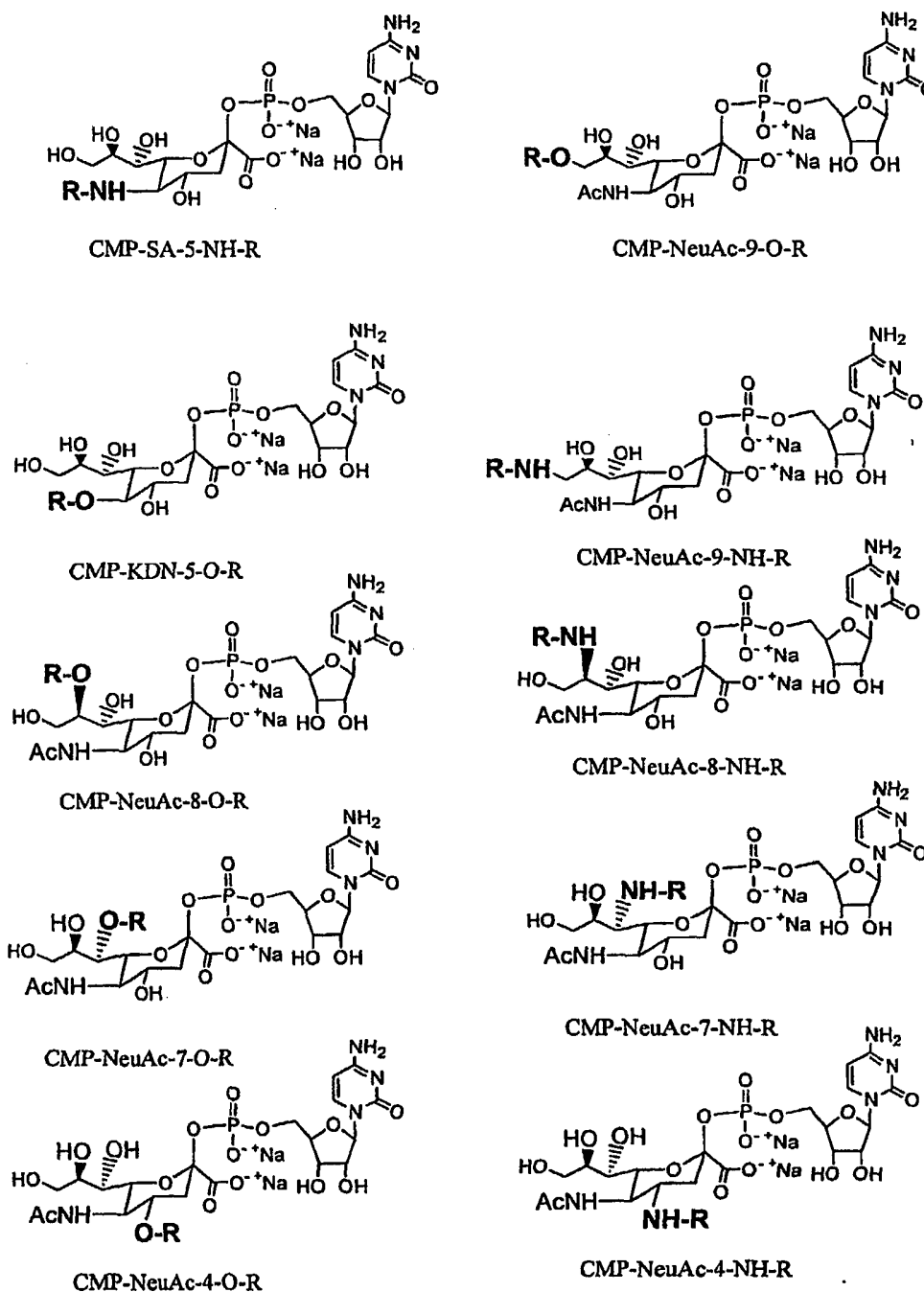
[0338] In Scheme 4, the amino glycoside **1**, is treated with the active ester of a protected amino acid (e.g., glycine) derivative, converting the sugar amine residue into the corresponding protected amino acid amide adduct. The adduct is treated with an aldolase to form α -hydroxy carboxylate **2**. Compound **2** is converted to the corresponding CMP derivative by the action of CMP-SA synthetase, followed by catalytic hydrogenation of the CMP derivative to produce compound **3**. The amine introduced via formation of the glycine adduct is utilized as a locus of PEG attachment by reacting compound **3** with an activated PEG or PPG derivative (e.g., PEG-C(O)NHS, PEG-OC(O)O-p-nitrophenyl), producing species such as **4** or **5**, respectively.

Scheme 4



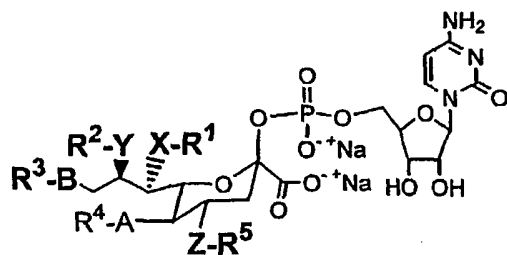
[0339] Table 1 sets forth representative examples of sugar monophosphates that are derivatized with a modifying group, such as a PEG or PPG moiety. Fibroblast Growth Factor peptides can be modified by the method of Scheme 1. Other derivatives are prepared by art-recognized methods. See, for example, Keppler et al., *Glycobiology* 11: 11R (2001); and Charter et al., *Glycobiology* 10: 1049 (2000)). Other amine reactive PEG and PPG analogues are commercially available, or they can be prepared by methods readily accessible to those of skill in the art.

Table 1



wherein R represents a modifying group, e.g., linear or branched PEG or $-L^x-R^x$ in which L^x is a linker selected from a bond (zero-order), substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl, and R^x is the modifying group.

[0340] The modified sugar phosphates of use in practicing the present invention can be substituted in other positions as well as those set forth above. Presently preferred substitutions of sialic acid are set forth in Formula 1:



(I)

in which X is a linking group, which is preferably selected from -O-, -N(H)-, -S, CH₂-, and -N(R)₂-, in which each R is a member independently selected from R¹-R⁵. The symbols Y, Z, A and B each represent a group that is selected from the group set forth above for the identity of X. X, Y, Z, A and B are each independently selected and, therefore, they can be the same or different. The symbols R¹, R², R³, R⁴ and R⁵ represent H, a water-soluble polymer, therapeutic moiety, biomolecule or other moiety. Alternatively, these symbols represent a linker that is bound to a water-soluble polymer, therapeutic moiety, biomolecule or other moiety.

[0341] Exemplary moieties attached to the conjugates disclosed herein include, but are not limited to, PEG derivatives (e.g., alkyl-PEG, acyl-PEG, acyl-alkyl-PEG, alkyl-acyl-PEG carbamoyl-PEG, aryl-PEG), PPG derivatives (e.g., alkyl-PPG, acyl-PPG, acyl-alkyl-PPG, alkyl-acyl-PPG carbamoyl-PPG, aryl-PPG), therapeutic moieties, diagnostic moieties, mannose-6-phosphate, heparin, heparan, SLe_x, mannose, mannose-6-phosphate, Sialyl Lewis X, FGF, VFGF, proteins, chondroitin, keratan, dermatan, albumin, integrins, antennary oligosaccharides, peptides and the like. Methods of conjugating the various modifying groups to a saccharide moiety are readily accessible to those of skill in the art (POLY (ETHYLENE GLYCOL CHEMISTRY : BIOTECHNICAL AND BIOMEDICAL APPLICATIONS, J. Milton Harris, Ed., Plenum Pub. Corp., 1992; POLY (ETHYLENE GLYCOL) CHEMICAL AND BIOLOGICAL APPLICATIONS, J. Milton Harris, Ed., ACS Symposium Series No. 680, American Chemical Society, 1997; Hermanson, BIOCONJUGATE TECHNIQUES, Academic Press, San Diego, 1996; and Dunn et al., Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991).

Cross-linking Groups

[0342] Preparation of the modified sugar for use in the methods of the present invention includes attachment of a modifying group to a sugar residue and forming a stable adduct, which is a substrate for a glycosyltransferase. The sugar and modifying group can be coupled by a zero- or higher-order cross-linking agent. Exemplary bifunctional compounds which can be used for attaching modifying groups to carbohydrate moieties include, but are not limited to, bifunctional poly(ethyleneglycols), polyamides, polyethers, polyesters and the like. General approaches for linking carbohydrates to other molecules are known in the literature. See, for example, Lee et al., Biochemistry 28: 1856 (1989); Bhatia et al., Anal. Biochem. 178: 408 (1989); Janda et al., J. Am. Chem. Soc. 112: 8886 (1990) and Bednarski et al., WO 92/18135. In the discussion that follows, the reactive groups are treated as benign on the sugar moiety of the nascent modified sugar. The focus of the discussion is for clarity of illustration. Those of skill in the art will appreciate that the discussion is relevant to reactive groups on the modifying group as well.

[0343] A variety of reagents are used to modify the components of the modified sugar with intramolecular chemical crosslinks (for reviews of crosslinking reagents and crosslinking procedures see: Wold, F., Meth. Enzymol. 25: 623-651, 1972; Weetall, H. H., and Cooney, D. A., In: ENZYMES AS DRUGS. (Holcenberg, and Roberts, eds.) pp. 395-442, Wiley, New York, 1981; Ji, T. H., Meth. Enzymol. 91: 580-609, 1983; Mattson et al., Mol. Biol. Rep. 17:167-183, 1993, all of which are incorporated herein by reference). Preferred crosslinking reagents are derived from various zero-length, homo-bifunctional, and hetero-bifunctional crosslinking reagents. Zero-length crosslinking reagents include direct conjugation of two intrinsic chemical groups with no introduction of extrinsic material. Agents that catalyze formation of a disulfide bond belong to this category. Another example is reagents that induce condensation of a carboxyl and a primary amino group to form an amide bond such as carbodiimides, ethylchloroformate, Woodward's reagent K (2-ethyl-5-phenylisoxazolium-3'-sulfonate), and carbonyldiimidazole. In addition to these chemical reagents, the enzyme transglutaminase (glutamyl-peptide γ -glutamyltransferase; EC 2.3.2.13) may be used as zero-length crosslinking reagent. This enzyme catalyzes acyl transfer reactions at carboxamide groups of protein-bound glutaminyl residues, usually with a primary amino group as substrate. Preferred homo- and hetero-bifunctional reagents contain two identical or two dissimilar sites, respectively, which may be reactive for amino, sulfhydryl, guanidino, indole, or nonspecific groups.

[0344] In still another embodiment, photoactivatable groups are selected from diazopyruvates. For example, the p-nitrophenyl ester of p-nitrophenyl diazopyruvate reacts with aliphatic amines to give diazopyruvic acid amides that undergo ultraviolet photolysis to form aldehydes. The photolyzed diazopyruvate-modified affinity component will react like formaldehyde or glutaraldehyde forming crosslinks.

Cleavable Linker Groups

[0345] In yet a further embodiment, the linker group is provided with a group that can be cleaved to release the modifying group from the sugar residue. Many cleaveable groups are known in the art. See, for example, Jung et al., Biochem. Biophys. Acta 761: 152-162 (1983); Joshi et al., J. Biol. Chem. 265: 14518-14525 (1990); Zarling et al., J. Immunol. 124: 913-920 (1980); Bouizar et al., Eur. J. Biochem. 155: 141-147 (1986); Park et al., J. Biol. Chem. 261: 205-210 (1986); Browning et al., J. Immunol. 143: 1859-1867 (1989). Moreover a broad range of cleavable, bifunctional (both homo- and hetero-bifunctional) linker groups is commercially available from suppliers such as Pierce.

[0346] Exemplary cleaveable moieties can be cleaved using light, heat or reagents such as thiols, hydroxylamine, bases, periodate and the like. Moreover, certain preferred groups are cleaved *in vivo* in response to being endocytized (e.g., cis-aconityl; see, Shen et al., Biochem. Biophys. Res. Commun. 102: 1048 (1991)). Preferred cleaveable groups comprise a cleaveable moiety which is a member selected from the group consisting of disulfide, ester, imide, carbonate, nitrobenzyl, phenacyl and benzoin groups.

Conjugation of Modified Sugars to Peptides

[0347] The modified sugars are conjugated to a glycosylated or non-glycosylated peptide using an appropriate enzyme to mediate the conjugation. Preferably, the concentrations of the modified donor sugar(s), enzyme(s) and acceptor peptide(s) are selected such that glycosylation proceeds until the acceptor is consumed. The considerations discussed below, while set forth in the context of a sialyltransferase, are generally applicable to other glycosyltransferase reactions.

[0348] A number of methods of using glycosyltransferases to synthesize desired oligosaccharide structures are known and are generally applicable to the instant invention. Exemplary methods are described, for instance, WO 96/32491, Ito et al., Pure Appl. Chem. 65: 753 (1993), and U.S. Pat. Nos. 5,352,670, 5,374,541, and 5,545,553.

[0349] The present invention is practiced using a single glycosyltransferase or a combination of glycosyltransferases. For example, one can use a combination of a sialyltransferase and a galactosyltransferase. In those embodiments using more than one enzyme, the enzymes and substrates are preferably combined in an initial reaction mixture, or the enzymes and reagents for a second enzymatic reaction are added to the reaction medium once the first enzymatic reaction is complete or nearly complete. By conducting two enzymatic reactions in sequence in a single vessel, overall yields are improved over procedures in which an intermediate species is isolated. Moreover, cleanup and disposal of extra solvents and by-products is reduced.

[0350] In a preferred embodiment, each of the first and second enzyme is a glycosyltransferase. In another preferred embodiment, one enzyme is an endoglycosidase. In an additional preferred embodiment, more than two enzymes are used to assemble the modified glycoprotein of the invention. The enzymes are used to alter a saccharide structure on the peptide at any point either before or after the addition of the modified sugar to the peptide.

[0351] In another embodiment, the method makes use of one or more exo- or endoglycosidase. The glycosidase is typically a mutant, which is engineered to form glycosyl bonds rather than cleave them. The mutant glycanase typically includes a substitution of an amino acid residue for an active site acidic amino acid residue. For example, when the endoglycanase is endo-H, the substituted active site residues will typically be Asp at position 130, Glu at position 132 or a combination thereof. The amino acids are generally replaced with serine, alanine, asparagine, or glutamine.

[0352] The mutant enzyme catalyzes the reaction, usually by a synthesis step that is analogous to the reverse reaction of the endoglycanase hydrolysis step. In these embodiments, the glycosyl donor molecule (e.g., a desired oligo- or mono-saccharide structure) contains a leaving group and the reaction proceeds with the addition of the donor molecule to a GlcNAc residue on the protein. For example, the leaving group can be a halogen, such as fluoride. In other embodiments, the leaving group is a Asn, or a Asn-peptide moiety. In yet further embodiments, the GlcNAc residue on the glycosyl donor molecule is modified. For example, the GlcNAc residue may comprise a 1,2 oxazoline moiety.

[0353] In a preferred embodiment, each of the enzymes utilized to produce a conjugate of the invention are present in a catalytic amount. The catalytic amount of a particular enzyme varies according to the concentration of that enzyme's substrate as well as to reaction conditions such as temperature, time and pH value. Means for determining the catalytic amount for a given enzyme under preselected substrate concentrations and reaction conditions are well known to those of skill in the art.

[0354] The temperature at which an above process is carried out can range from just above freezing to the temperature at which the most sensitive enzyme denatures. Preferred temperature ranges are about 0 °C to about 55 °C, and more preferably about 20 °C to about 30 °C. In another exemplary embodiment, one or more components of the present method are conducted at an elevated temperature using a thermophilic enzyme.

[0355] The reaction mixture is maintained for a period of time sufficient for the acceptor to be glycosylated, thereby forming the desired conjugate. Some of the conjugate can often be detected after a few hours, with recoverable amounts usually being obtained within 24 hours or less. Those of skill in the art understand that the rate of reaction is dependent on a number of variable factors (e.g, enzyme concentration, donor concentration, acceptor concentration, temperature,

solvent volume), which are optimized for a selected system.

[0356] The present invention also provides for the industrial-scale production of modified peptides. As used herein, an industrial scale generally produces at least about 250 mg, preferably at least about 500 mg, and more preferably at least about 1 gram of finished, purified conjugate, preferably after a single reaction cycle, *i.e.*, the conjugate is not a combination the reaction products from identical, consecutively iterated synthesis cycles.

[0357] In the discussion that follows, the invention is exemplified by the conjugation of modified sialic acid moieties to a glycosylated peptide. The exemplary modified sialic acid is labeled with m-PEG. The focus of the following discussion on the use of PEG-modified sialic acid and glycosylated peptides is for clarity of illustration and is not intended to imply that the invention is limited to the conjugation of these two partners. One of skill understands that the discussion is generally applicable to the additions of modified glycosyl moieties other than sialic acid. Moreover, the discussion is equally applicable to the modification of a glycosyl unit with agents other than m-PEG including other water-soluble polymers, therapeutic moieties, and biomolecules.

[0358] An enzymatic approach can be used for the selective introduction of PEGylated or PPGylated carbohydrates onto a peptide or glycopeptide. The method utilizes modified sugars containing PEG, PPG, or a masked reactive functional group, and is combined with the appropriate glycosyltransferase or glycosynthase. By selecting the glycosyltransferase that will make the desired carbohydrate linkage and utilizing the modified sugar as the donor substrate, the PEG or PPG can be introduced directly onto the peptide backbone, onto existing sugar residues of a glycopeptide or onto sugar residues that have been added to a peptide.

[0359] An acceptor for the sialyltransferase is present on the peptide to be modified by the methods of the present invention either as a naturally occurring structure or one placed there recombinantly, enzymatically or chemically. Suitable acceptors, include, for example, galactosyl acceptors such as Gal β 1,4GlcNAc, Gal β 1,4GalNAc, Gal β 1,3GalNAc, lacto-N-tetraose, Gal β 1,3GlcNAc, GalNAc, Gal β 1,3GalNAc, Gal β 1,6GlcNAc, Gal β 1,4Glc (lactose), and other acceptors known to those of skill in the art (see, *e.g.*, Paulson et al., J. Biol. Chem. 253: 5617-5624 (1978)).

[0360] In one embodiment, an acceptor for the sialyltransferase is present on the glycopeptide to be modified upon *in vivo* synthesis of the glycopeptide. Such glycopeptides can be sialylated using the claimed methods without prior modification of the glycosylation pattern of the glycopeptide. Alternatively, the methods of the invention can be used to sialylate a peptide that does not include a suitable acceptor; one first modifies the peptide to include an acceptor by methods known to those of skill in the art. In an exemplary embodiment, a GalNAc residue is added by the action of a GalNAc transferase.

[0361] In an exemplary embodiment, the galactosyl acceptor is assembled by attaching a galactose residue to an appropriate acceptor linked to the peptide, *e.g.*, a GalNAc. The method includes incubating the peptide to be modified with a reaction mixture that contains a suitable amount of a galactosyltransferase (*e.g.*, Gal β 1,3 or Gal β 1,4), and a suitable galactosyl donor (*e.g.*, UDP-galactose). The reaction is allowed to proceed substantially to completion or, alternatively, the reaction is terminated when a preselected amount of the galactose residue is added. Other methods of assembling a selected saccharide acceptor will be apparent to those of skill in the art.

[0362] In yet another embodiment, glycopeptide-linked oligosaccharides are first "trimmed," either in whole or in part, to expose either an acceptor for the sialyltransferase or a moiety to which one or more appropriate residues can be added to obtain a suitable acceptor. Enzymes such as glycosyltransferases and endoglycosidases (see, for example U.S. Patent No. 5,716,812) are useful for the attaching and trimming reactions.

[0363] In the discussion that follows, the method of the invention is exemplified by the use of modified sugars having a water-soluble polymer attached thereto. The focus of the discussion is for clarity of illustration. Those of skill will appreciate that the discussion is equally relevant to those embodiments in which the modified sugar bears a therapeutic moiety, biomolecule or the like.

[0364] In an exemplary embodiment, an O-linked carbohydrate residue is "trimmed" prior to the addition of the modified sugar. For example a GalNAc-Gal residue is trimmed back to GalNAc. A modified sugar bearing a water-soluble polymer is conjugated to one or more of the sugar residues exposed by the "trimming." In one example, a glycopeptide is "trimmed" and a water-soluble polymer is added to the resulting O-side chain amino acid or glycopeptide glycan via a saccharyl moiety, *e.g.*, Sia, Gal, or GalNAc moiety conjugated to the water-soluble polymer. The modified saccharyl moiety is attached to an acceptor site on the "trimmed" glycopeptide. Alternatively, an unmodified saccharyl moiety, *e.g.*, Gal can be added the terminus of the O-linked glycan.

[0365] In another exemplary embodiment, a water-soluble polymer is added to a GalNAc residue via a modified sugar having a galactose residue. Alternatively, an unmodified Gal can be added to the terminal GalNAc residue.

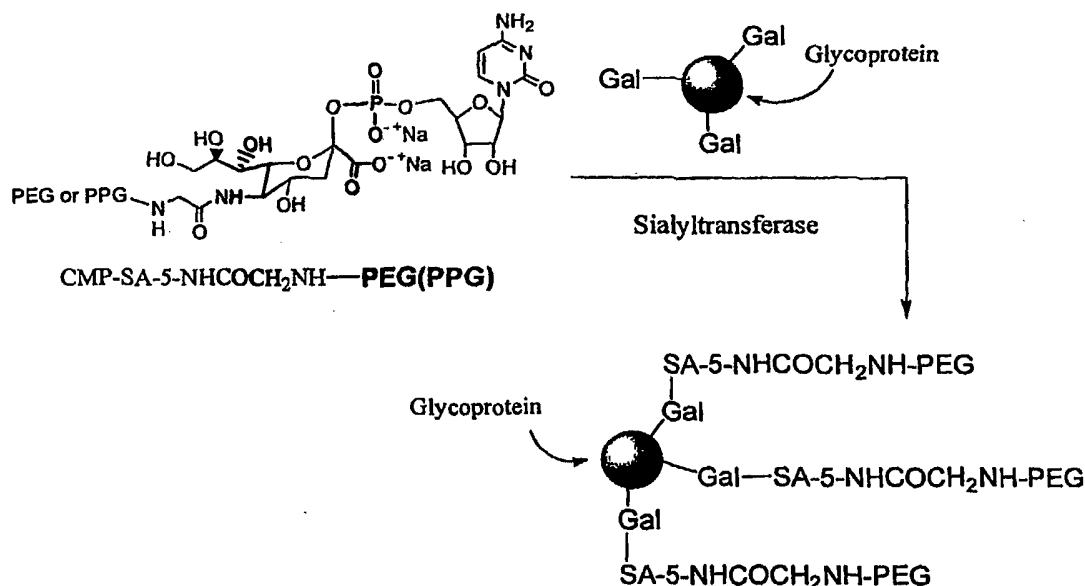
[0366] In yet a further example, a water-soluble polymer is added onto a Gal residue using a modified sialic acid.

[0367] In another exemplary embodiment, an O-linked glycosyl residue is "trimmed back" to the GalNAc attached to the amino acid. In one example, a water-soluble polymer is added via a Gal modified with the polymer. Alternatively, an unmodified Gal is added to the GalNAc, followed by a Gal with an attached water-soluble polymer. In yet another embodiment, one or more unmodified Gal residue is added to the GalNAc, followed by a sialic acid moiety modified with a water-soluble polymer.

[0368] Using the methods of the invention, it is possible to "trim back" and build up a carbohydrate residue of substantially any desired structure. The modified sugar can be added to the termini of the carbohydrate moiety as set forth above, or it can be intermediate between the peptide core and the terminus of the carbohydrate.

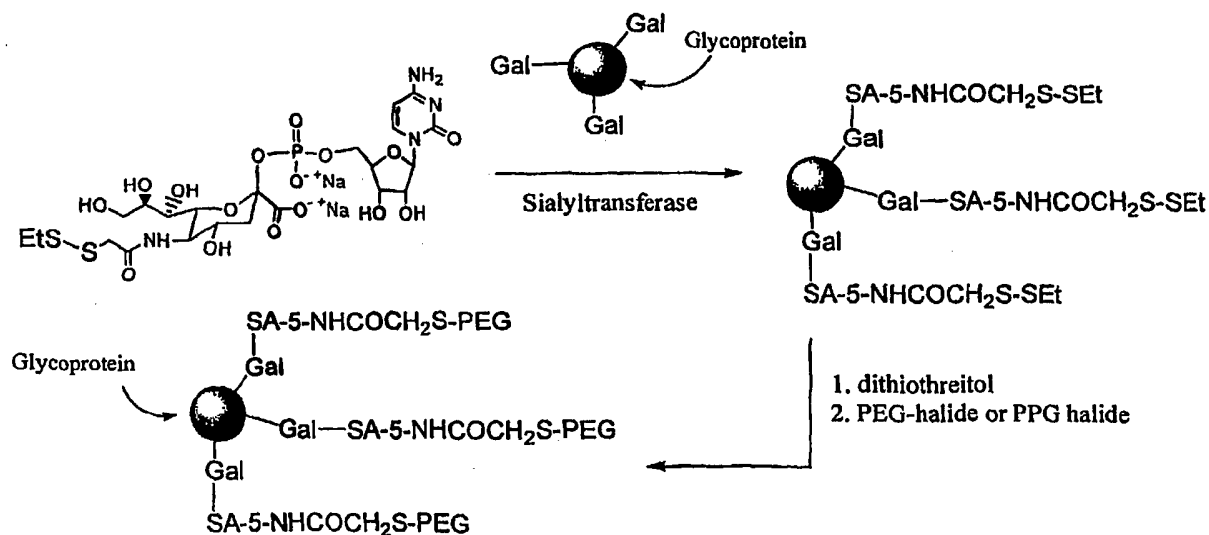
[0369] In an exemplary embodiment, the water-soluble polymer is added to a terminal Gal residue using a polymer modified sialic acid. An appropriate sialyltransferase is used to add a modified sialic acid. The approach is summarized in Scheme 5.

Scheme 5



[0370] In yet a further approach, summarized in Scheme 6, a masked reactive functionality is present on the sialic acid. The masked reactive group is preferably unaffected by the conditions used to attach the modified sialic acid to the peptide. After the covalent attachment of the modified sialic acid to the peptide, the mask is removed and the peptide is conjugated with an agent such as PEG, PPG, a therapeutic moiety, biomolecule or other agent. The agent is conjugated to the peptide in a specific manner by its reaction with the unmasked reactive group on the modified sugar residue.

Scheme 6



[0371] Any modified sugar can be used with its appropriate glycosyltransferase, depending on the terminal sugars of the oligosaccharide side chains of the glycopeptide (Table 2). As discussed above, the terminal sugar of the glycopeptide required for introduction of the PEG-ylated or PPG-ylated structure can be introduced naturally during expression or it

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can be produced post expression using the appropriate glycosidase(s), glycosyltransferase(s) or mix of glycosidase(s) and glycosyltransferase(s).

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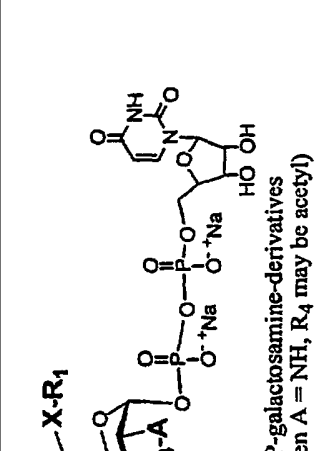
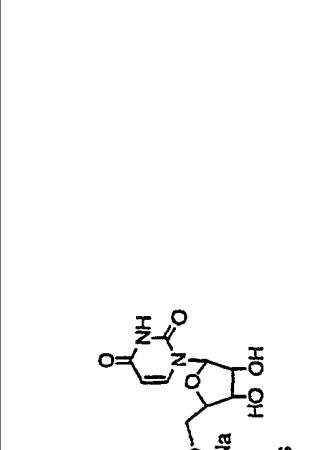
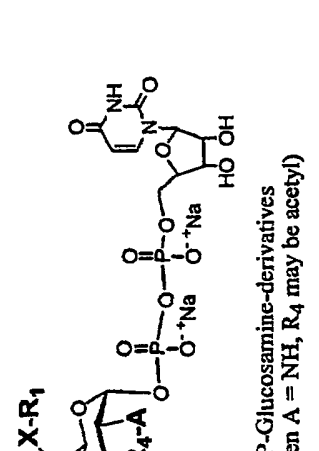
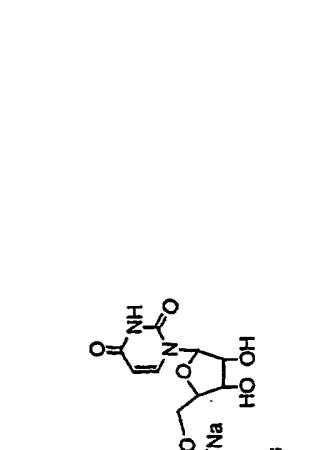
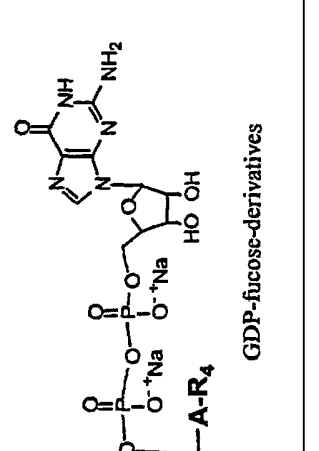
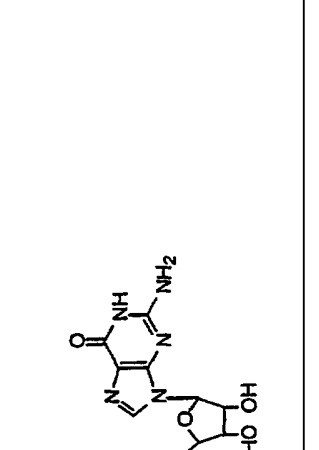
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Table 2

 UDP-galactosamine-derivatives (when A = NH, R₄ may be acetyl)	 UDP-galactose-derivatives
 UDP-Glucosamine-derivatives (when A = NH, R₄ may be acetyl)	 UDP-Glucose-derivatives
 GDP-fucose-derivatives	 GDP-Mannose-derivatives

X = O, NH, S, CH₂, N-(R₁-5)₂.

Y = X; Z = X; A = X; B = X.

Q = H₂, O, S, NH, N-R.R, R₁₋₄ = H, Linker-M, M.

M = Ligand of interest

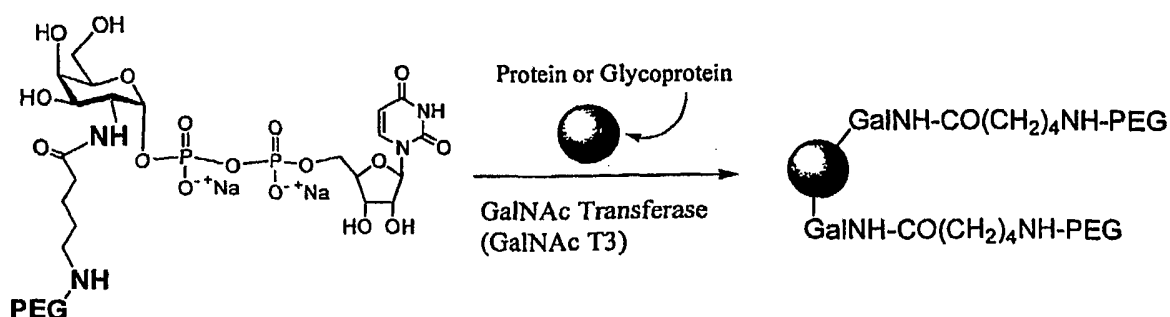
Ligand of interest = acyl-PEG, acyl-PPG, alkyl-PEG, acyl-alkyl-PEG, acyl-alkyl-PEG, carbamoyl-PEG, PEG, PPG, acyl-aryl-PEG, acyl-aryl-PPG, aryl-PEG, aryl-PPG, Mannose-6-phosphate, heparin, heparan, SLEX, Mannose, FGF, VEGF, protein, chondroitin, keratan, dermatan, albumin, integrins, peptides, etc.

[0372] In a further exemplary embodiment, UDP-galactose-PEG is reacted with bovine milk β 1,4-galactosyltransferase, thereby transferring the modified galactose to the appropriate terminal N-acetylglucosamine structure. The terminal GlcNAc residues on the glycopeptide may be produced during expression, as may occur in such expression systems as mammalian, insect, plant or fungus, but also can be produced by treating the glycopeptide with a sialidase and/or

[0373] In another exemplary embodiment, a GlcNAc transferase, such as GNT1-5, is utilized to transfer PEGylated-GlcN to a terminal mannose residue on a glycopeptide. In a still further exemplary embodiment, an the N- and/or O-linked glycan structures are enzymatically removed from a glycopeptide to expose an amino acid or a terminal glycosyl residue that is subsequently conjugated with the modified sugar. For example, an endoglycanase is used to remove the N-linked structures of a glycopeptide to expose a terminal GlcNAc as a GlcNAc-linked-Asn on the glycopeptide. UDP-Gal-PEG and the appropriate galactosyltransferase is used to introduce the PEG- or PPG-galactose functionality onto the exposed GlcNAc.

[0374] In an alternative embodiment, the modified sugar is added directly to the peptide backbone using a glycosyl-transferase known to transfer sugar residues to the peptide backbone. This exemplary embodiment is set forth in Scheme 7. Exemplary glycosyltransferases useful in practicing the present invention include, but are not limited to, GalNAc transferases (GalNAc T1-20), GlcNAc transferases, fucosyltransferases, glucosyltransferases, xylosyltransferases, mannosyltransferases and the like. Use of this approach allows the direct addition of modified sugars onto peptides that lack any carbohydrates or, alternatively, onto existing glycopeptides. In both cases, the addition of the modified sugar occurs at specific positions on the peptide backbone as defined by the substrate specificity of the glycosyltransferase and not in a random manner as occurs during modification of a protein's peptide backbone using chemical methods. An array of agents can be introduced into proteins or glycopeptides that lack the glycosyltransferase substrate peptide sequence by engineering the appropriate amino acid sequence into the polypeptide chain.

Scheme 7



[0375] In each of the exemplary embodiments set forth above, one or more additional chemical or enzymatic modification steps can be utilized following the conjugation of the modified sugar to the peptide. In an exemplary embodiment, an enzyme (e.g., fucosyltransferase) is used to append a glycosyl unit (e.g., fucose) onto the terminal modified sugar attached to the peptide. In another example, an enzymatic reaction is utilized to "cap" (e.g., sialylate) sites to which the modified sugar failed to conjugate. Alternatively, a chemical reaction is utilized to alter the structure of the conjugated modified sugar. For example, the conjugated modified sugar is reacted with agents that stabilize or destabilize its linkage with the peptide component to which the modified sugar is attached. In another example, a component of the modified sugar is deprotected following its conjugation to the peptide. One of skill will appreciate that there is an array of enzymatic and chemical procedures that are useful in the methods of the invention at a stage after the modified sugar is conjugated to the peptide. Further elaboration of the modified sugar-peptide conjugate is within the scope of the invention.

Enzyme Classes

[0376] Aspects of the present invention make use of enzymes that form a bond between an activated acyl moiety and a heteroatom found on a sugar nucleus. The enzymes useful in practicing the present invention include, but are not limited to, wild-type and mutant proteases, lipases, esterases, acylases, acyltransferases, glycosyltransferases, sulfotransferases, glycosidases, and the like. An exemplary mutant is one in which one or more amino acid residues in the active site are altered to provide an enzyme with synthetic activity that is improved relative to the activity in the corresponding wild-type enzyme.

Acyl Transfer

[0377] The discovery that some enzymes are catalytically active in organic solvents has greatly expanded their use as biocatalysts. In this medium these enzymes show a new catalytic behavior. For example lipases catalyze esterification and transesterification reactions in organic media. These properties enable the production of compounds which are difficult to obtain using chemical methods.

Proteases

[0378] A protease is employed in some embodiments of the invention. Proteases are known in the art to catalyze the attachment of amino acids to sugars through esterification. (Davis, (WO 03/014371, published Feb. 20, 2003). In this publication, a vinyl ester amino acid group was reacted with a carbohydrate acyl acceptor in the presence of the serine protease subtilisin derived from *Bacillus lentus*. Wild-type proteases can be additionally be isolated from *Bacillus amyloliquefaciens*. Mutant proteases can be made according to the teachings of, for example, PCT Publication Nos. WO 95/10615 and WO 91/06637, which are hereby incorporated by reference. Other proteases of use in this invention include serine proteases (such as chymotrypsin, plasmin, and thrombin), cysteine proteases (such as cathepsin B and papain), and aspartic endopeptidases (such as pepsin A, chymosin, cathepsin D, asparagenase).

[0379] In an exemplary embodiment, utilizing a protease, the link between the sugar moiety and the modifying group is an amino acid that is derivatized with the modifying group. The sugar and amino acid are linked through an amide moiety formed by the protease.

Lipases

[0380] A lipase is used in some embodiments of the invention. The use of lipases in the acylation of saccharides has been previously reported. For example, regioselective acylations of alkyl β -D-xylopyranosides using lipase PS in organic solvents was reported by Lopez. (Lopez et al., J. Org. Chem., 59, 7027-7032 (1994). Another group also utilized lipase PS in order to catalyze the transfer of acetyl groups onto sialic acids in vinyl acetate. (Lo et al., Bioorg. Med. Chem. Lett., 9, 709-712 (1999)). Regioselective disaccharide acylation in *tert*-butyl alcohol catalyzed by *Candida antarctica* lipase has also been reported. (Woudenberg van-Oosterom et al., Biotechnol. Bioeng., 49, 328-333 (1996)). Immobilized versions of the *Candida antarctica* lipase have also been used to acylate hydroxypropyl cellulose in *tert*-butanol. (Sereti et al., Biotechnol Bioeng., 72(4), 495-500 (2001)). Other lipases of use in this invention include lipoprotein lipase, triacylglycerol lipase, diglyceride lipase, and postheparin lipase.

Esterases

[0381] Esterases can also be used in some embodiments of the invention. Acetylation of cellobiose and cellulose was shown to be catalyzed in aqueous medium in the presence of isopropenyl acetate by an intracellular carboxylesterase from *Arthrobacter viscosus*. (Cui et al., Enzyme Microb. Technol., 24, 200-208 (1999)). Another group acetylated the amino groups of chitobiose and chitotetraose in an aqueous solution of 3M sodium acetate using a chitin deacetylase from *Colletotrichum lindemuthianum* (Tokuyasu et al., Carbohydr. Res., 322, 26-31 (1999)). A third group utilized acetyl-xylan esterase (AcXE) from *Schizophyllum commune* to catalyze acetyl group transfer to methyl β -D-xylopyranoside, methyl β -D-cellobioside, methyl β -D-glucopyranoside, cellotetraose, 2-deoxy-D-glucose, D-mannose, β -1,4-mannobiose, β -1,4-mannopentaose, β -1,4-mannohexaose, β -1,4-xylobiose, and β -1,4-xylopentaose. (Biely et al., Biochimica et Biophysica Acta, 1623, 62-71 (2003)). Acetylation of secondary alcohols was also achieved by transesterification from vinyl acetate by a feruloyl esterase from *Humicola insolens*. (Hatzakis et al., J. Mol. Catal., B Enzym. 21, 309-311 (2003)). Other esterases of use in this invention include choline esterase, sterol esterase, hydroxycinnamoyl esterase, acetyl-salicylic acid esterase, and polyneuridine esterase.

Acylases

[0382] Acylases can also be used in some embodiments of the invention. Exemplary acylases of use in this invention include aminoacylase I, L-amino-acid acylase, penicillin acylase, acetyl-CoA acylase, acyl-lysine deacylase, aculeacin A acylase, succinyl-CoA acylase, and acetyl-aspartic deaminase.

Acetyltransferases

[0383] In another embodiment of the invention, acyl transfer is accomplished by an acetyltransferase. The use of acetyltransferases in the acylation of saccharides has been previously reported. O-acetylation at the 9 position of sialic

acid has been shown to occur from the product of several genes in the COS cell system (Shi et al., *Glycobiology*, 8(2), 199-205 (1998)). Maltose O-acetyltransferase (MAT) from *Escherichia coli* is known to catalyze acetyl group transfer to the C6 positions of glucose and maltose. (Leggio et al., *Biochemistry*, 42, 5225-5235 (2003)). This same group also utilized galactoside acetyltransferase (GAT) to catalyze acetyl group transfer to galactosyl units. Other acetyltransferases of use in this invention include spermidine acetyltransferase, diamine N-acetyltransferase, and sialate O-acetyltransferase.

Sugar Transfer

[0384] In addition to the enzymes discussed above in the context of forming the acyl-linked conjugate, the glycosylation pattern of the conjugate and the starting substrates (e.g., peptides, lipids) can be elaborated, trimmed back or otherwise modified by methods utilizing other enzymes. The methods of remodeling peptides and lipids using enzymes that transfer a sugar donor to an acceptor are discussed in great detail in DeFrees, WO 03/031464 A2, published April 17, 2003. A brief summary of selected enzymes of use in the present method is set forth below.

Glycosyltransferases

[0385] Glycosyltransferases catalyze the addition of activated sugars (donor NDP-sugars), in a step-wise fashion, to a protein, glycopeptide, lipid or glycolipid or to the non-reducing end of a growing oligosaccharide. N-linked glycopeptides are synthesized via a transferase and a lipid-linked oligosaccharide donor Dol-PP-NAG₂Glc₃Man₉ in an en block transfer followed by trimming of the core. In this case the nature of the "core" saccharide is somewhat different from subsequent attachments. A very large number of glycosyltransferases are known in the art.

[0386] For enzymatic saccharide syntheses that involve glycosyltransferase reactions, glycosyltransferase can be cloned, or isolated from any source. Many cloned glycosyltransferases are known, as are their polynucleotide sequences. See, e.g., "The WWW Guide To Cloned Glycosyltransferases," (http://www.vei.co.uk/TGN/gt_guide.htm). Glycosyltransferase amino acid sequences and nucleotide sequences encoding glycosyltransferases from which the amino acid sequences can be deduced are also found in various publicly available databases, including GenBank, Swiss-Prot, EMBL, and others.

[0387] Glycosyltransferases that can be employed in the methods of the invention include, but are not limited to, galactosyltransferases, fucosyltransferases, glucosyltransferases, N-acetylgalactosaminyltransferases, N-acetylglucosaminyltransferases, glucuronyltransferases, sialyltransferases, mannosyltransferases, glucuronic acid transferases, galacturonic acid transferases, and oligosaccharyltransferases. Suitable glycosyltransferases include those obtained from eukaryotes, as well as from prokaryotes.

Fucosyltransferases

[0388] In some embodiments, a glycosyltransferase used in the method of the invention is a fucosyltransferase. Fucosyltransferases are known to those of skill in the art. Exemplary fucosyltransferases include enzymes, which transfer L-fucose from GDP-fucose to a hydroxy position of an acceptor sugar. Fucosyltransferases that transfer non-nucleotide sugars to an acceptor are also of use in the present invention.

[0389] In some embodiments, the acceptor sugar is, for example, the GlcNAc in a Gal β (1 $\rightarrow$ 3,4)GlcNAc β - group in an oligosaccharide glycoside. Suitable fucosyltransferases for this reaction include the Gal β (1 $\rightarrow$ 3,4)GlcNAc β 1- α (1 $\rightarrow$ 3,4)fucosyltransferase (FTIII E.C. No. 2.4.1.65), which was first characterized from human milk (see, Palcic, et al., *Carbohydrate Res.* 190:1-11 (1989); Prieels, et al., *J. Biol. Chem.* 256: 10456-10463 (1981); and Nunez, et al., *Can. J. Chem.* 59: 2086-2095 (1981)) and the Gal β (1 $\rightarrow$ 4)GlcNAc β - α fucosyltransferases (FTIV, FTV, FTVI) which are found in human serum. FTVII (E.C. No. 2.4.1.65), a sialyl α (2 $\rightarrow$ 3)Gal β ((1 $\rightarrow$ 3)GlcNAc β fucosyltransferase, has also been characterized. A recombinant form of the Gal β (1 $\rightarrow$ 3,4)GlcNAc β - α (1 $\rightarrow$ 3,4)fucosyltransferase has also been characterized (see, Dumas, et al., *Bioorg. Med. Letters* 1: 425-428 (1991) and Kukowska-Latallo, et al., *Genes and Development* 4: 1288-1303 (1990)). Other exemplary fucosyltransferases include, for example, α 1,2 fucosyltransferase (E.C. No. 2.4.1.69). Enzymatic fucosylation can be carried out by the methods described in Mollicone, et al., *Eur. J. Biochem.* 191: 169-176 (1990) or U.S. Patent No. 5,374,655. Cells that are used to produce a fucosyltransferase will also include an enzymatic system for synthesizing GDP-fucose.

Galactosyltransferases

[0390] In another group of embodiments, the glycosyltransferase is a galactosyltransferase. Exemplary galactosyltransferases include α (1,3) galactosyltransferases (E.C. No. 2.4.1.151, see, e.g., Dabkowski et al., *Transplant Proc.* 25:2921 (1993) and Yamamoto et al. *Nature* 345: 229-233 (1990), bovine (GenBank j04989, Joziassse et al., *J. Biol.*

Chem. 264: 14290-14297 (1989)), murine (GenBank m26925; Larsen et al., Proc. Nat'l. Acad. Sci. USA 86: 8227-8231 (1989)), porcine (GenBank L36152; Strahan et al., Immunogenetics 41: 101-105 (1995)). Another suitable α 1,3 galactosyltransferase is that which is involved in synthesis of the blood group B antigen (EC 2.4.1.37, Yamamoto et al., J. Biol. Chem. 265: 1146-1151 (1990) (human)). Yet a further exemplary galactosyltransferase is core Gal-T1.

[0391] Also suitable for use in the methods of the invention are β (1,4) galactosyltransferases, which include, for example, EC 2.4.1.90 (LacNAc synthetase) and EC 2.4.1.22 (lactose synthetase) (bovine (D'Agostaro et al., Eur. J. Biochem. 183: 211-217 (1989)), human (Masri et al., Biochem. Biophys. Res. Commun. 157: 657-663 (1988)), murine (Nakazawa et al., J. Biochem. 104: 165-168 (1988)), as well as E.C. 2.4.1.38 and the ceramide galactosyltransferase (EC 2.4.1.45, Stahl et al., J. Neurosci. Res. 38: 234-242 (1994)). Other suitable galactosyltransferases include, for example, α 1,2 galactosyltransferases (from e.g., *Schizosaccharomyces pombe*, Chapell et al., Mol. Biol. Cell 5: 519-528 (1994)).

Sialyltransferases

[0392] Sialyltransferases are another type of glycosyltransferase that is useful in the recombinant cells and reaction mixtures of the invention. Cells that produce recombinant sialyltransferases will also produce CMP-sialic acid, which is a sialic acid donor for sialyltransferases. Examples of sialyltransferases that are suitable for use in the present invention include ST3Gal III (e.g., a rat or human ST3Gal III), ST3Gal IV, ST3Gal I, ST6Gal I, ST3Gal V, ST6Gal II, ST6GalNAc I, ST6GalNAc II, and ST6GalNAc III (the sialyltransferase nomenclature used herein is as described in Tsuji et al., Glycobiology 6: v-xiv (1996)). An exemplary α (2,3)sialyltransferase referred to as α (2,3)sialyltransferase (EC 2.4.99.6) transfers sialic acid to the non-reducing terminal Gal of a Gal β 1 $\rightarrow$ 3Glc disaccharide or glycoside. See, Van den Eijnden et al., J. Biol. Chem. 256: 3159 (1981), Weinstein et al., J. Biol. Chem. 257: 13845 (1982) and Wen et al., J. Biol. Chem. 267: 21011 (1992). Another exemplary α 2,3-sialyltransferase (EC 2.4.99.4) transfers sialic acid to the non-reducing terminal Gal of the disaccharide or glycoside. see, Rearick et al., J. Biol. Chem. 254: 4444 (1979) and Gillespie et al., J. Biol. Chem. 267: 21004 (1992). Further exemplary enzymes include Gal β -1,4-GlcNAc α -2,6 sialyltransferase (See, Kurosawa et al. Eur. J. Biochem. 219: 375-381 (1994)).

[0393] Preferably, for glycosylation of carbohydrates of glycopeptides the sialyltransferase will be able to transfer sialic acid to the sequence Gal β 1,4GlcNAc-, the most common penultimate sequence underlying the terminal sialic acid on fully sialylated carbohydrate structures (see, Table 3).

Table 3: Sialyltransferases which use the Gal β 1,4GlcNAc sequence as an acceptor substrate

Sialyltransferase	Source	Sequence(s) formed	Ref.
ST6Gal I	Mammalian	NeuAc α 2,6Gal β 1,4GlcNAc-	1
ST3Gal III	Mammalian	NeuAc α 2,3Gal β 1,4GlcNAc- NeuAc α 2,3Gal β 1,3GlcNAc-	1
ST3Gal IV	Mammalian	NeuAc α 2,3Gal β 1,4GlcNAc- NeuAc α 2,3Gal β 1,3GlcNAc-	1
ST6Gal II	Mammalian	NeuAc α 2,6Gal β 1,4GlcNAc-	
ST6Gal II	photobacterium	NeuAc α 2,6Gal β 1,4GlcNAc-	2
ST3Gal V	<i>N. meningitidis N. gonorrhoeae</i>	NeuAc α 2,3Gal β 1,4GlcNAc-	3
1) Goochee et al., Bio/Technology 9: 1347-1355 (1991) 2) Yamamoto et al., J. Biochem. 120: 104-110 (1996) 3) Gilbert et al., J. Biol. Chem. 271: 28271-28276 (1996)			

[0394] An example of a sialyltransferase that is useful in the claimed methods is ST3Gal III, which is also referred to as α (2,3)sialyltransferase (EC 2.4.99.6). This enzyme catalyzes the transfer of sialic acid to the Gal of a Gal β 1,3GlcNAc or Gal β 1,4GlcNAc glycoside (see, e.g., Wen et al., J. Biol. Chem. 267: 21011 (1992); Van den Eijnden et al., J. Biol. Chem. 256: 3159 (1991)) and is responsible for sialylation of asparagine-linked oligosaccharides in glycopeptides. The sialic acid is linked to a Gal with the formation of an α -linkage between the two saccharides. Bonding (linkage) between the saccharides is between the 2-position of NeuAc and the 3-position of Gal. This particular enzyme can be isolated from rat liver (Weinstein et al., J. Biol. Chem. 257: 13845 (1982)); the human cDNA (Sasaki et al. (1993) J. Biol. Chem. 268: 22782-22787; Kitagawa & Paulson (1994) J. Biol. Chem. 269: 1394-1401) and genomic (Kitagawa et al. (1996) J. Biol. Chem. 271: 931-938) DNA sequences are known, facilitating production of this enzyme by recombinant expression.

In a preferred embodiment, the claimed sialylation methods use a rat ST3Gal III.

[0395] Other exemplary sialyltransferases of use in the present invention include those isolated from *Campylobacter jejuni*, including the $\alpha(2,3)$. See, e.g., WO99/49051.

[0396] Sialyltransferases other than those listed in Table 3, are also useful in an economic and efficient large-scale process for sialylation of commercially important glycopeptides. As a simple test to find out the utility of these other enzymes, various amounts of each enzyme (1-100 mU/mg protein) are reacted with asialo- α_1 AGP (at 1-10 mg/ml) to compare the ability of the sialyltransferase of interest to sialylate glycopeptides relative to either bovine ST6Gal I, ST3Gal III or both sialyltransferases. Alternatively, other glycopeptides or glycopeptides, or N-linked oligosaccharides enzymatically released from the peptide backbone can be used in place of asialo- α_1 AGP for this evaluation. Sialyltransferases with the ability to sialylate N-linked oligosaccharides of glycopeptides more efficiently than ST6Gal I are useful in a practical large-scale process for peptide sialylation.

[0397] FIG. 2 provides a listing of exemplary sialyltransferases of use in the present invention.

GalNAc transferases

[0398] N-acetylgalactosaminyltransferases are of use in practicing the present invention, particularly for binding a GalNAc moiety to an amino acid of the O-linked glycosylation site of the peptide. Suitable N-acetylgalactosaminyltransferases include, but are not limited to, $\alpha(1,3)$ N-acetylgalactosaminyltransferase, $\beta(1,4)$ N-acetylgalactosaminyltransferases (Nagata et al., J. Biol. Chem. 267: 12082-12089 (1992) and Smith et al., J. Biol. Chem. 269: 15162 (1994)) and polypeptide N-acetylgalactosaminyltransferase (Homa et al., J. Biol. Chem. 268: 12609 (1993)).

[0399] Production of proteins such as the enzyme GalNAc T_{I-XX} from cloned genes by genetic engineering is well known. See, e.g., U.S. Pat. No. 4,761,371. One method involves collection of sufficient samples, then the amino acid sequence of the enzyme is determined by N-terminal sequencing. This information is then used to isolate a cDNA clone encoding a full-length (membrane bound) transferase which upon expression in the insect cell line Sf9 resulted in the synthesis of a fully active enzyme. The acceptor specificity of the enzyme is then determined using a semiquantitative analysis of the amino acids surrounding known glycosylation sites in 16 different proteins followed by in vitro glycosylation studies of synthetic peptides. This work has demonstrated that certain amino acid residues are overrepresented in glycosylated peptide segments and that residues in specific positions surrounding glycosylated serine and threonine residues may have a more marked influence on acceptor efficiency than other amino acid moieties.

Cell-Bound Glycosyltransferases

[0400] In another embodiment, the enzymes utilized in the method of the invention are cell-bound glycosyltransferases. Although many soluble glycosyltransferases are known (see, for example, U.S. Pat. No. 5,032,519), glycosyltransferases are generally in membrane-bound form when associated with cells. Many of the membrane-bound enzymes studied thus far are considered to be intrinsic proteins; that is, they are not released from the membranes by sonication and require detergents for solubilization. Surface glycosyltransferases have been identified on the surfaces of vertebrate and invertebrate cells, and it has also been recognized that these surface transferases maintain catalytic activity under physiological conditions. However, the more recognized function of cell surface glycosyltransferases is for intercellular recognition (Roth, MOLECULAR APPROACHES to SUPRACELLULAR PHENOMENA, 1990).

[0401] Methods have been developed to alter the glycosyltransferases expressed by cells. For example, Larsen et al., Proc. Natl. Acad. Sci. USA 86: 8227-8231 (1989), report a genetic approach to isolate cloned cDNA sequences that determine expression of cell surface oligosaccharide structures and their cognate glycosyltransferases. A cDNA library generated from mRNA isolated from a murine cell line known to express UDP-galactose: β -D-galactosyl-1,4-N-acetyl-D-glucosaminide α -1,3-galactosyltransferase was transfected into COS-1 cells. The transfected cells were then cultured and assayed for α 1-3 galactosyltransferase activity.

[0402] Francisco et al., Proc. Natl. Acad. Sci. USA 89: 2713-2717 (1992), disclose a method of anchoring β -lactamase to the external surface of *Escherichia coli*. A tripartite fusion consisting of (i) a signal sequence of an outer membrane protein, (ii) a membrane-spanning section of an outer membrane protein, and (iii) a complete mature β -lactamase sequence is produced resulting in an active surface bound β -lactamase molecule. However, the Francisco method is limited only to procaryotic cell systems and as recognized by the authors, requires the complete tripartite fusion for proper functioning.

Sulfotransferases

[0403] The invention also provides methods for producing peptides that include sulfated molecules, including, for example sulfated polysaccharides such as heparin, heparan sulfate, carragenen, and related compounds. Suitable sulfotransferases include, for example, chondroitin-6-sulphotransferase (chicken cDNA described by Fukuta et al., J.

Biol. Chem. 270: 18575-18580 (1995); GenBank Accession No. D49915), glycosaminoglycan N-acetylglucosamine N-deacetylase/N-sulphotransferase 1 (Dixon et al., Genomics 26: 239-241 (1995); UL18918), and glycosaminoglycan N-acetylglucosamine N-deacetylase/N-sulphotransferase 2 (murine cDNA described in Orellana et al., J. Biol. Chem. 269: 2270-2276 (1994) and Eriksson et al., J. Biol. Chem. 269: 10438-10443 (1994); human cDNA described in GenBank Accession No. U2304).

Glycosidases

[0404] This invention also encompasses the use of wild-type and mutant glycosidases. Mutant β -galactosidase enzymes have been demonstrated to catalyze the formation of disaccharides through the coupling of an α -glycosyl fluoride to a galactosyl acceptor molecule. (Withers, U.S. Pat. No. 6,284,494; issued Sept. 4, 2001). Other glycosidases of use in this invention include, for example, β -glucosidases, β -galactosidases, β -mannosidases, β -acetyl glucosaminidases, β -N-acetyl galactosaminidases, β -xylosidases, β -fucosidases, cellulases, xylanases, galactanases, mannanases, hemi-cellulases, amylases, glucoamylases, α -glucosidases, α -galactosidases, α -mannosidases, α -N-acetyl glucosaminidases, α -N-acetyl galactose-aminidases, α -xylosidases, α -fucosidases, and neuraminidases/sialidases.

Immobilized Enzymes

[0405] The present invention also provides for the use of enzymes that are immobilized on a solid and/or soluble support. In an exemplary embodiment, there is provided a glycosyltransferase that is conjugated to a PEG via an intact glycosyl linker according to the methods of the invention. The PEG-linker-enzyme conjugate is optionally attached to solid support. The use of solid supported enzymes in the methods of the invention simplifies the work up of the reaction mixture and purification of the reaction product, and also enables the facile recovery of the enzyme. The glycosyltransferase conjugate is utilized in the methods of the invention. Other combinations of enzymes and supports will be apparent to those of skill in the art.

Glycosylation by Recombinant Methods

[0406] FGF peptide conjugates may also be prepared intracellularly by recombinant means. A polynucleotide sequence encoding a FGF, which comprises at least one newly introduced N- or O-linked glycosylation site, may be transfected into a suitable host cell line, e.g., a eukaryotic cell line derived from yeast, insect, or mammalian origin. The Fibroblast Growth Factor recombinantly produced from such a cell line is glycosylated by the host cell glycosylation machinery.

Purification of FGF peptide conjugates

[0407] The FGF peptide conjugate produced by the above processes is preferably purified before use. Standard, well known techniques such as thin or thick layer chromatography, column chromatography, ion exchange chromatography, or membrane filtration can be used. It is preferred to use membrane filtration, more preferably utilizing a reverse osmotic membrane, or one or more column chromatographic techniques for the recovery as is discussed hereinafter and in the literature cited herein.

[0408] If the FGF peptide conjugate is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration; optionally, the protein may be concentrated with a commercially available protein concentration filter, followed by separating the polypeptide variant from other impurities by one or more steps selected from immunoaffinity chromatography, ion-exchange column fractionation (e.g., on diethylaminoethyl (DEAE) or matrices containing carboxymethyl or sulfopropyl groups), chromatography on Blue-Sepharose, CM Blue-Sepharose, MONO-Q, MONO-S, lentil lectin-Sepharose, WGA-Sepharose, Con A-Sepharose, Ether Toyopearl, Butyl Toyopearl, Phenyl Toyopearl, SP-Sepharose, or protein A Sepharose, SDS-PAGE chromatography, silica chromatography, chromatofocusing, reverse phase HPLC (e.g., silica gel with appended aliphatic groups), gel filtration using, e.g., Sephadex molecular sieve or size-exclusion chromatography, chromatography on columns that selectively bind the polypeptide, and ethanol or ammonium sulfate precipitation.

[0409] A FGF peptide conjugate produced in culture is usually isolated by initial extraction from cells, cell lysate, culture media, etc., followed by one or more concentration, salting-out, aqueous ion-exchange, or size-exclusion chromatography steps. Additionally, the glycoprotein may be purified by affinity chromatography. Finally, HPLC may be employed for final purification steps.

[0410] A protease inhibitor, e.g., methylsulfonylfluoride (PMSF) may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

[0411] In some cases, supernatants from systems that produce the FGF peptide conjugates of the invention are first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon

ultrafiltration unit. Following the concentration step, the concentrate may be applied to a suitable purification matrix. For example, a suitable affinity matrix may comprise a ligand for the peptide, a lectin or antibody molecule bound to a suitable support. Alternatively, an anion-exchange resin may be employed, for example, a matrix or substrate having pendant DEAE groups. Suitable matrices include acrylamide, agarose, dextran, cellulose, or other types commonly employed in protein purification. Also, a cation-exchange step may be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are particularly preferred.

[0412] Finally, one or more RP-HPLC steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, may be employed to further purify a FGF peptide conjugate. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a glycoprotein.

[0413] The FGF peptide conjugate of the invention resulting from a large-scale fermentation may be purified by methods analogous to those disclosed by Urdal et al., J. Chromatog. 296: 171 (1984). This reference describes two sequential, RP-HPLC steps for purification of recombinant human IL-2 on a preparative HPLC column. Alternatively, techniques such as affinity chromatography, may be utilized to purify the glycoprotein.

[0414] Following the production and, preferably, purification of a glycosylated mutant Fibroblast Growth Factor, the biological functions of the glycoprotein are tested using several methods known in the art. The functional assays are based on various characteristics of Fibroblast Growth Factor.

Pharmaceutical Composition and Administration

[0415] The FGF peptide conjugates having desired oligosaccharide determinants described above can be used as therapeutics for treating a variety of diseases and conditions related to deficiency in growth hormone. Growth-related conditions that can be treated with the FGF peptide conjugates of the present invention include: dwarfism, short-stature in children and adults, cachexia/muscle wasting, general muscular atrophy, and sex chromosome abnormality (e.g., Turner's Syndrome). Other conditions may be treated using the FGF peptide conjugates of the present invention include: short-bowel syndrome, lipodystrophy, osteoporosis, uraemia, burns, female infertility, bone regeneration, general diabetes, type II diabetes, osteo-arthritis, chronic obstructive pulmonary disease (COPD), and insomnia. The FGF peptide conjugates of the invention may also be used to promote various healing processes, e.g., general tissue regeneration, bone regeneration, and wound healing, or as a vaccine adjunct. Thus, the present invention also provides pharmaceutical compositions comprising an effective amount of FGF peptide conjugate, which is produced according to the methods described above.

[0416] Pharmaceutical compositions of the invention are suitable for use in a variety of drug delivery systems. Suitable formulations for use in the present invention are found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). For a brief review of methods for drug delivery, see, Langer, Science 249: 1527-1533 (1990).

[0417] The pharmaceutical compositions are intended for parenteral, intranasal, topical, oral, or local administration, such as by subcutaneous injection, aerosol inhalation, or transdermal adsorption, for prophylactic and/or therapeutic treatment. Commonly, the pharmaceutical compositions are administered parenterally, e.g., subcutaneously or intravenously. Thus, the invention provides compositions for parenteral administration which comprise the FGF peptide conjugate dissolved or suspended in an acceptable carrier, preferably an aqueous carrier, e.g., water, buffered water, saline, PBS and the like. The compositions may also contain detergents such as Tween 20 and Tween 80; stabilizers such as mannitol, sorbitol, sucrose, and trehalose; and preservatives such as EDTA and m-cresol. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents, detergents and the like.

[0418] These compositions may be sterilized by conventional sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous carrier prior to administration. The pH of the preparations typically will be between 3 and 11, more preferably from 5 to 9, and most preferably from 7 and 8.

[0419] The compositions containing the FGF peptide conjugates can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a patient already suffering from a disease or condition related to growth hormone deficiency, in an amount sufficient to cure or at least partially arrest the symptoms of the disease and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend on the severity of the disease or condition and the weight and general state of the patient, but generally range from about 0.1 mg to about 2,000 mg of FGF peptide conjugates per day for a 70 kg patient, with dosages of from about 5 mg to about 200 mg of the compounds per day being more commonly used.

[0420] In prophylactic applications, compositions containing the FGF peptide conjugate of the invention are administered to a patient susceptible to or otherwise at risk of a particular disease. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend on the patient's state of health and weight, but generally range from about 0.1 mg to about 1,000 mg per 70 kilogram patient, more commonly from about 5 mg to about

200 mg per 70 kg of body weight.

[0421] Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of the FGF peptide conjugate of this invention sufficient to effectively treat the patient.

EXAMPLES

[0422] The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of non-critical parameters that could be changed or modified to yield essentially similar results. Though the method is exemplified by reference to FGF-20 and FGF-21, those of skill will appreciate that glycosylation sites can be incorporated into the peptide sequences of other FGFs, e.g. FGF-9 and FGF-18, in the manner set forth below.

Fibroblast Growth Factor-20 sequence information

[0423] A Fibroblast Growth Factor-20 sequence displaying the different regions of the protein is shown in Table 5. The wild-type FGF-20 is thought to be un-glycosylated and may be produced in *Escherichia coli* as a therapeutic. The amino acid sequence is shown in Table 4, below.

Table 4. Human Fibroblast Growth Factor-20, (SEQ ID NO:1)

MAPLAEVGGF LGGLEGLGQQ VGSHFLLPPA GERPPLLGER RSAAERSARG GPGAAQLAHL HGILRRRQLY CRTGFHLQIL PDGSVQGTRQ DHSFLGILEF ISVAVGLVSI RGVDSGLYLG MNDKGELYGS EKL TSECIFR EQFEENWYNT YSSNIYKHGD TGRRYFVALN KDGTPRDGAR SKRHQKFTHF LPRPVDPERV PELYKDLLMY T

[0424] Regions of FGF-20 that are suited to mutation for the purpose of creating glycosylation sites are shown in Table 5. These regions are indicated either in bold, or in italics when one region is contiguous to another.

Table 5: Wild-type Human FGF-20 Sequence Showing Different Protein Regions

MAPLAEVGGF LGGLEGLGQQ VGSHFLLPPA GERPPLLGER RSAAERSARG GPGAAQLAHL

region 1	region 2	region 3
1.....10.....20.....30.....40.....50.....60		

HGILRRRQLY CRTGFHLQIL PDGSVQGTRQ DHSFLGILEF ISVAVGLVSI RGVDSGLYLG MN

region 4
61.....70.....80.....90.....100.....110.....120...

DKGELYGSEKLTSECIFR EQFEENWYNTYSSNIYKHGD TGRRYFVALN KDGTPRDGAR SKRH

region 5
123... 130.....140.....150.....160.....170.....180...

QKFTHF LPRPVDPERV PELYKDLLMY T

region 6	region 7
185...190.....200.....210.211	

Fibroblast Growth Factor-21 sequence information

[0425] A Fibroblast Growth Factor-21 sequence displaying the different regions of the protein is shown in Table 7. The wild-type FGF-21 is thought to be un-glycosylated and may be produced in *Escherichia coli* as a therapeutic. The amino acid sequence is shown in Table 6, below.

Table 6. Human Fibroblast Growth Factor-21, (SEQ ID NO:146)

MHP IPDSSPLLQF GGQVRQRYLY TDDAQQTEAH LEIREDGTVG
 GAADQSPESL LQLKALKPGV IQILGVKTSR FLCQRPD GAL YGSLHFDPEA
 CSFRELLED GYNVYQSEAH GLPLHLPNGK SPHRDPAPRG PARFLPLPGL
 PPALPEPPGI LAPQPPDVGS SDPLSMVGPS QGRSPSYAS

[0426] Regions of FGF-21 that are suited to mutation for the purpose of creating glycosylation sites are shown in Table 7. These regions are indicated either in bold, or in italics when one region is contiguous to another.

Table 7: Wild-type Human FGF-21 Sequence Showing Different Protein Regions

MHP IPDSSPLLQF GGQVRQRYLY TDDAQQTEAH LEIREDGTVG GAADQSPESL
 region 1 region 2 region 3
 1.....10.....20.....30.....40.....50.....
 LQLKALKPGV IQILGVKTSR FLCQRPD GAL YGSLHFDPEA CSFRELLED GYNVYQS
 region 4 region 5 region 6
 55.....60.....70.....80.....90.....100.....110
 EAHGLPLHLPNGK SPHRDPAPRG PARFLPLPGL PPALPEPPGI LAPQPP
 region 7 region 8
 111.....120.....130.....140.....150.....159
 DVGSSDPLSMVGPS QGRSPSYAS
 region 9 region 10 region 11
 160.....170.....180.182

[0427] FGF or mutated FGF can be glycosylated or glycoconjugated (see WO 03/31464, incorporated herein by reference). Preferably, a mutated FGF is glycoPEGylated, wherein a polyethylene glycol (PEG) moiety is conjugated to the mutated FGF polypeptide via a glycosyl linkage (see WO 03/31464, incorporated herein by reference). GlycoPEGylation of the FGF is expected to result in improved biophysical properties that may include but are not limited to improved half-life, improved area under the curve (AUC) values, reduced clearance, and reduced immunogenicity.

EXAMPLE 1

[0428] Exemplary regions on FGF-20 that are suited for the introduction of glycosylation sites by mutation are shown in Table 5, above. In all cases, the N-terminal Met may be present or absent on any FGF mutant. The numbering of the amino acid residues is based on the initial unmodified sequence in which the left most residue, methionine, is numbered as position 1. To highlight how the mutant sequence differs in respect to the unmodified sequence, the numbering of unmodified amino acids as they appear in the sequences below remains unchanged following the modification. More than one of the described sequence modifications may be present in an FGF mutant of the present invention. Specifically, the preferred regions for introduction of mutations to create a glycosylation site(s) not present in the wild-type peptide are the nucleotide sequences that encode: amino acids 1-7 (REGION 1; SEQ ID NO:2), amino acids 20-42 (REGION 2; SEQ ID NO:3), amino acids 43-60 (REGION 3; SEQ ID NO:4), amino acids 73-90 (REGION 4; SEQ ID NO:5), amino acids 159-174 (REGION 5; SEQ ID NO:6), amino acids 177-198 (REGION 6; SEQ ID NO:7) or amino acids 199-201 (REGION 7; SEQ ID NO:8) of the wild-type FGF amino acid sequence (see Table 5) can be mutated so that either an N-linked or an O-linked glycosylation site is introduced into the resulting mutated FGF-20 polypeptide.

[0429] The following example describes amino acid sequence mutations introducing N-linked e.g., asparagine residues, and O-linked glycosylation sites, e.g., serine or threonine residues, into a preferably proline-containing site of a

wild-type Fibroblast Growth Factor-20 sequence or any modified version thereof.

1. Region 1

5 **[0430]** In the Region 1 mutants, the N-terminus of a wild-type FGF-20, MAP³LAEV; SEQ ID NO:2, is replaced with MXY_aZ_bP³BJO1234, wherein 1, 2, 3, 4, X, Y, Z, B, J and O are independently selected from any uncharged amino acid, or glutamic acid (E), and wherein at least one is threonine (T) or serine (S), and is a substrate for GalNAc transferase where GalNAc is added to at least threonine or serine to create an O-linked glycosylation site. The symbols a and b independently represent 0 or 1. To clarify, sequences designated as SEQ ID NO:12-14, 338-344 contain amino acid
10 insertions between P³ and L⁴ of the native FGF-20 sequence. Preferred examples include:

	MAPTP ³ LAEV;	SEQ ID NO:9
	MVTP ³ LAEV;	SEQ ID NO:10
	MAP ³ TTEV;	SEQ ID NO:11
15	MAP ³ TQGAMPL ⁴ AEV;	SEQ ID NO:12
	MAP ³ TSSL ⁴ AEV;	SEQ ID NO:13
	MAP ³ TALPL ⁴ AEV;	SEQ ID NO:14
	MAP ³ TQAPL ⁴ AEV;	SEQ ID NO:338
20	MAP ³ TEIPL ⁴ AEV;	SEQ ID NO:339
	MAP ³ TINTPL ⁴ AEV;	SEQ ID NO:340
	MAP ³ TINTL ⁴ AEV;	SEQ ID NO:341
	MAP ³ TTVSL ⁴ AEV;	SEQ ID NO:342
	MAP ³ TQEV ⁴ L ⁴ AEV;	SEQ ID NO:343
25	MAP ³ TQAVL ⁴ AEV;	SEQ ID NO:344

2. Region 2

30 **[0431]** In these mutants, the wild-type QVGSHFLLP²⁸P²⁹A³⁰GERPPLLGERRS; SEQ ID NO:3, is subdivided into three regions: Region 2(a) VGSHFLLP²⁸P²⁹A³⁰GERPP, SEQ ID NO:15; Region 2(b) P²⁸P²⁹AGERPP, SEQ ID NO:16; and Region 2(c) P³⁴P³⁵PLLGERRS, SEQ ID NO:17. Mutations in each region are considered separately below.

35 **[0432]** Region 2(a): in these mutants the wild-type VGSHFLLP²⁸P²⁹A³⁰GERPP (SEQ ID NO:15) is replaced with 1234XYZ P²⁸P²⁹A³⁰ wherein 1, 2, 3, 4, X, Y, Z, are independently selected from any uncharged amino acid, or glutamic acid (E), wherein at least one is threonine (T) or serine (S), and is a substrate for GalNAc transferase where GalNAc is added to at least threonine or serine to create an O-linked glycosylation site. The symbols a and b independently represent 0 or 1. Preferred mutations include:

	TET P ²⁸ P ²⁹ A ³⁰ GERPP;	SEQ ID NO:18
40	GTET P ²⁸ P ²⁹ A ³⁰ GERPP;	SEQ ID NO:19
	VGTET P ²⁸ P ²⁹ A ³⁰ GERPP;	SEQ ID NO:20
	TGT P ²⁸ P ²⁹ AEERPP;	SEQ ID NO:21
	TAT P ²⁸ P ²⁹ AEERPP;	SEQ ID NO:22

45 **[0433]** Region 2(b): in these mutants the wild-type P²⁸P²⁹A³⁰GERPP (SEQ ID NO:16) is replaced with P²⁸P²⁹1234(S)_aPP wherein 1, 2, 3, 4, X, Y, Z, are as described for Region 2(a). Preferred mutations include:

	P ²⁸ P ²⁹ TGEAPP;	SEQ ID NO:23
50	P ²⁸ P ²⁹ TGEVPP;	SEQ ID NO:24
	P ²⁸ P ²⁹ TQGAPP;	SEQ ID NO:25
	P ²⁸ P ²⁹ ATVAPP;	SEQ ID NO:26
	P ²⁸ P ²⁹ ATILPP;	SEQ ID NO:27
	P ²⁸ P ²⁹ AGTAPP;	SEQ ID NO:28
55	P ²⁸ P ²⁹ TQGAMPP;	SEQ ID NO:29
	P ²⁸ P ²⁹ GSTAPP;	SEQ ID NO:30
	P ²⁸ P ²⁹ AGTSPP;	SEQ ID NO:31

(continued)

P²⁸P²⁹AGETPP; SEQ ID NO:32
P²⁸P²⁹ATETPP; SEQ ID NO:33
P²⁸P²⁹GTETPP; SEQ ID NO:34
P²⁸P²⁹TGERPP; SEQ ID NO:35
P²⁸P²⁹TINTPP; SEQ ID NO:345
P²⁸P²⁹TTVSPP; SEQ ID NO:346
P²⁸P²⁹TQALPP; SEQ ID NO:347

[0434] Region 2(c): in these mutants the wild-type P³⁴P³⁵PLLGERRS (SEQ ID NO:17) is replaced with P³⁴P³⁵123456 wherein 1, 2, 3, 4, 5, 6, are independently selected from any uncharged amino acid, or glutamic acid (E), and wherein at least one is threonine (T) or serine (S), and is a substrate for GalNAc transferase where GalNAc is added to at least threonine or serine to create an O-linked glycosylation site. Preferred mutations include:

P³⁴P³⁵TQGAMP; SEQ ID NO:36
P³⁴P³⁵TQGAMRS; SEQ ID NO:37
P³⁴P³⁵TQGAMAS; SEQ ID NO:38
P³⁴P³⁵TQGAMFS; SEQ ID NO:39
P³⁴P³⁵TSSSTRS; SEQ ID NO:40
P³⁴P³⁵TSSSTKS; SEQ ID NO:41
P³⁴P³⁵TGERRS; SEQ ID NO:42
P³⁴P³⁵TIGVRRS; SEQ ID NO:43
P³⁴P³⁵TTGEARS; SEQ ID NO:44
P³⁴P³⁵TAGERRS; SEQ ID NO:45
P³⁴P³⁵TINTRRS; SEQ ID NO:348
P³⁴P³⁵TTVSRRS; SEQ ID NO:349

3. Region 3

[0435] In these mutants, the amino acid sequence surrounding P⁵²,

[0436] AAERSARGGP⁵²GAAQLAHL; SEQ ID NO:4, is subdivided into two regions; Region 3(a) RSARGGP⁵²; SEQ ID NO:46 and Region 3(b) P⁵²GAAQLA, SEQ ID NO:47. Mutations in each region are considered separately, below.

[0437] Region 3(a): in these mutants the wild-type RSARGG P⁵² (SEQ ID NO:46) is replaced with 123456P⁵² wherein 1, 2, 3, 4, 5, 6, are independently selected from any uncharged amino acid, or glutamic acid (E), wherein at least one is threonine (T) or serine (S), and is a substrate for GalNAc transferase where GalNAc is added to at least threonine or serine to create an O-linked glycosylation site. Preferred examples include:

RSATETP⁵²; SEQ ID NO:48
RSGTETP⁵²; SEQ ID NO:49
RSGTETP⁵²; SEQ ID NO:50
RVGTETP⁵²; SEQ ID NO:51
GVGTETP⁵²; SEQ ID NO:52
GSATETP⁵²; SEQ ID NO:53
GVGVETP⁵²; SEQ ID NO:54
GVTETP⁵²; SEQ ID NO:55
QTELP⁵²; SEQ ID NO:56
GVTSAP⁵²; SEQ ID NO:57
SVVTP⁵²; SEQ ID NO:58

[0438] Region 3(b): in these mutants the wild-type P⁵²GAAQLA (SEQ ID NO:47) is replaced with P⁵²123456 wherein 1, 2, 3, 4, 5, 6, are independently selected from any uncharged amino acid, or glutamic acid (E), wherein at least one is threonine (T) or serine (S), and is a substrate for GalNAc transferase where GalNAc is added to at least threonine or serine to create an O-linked glycosylation site. Preferred examples include:

	P ⁵² TGAQLA;	SEQ ID NO:59
	P ⁵² TQGAMP;	SEQ ID NO:60
	P ⁵² TQGAMA;	SEQ ID NO:61
5	P ⁵² TTAQLA;	SEQ ID NO:62
	P ⁵² GATQLA;	SEQ ID NO:63
	P ⁵² TSSSTA;	SEQ ID NO:64
	P ⁵² TSSSLA;	SEQ ID NO:65
10	P ⁵² TINTLA;	SEQ ID NO:350
	P ⁵² TTVSLA;	SEQ ID NO:351
	P ⁵² TQAQLA;	SEQ ID NO:352

4. Region 4

[0439] In these mutants, the wild-type TGFHLQIL P⁸¹DGSVQGTRQ; SEQ ID NO:5, is subdivided into three regions; Region 4(a) HLQILP⁸¹; SEQ ID NO:66; Region 4(b) P⁸¹DGSVQGT; SEQ ID NO:67; and Region 4(c) P⁸¹NGS SEQ ID NO:68. Mutations in each region are considered separately below.

[0440] Region 4(a): in these mutants the wild-type HLQILP⁸¹ (SEQ ID NO:66) is replaced with 12345 P⁸¹ wherein 1, 2, 3, 4, 5, are independently selected from any uncharged amino acid, or glutamic acid (E), wherein at least one is threonine (T) or serine (S), and is a substrate for GalNAc transferase where GalNAc is added to at least threonine or serine to create an O-linked glycosylation site. Preferred examples include:

	QTELP ⁸¹ ;	SEQ ID NO:69
25	LIVTP ⁸¹ ;	SEQ ID NO:70
	LTELP ⁸¹ ;	SEQ ID NO:71
	LTELP ⁸¹ ;	SEQ ID NO:72
	GVTAP ⁸¹ ;	SEQ ID NO:73
30	HLTETP ⁸¹ ;	SEQ ID NO:74
	VLTETP ⁸¹ ;	SEQ ID NO:75
	VGTETP ⁸¹ ;	SEQ ID NO:76
	VGVGTETP ⁸¹ ;	SEQ ID NO:77
35	VTSAP ⁸¹ ;	SEQ ID NO:78

	VSTP ⁸¹ ;	SEQ ID NO:79
	EATP ⁸¹ ;	SEQ ID NO:80

[0441] Region 4(b): in these mutants the wild-type P⁸¹DGSVQGT (SEQ ID NO:67) is replaced with P⁸¹12345GT wherein 1, 2, 3, 4 and 5 are independently selected from any uncharged amino acid, or glutamic acid (E), wherein at least one is threonine (T) or serine (S), and is a substrate for GalNAc transferase where GalNAc is added to at least threonine or serine to create an O-linked glycosylation site. Preferred examples include:

	P ⁸¹ TGSGVT;	SEQ ID NO:81
	P ⁸¹ TQGVQGT;	SEQ ID NO:82
	P ⁸¹ TGSGVPGT;	SEQ ID NO:83
50	P ⁸¹ TQGAMPGT;	SEQ ID NO:84
	P ⁸¹ TTSVQGT;	SEQ ID NO:85
	P ⁸¹ TTAVQGT;	SEQ ID NO:86
	P ⁸¹ TINTQGT;	SEQ ID NO:353
55	P ⁸¹ TTVSQGT;	SEQ ID NO:354

[0442] Region 4(c): in these mutants the wild-type P⁸¹DGS (SEQ ID NO:68) is mutated to create an N-linked glycosylation site. Preferred examples include:

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	IL P ⁸¹ NGSVH;	SEQ ID NO:87
	IF P ⁸¹ NGSV;	SEQ ID NO:88
	P ⁸¹ NGT;	SEQ ID NO:89
5	L P ⁸¹ NGTVH;	SEQ ID NO:90
	P ⁸¹ NGTV;	SEQ ID NO:91
	IL P ⁸¹ NGT;	SEQ ID NO:92
	QIL P ⁸¹ NGT;	SEQ ID NO:93
10	QIL P ⁸¹ NGTVH;	SEQ ID NO:94

5. Region 5

[0443] In these mutants the wild-type LN KDGTP¹⁷⁵RDGAR SKRH, SEQ ID NO:6 is replaced with 12345 P¹⁷⁵67891011 wherein 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, are independently selected from any uncharged amino acid, or glutamic acid (E), wherein at least one is threonine (T) or serine (S), and is a substrate for GalNAc transferase where GalNAc is added to at least threonine or serine to create an O-linked glycosylation site. Preferred examples include:

	LNVTEP ¹⁷⁵ RDGARSKRH;	SEQ ID NO:95
20	LNVTEP ¹⁷⁵ DDGARSKRH;	SEQ ID NO:96
	LNVTEP ¹⁷⁵ LDGARSKRH;	SEQ ID NO:97
	LNAITP ¹⁷⁵ RDGARSKRH;	SEQ ID NO:98
	LNAITP ¹⁷⁵ LDGARSKRH;	SEQ ID NO:99
25	LNQEAT P ¹⁷⁵ LDGARSKRH;	SEQ ID NO:100
	LNQTEL P ¹⁷⁵ LDGARSKRH;	SEQ ID NO:101
	LNQTEL P ¹⁷⁵ ADGARSKRH;	SEQ ID NO:102
	LNKDGT P ¹⁷⁵ TDGARSKRH;	SEQ ID NO:103
	LNKDGT P ¹⁷⁵ TSGARSKRH;	SEQ ID NO:104
30	LNKDGT P ¹⁷⁵ IDGAASKRH;	SEQ ID NO:105
	LNKDGT P ¹⁷⁵ TSGAASKRH;	SEQ ID NO:106
	LNKDGT P ¹⁷⁵ TQGAMPKRH;	SEQ ID NO:107
	LNKDGT P ¹⁷⁵ TQGAMSKRH;	SEQ ID NO:108
35	LNKDGT P ¹⁷⁵ TTTARSKRH;	SEQ ID NO:109
	LN KDGTP ¹⁷⁵ TINTRSKRH;	SEQ ID NO:355
	LN KDGTP ¹⁷⁵ TINTSSKRH;	SEQ ID NO:356
	LN KDGTP ¹⁷⁵ TTVSRSKRH;	SEQ ID NO:357
40	LN KDGTP ¹⁷⁵ TTVSASKRH;	SEQ ID NO:358

6. Region 6

[0444] In these mutants, the wild-type sequence, FTHFL P¹⁹²RPVD P¹⁹⁷ERVP²⁰¹ELYKDLL; SEQ ID NO:7, is subdivided into two regions; Region 6(a) LP¹⁹²RPVD P¹⁹⁷ERV P²⁰¹ELYKD; SEQ ID NO:110 and Region 6(b) P¹⁹⁷ERVP²⁰¹ELYKD, SEQ ID NO:111. Mutations in each region are considered separately, below.

[0445] Region 6(a): Region 6(a) in these mutants the wild-type LP¹⁹²RPVD P¹⁹⁷ERVP²⁰¹ELYKD (SEQ ID NO:110) is replaced with P¹⁹²IP23 P¹⁹⁷ wherein 1, 2, 3, are independently selected from any uncharged amino acid, or glutamic acid (E), wherein at least one is threonine (T) or serine (S), and is a substrate for GalNAc transferase where GalNAc is added to at least threonine or serine to create an O-linked glycosylation site. Preferred examples include:

	LP ¹⁹² APTD P ¹⁹⁷ ERVP ²⁰¹ ELYKD;	SEQ ID NO:112
	LP ¹⁹² NPTA P ¹⁹⁷ ERVP ²⁰¹ ELYKD;	SEQ ID NO:113
	LP ¹⁹² RPTA P ¹⁹⁷ ERVP ²⁰¹ ELYKD;	SEQ ID NO:114
55	LP ¹⁹² APTQ P ¹⁹⁷ ERVP ²⁰¹ ELYKD;	SEQ ID NO:115
	LP ¹⁹² TPVD P ¹⁹⁷ ERVP ²⁰¹ ELYKD;	SEQ ID NO:116
	LP ¹⁹² TPSD P ¹⁹⁷ ERVP ²⁰¹ ELYKD;	SEQ ID NO:117

(continued)

LP¹⁹²VPTD P¹⁹⁷ERVP²⁰¹ELYKD; SEQ ID NO:118LP¹⁹²TPAD P¹⁹⁷ERVP²⁰¹ELYKD; SEQ ID NO:119

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[0446] Region 6(b): Region 6(b) in these mutants the wild-type P¹⁹⁷ERVP²⁰¹ELYKD (SEQ ID NO:111) is replaced with P¹⁹⁷123P²⁰¹45678 wherein 1, 2, 3, 4, 5, 6, 7, 8, are independently selected from any uncharged amino acid, or glutamic acid (E), wherein at least one is threonine (T) or serine (S), and is a substrate for GalNAc transferase where GalNAc is added to at least threonine or serine to create an O-linked glycosylation site. Preferred examples include:

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P¹⁹⁷TAS P²⁰¹ELYKD; SEQ ID NO:120P¹⁹⁷TAS P²⁰¹ALYKD; SEQ ID NO:121P¹⁹⁷NLT P²⁰¹ELYKD; SEQ ID NO:122P¹⁹⁷ETV P²⁰¹ELYKD; SEQ ID NO:123

15

P¹⁹⁷QET P²⁰¹ELYKD; SEQ ID NO:124P¹⁹⁷TQG P²⁰¹ELYKD; SEQ ID NO:125P¹⁹⁷TQG P²⁰¹ALYKD; SEQ ID NO:126P¹⁹⁷QGT P²⁰¹ALYKD; SEQ ID NO:127

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P¹⁹⁷ATE P²⁰¹ELYKD; SEQ ID NO:128P¹⁹⁷TTQ P²⁰¹ELYKD; SEQ ID NO:129P¹⁹⁷TTE P²⁰¹ELYKD; SEQ ID NO:130P¹⁹⁷ERVP²⁰¹TLYKD; SEQ ID NO:131P¹⁹⁷ERVP²⁰¹TLYAD; SEQ ID NO:132

25

P¹⁹⁷ERVP²⁰¹TQGAD; SEQ ID NO:133P¹⁹⁷ERVP²⁰¹TQGAMP; SEQ ID NO:134P¹⁹⁷ERVP²⁰¹TQGA; SEQ ID NO:135P¹⁹⁷TQAP²⁰¹ELYKD; SEQ ID NO:359

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P¹⁹⁷TEIP²⁰¹ELYKD; SEQ ID NO:360

7. Region 7

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[0447] In these mutants the wild-type L²⁰⁸MY T²¹¹ (SEQ ID NO: 8) is replaced with 123(4)_a(5)_b(6)_c(x) wherein 1, 2, 3, 4, 5, are independently selected from any uncharged amino acid, or glutamic acid (E), wherein at least one is threonine (T) or serine (S), and is a substrate for GalNAc transferase where GalNAc is added to at least threonine or serine to create an O-linked glycosylation site. The symbols a, b, and c independently represent 0 or 1, and (x) is selected from OH, NH₂, glycine, alanine, leucine, and asparagine. Preferred examples include:

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L²⁰⁸MY T²¹¹P(x); SEQ ID NO:136L²⁰⁸TE T²¹¹P(x); SEQ ID NO:137VTE T²¹¹P(x); SEQ ID NO:138GVTE T²¹¹PL(x); SEQ ID NO:139

45

PELVGVTC T²¹¹PL(x); SEQ ID NO:140L²⁰⁸MY T²¹¹(x); SEQ ID NO:141L²⁰⁸MY T²¹¹PTASP; SEQ ID NO:142L²⁰⁸MY T²¹¹PATEP; SEQ ID NO:143L²⁰⁸MY T²¹¹PTP(x); SEQ ID NO:144

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L²⁰⁸MY T²¹¹PTAP(x); SEQ ID NO:145

[0448] The numbering of the amino acid residues is based on the initial unmodified sequence in which the most N-terminal residue is numbered 1. The numbering of unmodified amino acids remains unchanged following the modification.

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More than one of the above described sequence modifications may be present in a FGF mutant of the present invention.

EXAMPLE 2

[0449] A library of FGF-20 peptides each with one potential O-linked glycosylation site as disclosed in Example 1, is expressed in *E. coli* or by using *in vitro* translation methods. Protein is purified using either a heparin binding or IMAC capture method and tested by for *in vitro* biological activity. Those protein sequences that retain *in vitro* activity are tested as acceptors for GlycoPEGylation. GlycoPEGylated FGF-20's (40 kDa branched) are purified for further biological evaluation as outlined above.

EXAMPLE 3

[0450] Exemplary regions on FGF-21 that are suited for the introduction of glycosylation sites by mutation are shown in Table 7, above. In all cases, the N-terminal Met may be present or absent on any FGF mutant. The numbering of the amino acid residues is based on the initial unmodified sequence in which the left most residue, methionine, is numbered as position 1. To highlight how the mutant sequence differs in respect to the unmodified sequence, the numbering of unmodified amino acids as they appear in the mutant sequences below remains unchanged following the modification. More than one of the described sequence modifications may be present in an FGF mutant of the present invention. Specifically, the preferred regions for introduction of mutations to create a glycosylation site(s) not present in the wild-type peptide are the nucleotide sequences that encode: amino acids 1-8 (REGION 1; SEQ ID NO:147), amino acids 9-13 (REGION 2; SEQ ID NO:148), amino acids 46-54 (REGION 3; SEQ ID NO:149), amino acids 60-65 (REGION 4; SEQ ID NO:150), amino acids 78-83 (REGION 5; SEQ ID NO:151), amino acids 86-91 (REGION 6; SEQ ID NO:152) or amino acids 112-141 (REGION 7; SEQ ID NO:153), amino acids 149-157 (REGION 8; SEQ ID NO:154), amino acids 160-166 (REGION 9; SEQ ID NO:155), amino acids 167-172 (REGION 10; SEQ ID NO:156), amino acids 173-182 (REGION 11; SEQ ID NO:157) of the wild-type FGF-121 amino acid sequence (see Table 7) can be mutated so that either an N-linked or an O-linked glycosylation site is introduced into the resulting mutated FGF-21 polypeptide.

[0451] The following example describes amino acid sequence mutations introducing N-linked *e.g.*, asparagine residues, and O-linked glycosylation sites, *e.g.*, serine or threonine residues, into a preferably proline-containing site of a wild-type Fibroblast Growth Factor-21 sequence or any modified version thereof.

1. Region 1

[0452] In the Region 1 mutants, the N-terminus of a wild-type FGF-21, M¹HPIPDSS (SEQ ID NO:147), is subdivided into three regions; Region 1(a) M¹HP³ (SEQ ID NO:158); Region 1(b) M¹HPIP (SEQ ID NO:159); and Region 1(c) P⁵DSS (SEQ ID NO:160). Mutations in each region are considered separately, below.

[0453] Region 1(a): In these mutants, the wild-type M¹HP³ (SEQ ID NO:158) is replaced with M¹X_nB_oO_rJ_qP³ wherein B, O, J are independently selected from any uncharged amino acid, and where X is any uncharged amino acid, or histidine (H), and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols n, o, q, r independently represent 0-3. Preferred mutations include:

M ¹ VTP ³	SEQ ID NO:161
M ¹ QTP ³ ;	SEQ ID NO:162
M ¹ ATP ³ ;	SEQ ID NO:163
M ¹ IATP ³ ;	SEQ ID NO:164

[0454] Region 1(b): in these mutants the wild-type M¹HPIP (SEQ ID NO:159) is replaced with M¹X_nPB_oP wherein B is independently selected from any uncharged amino acid, and where X is any uncharged amino acid, or histidine (H), and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols n, o independently represent 0-3. Preferred mutations include:

M ¹ FPTP;	SEQ ID NO:165
M ¹ HPTP;	SEQ ID NO:166
M ¹ APTP;	SEQ ID NO:167
M ¹ FPSP;	SEQ ID NO:168
M ¹ HPSP;	SEQ ID NO:169
M ¹ APSP;	SEQ ID NO:170
M ¹ SPTP;	SEQ ID NO:171

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[0455] Region 1(c): in these mutants the wild-type P⁵DSS (SEQ ID NO:160) is replaced with P⁵B_oO_rJ_q wherein B, O, J are independently selected from any uncharged amino acid, and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols o, q, r independently represent 0-3. Preferred mutations include:

P⁵TSS; SEQ ID NO:172
P⁵TQA; SEQ ID NO:173
P⁵TAQ; SEQ ID NO:174
P⁵TIE; SEQ ID NO:175
P⁵SSS; SEQ ID NO:176

2. Region 2

[0456] In these mutants, the wild-type P⁹L¹⁰LQF (SEQ ID NO:148) is replaced with P⁹J_qX_nO_rU_s wherein X, J, O, U are independently selected from any uncharged amino acid, and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols q, n, r, s independently represent 0-3. Preferred examples include:

P⁹T¹⁰TQF; SEQ ID NO:177
P⁹T¹⁰INT; SEQ ID NO:178
P⁹T¹⁰QGA; SEQ ID NO:179
P⁹T¹⁰QGF; SEQ ID NO:180
P⁹T¹⁰TVS; SEQ ID NO:181
P⁹T¹⁰QAF; SEQ ID NO:182

3. Region 3

[0457] In these mutants, the wild-type ADQSP⁵⁰ESLL (SEQ ID NO:149) is replaced with 1_tØZ_mB_oP⁵⁰J_qX_nO_rU_s wherein Ø, Z, X, B, J, O, U, 1, 2, 3 are independently selected from any uncharged amino acid, where Z or J is independently selected as glutamic acid (E), where 2 and X may be independently selected as lysine (K) or arginine (R), and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols t, m, n, o, q, r, s, t independently represent 0-3. Preferred examples include:

ADQSP⁵⁰TSLL; SEQ ID NO:183
ADQSP⁵⁰TTVS; SEQ ID NO:184
ADQSP⁵⁰TINT; SEQ ID NO:185
ADQSP⁵⁰TQAL; SEQ ID NO:186
ADQSP⁵⁰TQGA; SEQ ID NO:187
ADQSP⁵⁰TQAL; SEQ ID NO:188
ATQSP⁵⁰ESLL; SEQ ID NO:189
ATESP⁵⁰ESLL; SEQ ID NO:190
ATETP⁵⁰ESLL; SEQ ID NO:191
VTQSP⁵⁰ESLL; SEQ ID NO:192
VTETP⁵⁰ESLL; SEQ ID NO:193
ATESP⁵⁰ASLL; SEQ ID NO:194

4. Region 4

[0458] In these mutants, the wild-type KP⁶¹GVIQ (SEQ ID NO:150) is replaced with B_oP⁶¹J_qX_nO_rU_s wherein B is selected from lysine (K) or any uncharged amino acid, where X, J, O, U are independently selected from any uncharged amino acid, and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols n, o, q, r, s independently represent 0-3. Preferred examples include:

SP⁶¹TVIQ; SEQ ID NO:195

(continued)

	AP ⁶¹ TVIQ;	SEQ ID NO:196
	SP ⁶¹ TTVS;	SEQ ID NO:197
5	SP ⁶¹ TINT;	SEQ ID NO:198
	SP ⁶¹ TQAA;	SEQ ID NO:199
	SP ⁶¹ TQGA;	SEQ ID NO:200
	SP ⁶¹ TVIA;	SEQ ID NO:201
10	AP ⁶¹ TTVS;	SEQ ID NO:202
	AP ⁶¹ TINT;	SEQ ID NO:203

5. Region 5

15 **[0459]** In these mutants, the wild-type RP⁷⁹DGAL (SEQ ID NO:151) is replaced with B_oP⁷⁹J_qX_nO_rU_s wherein X, B, J, O, U are independently selected from any uncharged amino acid, where B may be independently selected as lysine (K) or arginine (R), and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols n, o, q, r, s independently represent 0-3. Preferred examples include:

20	SP ⁷⁹ TGAL;	SEQ ID NO:204
	AP ⁷⁹ TGAL;	SEQ ID NO:205
	SP ⁷⁹ TINT;	SEQ ID NO:206
	SP ⁷⁹ TTVS;	SEQ ID NO:207
	SP ⁷⁹ TQAL;	SEQ ID NO:208
25	AP ⁷⁹ TQAL;	SEQ ID NO:209
	SP ⁷⁹ TQGA;	SEQ ID NO:210
	SP ⁷⁹ TQGAM;	SEQ ID NO:211

30 6. Region 6

[0460] In these mutants, the wild-type SLHFDP⁹¹ (SEQ ID NO:152) is replaced with 21_tØZ_mB_oP⁹¹ wherein Ø, Z, B, 1, 2 are independently selected from any uncharged amino acid, and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols t, m, o independently represent 0-3. Preferred examples include:

	SLTFTP ⁹¹ ;	SEQ ID NO:212
	SLTETP ⁹¹ ;	SEQ ID NO:213
40	SVTETP ⁹¹ ;	SEQ ID NO:214

7. Region 7

45 **[0461]** In these mutants, the wild-type A¹¹²HGLPLHLP¹²⁰GNKSPHRDPAPRGPARFLPLP (SEQ ID NO:153) is subdivided into five regions; Region 7(a) AHGLP¹¹⁶LHLP¹²⁰ (SEQ ID NO:215); Region 7(b) HLP¹²⁰GNKSP¹²⁵HR (SEQ ID NO:216); Region 7(c) KSP¹²⁵HRDP¹²⁹APR (SEQ ID NO:217); Region 7(d) RGP¹³⁴ARFLP¹³⁹LP (SEQ ID NO:218); and Region 7(e) RGP¹³⁴ARFLP¹³⁹LP (SEQ ID NO:219). Mutations in each region are considered separately, below.

50 **[0462] Region 7(a):** In these mutants, the wild-type AHGLP¹¹⁶LHLP¹²⁰ (SEQ ID NO:215) is replaced with 1_tØZ_mB_oP¹¹⁶J_qX_nO_rP¹²⁰ wherein 1, Ø, Z, X, B, J, O are independently selected from any uncharged amino acid, and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols t, m, o, q, n, r independently represent 0-3. Preferred mutations include:

	ATGTP ¹¹⁶ LHLP ¹²⁰ ;	SEQ ID NO:220
	ATETP ¹¹⁶ LHLP ¹²⁰ ;	SEQ ID NO:221
55	VTETP ¹¹⁶ LHLP ¹²⁰ ;	SEQ ID NO:222
	VTGLP ¹¹⁶ LHLP ¹²⁰ ;	SEQ ID NO:223
	ATGLP ¹¹⁶ LHLP ¹²⁰ ;	SEQ ID NO:224

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(continued)

	AHGLP ¹¹⁶ TQAP ¹²⁰ ;	SEQ ID NO:225
	AHGLP ¹¹⁶ TAQP ¹²⁰ ;	SEQ ID NO:226
5	AHGLP ¹¹⁶ TEIP ¹²⁰ ;	SEQ ID NO:227
	AHGLP ¹¹⁶ TSSP ¹²⁰ ;	SEQ ID NO:228
	AHGLP ¹¹⁶ TALP ¹²⁰ ;	SEQ ID NO:229
	ASGLP ¹¹⁶ TQAP ¹²⁰ ;	SEQ ID NO:230
10	ASGLP ¹¹⁶ TEIP ¹²⁰ ;	SEQ ID NO:231

[0463] Region 7(b): In these mutants, the wild-type HLP¹²⁰GNKSP¹²⁵HR (SEQ ID NO:216) is replaced with $1_tLP^{120}X_nO_rU_s2_aP^{125}B_oJ_q$ wherein X, B, J, O, U, 1, 2 are independently selected from any uncharged amino acid, where B, J, 1 are independently selected as histidine (H), lysine (K), or arginine (R), and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols t, n, r, s, a, o, q independently represent 0-3. Preferred mutations include:

	HLP ¹²⁰ TTAVP ¹²⁵ HR;	SEQ ID NO:232
	HLP ¹²⁰ TSGEP ¹²⁵ HR;	SEQ ID NO:233
20	HLP ¹²⁰ GSTAP ¹²⁵ HR;	SEQ ID NO:234
	HLP ¹²⁰ GNTSP ¹²⁵ HR;	SEQ ID NO:235
	HLP ¹²⁰ GTESP ¹²⁵ HR;	SEQ ID NO:236
	HLP ¹²⁰ LTQTP ¹²⁵ HR;	SEQ ID NO:237
	HLP ¹²⁰ GTQTP ¹²⁵ HR;	SEQ ID NO:238
25	HLP ¹²⁰ LTQTP ¹²⁵ AR;	SEQ ID NO:239
	HLP ¹²⁰ TNASP ¹²⁵ HR;	SEQ ID NO:240
	HLP ¹²⁰ TQGSP ¹²⁵ HR;	SEQ ID NO:241
	HLP ¹²⁰ VTSQP ¹²⁵ HR;	SEQ ID NO:242
30	HLP ¹²⁰ TINTP ¹²⁵ HR;	SEQ ID NO:243
	HLP ¹²⁰ TSVSP ¹²⁵ HR;	SEQ ID NO:244

[0464] Region 7(c): In these mutants, the wild-type KSP¹²⁵HRDP¹²⁹APR (SEQ ID NO:217) is replaced with $1_tSP^{125}X_nO_rU_sP^{129}B_oPJ_q$ wherein B, U, 1 are independently selected from any uncharged amino acid, where X, O, J are independently selected from any uncharged amino acid or histidine (H), lysine (K), or arginine (R), and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols t, n, r, s, o, q independently represent 0-3. Preferred mutations include:

	KSP ¹²⁵ TAQP ¹²⁹ APR;	SEQ ID NO:245
40	KSP ¹²⁵ TADP ¹²⁹ APR;	SEQ ID NO:246
	ASP ¹²⁵ TAQP ¹²⁹ APR;	SEQ ID NO:247
	SSP ¹²⁵ TADP ¹²⁹ APR;	SEQ ID NO:248
	KSP ¹²⁵ TSDP ¹²⁹ APR;	SEQ ID NO:249
45	KSP ¹²⁵ TEIP ¹²⁹ APR;	SEQ ID NO:250
	KSP ¹²⁵ TEIP ¹²⁹ APR;	SEQ ID NO:251
	KSP ¹²⁵ TEDP ¹²⁹ APR;	SEQ ID NO:252
	ASP ¹²⁵ TEDP ¹²⁹ APR;	SEQ ID NO:253
	SSP ¹²⁵ TADP ¹²⁹ APR;	SEQ ID NO:254
50	SSP ¹²⁵ TAQP ¹²⁹ APR;	SEQ ID NO:255
	KSP ¹²⁵ TQAP ¹²⁹ APR;	SEQ ID NO:256
	SSP ¹²⁵ TQAP ¹²⁹ APR;	SEQ ID NO:257
	ASP ¹²⁵ TEIP ¹²⁹ APR;	SEQ ID NO:258
55	KSP ¹²⁵ HRDP ¹²⁹ TPR;	SEQ ID NO:259
	KSP ¹²⁵ HRDP ¹²⁹ SPR;	SEQ ID NO:260
	KSP ¹²⁵ HRDP ¹²⁹ TPA;	SEQ ID NO:261
	KSP ¹²⁵ HRDP ¹²⁹ TPS;	SEQ ID NO:262

(continued)

KSP¹²⁵HSDP¹²⁹TPA; SEQ ID NO:263
 KSP¹²⁵HADP¹²⁹TPS; SEQ ID NO:264
 KSP¹²⁵HADP¹²⁹TPA; SEQ ID NO:265

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[0465] Region 7(d): In these mutants, the wild-type RGP¹³⁴ARFLP¹³⁹LP (SEQ ID NO:218) is replaced with $1_tGP^{134}X_nO_rU_s2_aP^{139}B_oP$ wherein X, B, O, U, 1,2 are independently selected from any uncharged amino acid, where O, 1 are independently selected from lysine (K) or arginine (R), and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols t, n, r, s, a, o independently represent 0-3. Preferred mutations include:

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RGP¹³⁴TSFLP¹³⁹LP; SEQ ID NO:266
 RGP¹³⁴TSGEF¹³⁹LP; SEQ ID NO:267
 RGP¹³⁴GSTAP¹³⁹LP; SEQ ID NO:268
 RGP¹³⁴ANTSP¹³⁹LP; SEQ ID NO:269
 RGP¹³⁴ATESP¹³⁹LP; SEQ ID NO:270
 RGP¹³⁴ATQTP¹³⁹LP; SEQ ID NO:271
 RGP¹³⁴ATQTP¹³⁹LP; SEQ ID NO:272
 RGP¹³⁴LTQTP¹³⁹LP; SEQ ID NO:273
 RGP¹³⁴TQFLP¹³⁹LP; SEQ ID NO:274
 RGP¹³⁴TSFLP¹³⁹LP; SEQ ID NO:275
 RGP¹³⁴VTSPQ¹³⁹LP; SEQ ID NO:276
 SGP¹³⁴TSFLP¹³⁹LP; SEQ ID NO:277
 AGP¹³⁴TSGEF¹³⁹LP; SEQ ID NO:278
 SGP¹³⁴TSALP¹³⁹LP; SEQ ID NO:279

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[0466] Region 7(e): In these mutants, the wild-type RGP¹³⁴ARFLP¹³⁹LP (SEQ ID NO:219) is replaced with $1_tGP^{134}X_nO_rU_s2_aP^{139}B_oP$ wherein X, B, O, U, 1,2 are independently selected from any uncharged amino acid, where O, 1 are independently selected from lysine (K) or arginine (R), and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols t, n, r, s, a, o independently represent 0-3. Preferred mutations include:

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RGP¹³⁴ARFLP¹³⁹TP; SEQ ID NO:280
 RGP¹³⁴ARFLP¹³⁹SP; SEQ ID NO:281
 RGP¹³⁴ASFLP¹³⁹TP; SEQ ID NO:282

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8. Region 8

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[0467] In these mutants, the wild-type EPP¹⁵¹GILAP¹⁵⁶Q (SEQ ID NO:154) is replaced with $B_oPP^{151}X_nO_rU_s2_aP^{156}1_t$ wherein B, X, O, U, 2, 1 are independently selected from any uncharged amino acid, and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols o, n, r, s, a, t independently represent 0-3. Preferred mutations include:

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TPP¹⁵¹GILAP¹⁵⁶Q; SEQ ID NO:283
 SPP¹⁵¹GILAP¹⁵⁶Q; SEQ ID NO:284
 EPP¹⁵¹TILAP¹⁵⁶Q; SEQ ID NO:285
 EPP¹⁵¹TTTAP¹⁵⁶Q; SEQ ID NO:286
 EPP¹⁵¹TQLAP¹⁵⁶Q; SEQ ID NO:287
 EPP¹⁵¹TQGAP¹⁵⁶Q; SEQ ID NO:288
 EPP¹⁵¹TSGEF¹⁵⁶Q; SEQ ID NO:289
 EPP¹⁵¹GSTAP¹⁵⁶Q; SEQ ID NO:290
 EPP¹⁵¹TTAVP¹⁵⁶Q; SEQ ID NO:291
 EPP¹⁵¹GNTSP¹⁵⁶Q; SEQ ID NO:292

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(continued)

	EPP ¹⁵¹ GTESP ¹⁵⁶ Q;	SEQ ID NO:293
	EPP ¹⁵¹ GTETP ¹⁵⁶ Q;	SEQ ID NO:294
5	EPP ¹⁵¹ VTSPQ ¹⁵⁶ Q;	SEQ ID NO:295
	EPP ¹⁵¹ AVQTP ¹⁵⁶ Q;	SEQ ID NO:296
	EPP ¹⁵¹ LTQTP ¹⁵⁶ Q;	SEQ ID NO:297
	EPP ¹⁵¹ VTSPQ ¹⁵⁶ Q;	SEQ ID NO:298
10	EPP ¹⁵¹ SSGAP ¹⁵⁶ Q;	SEQ ID NO:299
	EPP ¹⁵¹ TINTP ¹⁵⁶ Q;	SEQ ID NO:300
	EPP ¹⁵¹ TTVSP ¹⁵⁶ Q;	SEQ ID NO:301
	EPP ¹⁵¹ TQAAP ¹⁵⁶ Q;	SEQ ID NO:302
	EPP ¹⁵¹ GILAP ¹⁵⁶ T;	SEQ ID NO:303
15	EPP ¹⁵¹ GILAP ¹⁵⁶ S;	SEQ ID NO:304

9. Region 9

[0468] In these mutants, the wild-type DVGSSDP¹⁶⁶ (SEQ ID NO:155) is replaced with X_nO_rU_s2_aB_oZ_mP¹⁶⁶ wherein Z, X, B, O, U, 2 are independently selected from any uncharged amino acid, glutamic acid (E), or aspartic acid (D), and where at least one is T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols n, r, s, a, o, m independently represent 0-3. Preferred mutations include:

	TVGSSDP ¹⁶⁶ ;	SEQ ID NO:305
25	DVGSSTP ¹⁶⁶ ;	SEQ ID NO:306
	DVGTETP ¹⁶⁶ ;	SEQ ID NO:307
	DAASAAP ¹⁶⁶ ;	SEQ ID NO:308
	DAATAAP ¹⁶⁶ ;	SEQ ID NO:309
30	DVGTS DP ¹⁶⁶ ;	SEQ ID NO:310
	DVATSDP ¹⁶⁶ ;	SEQ ID NO:311
	TGDSSDP ¹⁶⁶ ;	SEQ ID NO:312
	TDASGAP ¹⁶⁶ ;	SEQ ID NO:313
	DVGTS GP ¹⁶⁶ ;	SEQ ID NO:314

10. Region 10

[0469] In these mutants, the wild-type LSMVGP¹⁷² (SEQ ID NO:156) is replaced with X_nO_rU_s2_aB_oP¹⁷² wherein B, O, U, 2 are independently selected from any uncharged amino acid, glutamic acid (E), or aspartic acid (D), where at least X is selected as either T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols n, r, s, a, o independently represent 0-3. Preferred mutations include:

	TSMVGP;	SEQ ID NO:315
45	TSGVGP;	SEQ ID NO:316
	TSGAMP;	SEQ ID NO:317
	TQGAMP;	SEQ ID NO:318
	TSMVGP;	SEQ ID NO:319
50	TQGAMP;	SEQ ID NO:320

11. Region 11

[0470] In these mutants, the wild-type SQGRSP¹⁷⁸SYAS (SEQ ID NO:157) is subdivided into two regions; Region 11(a) SQGRSP¹⁷⁸ (SEQ ID NO:321); and the carboxy terminal Region 11(b) RSO¹⁷⁸SYAS (SEQ ID NO:322). Mutations in each region are considered separately, below.

[0471] Region 11(a): In these mutants, the wild-type SQGRSP¹⁷⁸ (SEQ ID NO:321) is replaced with X_nO_rU_s2_aB_oP¹⁷⁸ wherein B, O, U, 2 are independently selected from any uncharged amino acid, glutamic acid (E), or aspartic acid (D),

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where at least X is selected as either T or S, and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols n, r, s, a, o independently represent 0-3. Preferred mutations include:

SQGASP ¹⁷⁸ ;	SEQ ID NO: 323
TQGASP ¹⁷⁸ ;	SEQ ID NO: 324
TQGAMP ¹⁷⁸ ;	SEQ ID NO: 325
TQGAMP ¹⁷⁸ ;	SEQ ID NO: 326

[0472] Region 11(b): In these mutants, the wild-type carboxy terminal RSP¹⁷⁸ SYAS (SEQ ID NO:322) is replaced with ZSP¹⁷⁸X_nO_rU_s1B_o23 wherein Z, X, B, O, U, 1, 2, 3 are independently selected from any uncharged amino acid, glutamic acid (E), or aspartic acid (D), where at least X is selected as either T or S, where Z may be independently selected as arginine (R) or lysine (K), and is a substrate for GalNAc transferase where GalNAc is added to at least T or S. The symbols n, r, s, o independently represent 0-3. Preferred mutations include:

ASP ¹⁷⁸ SYAS;	SEQ ID NO: 327
RSP ¹⁷⁸ TSVAA;	SEQ ID NO: 328
ASP ¹⁷⁸ TSVAA;	SEQ ID NO: 329
ASP ¹⁷⁸ SSGAPPPS;	SEQ ID NO: 330
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RSP ¹⁷⁸ SSGAPPPS;	SEQ ID NO: 333
ASP ¹⁷⁸ TINT;	SEQ ID NO: 334
ASP ¹⁷⁸ TSVS;	SEQ ID NO: 335
ASP ¹⁷⁸ TQAF;	SEQ ID NO: 336
ASP ¹⁷⁸ TINTP;	SEQ ID NO: 337

EXAMPLE 4

Soluble Expression of FGF-20 and FGF-21 in *E. coli*

[0473] Therapeutic proteins are commonly expressed in *E. coli* as inactive, insoluble inclusion bodies. Following inclusion body purification, soluble therapeutics are obtained by a protein refolding reaction. This refolding process is typically enhanced by the inclusion of compounds that facilitate the reshuffling of disulfide bonds.

[0474] The *E. coli* cytoplasm, the site of the protein expression and inclusion body formation, is a chemically reducing environment that inhibits the formation of disulfide bonds. A strain that has a less reducing, more oxidizing cytoplasm would theoretically permit disulfide bond formation, facilitating the expression of therapeutic proteins in a soluble form.

Experimental:

[0475] Therapeutic proteins tested were human FGF-20 and FGF-21. The FGF-21 construct lacked its N-terminal signal sequence. Genes encoding these therapeutic proteins were cloned into up to four different vector backbones (Vector #1, Vector #2, Vector #3, and pET24a) as indicated in Table 8. These constructs were tested in one or two of four different bacterial strains (W3110, BL21 DE3, *E. coli*_(trxb,gor,supp)-2, and *E. coli*_(trxb,gor,supp)-2 DE3) as indicated in Table 8.

[0476] For protein expression, an overnight small scale culture was used to inoculate a 100 mL culture of prewarmed martone LB containing 50 µg/mL kanamycin. The culture was incubated at 37°C with shaking, and monitored for OD₆₂₀. When the OD₆₂₀ reached 0.4-0.6, the cultures were split and transferred to a 37°C or 20°C shaking incubator for 15-20 minutes. IPTG was then added to 0.1 -1.0 mM final concentration, and shaking incubation was continued for 1.5 hours up to overnight. Cells were harvested by centrifugation at 4°C, 7000xg for 15 mins in a Sorvall RC3C+.

[0477] For whole cell extract analysis of protein expression, cells from a 150 µL aliquot of the induced cultures were collected by centrifugation and lysed in 1xPBS/0.1% SDS. Following heating with 100 mM DTT and 1x protein sample buffer, samples were resolved by SDS-PAGE, and stained with Coomassie fluorescent orange.

[0478] For the analysis of protein solubility, bacterial cell pellets from 50-100 mL of induced cultures were resuspended using ~30 mL of lysis buffer (eg 1x PBS, 5mM EDTA), and lysed by mechanical disruption with three passes through a microfluidizer. Small samples were taken and insoluble material was pelleted by centrifugation for 10 minutes at top

speed at 4°C in a microcentrifuge. Following the spin, the supernatant was separated from the pellet, and both were analyzed by SDS-PAGE and protein staining. Western blot analysis with antibodies specific for the therapeutic proteins was also carried out to verify the identity of the observed soluble proteins.

Results:

FGF-20

[0479] Vectors bearing FGF-20 were transformed into different bacterial strains as indicated in Table 8. 50-100 mL induction cultures, varying by temperature, aeration (rpm), IPTG concentration, and time, were analyzed by whole cell extract (WCE) SDS-PAGE. As shown in **FIG. 1a**, moderate expression was observed in Vector #2, Vector #3, and pET24a vectors, but not in Vector #1. Expression was observed as soon as 1.5 hours after induction, and greater levels of expression were at 37°C than 20°C.

[0480] To determine whether FGF-20 was expressed as a soluble protein, induced cell pellets of BL21 DE3 and *E. coli*_(trxb,gor,supp)-2 DE3 strains bearing pET24a FGF-20 were lysed, centrifuged, and analyzed by SDS-PAGE. As shown in **FIG. 1b**, the majority of FGF-20 was soluble in the *E. coli*_(trxb,gor,supp)-2 DE3 cells when grown at 20°C. Growth at 37°C yielded approximately equal amount of soluble and insoluble protein in both BL21 DE3 and *E. coli*_(trxb,gor,supp)-2 DE3 cells.

FGF-21

[0481] Vectors bearing FGF-21 were transformed into different bacterial strains as indicated in Table 8. 100 mL induction cultures, varying by temperature and time, were analyzed by WCE SDS-PAGE. As shown in **FIG. 1c**, expression was observed in Vector #2, Vector #3, and pET24a vectors, but not in Vector #1. Expression was observed as soon as 1.5 hours after induction. As rFGF-21 was observed to resolve by SDS-PAGE approximately 3-5 kDa larger than the expected ~19.7 kDa, the identity of the induced band was confirmed by Western blot (**FIG. 1c**).

[0482] To determine whether FGF-21 was expressed as a soluble protein, induced cell pellets of W3110 and *E. coli*_(trxb,gor,supp) 2 strains bearing Vector #3 FGF-21 were lysed, centrifuged, and analyzed by SDS-PAGE. As shown in **FIG. 1d**, the majority of FGF-21 was soluble only in the *E. coli*_(trxb,gor,supp) 2 cells when induced at 20°C. Induction in *E. coli*_(trxb,gor,supp) 2 cells at 37°C, or in W3110 cells at either temperature yielded predominantly insoluble protein.

[0483] This study demonstrated a method for expressing the therapeutic proteins FGF-20 and FGF-21 in bacteria as soluble proteins. The expression technique using *E. coli*_(trxb,gor,supp)-2 described here should be applicable for the soluble expression of other therapeutic proteins.

[0484] Some embodiments of the invention are disclosed in the following items:

1. A Fibroblast Growth Factor (FGF) conjugate comprising: a FGF peptide; and a modifying group, wherein said modifying group is covalently attached to said peptide at a preselected glycosyl or amino acid residue of said peptide via an intact glycosyl linking group.

2. The FGF conjugate of item 1, wherein said FGF peptide is a member selected from FGF-I, FGF-2, FGF-18, FGF-20 and FGF-21.

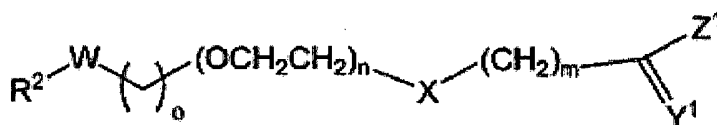
3. The FGF conjugate of item 1, wherein said FGF peptide is at least 95% homologous to at least one amino acid sequence selected from the group consisting of SEQ ID NOs: 1,9-14, 18-45, 48-65, 69-109, 112-146, 161-214, 220-320 and 323-360.

4. The FGF conjugate of item 1, wherein said modifying group is covalently attached at said preselected glycosyl residue.

5. The FGF conjugate of item 4, wherein said modifying group is a non- glycosidic modifying group.

6. The FGF conjugate of item 5, wherein said non-glycosidic modifying group is a member selected from linear PEG and branched PEG.

7. The FGF conjugate of item 6, wherein said PEG moiety is linear PEG and said linear PEG has a structure according to the following formula:



in which

R^2 is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted heteroalkyl, e.g., acetal, $OHC-$, $H_2N-CH_2CH_2-$, $HS-CH_2CH_2-$, and $-(CH_2)_qC(Y^1)Z^2$;

-sugar- nucleotide, or protein;

n is an integer selected from 1 to 2500; m , o , and q are integers independently selected from 0 to 20;

Z is a member selected from OH , NH_2 , halogen, $S-R^3$, the alcohol portion of activated esters, $-(CH_2)_pC(Y^2)V$, $-(CH_2)_pU(CH_2)_sC(Y^2)_v$, sugar-nucleotide, protein, and leaving groups, e.g., imidazole, p -nitrophenyl, HOBT, tetrazole, halide;

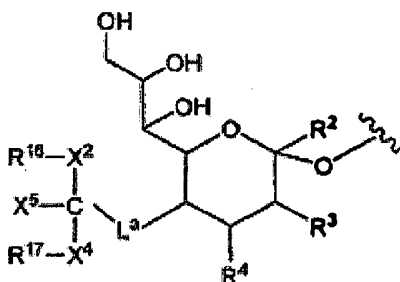
X , Y^1 , Y^2 , W and U are independently selected from O , S , $N-R^4$;

V is a member selected from OH , NH_2 , halogen, $S-R^5$, the alcohol component of activated esters, the amine component of activated amides, sugar-nucleotides, and proteins;

p , s and v are integers independently selected from 0 to 20; and

R^3 , R^4 and R^5 are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heterocycloalkyl and substituted or unsubstituted heteroaryl.

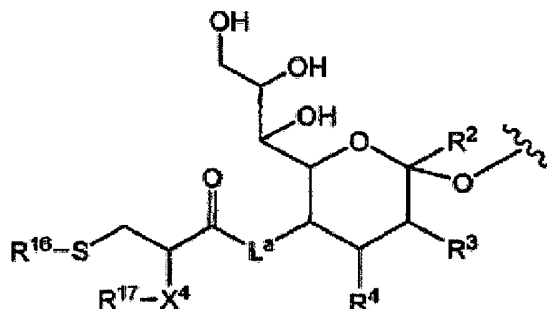
8. The FGF conjugate of item 1, wherein said glycosyl linking group has a structure according to the following formula:



wherein R^2 is H, CH_2OR^7 , $COOR^7$ or OR^7 wherein R^7 represents H, substituted or unsubstituted alkyl or substituted or unsubstituted heteroalkyl; R^3 and R^4 are members independently selected from H, substituted or unsubstituted alkyl, OR^8 , $NHC(O)R^9$ wherein

R^8 and R^9 are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl or sialic acid; L^a is a linker selected from a bond, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl; R^{16} and R^{17} are independently selected polymeric arms; X^2 and X^4 are independently selected linkage fragments joining polymeric moieties R^{16} and R^{17} to C; and X^5 is a non-reactive group.

9. The FGF conjugate of item 8, wherein said glycosyl linking group has a structure according to the following formula:



10. An isolated nucleic acid comprising a polynucleotide sequence encoding a mutant Fibroblast Growth Factor, wherein the mutant Fibroblast Growth Factor comprises a newly introduced N-linked or O-linked glycosylation site that does not exist in the corresponding wild-type Fibroblast Growth Factor.

11. The nucleic acid of item 10, wherein the nucleic acid encodes a mutant Fibroblast Growth Factor-20 that has a corresponding wild-type Fibroblast Growth Factor-20 amino acid sequence that is at least 95% homologous to SEQ ID NO: 1.

12. The nucleic acid of item 10, wherein the newly introduced glycosylation site is near a proline residue.

13. The nucleic acid of item 12, wherein the proline residue is located at position 3, 28, 29, 34, 35, 52, 81, 175, 192, 197, or 201 of SEQ ID NO:1.

14. The nucleic acid of item 10, wherein the mutant Fibroblast Growth Factor is at least 95% homologous to at least one amino acid sequence selected from the group consisting of SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, 112-145, 161-214, 220-320 and 323-360.

15. The nucleic acid of item 10, wherein the mutant Fibroblast Growth Factor comprises more than one newly introduced glycosylation site.

16. An expression cassette comprising the nucleic acid of item 10.

17. A cell comprising the nucleic acid of item 10.

18. A mutant Fibroblast Growth Factor, comprising a newly introduced N- linked or O-linked glycosylation site that does not exist in the corresponding wild-type Fibroblast Growth Factor.

19. The mutant Fibroblast Growth Factor of item 18, wherein the corresponding wild-type Fibroblast Growth Factor has the amino acid sequence that is at least 95% homologous to SEQ ID NO: 1.

20. The mutant Fibroblast Growth Factor of item 18, wherein the newly introduced glycosylation site is proximate to a proline residue.

21. The mutant Fibroblast Growth Factor of item 20, wherein the proline residue is located at position 3, 28, 29, 34, 35, 52, 81, 175, 192, 197, or 201 of SEQ ID NO:1.

22. The mutant Fibroblast Growth Factor of item 18, wherein said mutant FGF is at least 95% homologous to at least one amino acid sequence selected from the group consisting of SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, 112-145, 161-214, 220-320 and 323- 360.

23. The mutant Fibroblast Growth Factor of item 18, wherein the mutant Fibroblast Growth Factor comprises more than one newly introduced glycosylation site.

24. The mutant Fibroblast Growth Factor of item 18, comprising a water- soluble polymer attached to a glycosylation site through a glycosyl linker.

25. The mutant Fibroblast Growth Factor of item 24, wherein said glycosyl linker is an intact glycosyl linker.

26. A method for making a mutant Fibroblast Growth Factor, which comprises a newly introduced N-linked or O-linked glycosylation that does not exist in the corresponding wild-type Fibroblast Growth Factor, comprising the steps of: (a) recombinantly producing the mutant Fibroblast Growth Factor; and (b) glycosylating the mutant Fibroblast Growth Factor at the newly introduced glycosylation site wherein said glycosylating is a cell free, in vitro process.

27. The method of item 26, wherein the corresponding wild-type Fibroblast Growth Factor has the amino acid sequence that is at least 95% homologous to SEQ ID NO: 1.

28. The method of item 26, wherein the newly introduced glycosylation site is near a proline residue.

29. The method of item 28, wherein the proline residue is located at position 3, 28, 29, 34, 35, 52, 81, 175, 192, 197, or 201 of SEQ ID NO:1.

30. The method of item 26, wherein the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from the group consisting of SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, 112-145, 161-214, 220-320 and 323-360.

31. The method of item 26, wherein the mutant Fibroblast Growth Factor comprises more than one newly introduced glycosylation site.

32. A pharmaceutical composition comprising an effective amount of a mutant Fibroblast Growth Factor, which comprises a newly introduced N-linked or O-linked glycosylation that does not exist in the corresponding wild-type Fibroblast Growth Factor.

33. The composition of item 32, wherein the corresponding wild-type Fibroblast Growth Factor has the amino acid sequence that is at least 95% homologous to SEQ ID NO: 1.

34. The composition of item 32, wherein the newly introduced glycosylation site is near a proline residue.

35. The composition of item 34, wherein the proline residue is located at position 3, 28, 29, 34, 35, 52, 81, 175, 192, 197, or 201 of SEQ ID NO:1.

36. The composition of item 32, wherein the mutant Fibroblast Growth Factor is at least 95% homologous to at least one amino acid sequence selected from the group consisting of SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, 112-145, 161-214, 220-320 and 323-360.

37. The composition of item 32, wherein the mutant Fibroblast Growth Factor comprises more than one newly introduced glycosylation site.

38. A method for treating Fibroblast Growth Factor deficiency in a patient, comprising the step of administering an effective amount of a mutant Fibroblast Growth Factor to the patient, wherein the mutant Fibroblast Growth Factor comprises a newly introduced N-linked or O-linked glycosylation that does not exist in the corresponding wild-type Fibroblast Growth Factor.

39. The method of item 38, wherein the corresponding wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO: 1.

40. The method of item 38, wherein the newly introduced glycosylation site is near a proline residue.

41. The method of item 40, wherein the proline residue is located at position 3, 28, 29, 34, 35, 52, 81, 175, 192, 197, or 201 of SEQ ID NO:1.

42. The method of item 38, wherein the mutant Fibroblast Growth Factor is at least 95% homologous to at least one amino acid sequence selected from the group consisting of SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, 112-145, 161-214, 220-320 and 323-360.

43. The method of item 38, wherein the mutant Fibroblast Growth Factor comprises more than one newly introduced glycosylation site.

44. A method for making a glycoconjugate of a mutant Fibroblast Growth Factor, which comprises a newly introduced N-linked or O-linked glycosylation that does not exist in the corresponding wild-type Fibroblast Growth Factor, comprising the steps of: (a) recombinantly producing the mutant Fibroblast Growth Factor, and (b) enzymatically glycosylating the mutant Fibroblast Growth Factor with a modified sugar at the newly introduced glycosylation site wherein said glycosylating is a cell free, in vitro process.

45. The method of item 44, wherein the modified sugar is modified with a water-soluble polymer.

46. The method of item 45, wherein the modified sugar is modified with a water soluble polymer selected from the

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group consisting of poly(ethylene glycol) and m- poly(ethylene glycol).

47. The method of item 44 wherein the corresponding wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO: 1.

48. The method of item 45, wherein the newly introduced glycosylation site is near a proline residue.

49. The method of item 44, wherein the proline residue is located at position 3, 28, 29, 34, 35, 52, 81, 175, 192, 197, or 201 of SEQ ID NO:1.

50. The method of item 44, wherein the mutant Fibroblast Growth Factor comprises at least one amino acid sequence selected from the group consisting of SEQ ID NOs: SEQ ID NOs: 9-14, 18-45, 48-65, 69-109, 112-145, 161-214, 220-320 and 323-360.

51. The method of item 44, wherein the mutant Fibroblast Growth Factor comprises more than one newly introduced glycosylation site.

SEQUENCE LISTING

[0485]

<110> Neose Technologies, Inc. DeFrees, Shawn

<120> REMODELING AND GLYCOPEGYLATION OF FIBROBLAST GROWTH FACTOR (FGF)

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35 His Leu Glu Ile Arg Glu Asp Gly Thr Val Gly Gly Ala Ala Asp Gln
 35 40 45

40 Ser Pro Glu Ser Leu Leu Gln Leu Lys Ala Leu Lys Pro Gly Val Ile
 50 55 60

45 Gln Ile Leu Gly Val Lys Thr Ser Arg Phe Leu Cys Gln Arg Pro Asp
 65 70 75 80

Gly Ala Leu Tyr Gly Ser Leu His Phe Asp Pro Glu Ala Cys Ser Phe
 85 90 95

50 Arg Glu Leu Leu Leu Glu Asp Gly Tyr Asn Val Tyr Gln Ser Glu Ala
 100 105 110

55 His Gly Leu Pro Leu His Leu Pro Gly Asn Lys Ser Pro His Arg Asp
 115 120 125

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Pro Ala Pro Arg Gly Pro Ala Arg Phe Leu Pro Leu Pro Gly Leu Pro
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Claims

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1. A Fibroblast Growth Factor (FGF) conjugate comprising:

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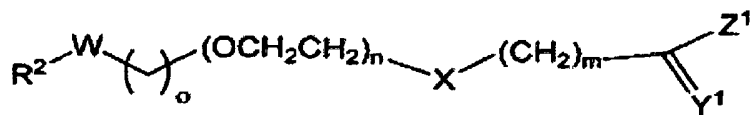
a mutant FGF-21 peptide comprising a newly introduced N-linked or O-linked glycosylation site that does not exist in the corresponding wild-type Fibroblast Growth Factor, wherein the corresponding wild-type Fibroblast Growth Factor has an amino acid sequence that is at least 95% homologous to SEQ ID NO: 146; and wherein the newly introduced N-linked or O-linked glycosylation site is less than 4 amino acids removed from a proline residue on the C- or N-terminal side of the proline residue, e.g.

55

the proline residue located at position 3, 5, 9, 50, 61, 79, 91, 116, 120, 125, 129, 134, 139, 151, 156, 166, 172, or 178 of SEQ ID NO: 146; and
a modifying group, wherein said modifying group is covalently attached to said peptide at a preselected glycosyl or amino acid residue of said mutant FGF peptide-21 via an intact glycosyl linking group, namely a glycosyl linking group in which the saccharide monomer that links the modifying group to the remainder of the molecule is not degraded.

2. The FGF conjugate of claim 1, wherein said mutant FGF-21 peptide comprises at least one amino acid sequence selected from the group consisting of SEQ ID NOs: 161-214, 220-320 and 323-337.

3. The FGF conjugate of any preceding claim, wherein said modifying group is covalently attached at said preselected glycosyl residue, optionally said modifying group is a non-glycosidic modifying group; optionally said non-glycosidic modifying group is a member selected from linear PEG and branched PEG; optionally said linear PEG has a structure according to the following formula:



in which

R^2 is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted heteroalkyl, e.g., acetal, OHC-, $H_2N-CH_2CH_2-$, $HS-CH_2CH_2-$, and $-(CH_2)_qC(Y^1)Z^2$; -sugar-nucleotide, or protein;

n is an integer selected from 1 to 2500;

m , o , and q are integers independently selected from 0 to 20;

Z is a member selected from OH, NH_2 , halogen, $S-R^3$, the alcohol portion of activated esters, $-(CH_2)_pC(Y^2)V$, $-(CH_2)_pU(CH_2)_sC(Y^2)_v$, sugar-nucleotide, protein, and leaving groups, e.g., imidazole, p-nitrophenyl, HOBT, tetrazole, halide;

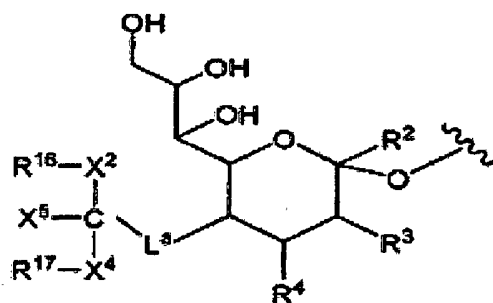
X , Y^1 , Y^2 , W and U are independently selected from O, S, $N-R^4$;

V is a member selected from OH, NH_2 , halogen, $S-R^5$, the alcohol component of activated esters, the amine component of activated amides, sugar-nucleotides, and proteins;

p , s and v are integers independently selected from 0 to 20; and

R^3 , R^4 and R^5 are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heterocycloalkyl and substituted or unsubstituted heteroaryl.

4. The FGF conjugate of any preceding claim, wherein said glycosyl linking group has a structure according to the following formula:



wherein

R^2 is H, CH_2OR^7 , $COOR^7$ or OR^7

wherein

R^7 represents H, substituted or unsubstituted alkyl or substituted or unsubstituted heteroalkyl;

R^3 and R^4 are members independently selected from H, substituted or unsubstituted alkyl, OR^8 , $NHC(O)R^9$

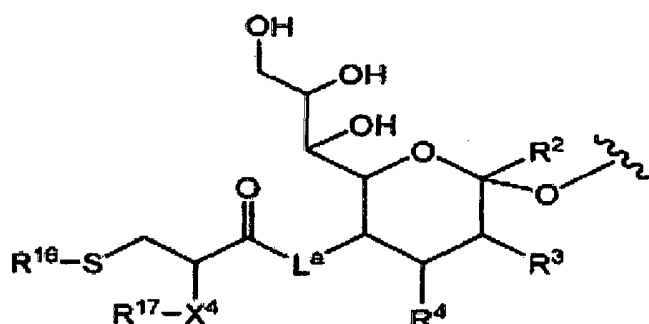
wherein

R^8 and R^9 are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl or sialic acid;

L^a is a linker selected from a bond, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl

R^{16} and R^{17} are independently selected polymeric arms;

X² and X⁴ are independently selected linkage fragments joining polymeric moieties R¹⁶ and R¹⁷ to C, and X⁵ a non-reactive group; optionally said glycosyl linking group has a structure according to the following formula:



5. An isolated nucleic acid comprising a polynucleotide sequence encoding a mutant Fibroblast Growth Factor-21, wherein the mutant Fibroblast Growth Factor-21 comprises a newly introduced N-linked or O-linked glycosylation site that does not exist in the corresponding wild-type Fibroblast Growth Factor, wherein the corresponding wild-type Fibroblast Growth Factor amino acid sequence is at least 95% homologous to SEQ ID NO: 146; and wherein the newly introduced glycosylation site is less than 4 amino acids removed from a proline residue on the C- or N-terminal side of the proline residue, e.g. the proline residue located at position 3, 5, 9, 50, 61, 79, 91, 116, 120, 125, 129, 134, 139, 151, 156, 166, 172, or 178 of SEQ ID NO: 146.
6. The nucleic acid of claim 5, wherein the mutant Fibroblast Growth Factor-21 comprises at least one amino acid sequence selected from the group consisting of SEQ ID NOs: 161-214, 220-320 and 323-337, and/or wherein the mutant Fibroblast Growth Factor-21 comprises more than one newly introduced glycosylation site.
7. An expression cassette or a cell comprising the nucleic acid of claim 5 or 6.
8. A mutant Fibroblast Growth Factor-21, comprising a newly introduced N-linked or O-linked glycosylation site that does not exist in the corresponding wild-type Fibroblast Growth Factor, wherein the corresponding wild-type Fibroblast Growth Factor has an amino acid sequence that is at least 95% homologous to SEQ ID NO: 146; and wherein the newly introduced glycosylation site is less than 4 amino acids removed from a proline residue on the C- or N-terminal side of the proline residue, e.g. the proline residue is located at position 3, 5, 9, 50, 61, 79, 91, 116, 120, 125, 129, 134, 139, 151, 156, 166, 172, or 178 of SEQ ID NO: 146.
9. The mutant Fibroblast Growth Factor-21 of claim 8, wherein said mutant FGF-21 comprises at least one amino acid sequence selected from the group consisting of SEQ ID NOs: 161-214, 220-320 and 323-337, and/or wherein the mutant Fibroblast Growth Factor-21 comprises more than one newly introduced glycosylation site.
10. The mutant Fibroblast Growth Factor-21 of claim 8 or 9, comprising a water-soluble polymer attached to a glycosylation site through a glycosyl linker, optionally said glycosyl linker is an intact glycosyl linker.
11. A method for making a mutant Fibroblast Growth Factor-21, which comprises a newly introduced N-linked or O-linked glycosylation site that does not exist in the corresponding wild-type Fibroblast Growth Factor, comprising the steps of:
 - (a) recombinantly producing the mutant Fibroblast Growth Factor-21; and
 - (b) glycosylating the mutant Fibroblast Growth Factor-21 at the newly introduced glycosylation site
 wherein
 said glycosylating is a cell free, in vitro process, wherein the corresponding wild-type Fibroblast Growth Factor has an amino acid sequence that is at least 95% homologous to SEQ ID NO: 146 and wherein the newly introduced glycosylation site is less than 4 amino acids removed from a proline residue on the C- or N-terminal side of the proline residue, e.g. the proline residue located at position 3, 5, 9, 50, 61, 79, 91, 116, 120, 125, 129, 134, 139, 151, 156, 166, 172, or 178 of SEQ ID NO: 146.

12. The method of claim 11, wherein the mutant Fibroblast Growth Factor-21 comprises at least one amino acid sequence selected from the group consisting of SEQ ID NOs: 161-214, 220-320 and 323-337, and/or wherein the mutant Fibroblast Growth Factor comprises more than one newly introduced glycosylation site.

13. A pharmaceutical composition comprising an effective amount of a product of any of claims 1 to 4 and 8 to 10.

14. A product of any of claims 1 to 4 and 8 to 10 for the use in treating Fibroblast Growth Factor deficiency.

15. A method for making a glycoconjugate of a mutant Fibroblast Growth Factor-21, which comprises a newly introduced N-linked or O-linked glycosylation site that does not exist in the corresponding wild-type Fibroblast Growth Factor, comprising the steps of:

(a) recombinantly producing the mutant Fibroblast Growth Factor-21, and

(b) enzymatically glycosylating the mutant Fibroblast Growth Factor-21 with a modified sugar at the newly introduced glycosylation site

wherein

said glycosylating is a cell free, in vitro process; wherein the corresponding wild-type Fibroblast Growth Factor has the amino acid sequence of SEQ ID NO: 146; and wherein the newly introduced glycosylation site is less than 4 amino acids removed from a proline residue on the C- or N-terminal side of the proline residue, e.g. the proline residue located at position 3, 5, 9, 50, 61, 79, 91, 116, 120, 125, 129, 134, 139, 151, 156, 166, 172, or 178 of SEQ ID NO: 146, and/or wherein the mutant Fibroblast Growth Factor-21 comprises at least one amino acid sequence selected from the group consisting of SEQ ID NOs: 161-214, 220-320 and 323-337, and/or the mutant Fibroblast Growth Factor-21 comprises more than one newly introduced glycosylation site; optionally the modified sugar is modified with a water-soluble polymer, optionally the water soluble polymer selected from the group consisting of poly(ethylene glycol) and m-poly(ethylene glycol).

Patentsprüche

1. Ein Fibroblasten-Wachstumsfaktor (FGF)-Konjugat umfassend:

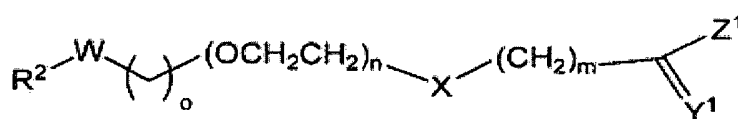
eine FGF-21-Peptidmutante umfassend eine neu eingeführte N-verknüpfte oder O-verknüpfte Glykosylierungsstelle, die nicht in dem dazugehörigen Wildtyp-Fibroblasten-Wachstumsfaktor existiert, wobei der dazugehörige Wildtyp-Fibroblasten-Wachstumsfaktor eine Aminosäuresequenz hat, die mindestens 95% homolog zu SEQ ID Nr: 146 ist;

und wobei die neu eingeführte N-verknüpfte oder O-verknüpfte Glykosylierungsstelle weniger als vier Aminosäuren von einem Prolinrest auf der C- oder der N-terminalen Seite von diesem Prolinrest entfernt ist, z.B. der Prolinrest, welcher sich an Position 3, 5, 9, 50, 61, 79, 91, 116, 120, 125, 129, 134, 139, 151, 156, 166, 172 oder 178 von SEQ ID Nr: 146 befindet; und

eine modifizierende Gruppe, wobei diese modifizierende Gruppe kovalent an das Peptid an einen vorgewählten Glykosyl- oder Aminosäurerest von der FGF-21-Peptidmutante über eine intakte Glykosyl-Linkergruppe angehängt ist, und zwar eine Glykosyl-Linkergruppe, in welcher das Saccharidmonomer, das die modifizierende Gruppe mit dem Rest des Moleküls verknüpft, nicht degradiert ist.

2. Das FGF-Konjugat von Anspruch 1, wobei die FGF-21-Peptidmutante mindestens eine Aminosäuresequenz ausgewählt aus der Gruppe bestehend aus SEQ ID Nr: 161-214, 220-320 und 323-337 umfasst.

3. Das FGF-Konjugat gemäß einem der vorangehenden Ansprüche, wobei die modifizierende Gruppe kovalent an den vorgewählten Glykosylrest angehängt ist, optional ist die modifizierende Gruppe eine nicht-glykosidische modifizierende Gruppe; optional ist die nicht-glykosidische modifizierende Gruppe ein Mitglied ausgewählt aus linearem PEG und verzweigtem PEG; optional hat das lineare PEG eine Struktur gemäß der folgenden Formel:



in welcher

R^2 ein Mitglied ausgewählt aus H, substituiertem oder unsubstituiertem Alkyl, substituiertem oder unsubstituiertem Aryl, substituiertem oder unsubstituiertem Heteroaryl, substituiertem oder unsubstituiertem Heterozykloalkyl, substituiertem oder unsubstituiertem Heteroalkyl, z.B. Acetal, OHC-, $H_2N-CH_2CH_2-$, $HS-CH_2CH_2-$ und $-(CH_2)_qC(Y^1)Z^2$; -Zucker-Nukleotid oder Protein ist;

n eine ganze Zahl ausgewählt aus 1 bis 2500 ist;

m, o, und q ganze Zahlen unabhängig voneinander ausgewählt aus 0 bis 20 sind;

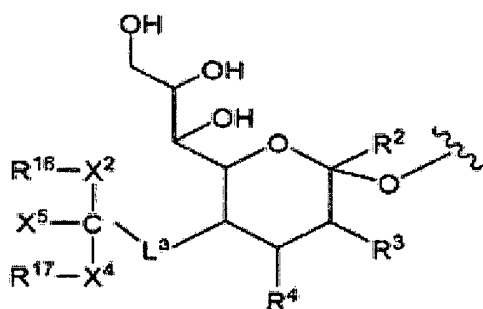
Z ein Mitglied ausgewählt aus OH, NH_2 , Halogen, $S-R^3$, dem Alkoholanteil von aktivierten Estern, $-(CH_2)_pC(Y^2)V$, $-(CH_2)_pU(CH_2)_sC(Y^2)_v$, Zuckernukleotid, Protein und Abgangsgruppen, wie z.B. Imidazol, p-Nitrophenyl, HOBT, Tetrazol, Halid, ist;

X, Y^1 , Y^2 , W und U unabhängig voneinander ausgewählt sind aus O, S, $N-R^4$; V ein Mitglied ausgewählt aus OH, NH_2 , Halogen, $S-R^5$, der Alkoholkomponente von aktivierten Estern, der Aminkomponente von aktivierten Amiden, Zuckernukleotiden und Proteinen ist;

p, s und v ganze Zahlen sind, die unabhängig voneinander ausgewählt sind aus 0 bis 20; und

R^3 , R^4 und R^5 unabhängig voneinander ausgewählt sind aus H, substituiertem oder unsubstituiertem Alkyl, substituiertem oder unsubstituiertem Heteroalkyl, substituiertem oder unsubstituiertem Aryl, substituiertem oder unsubstituiertem Heterozykloalkyl und substituiertem oder unsubstituiertem Heteroaryl.

4. Das FGF-Konjugat gemäß einem der vorangehenden Ansprüche, wobei besagte Glykosyl-Linkergruppe eine Struktur gemäß der folgenden Formel hat:



wobei

R^2 H, CH_2OR^7 , $COOR^7$ oder OR^7 ist,

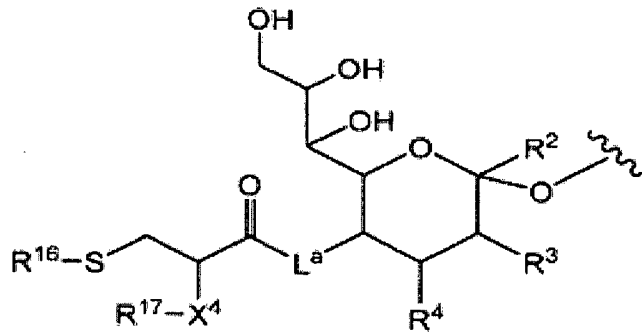
wobei

R^7 H, substituiertes oder unsubstituiertes Alkyl oder substituiertes oder unsubstituiertes Heteroalkyl repräsentiert; R^3 und R^4 unabhängig voneinander ausgewählte Mitglieder aus H, substituiertem oder unsubstituiertem Alkyl, OR^8 , $NHC(O)R^9$ sind, wobei

R^8 und R^9 unabhängig voneinander ausgewählt aus H, substituiertem oder unsubstituiertem Alkyl, substituiertem oder unsubstituiertem Heteroalkyl oder Sialinsäure sind;

L^a ein Linker ist, ausgewählt aus einer Bindung, substituiertem oder unsubstituiertem Alkyl und substituiertem oder unsubstituiertem Heteroalkyl, R^{16} und R^{17} unabhängig voneinander ausgewählte polymere Arme sind; X^2 und X^4 unabhängig voneinander ausgewählte Linkerfragmente sind, die die polymeren Einheiten R^{16} und R^{17} mit C verbinden, und

X^5 ist eine nicht-reaktive Gruppe; optional hat die Glykosyl-Linkergruppe eine Struktur gemäß der folgenden Formel:



5. Eine isolierte Nukleinsäure umfassend eine Polynukleotidsequenz, die eine Fibroblasten-Wachstumsfaktor-21-Mutante codiert, wobei die Fibroblasten-Wachstumsfaktor-21-Mutante eine neu eingeführte N-verknüpfte oder O-verknüpfte Glykosylierungsstelle umfasst, die nicht in dem dazugehörigen Wildtyp-Fibroblasten-Wachstumsfaktor existiert, wobei die Aminosäuresequenz von dem dazugehörigen Wildtyp-Fibroblasten-Wachstumsfaktor mindestens 95% homolog zu der SEQ ID Nr: 146 ist; und wobei die neu eingeführte Glykosylierungsstelle weniger als vier Aminosäuren von einem Prolinrest auf der C- oder N-terminalen Seite von dem Prolinrest entfernt ist, z.B. dem Prolinrest, der sich an Position 3, 5, 9, 50, 61, 79, 91, 116, 120, 125, 129, 134, 139, 151, 156, 166, 172 oder 178 von SEQ ID Nr: 146 befindet.
6. Die Nukleinsäure von Anspruch 5, wobei die Fibroblasten-Wachstumsfaktor-21-Mutante mindestens eine Aminosäuresequenz ausgewählt aus der Gruppe bestehend aus SEQ ID Nr: 161-214, 220-230 und 323-337 umfasst, und/oder wobei die Fibroblasten-Wachstumsfaktor-21-Mutante mindestens eine neu eingeführte Glykosylierungsstelle umfasst.
7. Eine Expressionskassette oder eine Zelle umfassend die Nukleinsäure gemäß Anspruch 5 oder 6.
8. Eine Fibroblasten-Wachstumsfaktor-21-Mutante umfassend eine neu eingeführte N-verknüpfte oder O-verknüpfte Glykosylierungsstelle, die nicht in dem dazugehörigen Wildtyp-Fibroblasten-Wachstumsfaktor existiert, wobei der dazugehörige Wildtyp-Fibroblasten-Wachstumsfaktor eine Aminosäuresequenz hat, die mindestens 95% homolog zu SEQ ID Nr: 146 ist; und wobei die neu eingeführte Glykosylierungsstelle weniger als vier Aminosäuren von einem Prolinrest auf der C- oder N-terminalen Seite von dem Prolinrest entfernt ist, z.B. befindet sich der Prolinrest an Position 3, 5, 9, 50, 61, 79, 91, 116, 120, 125, 129, 134, 139, 151, 156, 166, 172 oder 178 von SEQ ID Nr: 146.
9. Die Fibroblasten-Wachstumsfaktor-21-Mutante gemäß Anspruch 8, wobei die FGF-21-Mutante mindestens eine Aminosäuresequenz ausgewählt aus der Gruppe bestehend aus SEQ ID Nr: 161-214, 220-230 und 323-337 umfasst, und/oder wobei die Fibroblasten-Wachstumsfaktor-21-Mutante mehr als eine neue eingeführte Glykosylierungsstelle umfasst.
10. Die Fibroblasten-Wachstumsfaktor-21-Mutante gemäß Anspruch 8 oder 9 umfassend ein wasserlösliches Polymer, welches an eine Glykosylierungsstelle über einen Glykosyl-Linker angehängt ist, optional ist dieser Glykosyl-Linker ein intakter Glykosyl-Linker.
11. Ein Verfahren zur Herstellung einer Fibroblasten-Wachstumsfaktor-21-Mutante, welche eine neu eingeführte N-verknüpfte oder O-verknüpfte Glykosylierungsstelle umfasst, die nicht in dem dazugehörigen Wildtyp-Fibroblasten-Wachstumsfaktor existiert, umfassend die Schritte:
 - (a) rekombinantes Produzieren der Fibroblasten-Wachstumsfaktor-21-Mutante; und
 - (b) Glykosylieren der Fibroblasten-Wachstumsfaktor-21-Mutante an der neu eingeführten Glykosylierungsstelle wobei die Glykosylierung ein zellfreier, *in vitro* Prozess ist, wobei der dazugehörige Wildtyp-Fibroblasten-Wachstumsfaktor eine Aminosäuresequenz hat, die mindestens 95% homolog zu SEQ ID Nr: 146 ist, und wobei die neu eingeführte Glykosylierungsstelle weniger als vier Aminosäurereste von einem Prolinrest auf der C- oder N-terminalen Seite von dem Prolinrest entfernt ist, z.B. dem Prolinrest, der sich an Position 3, 5, 9, 50, 61, 79, 91,

116, 120, 125, 129, 134, 139, 151, 156, 166, 172 oder 178 von SEQ ID Nr: 146 befindet.

12. Das Verfahren gemäß Anspruch 11, wobei die Fibroblasten-Wachstumsfaktor-21-Mutante mindestens eine Aminosäuresequenz ausgewählt aus der Gruppe bestehend aus SEQ ID Nr: 161-214, 220-320 und 323-337 ist, und/oder wobei die Fibroblasten-Wachstumsfaktor-21-Mutante mehr als eine neu eingeführte Glykosylierungsstelle umfasst.
13. Eine pharmazeutische Zusammensetzung umfassend eine effektive Menge von einem Produkt gemäß einem der Ansprüche 1 bis 4 und 8 bis 10.
14. Ein Produkt von einem der Ansprüche 1 bis 4 und 8 bis 10 zur Verwendung in der Behandlung einer Fibroblasten-Wachstumsfaktor-Defizienz.
15. Ein Verfahren zur Herstellung eines Glykokonjugats von einer Fibroblasten-Wachstumsfaktor-21-Mutante, welche eine neu eingeführte N-verknüpfte oder O-verknüpfte Glykosylierungsstelle umfasst, die nicht in dem dazugehörigen Wildtyp-Fibroblasten-Wachstumsfaktor existiert, umfassend die Schritte:
 - (a) rekombinantes Produzieren der Fibroblasten-Wachstumsfaktor-21-Mutante; und
 - (b) enzymatisches Glykosylieren der Fibroblasten-Wachstumsfaktor-21-Mutante mit einem modifizierten Zucker an der neu eingeführten Glykosylierungsstelle wobei das Glykosylieren ein zellfreier, *in vitro* Prozess ist; wobei der dazugehörige Wildtyp-Fibroblasten-Wachstumsfaktor eine Aminosäuresequenz nach SEQ ID Nr: 146 hat; und wobei die neu eingeführte Glykosylierungsstelle weniger als vier Aminosäuren von einem Prolinrest auf der C- oder N-terminalen Seite von dem Prolinrest entfernt ist, z.B. ist der Prolinrest lokalisiert an Position 3, 5, 9, 50, 61, 79, 91, 116, 120, 125, 129, 134, 139, 151, 156, 166, 172 oder 178 gemäß SEQ ID Nr: 146, und/oder wobei die Fibroblasten-Wachstumsfaktor-21-Mutante mindestens eine Aminosäuresequenz ausgewählt aus der Gruppe bestehend aus SEQ ID Nr: 161-214, 220-320 und 323-337 umfasst, und/oder die Fibroblasten-Wachstumsfaktor-21-Mutante mehr als eine neu eingeführte Glykosylierungsstelle umfasst; optional ist der modifizierte Zucker mit einem wasserlöslichen Polymer modifiziert, optional ist das wasserlösliche Polymer ausgewählt aus der Gruppe bestehend aus Poly(ethylenglykol) und m-Poly(ethylenglykol).

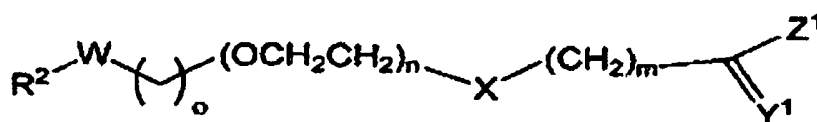
Revendications

1. Conjugué de facteur de croissance de fibroblastes (FGF) comprenant :

un peptide de FGF-21 mutant comprenant un site de glycosylation à liaison N ou à liaison O nouvellement introduit qui n'existe pas dans le facteur de croissance de fibroblastes de type sauvage correspondant, sachant que le facteur de croissance de fibroblastes de type sauvage correspondant comporte une séquence d'acides aminés qui est au moins à 95 % homologue de SEQ ID N° 146 ;

et sachant que le site de glycosylation à liaison N ou à liaison O nouvellement introduit est déplacé de moins de 4 acides aminés d'un résidu de proline du côté C-terminal ou N-terminal du résidu de proline, par ex. le résidu de proline situé en position 3, 5, 9, 50, 61, 79, 91, 116, 120, 125, 129, 134, 139, 151, 156, 166, 172, ou 178 de SEQ ID N° 146 ; et

un groupe modificateur, sachant que ledit groupe modificateur est lié de façon covalente audit peptide au niveau d'un résidu de glycosyl ou d'acide aminé présélectionné dudit peptide de FGF-21 mutant via un groupe de liaison de glycosyl intact, à savoir un groupe de liaison de glycosyl dans lequel le monomère de saccharide qui lie le groupe modificateur au reste de la molécule n'est pas dégradé.
2. Le conjugué de FGF de la revendication 1, sachant que ledit peptide de FGF-21 mutant comprend au moins une séquence d'acides aminés sélectionnée à partir du groupe constitué par SEQ ID N° 161 à 214, 220 à 320 et 323 à 337.
3. Le conjugué de FGF de l'une quelconque revendication précédente, sachant que ledit groupe modificateur est lié de façon covalente au niveau dudit résidu de glycosyl présélectionné ; facultativement, ledit groupe modificateur est un groupe modificateur non glycosidique ; facultativement, ledit groupe modificateur non glycosidique est un élément sélectionné parmi PEG linéaire et PEG ramifié ; facultativement, ledit PEG linéaire présente une structure selon la formule suivante :



dans lequel

R² est un élément sélectionné parmi H, de l'alkyle substitué ou non substitué, de l'aryle substitué ou non substitué, de l'hétéroaryle substitué ou non substitué, de l'hétérocycloalkyle substitué ou non substitué, de l'hétéroalkyle substitué ou non substitué, par ex. de l'acétal, OHC-, H₂N-CH₂CH₂-, HS-CH₂CH₂-, et-(CH₂)_qC(Y¹)Z² ; du nucléotide de sucre, ou une protéine ;

n est un entier sélectionné de 1 à 2500 ;

m, o, et q sont des entiers sélectionnés indépendamment de 0 à 20 ;

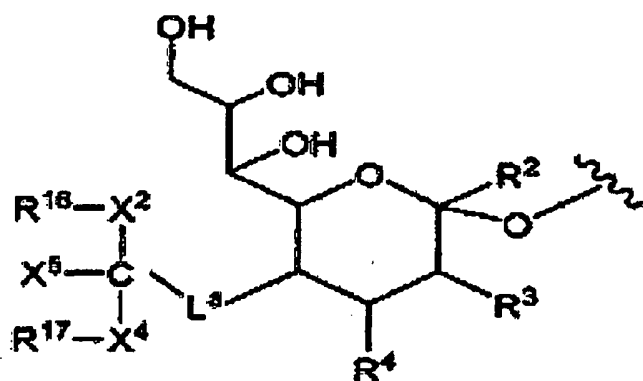
Z est un élément sélectionné parmi OH, NH₂, l'halogène, S-R³, la partie alcool d'esters activés, -(CH₂)_pC(Y²)V, -(CH₂)_pU(CH₂)_s(Y²)_v, du nucléotide de sucre, une protéine, et des groupes partants, par ex. de l'imidazole, du p-nitrophényle, HOBT, du tétrazole, de l'halogénure ;

X, Y¹, Y², W et U sont sélectionnés indépendamment parmi O, S, N-R⁴ ; V est un élément sélectionné parmi OH, NH₂, l'halogène, S-R⁵, le composant alcool d'esters activés, le composant amine d'amides activés, les nucléotides de sucre, et les protéines ;

p, s et v sont des entiers sélectionnés indépendamment de 0 à 20 ; et

R³, R⁴ et R⁵ sont sélectionnés indépendamment parmi H, de l'alkyle substitué ou non substitué, de l'hétéroalkyle substitué ou non substitué, de l'aryle substitué ou non substitué, de l'hétérocycloalkyle substitué ou non substitué et de l'hétéroaryle substitué ou non substitué.

4. Le conjugué de FGF de l'une quelconque revendication précédente, sachant que ledit groupe de liaison de glycosyl présente une structure conformément à la formule suivante :



sachant que

R² est H, CH₂OR⁷, COOR⁷ ou OR⁷

sachant que

R⁷ représente H, de l'alkyle substitué ou non substitué ou de l'hétéroalkyle substitué ou non substitué ;

R³ et R⁴ sont des éléments sélectionnés indépendamment parmi H, de l'alkyle substitué ou non substitué, OR⁸, NHC(O)R⁹

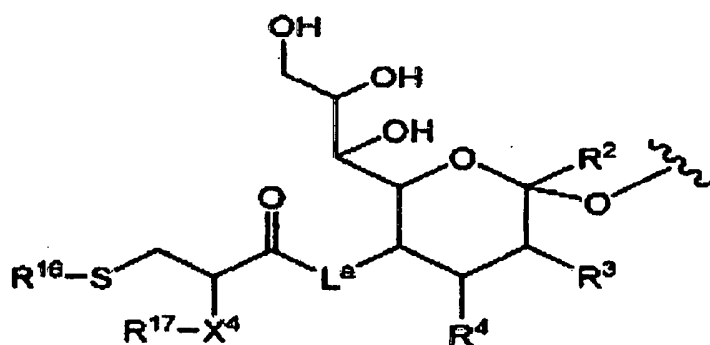
sachant que

R⁸ et R⁹ sont sélectionnés indépendamment parmi H, de l'alkyle substitué ou non substitué, de l'hétéroalkyle substitué ou non substitué ou de l'acide sialique ;

L^a est un lien sélectionné parmi une liaison, de l'alkyle substitué ou non substitué et de l'hétéroalkyle substitué ou non substitué ;

R¹⁶ et R¹⁷ sont des bras polymériques sélectionnés indépendamment ; X² et X⁴ sont des fragments de liaison sélectionnés indépendamment reliant des groupes fonctionnels polymériques R¹⁶ et R¹⁷ à C, et

X⁵ est un groupe non réactif ; facultativement, ledit groupe de liaison de glycosyl présente une structure selon la formule suivante :



5. Acide nucléique isolé comprenant une séquence polynucléotidique codant un facteur de croissance de fibroblastes 21 mutant, sachant que le facteur de croissance de fibroblastes 21 mutant comprend un site de glycosylation à liaison N ou à liaison O nouvellement introduit qui n'existe pas dans le facteur de croissance de fibroblastes de type sauvage correspondant, sachant que la séquence d'acides aminés de facteur de croissance de fibroblastes de type sauvage correspondant est au moins à 95 % homologue de SEQ ID N° 146 ; et sachant que le site de glycosylation nouvellement introduit est déplacé de moins de 4 acides aminés d'un résidu de proline du côté C-terminal ou N-terminal du résidu de proline, par ex. le résidu de proline situé en position 3, 5, 9, 50, 61, 79, 91, 116, 120, 125, 129, 134, 139, 151, 156, 166, 172, ou 178 de SEQ ID N° 146.
6. L'acide nucléique de la revendication 5, sachant que le facteur de croissance de fibroblastes 21 mutant comprend au moins une séquence d'acides aminés sélectionnée à partir du groupe constitué par SEQ ID N° 161 à 214, 220 à 320 et 323 à 337, et/ou sachant que le facteur de croissance de fibroblastes 21 mutant comprend plus d'un site de glycosylation nouvellement introduit.
7. Cassette d'expression ou cellule comprenant l'acide nucléique de la revendication 5 ou 6.
8. Facteur de croissance de fibroblastes 21 mutant, comprenant un site de glycosylation à liaison N ou à liaison O nouvellement introduit qui n'existe pas dans le facteur de croissance de fibroblastes de type sauvage correspondant, sachant que le facteur de croissance de fibroblastes de type sauvage correspondant comporte une séquence d'acides aminés qui est au moins à 95 % homologue de SEQ ID N° 146 ; et sachant que le site de glycosylation nouvellement introduit est déplacé de moins de 4 acides aminés d'un résidu de proline du côté C-terminal ou N-terminal du résidu de proline, par ex. le résidu de proline est situé en position 3, 5, 9, 50, 61, 79, 91, 116, 120, 125, 129, 134, 139, 151, 156, 166, 172, ou 178 de SEQ ID N° 146.
9. Le facteur de croissance de fibroblastes 21 mutant de la revendication 8, sachant que ledit FGF 21 mutant comprend au moins une séquence d'acides aminés sélectionnée à partir du groupe constitué par SEQ ID N° 161 à 214, 220 à 320 et 323 à 337, et/ou sachant que le facteur de croissance de fibroblastes 21 mutant comprend plus d'un site de glycosylation nouvellement introduit.
10. Le facteur de croissance de fibroblastes 21 mutant de la revendication 8 ou 9, comprenant un polymère hydrosoluble lié à un site de glycosylation via un lieu de glycosyl, ledit lieu de glycosyl étant facultativement un lieu de glycosyl intact.
11. Procédé de fabrication d'un facteur de croissance de fibroblastes 21 mutant, lequel comprend un site de glycosylation à liaison N ou à liaison O nouvellement introduit qui n'existe pas dans le facteur de croissance de fibroblastes de type sauvage correspondant, comprenant les étapes de :
 - (a) production recombinante du facteur de croissance de fibroblastes 21 mutant ; et
 - (b) glycosylation du facteur de croissance de fibroblastes 21 mutant au niveau du site de glycosylation nouvellement introduit sachant que ladite glycosylation est un processus in vitro sans cellule, sachant que le facteur de croissance de fibroblastes de type sauvage correspondant comporte une séquence d'acides aminés qui est au moins à 95 % homologue

de SEQ ID N° 146 et sachant que le site de glycosylation nouvellement introduit est déplacé de moins de 4 acides aminés d'un résidu de proline du côté C-terminal ou N-terminal du résidu de proline, par ex. le résidu de proline situé en position 3, 5, 9, 50, 61, 79, 91, 116, 120, 125, 129, 134, 139, 151, 156, 166, 172, ou 178 de SEQ ID N° 146.

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12. Le procédé de la revendication 11, sachant que le facteur de croissance de fibroblastes 21 mutant comprend au moins une séquence d'acides aminés sélectionnée à partir du groupe constitué par SEQ ID N° 161 à 214, 220 à 320 et 323 à 337, et/ou sachant que le facteur de croissance de fibroblastes mutant comprend plus d'un site de glycosylation nouvellement introduit.

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13. Composition pharmaceutique comprenant une quantité efficace d'un produit de l'une quelconque des revendications 1 à 4 et 8 à 10.

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14. Produit de l'une quelconque des revendications 1 à 4 et 8 à 10, destiné à être utilisé dans le traitement de la déficience du facteur de croissance de fibroblastes.

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15. Procédé de fabrication d'un glycoconjugué d'un facteur de croissance de fibroblastes 21 mutant, lequel comprend un site de glycosylation à liaison N ou à liaison O nouvellement introduit qui n'existe pas dans le facteur de croissance de fibroblastes de type sauvage correspondant, comprenant les étapes de :

- (a) production recombinante du facteur de croissance de fibroblastes 21 mutant, et
- (b) glycosylation enzymatique du facteur de croissance de fibroblastes 21 mutant avec un sucre modifié sur le site de glycosylation nouvellement introduit sachant que

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ladite glycosylation est un processus in vitro sans cellule ; sachant que le facteur de croissance de fibroblastes de type sauvage correspondant comporte la séquence d'acides aminés de SEQ ID N° 146 ; et sachant que le site de glycosylation nouvellement introduit est déplacé de moins de 4 acides aminés d'un résidu de proline du côté C-terminal ou N-terminal du résidu de proline, par ex. le résidu de proline situé en position 3, 5, 9, 50, 61, 79, 91, 116, 120, 125, 129, 134, 139, 151, 156, 166, 172, ou 178 de SEQ ID N° 146, et/ou le facteur de croissance de fibroblastes 21 mutant comprend au moins une séquence d'acides aminés sélectionnée à partir du groupe constitué par SEQ ID N° 161 à 214, 220 à 320 et 323 à 337, et/ou le facteur de croissance de fibroblastes 21 mutant comprend plus d'un site de glycosylation nouvellement introduit ; facultativement, le sucre modifié est modifié avec un polymère hydrosoluble, le polymère hydrosoluble étant facultativement sélectionné à partir du groupe constitué par le poly(éthylène glycol) et le m-poly(éthylène glycol).

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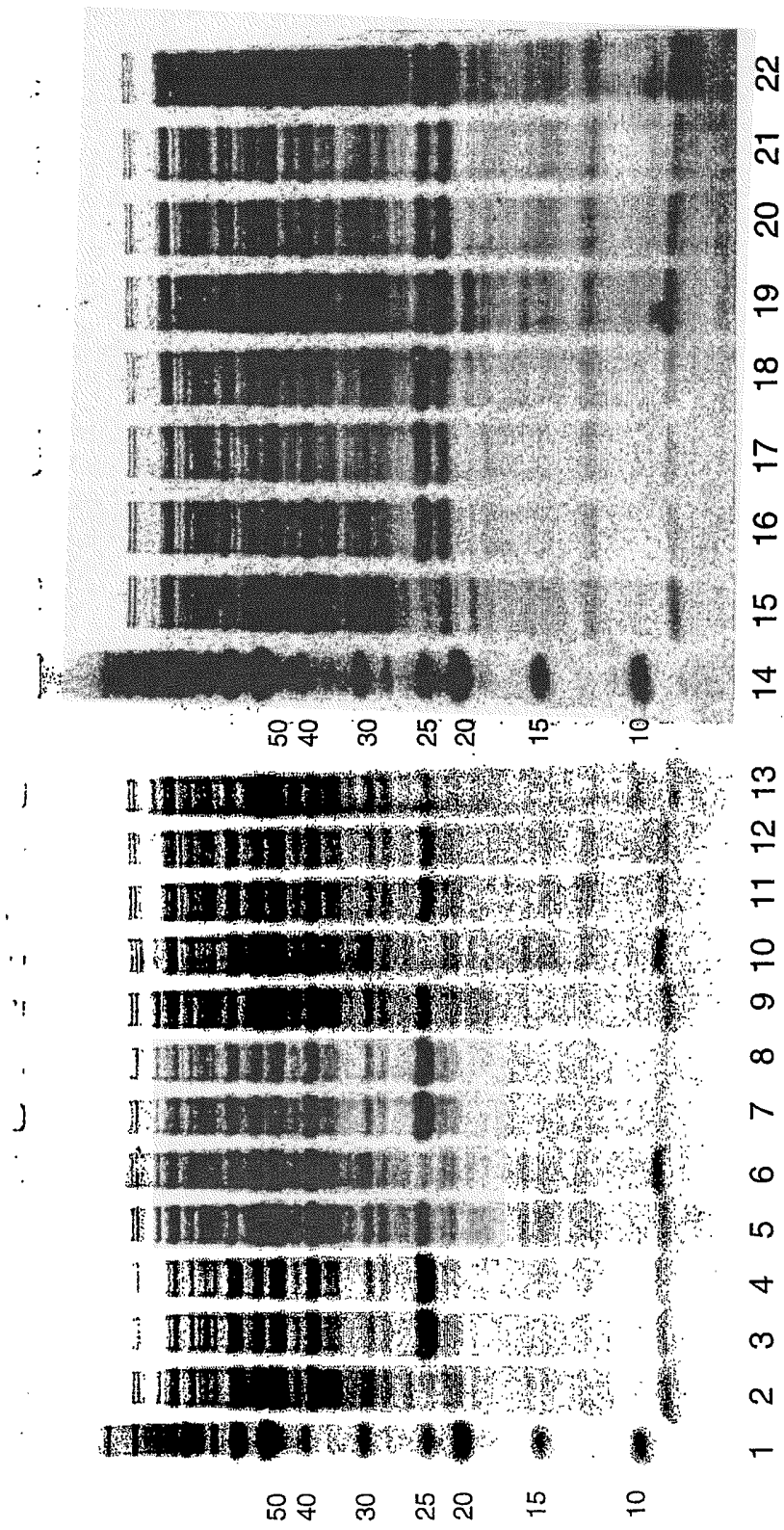
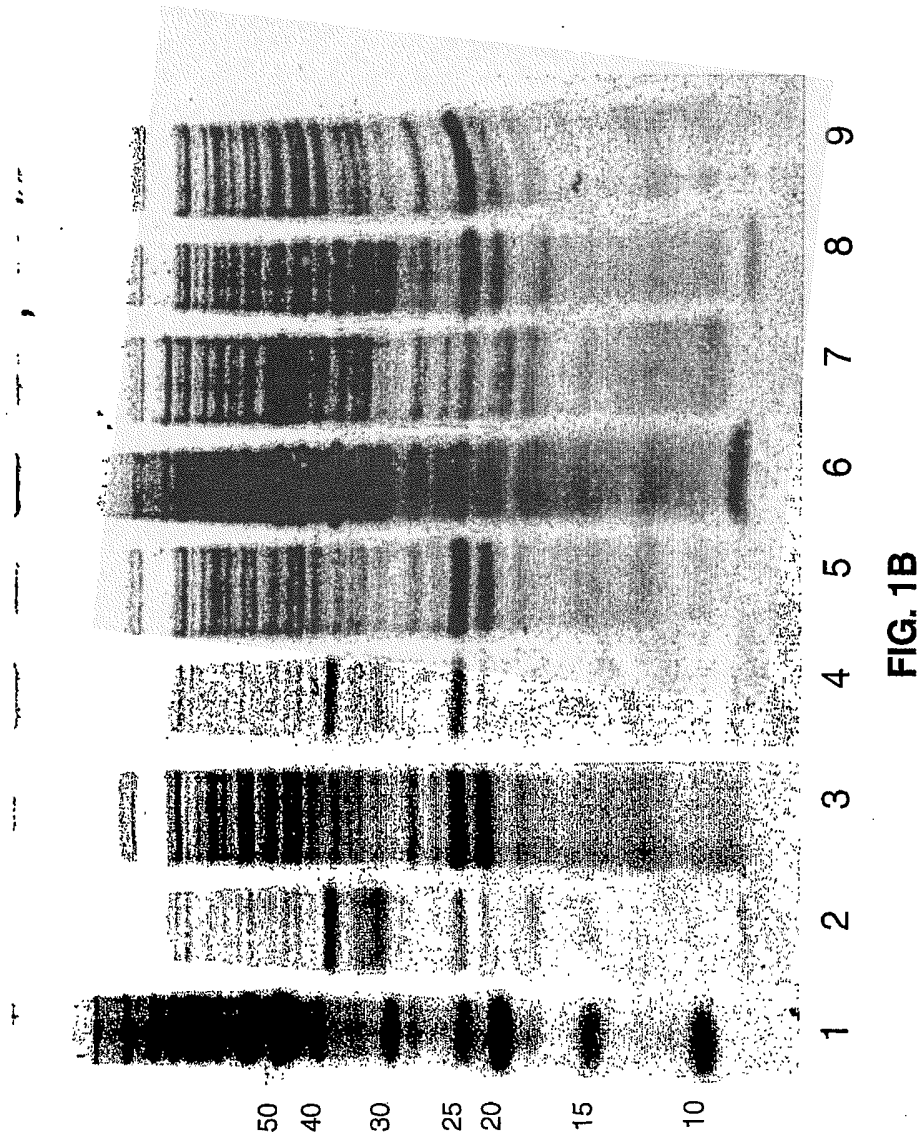


FIG. 1A



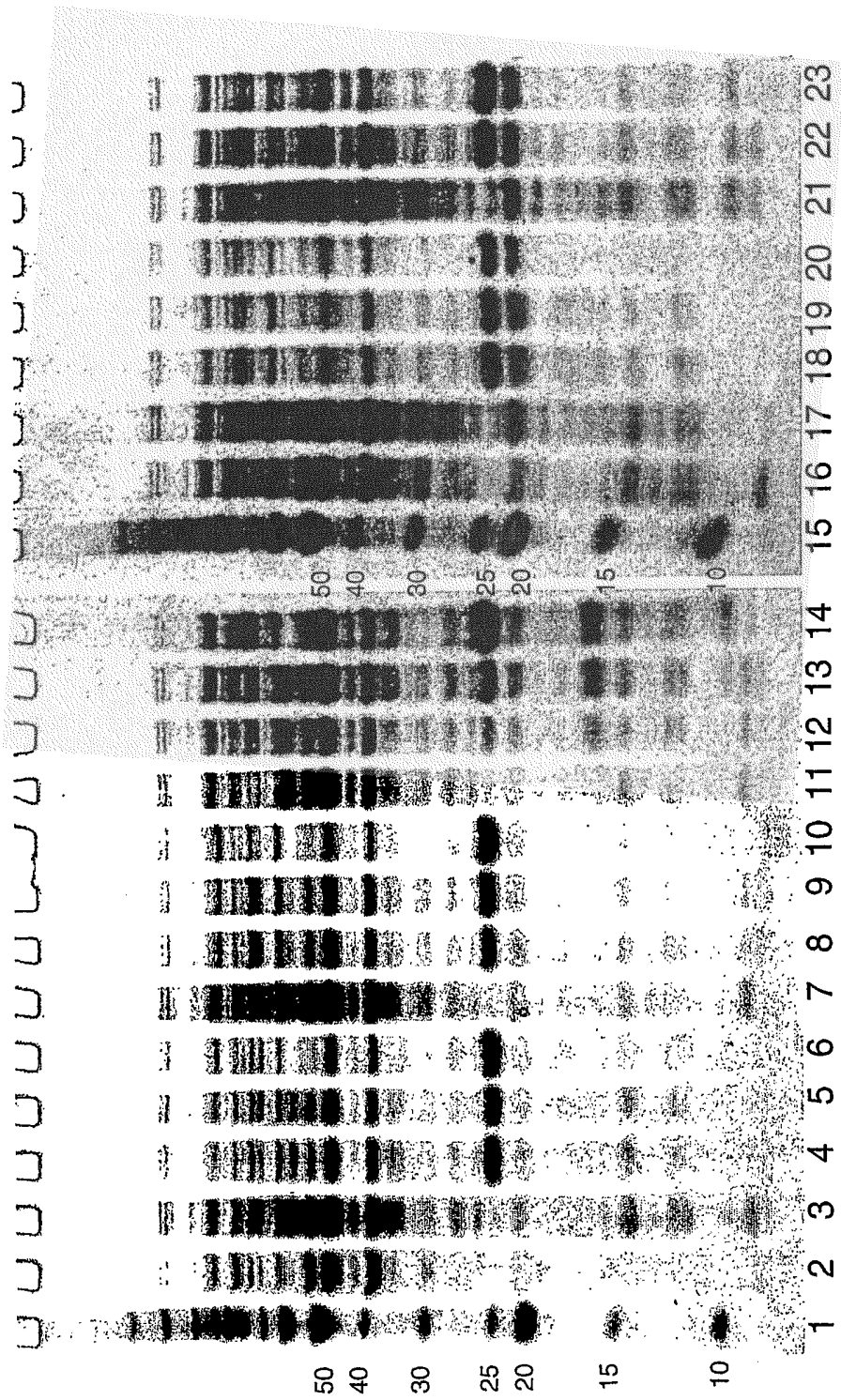


FIG. 1C

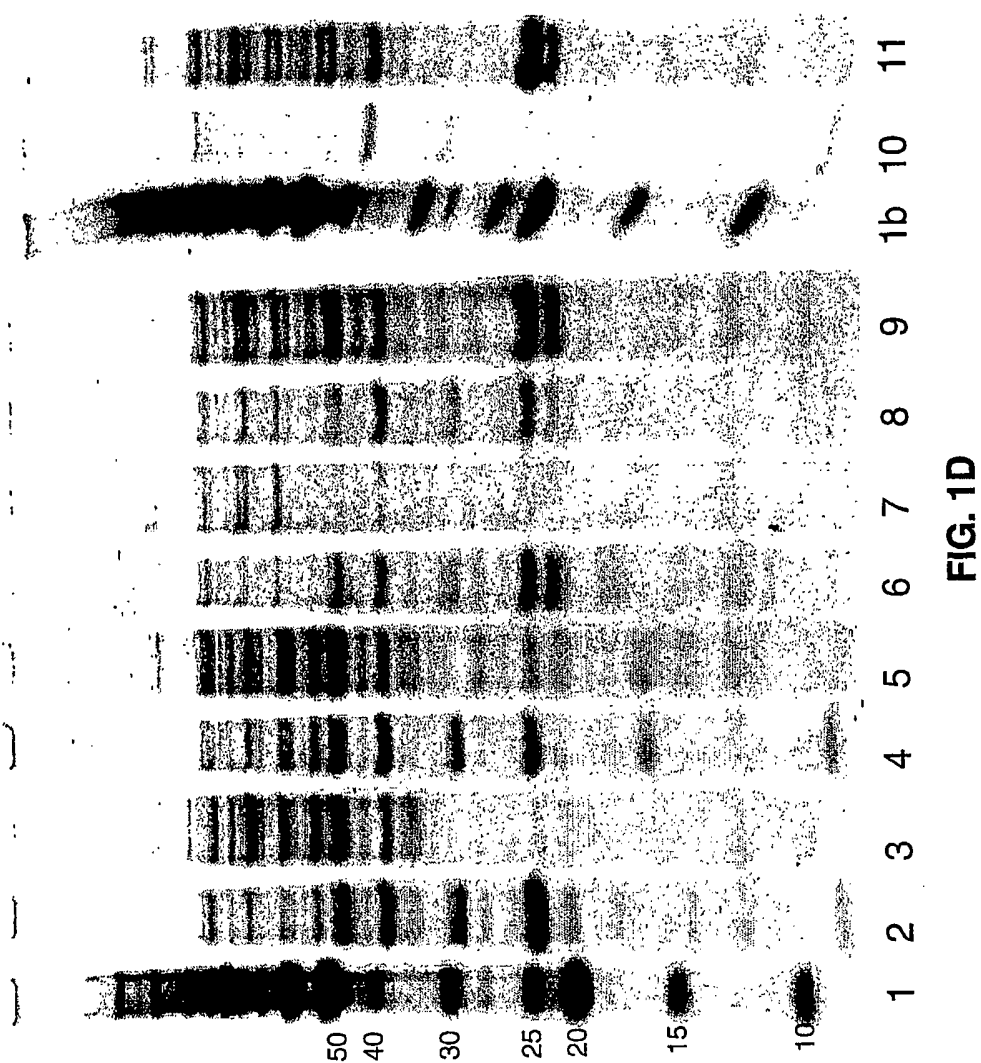


FIG. 1D

FIG. 2A

Protein	Organism	EC#	GenBank / GenPept		SwissProt	PDB / SD
At1g08280	<i>Arabidopsis thaliana</i>	n.d.	AC011438 BT004583 NC_003070	AAF18241.1 AAO42829.1 NP_172305.1	Q84W00 Q8SGD2	
At1g08680/F22O13.14	<i>Arabidopsis thaliana</i>	n.d.	AC003981 AY084135 AY124807 NC_003070 NM_160808	AAF89778.1 AAL38042.1 AAM70518.1 NP_172342.1 NP_650640.1	Q8VZJ0 Q8FRR9	
At3g48820/T21J18_90	<i>Arabidopsis thaliana</i>	n.d.	AY080589 AY138818 AL132983 NM_114741	AAL85988.1 AAM91750.1 CAB87910.1 NP_180451.1	Q8FY00 Q9M301	
α -2,3-sialyltransferase (ST3Gal-IV)	<i>Bos taurus</i>	n.d.	AJ584673	CAE48298.1		
α -2,3-sialyltransferase (ST3Gal-V)	<i>Bos taurus</i>	n.d.	AJ585768	CAE51392.1		
α -2,6-sialyltransferase (Siat7b)	<i>Bos taurus</i>	n.d.	AJ620851	CAF05880.1		
α -2,8-sialyltransferase (SIAT8A)	<i>Bos taurus</i>	2.4.99.8	AJ699418	CAG27880.1		
α -2,8-sialyltransferase (Siat8D)	<i>Bos taurus</i>	n.d.	AJ699421	CAG27883.1		
α -2,8-sialyltransferase ST8Sia-III (Siat8C)	<i>Bos taurus</i>	n.d.	AJ704563	CAG28698.1		
CMP α -2,6-sialyltransferase (ST6Gal I)	<i>Bos taurus</i>	2.4.99.1	Y15111 NM_177517	CAA75385.1 NP_803483.1	O18974	
sialyltransferase 8 (fragment)	<i>Bos taurus</i>	n.d.	AF450088	AAL47018.1	Q8WN13	
sialyltransferase ST3Gal-II (Siat4B)	<i>Bos taurus</i>	n.d.	AJ748841	CAG44450.1		
sialyltransferase ST3Gal-III (Siat5)	<i>Bos taurus</i>	n.d.	AJ748842	CAG44451.1		
sialyltransferase ST3Gal-VI (Siat10)	<i>Bos taurus</i>	n.d.	AJ748843	CAG44452.1		
ST3Gal I	<i>Bos taurus</i>	n.d.	AJ305088	CAC24698.1	Q9BEG4	
ST6GalNAc-VI	<i>Bos taurus</i>	n.d.	AJ620949	CAF05586.1		
CDS4	<i>Branchiostoma floridae</i>	n.d.	AF391289	AAM18678.1	Q8T771	
polysialyltransferase (PST) (fragment) ST8Sia IV	<i>Cercopithecus aethiops</i>	2.4.99.-	AF210729	AAF17105.1	Q9TT09	
polysialyltransferase (STX) (fragment) ST8Sia II	<i>Cercopithecus aethiops</i>	2.4.99.-	AF210318	AAF17104.1	Q9TT10	
α -2,3-sialyltransferase ST3Gal I (Siat4)	<i>Clona intestinalis</i>	n.d.	AJ626815	CAF25173.1		
α -2,3-sialyltransferase ST3Gal I (Siat4)	<i>Clona savignyi</i>	n.d.	AJ626814	CAF25172.1		
α -2,8-polysialyltransferase ST8Sia IV	<i>Cricetulus griseus</i>	2.4.99.-	Z48801	AAE28834 CAA88822.1	Q64890	
Gal β -1,3/4-GlcNAc α -2,3-sialyltransferase St3Gal I	<i>Cricetulus griseus</i>	n.d.	AY268676	AAP22942.1	Q80WLO	
Gal β -1,3/4-GlcNAc α -2,3-sialyltransferase St3Gal II (fragment)	<i>Cricetulus griseus</i>	n.d.	AY268678	AAP22943.1	Q80WK9	
α -2,3-sialyltransferase ST3Gal I (Siat4)	<i>Danio rerio</i>	n.d.	AJ783740	CAH04017.1		
α -2,3-sialyltransferase ST3Gal II (Siat5)	<i>Danio rerio</i>	n.d.	AJ783741	CAH04018.1		

FIG. 2B

Protein	Organism	EC#	GenBank / GenPept	SwissProt	PDB / 3D
α -2,3-sialyltransferase ST3Gal II (Siat8)	<i>Danio rerio</i>	n.d.	AJ626821 CAF25178.1		
α -2,3-sialyltransferase ST3Gal IV (Siat4c)	<i>Danio rerio</i>	n.d.	AJ744809 CAG32845.1		
α -2,3-sialyltransferase ST3Gal V-r (Siat5-related)	<i>Danio rerio</i>	n.d.	AJ783742 CAH04018.1		
α -2,6-sialyltransferase ST6Gal I (Siat1)	<i>Danio rerio</i>	n.d.	AJ744801 CAG32857.1		
α -2,6-sialyltransferase ST6GalNAc II (Siat7B)	<i>Danio rerio</i>	n.d.	AJ834459 CAG25680.1		
α -2,6-sialyltransferase ST6GalNAc V (Siat7E) (fragment)	<i>Danio rerio</i>	n.d.	AJ846874 CAG26703.1		
α -2,6-sialyltransferase ST6GalNAc VI (Siat7F) (fragment)	<i>Danio rerio</i>	n.d.	AJ846883 CAG26712.1		
α -2,6-sialyltransferase ST8Sia I (Siat 8A) (fragment)	<i>Danio rerio</i>	n.d.	AJ715535 CAG26374.1		
α -2,6-sialyltransferase ST8Sia III (Siat 8C) (fragment)	<i>Danio rerio</i>	n.d.	AJ715543 CAG26382.1		
α -2,6-sialyltransferase ST8Sia IV (Siat 8D) (fragment)	<i>Danio rerio</i>	n.d.	AJ715545 CAG26384.1		
α -2,6-sialyltransferase ST8Sia V (Siat 8E) (fragment)	<i>Danio rerio</i>	n.d.	AJ715546 CAG26385.1		
α -2,6-sialyltransferase ST8Sia VI (Siat 8F) (fragment)	<i>Danio rerio</i>	n.d.	AJ715551 CAG26390.1		
β -galactosamide α -2,6-sialyltransferase II (ST6Gal II)	<i>Danio rerio</i>	n.d.	AJ827827 CAF26486.1		
N-glycan α -2,6-sialyltransferase	<i>Danio rerio</i>	n.d.	BC050483 AAH50483.1 AY055462 AAL17875.1 NM_153882 NP_705948.1	Q7ZU51 Q8QH83	
ST3Gal III-related (sial8r)	<i>Danio rerio</i>	n.d.	BC053179 AAH53179.1 AJ828820 CAF25178.1 NM_200355 NP_956849.1	Q7T3B9	
ST3Gal-V	<i>Danio rerio</i>	n.d.	AJ818880 CAF04081.1		
st6GalNAc-VI	<i>Danio rerio</i>	n.d.	BC060932 AAH60932.1 AJ820847 CAF08584.1		
α -2,6-sialyltransferase (CG4871) ST6Gal I	<i>Drosophila melanogaster</i>	2.4.99.1	AE003485 AAF47268.1 AF218237 AAG13185.1 AF397532 AAK92126.1 AE003485 AAM70791.1 NM_079129 NP_523853.1 NM_188884 NP_728474.1	Q8GU23 Q9W121	
α -2,3-sialyltransferase (ST3Gal-VI)	<i>Gallus gallus</i>	n.d.	AJ585787 CAE51391.1 AJ827204 CAF25503.1		
α -2,3-sialyltransferase ST3Gal I	<i>Gallus gallus</i>	2.4.99.4	X80503 CAA56688.1 NM_205217 NP_990548.1	Q11200	
α -2,3-sialyltransferase ST3Gal IV (fragment)	<i>Gallus gallus</i>	2.4.99.-	AF035250 AAC14163.1	Q73724	
α -2,3-sialyltransferase (ST3GAL-II)	<i>Gallus gallus</i>	n.d.	AJ585781 CAE51385.2		
α -2,6-sialyltransferase (Siat7b)	<i>Gallus gallus</i>	n.d.	AJ620853 CAF05652.1		
α -2,6-sialyltransferase ST6Gal I	<i>Gallus gallus</i>	2.4.99.1	X75558 CAA53235.1 NM_205241 NP_990572.1	Q92182	
α -2,6-sialyltransferase	<i>Gallus gallus</i>	2.4.99.3	- AAE68028.1	Q92183	

FIG. 2C

Protein	Organism	EC#	GenBank / GenPept	SwissProt / PDB / 3D	
ST6GalNAc I			X74948 NM_205240	AAE88028.1 CAA52902.1 NP_090871.1	
α -2,8-sialyltransferase ST6GalNAc II	<i>Gallus gallus</i>	2.4.99.-	X77778 NM_205233	AAE88030.1 CAA54813.1 NP_090584.1	
α -2,8-sialyltransferase ST6GalNAc III (SIAT7C) (fragment)	<i>Gallus gallus</i>	n.d.	AJ634455	CAG28677.1	
α -2,8-sialyltransferase ST6GalNAc V (SIAT7E) (fragment)	<i>Gallus gallus</i>	n.d.	AJ646877	CAG28708.1	
α -2,8-sialyltransferase (GDS Synthase) ST8Sia	<i>Gallus gallus</i>	2.4.99.-	U73176	AAC28888.1	
α -2,8-sialyltransferase (SIAT8B)	<i>Gallus gallus</i>	n.d.	AJ699419	CAG27881.1	
α -2,8-sialyltransferase (SIAT8C)	<i>Gallus gallus</i>	n.d.	AJ699420	CAG27882.1	
α -2,8-sialyltransferase (SIAT8F)	<i>Gallus gallus</i>	n.d.	AJ699424	CAG27888.1	
α -2,8-sialyltransferase ST8Sia-V (SIAT8C)	<i>Gallus gallus</i>	n.d.	AJ704584	CAG28697.1	
β -galactosamide α -2,8- sialyltransferase II (ST6Gal II)	<i>Gallus gallus</i>	n.d.	AJ627829	CAF29497.1	
GM3 synthase (SIAT9)	<i>Gallus gallus</i>	2.4.99.9	AY515255	AA883519.1	
polysialyltransferase ST8Sia IV	<i>Gallus gallus</i>	2.4.99.-	AF008194	AAB95120.1	
α -2,3-sialyltransferase ST3Gal I	<i>Homo sapiens</i>	2.4.99.4	L29555 AF059321 L13972 AF165238 AF186181 BC018357 NM_003033 NM_173344	AAA36612.1 AAC17874.1 AAC37574.1 AAD39238.1 AAG29878.1 AAH18357.1 NP_003024.1 NP_775479.1	
α -2,3-sialyltransferase ST3Gal II	<i>Homo sapiens</i>	2.4.99.4	U63090 BC036777 X95687 NM_006927	AAB40389.1 AAH36777.1 CAA85447.1 NP_008858.1	
α -2,3-sialyltransferase ST3Gal III (Siat6)	<i>Homo sapiens</i>	2.4.99.6	L23768 BC050380 AF425851 AF425852 AF425853 AF425854 AF425855 AF425856 AF425857 AF425858 AF425859 AF425860 AF425861 AF425862 AF425863 AF425864 AF425865 AF425866 AY187992 AY187993 AY187994	AAA35778.1 AAH50380.1 AAO13869.1 AAO13860.1 AAO13861.1 AAO13862.1 AAO13863.1 AAO13864.1 AAO13865.1 AAO13866.1 AAO13867.1 AAO13868.1 AAO13869.1 AAO13870.1 AAO13871.1 AAO13872.1 AAO13873.1 AAO13874.1 AAO13875.1 AAO38806.1 AAO38807.1 AAO38808.1	Q11203 Q86UR6 Q86UR7 Q86UR8 Q86UR9 Q86US0 Q86US1 Q86US2 Q8IX43 Q8IX44 Q8IX45 Q8IX46 Q8IX47 Q8IX48 Q8IX49 Q8IX50 Q8IX51 Q8IX52 Q8IX53 Q8IX54 Q8IX55 Q8IX56

FIG. 2D

Protein	Organism	EC#	GenBank / GenPept	SwissProt / PDB / 3D
			AY187995 AAO38809.1 AY187996 AAO38810.1 AY187997 AAO38811.1 AY187998 AAO38812.1 NM_008270 NP_008270.1 NM_174984 NP_777824.1 NM_174985 NP_777825.1 NM_174986 NP_777826.1 NM_174987 NP_777827.1 NM_174988 NP_777828.1 NM_174970 NP_777830.1 NM_174972 NP_777832.1	Q8IX57 Q8IX58
α -2,3-sialyltransferase ST3Gal IV	<i>Homo sapiens</i>	2.4.99.-	L23767 AAA16480.1 AF035249 AAC14182.1 BC010645 AAH10645.1 AY040828 AAK93790.1 AF516602 AAM86431.1 AF516603 AAM86432.1 AF516604 AAM86433.1 AF525084 AAM81378.1 X74570 CAA52662.1 CF456858 CAG33139.1 NM_006278 NP_006289.1	Q11208 Q60497 Q86Q09 Q8N8A8 Q8N8A7 Q8NFD3 Q8NFG7
α -2,3-sialyltransferase ST3Gal VI	<i>Homo sapiens</i>	2.4.99.4	AF119391 AAD39131.1 BC023312 AAH23312.1 AB022918 BAA77809.1 AX877828 CAE88895.1 AX888023 CAF00181.1 NM_008100 NP_008091.1	Q8Y274
α -2,6-sialyltransferase (ST6Gal II ; KIAA1877)	<i>Homo sapiens</i>	n.d.	BC008680 AAH08680.1 AB058780 BAB47508.1 AB059555 BAC24793.1 AJ512141 CAD54408.1 AX795193 CAE48260.1 AX795193 CAE48261.1 NM_032528 NP_115917.1	Q86Y44 Q8IUG7 Q86HE4 Q86JF0
α -2,6-sialyltransferase (ST6GALNAC III)	<i>Homo sapiens</i>	n.d.	BC059383 AAH59383.1 AY358540 AAQ88904.1 AK091215 BAC03611.1 AJ507291 CAD45371.1 NM_152898 NP_694541.1	Q8N259 Q8NDV1
α -2,6-sialyltransferase (ST6GalNAc V)	<i>Homo sapiens</i>	n.d.	BC001201 AAH01201.1 AK056241 BAB71127.1 AL035408 CAB72344.1 AJ507292 CAD45372.1 NM_030865 NP_112227.1	Q8BVH7
α -2,6-sialyltransferase (SThM) ST6GalNAc II	<i>Homo sapiens</i>	2.4.99.-	U14550 AAA52228.1 BC040455 AAH40455.1 AJ251053 CAB81434.1 NM_008458 NP_008447.1	Q8UJ37 Q12971
α -2,6-sialyltransferase ST6Gal I	<i>Homo sapiens</i>	2.4.99.1	BC031476 AAH31476.1 BC040009 AAH40009.1 A17362 CAA01327.1 A23899 CAA01888.1 X17247 CAA35111.1 X54383 CAA38248.1 X62822 CAA44634.1 NM_003032 NP_003023.1 NM_173218 NP_775323.1	P15907
α -2,6-sialyltransferase ST6GalNAc I	<i>Homo sapiens</i>	2.4.99.3	BC022462 AAH22462.1 AY096001 AAM22800.1 AY358918 AAQ89277.1 AK000113 BAA90953.1 Y11339 CAA72179.2	Q8TBJ8 Q8NSC7 Q8NXQ7

FIG. 2E

Protein	Organism	EC#	GenBank / GenPept	SwissProt	PDB / SD
			NM_018414 NP_080884.1		
α -2,8-polysialyltransferase ST8Sia IV	<i>Homo sapiens</i>	2.4.99.-	L41680 AAC41776.1 BC027888 AAH27888.1 BC053687 AAH53687.1 NM_005668 NP_005669.1	Q8N1F4 Q92187 Q92693	
α -2,8-sialyltransferase (GD3 synthase) ST8Sia I	<i>Homo sapiens</i>	2.4.99.9	L32887 AAA62388.1 L43494 AAC37989.1 BC046168 AAH46168.1 AAQ53140.1 AY568975 AA876783.1 D28360 BAA05391.1 X77922 CAA54891.1 NM_003034 NP_003025.1	Q8BX71 Q92185 Q92084	
α -2,8-sialyltransferase ST8Sia II	<i>Homo sapiens</i>	2.4.99.-	L29556 AAA36813.1 U82782 AAB51242.1 U33551 AAC24458.1 BC089584 AAH89584.1 NM_006011 NP_006002.1	Q92188 Q92470 Q92748	
α -2,8-sialyltransferase ST8Sia III	<i>Homo sapiens</i>	2.4.99.-	AF004888 AAB87842.1 AF003082 AAC15901.2 NM_015878 NP_058993.1	Q43173 Q9NS41	
α -2,8-sialyltransferase ST8Sia V	<i>Homo sapiens</i>	2.4.99.-	U91841 AAC61727.1 CF457037 CAG33318.1 NM_013305 NP_037437.1	O15468	
ENSP00000020221 (fragment)		n.d.	AC023285		
lactosylceramide α -2,3-sialyltransferase (ST3Gal V)	<i>Homo sapiens</i>	2.4.99.9	AF105028 AAD14834.1 AF119415 AAF68148.1 BC065836 AAH85836.1 AY152815 AAO16888.1 AAP85068 AAP85068.1 AY359105 AAQ89463.1 AB018356 BAA33950.1 AX876536 CAE89320.1 NM_003886 NP_003887.2	Q9UNP4 Q94902	
N-acetylgalactosaminide α -2,6-sialyltransferase (ST6GalNAc VI)	<i>Homo sapiens</i>	2.4.99.-	BC008684 AAH08584.1 BC007802 AAH07802.1 BC018299 AAH16299.1 AY358672 AAQ89035.1 AB035173 BAA87035.1 AK023900 BAB14715.1 AJ607293 CAD46373.1 AX880950 CAE91145.1 CF457318 CAG33599.1 NM_013443 NP_038471.2	Q969X2 Q9H8A2 Q9ULB8	
N-acetylgalactosaminide α -2,6-sialyltransferase IV (ST6GalNAc IV)	<i>Homo sapiens</i>	2.4.99.-	AF127142 AAF00102.1 BC038705 AAH38705.1 AAP83349.1 AB035172 BAA87034.1 AK000800 BAA91281.1 Y17481 CAB44354.1 AJ271734 CAC07404.1 AX081820 CAC24981.1 AX088265 CAC27250.1 AX889252 CAF14380.1 NM_014403 NP_055218.3 NM_175039 NP_778204.1	Q9H4F1 Q9NWU8 Q9UKU1 Q9ULB9 Q9Y3G3 Q9Y3G4	
ST8Sia-VI (fragment)	<i>Homo sapiens</i>	n.d.	AJ821589 CAF21722.1 XM_291725 XP_291725.2		
unnamed protein product	<i>Homo sapiens</i>	n.d.	AK021929 BAB13940.1 AX881888 CAE91353.1	Q9HAA9	
Gal β 1,3/4-GlcNAc α -	<i>Mesocricetus</i>	2.4.99.6	AJ245689 CAB53394.1	Q9QXF6	

FIG. 2F

Protein	Organism	EC#	GenBank / GenPept	SwissProt	PDB / SD
2,3-sialyltransferase (ST3Gal III)	<i>auratus</i>				
Gal β 1,3/4-GlcNAc α -2,3-sialyltransferase (ST3Gal IV)	<i>Mesocricetus auratus</i>	2.4.99.8	AJ245700	CAB53995.1	Q9QXF6
GD3 synthase (fragment) ST8Sia I	<i>Mesocricetus auratus</i>	n.d.	AF141657	AAD33879.1	Q9WUL1
polysialyltransferase (ST8Sia IV)	<i>Mesocricetus auratus</i>	2.4.99.-	AJ245701	CAB53996.1	Q9QXF4
α -2,3-sialyltransferase ST3Gal I	<i>Mus musculus</i>	2.4.99.4	AF214028 AK031944 AK078469 X73523 NM_009177	AAF80073.1 BAC27368.1 BAC37280.1 CAA51919.1 NP_033203.1	P64751 Q11202 Q8JL30
α -2,3-sialyltransferase ST3Gal II	<i>Mus musculus</i>	2.4.99.4	BC016264 BC068084 AK034564 AK034883 AK053827 X76989 NM_009179 NM_178048	AAH15264.1 AAH68084.1 BAC28752.1 BAC28859.1 BAC35543.1 CAA54294.1 NP_033205.1 NP_836149.1	Q11204 Q8BPL0 Q8BSA0 Q8BSE9 Q91WH6
α -2,3-sialyltransferase ST3Gal III	<i>Mus musculus</i>	2.4.99.-	BC006710 AK005053 AK013018 X84234 NM_009176	AAH08710.1 BAB23779.1 BAB28598.1 CAA59013.1 NP_033202.2	P97325 Q922X6 Q9CZ48 Q9DBB6
α -2,3-sialyltransferase ST3Gal IV	<i>Mus musculus</i>	2.4.99.4	BC011121 BC050773 D28941 AK008543 AB081305 X95809 NM_009178	AAH11121.1 AAH50773.1 BAA06088.1 BAB25732.1 BAB47508.1 CAA85078.1 NP_033204.2	P97354 Q81325 Q91Y74 Q921R6 Q9CVE8
α -2,3-sialyltransferase ST3Gal VI	<i>Mus musculus</i>	2.4.99.4	AF118390 BC052338 AB063328 AK033562 AK041173 NM_018784	AAD39130.1 AAH52338.1 BAB79494.1 BAC28360.1 BAC30851.1 NP_081254	Q80UR7 Q8BLV1 Q8VIB3 Q9WVG2
α -2,6-sialyltransferase ST6GalNAc II	<i>Mus musculus</i>	2.4.99.-	NM_009180 BC010208 AB027198 AK004813 X93989 X94000 NM_009180	6677963 AAH10208.1 BAB00637.1 BAB23410.1 CAA63821.1 CAA63822.1 NP_033206.2	P70277 Q8DC24 Q8JIM5
α -2,6-sialyltransferase ST6Gal I	<i>Mus musculus</i>	2.4.99.1	BC027833 D16106 AK034768 AK084124 NM_145933	AAE88031.1 AAH27833.1 BAA03680.1 BAC28828.1 BAC39120.1 NP_668046.1	Q64685 Q8BM62 Q8K1L1
α -2,6-sialyltransferase ST6Gal II	<i>Mus musculus</i>	n.d.	AK082568 AB095083 AK129482 NM_172829	BAC38534.1 BAC57752.1 BAC98272.1 NP_768417.1	Q8BUU4
α -2,6-sialyltransferase ST6GalNAc I	<i>Mus musculus</i>	2.4.99.3	Y11274 NM_011371	CAA72137.1 NP_035501.1	Q8QZ39 Q8JJP5
α -2,6-sialyltransferase ST6GalNAc III	<i>Mus musculus</i>	n.d.	BC058387 AK034804 Y11342 Y11343	AAH58387.1 BAC28838.1 CAA72181.2 CAB95031.1	Q8WUV2 Q8JHP5

FIG. 2G

Protein	Organism	EC#	GenBank / GenPept	SwissProt	PDB / 3D
α -2,8-sialyltransferase ST6GalNAc IV	St6galnac4 <i>Mus musculus</i>	2.4.99.7	NM_011372 BC058451 AK085730 AJ007310 Y15779 Y15780 Y19055 Y19057 NM_011373	NP_035502 AAH58451.1 BAC39523.1 CAA07446.1 CAB43507.1 GAB43514.1 CAB83246.1 CAB83248.1 NP_035503.1	Q8C3J2 Q8JHP2 Q8R2B6 Q88725 Q8JHP0 Q8QUP9 Q8R2B5
α -2,8-sialyltransferase (GD3 synthase) ST6Sia I	St6sia1 <i>Mus musculus</i>	2.4.99.8	L38677 BC024821 AK048188 AK052444 X84235 AJ401102 NM_011374	AAH1889.1 AAH24821.1 BAC32825.1 BAC34994.1 CAA58014.1 CAC20706.1 NP_035504.1	Q84488 Q84687 Q8BL76 Q8BW10 Q8K1C1 Q9EPK0
α -2,8-sialyltransferase (ST6Sia VI)	St6sia6 <i>Mus musculus</i>	n.d.	AB059554 AK085105 NM_145838	BAC01285.1 BAC39387.1 NP_685837.1	Q8BI43 Q8K4T1
α -2,8-sialyltransferase ST6Sia II	St6sia2 <i>Mus musculus</i>	2.4.99.-	X83582 X89848 X89847 X89848 X89849 X89850 X89851 NM_009181	CAA58548.1 CAA67965.1 CAA67965.1 CAA67965.1 CAA67965.1 CAA67965.1 CAA67965.1 NP_033207.1	Q35698
α -2,8-sialyltransferase ST6Sia IV	St6sia4 <i>Mus musculus</i>	2.4.99.8	BC060112 AK003890 AK041723 AJ223958 X85000 Y09484 NM_008183	AAH60112.1 BAB22941.1 BAC31044.1 CAA11685.1 CAA59992.1 CAA70892.1 NP_033208.1	Q64692 Q8BY70
α -2,8-sialyltransferase ST6Sia V	St6sia5 <i>Mus musculus</i>	2.4.99.-	BC034855 AK078670 X98014 X98014 X98014 NM_013866 NM_153124 NM_177416	AAH34855.1 BAC37354.1 CAA86642.1 CAA86643.1 CAA86644.1 NP_038694.1 NP_694764.1 NP_803135.1	P70128 P70127 P70128 Q8BJW0 Q8JZQ3
α -2,8-sialyltransferase ST6Sia III	St6sia3 <i>Mus musculus</i>	2.4.99.-	BC076645 AK015874 X80502 NM_009182	AAH75645.1 BAB30012.1 CAA58885.1 NP_033208.1	Q84589 Q8CUJ8
GD1 synthase (ST6GalNAc V)	St6galnac5 <i>Mus musculus</i>	n.d.	BC055737 AB030838 AB028840 AK034387 AK038434 AK042883 NM_012028	AAH55737.1 BAA85747.1 BAA89292.1 BAC28693.1 BAC29997.1 BAC31331.1 NP_038158.2	Q8CAM7 Q8CBX1 Q9QYJ1 Q9R0K6
GM3 synthase (α -2,3- sialyltransferase) ST3Gal V	St3gal5 <i>Mus musculus</i>	2.4.99.9	AF119418 AB018048 AB013302 AK012961 Y15003 NM_011375	AAF66147.1 AAP65063.1 BAA33491.1 BAA76467.1 BAB26571.1 CAA75235.1 NP_035505.1	Q88829 Q8CZ85 Q9QWF9
N-acetylgalactosaminide α -2,6-sialyltransferase (ST6GalNAc VI)	St6galnac6 <i>Mus musculus</i>	2.4.99.-	BC038985 AB035174 AB035123 AK030648	AAH36985.1 BAA87036.1 BAA95940.1 BAC27064.1	Q8CDC3 Q8JZW3 Q8JM95 Q9R0Q9

FIG. 2H

Protein	Organism	EC#	GenBank / GenPept	SwissProt	PDB / 3D
			NM_016973/ NP_058888.1		
M138L	<i>Myxoma virus</i>	n.d.	U48578 AF170726 NC_001132 AAE00089.1 AAE61323.1 AAE61326.1 AAF18026.1 NP_051852.1		
α -2,3-sialyltransferase (St3Gal-I)	<i>Oncorhynchus mykiss</i>	n.d.	AJ585780	CAE51384.1	
α -2,6-sialyltransferase (Siat1)	<i>Oncorhynchus mykiss</i>	n.d.	AJ620849	CAF05848.1	
α -2,8-polysialyltransferase IV (ST8Sia IV)	<i>Oncorhynchus mykiss</i>	n.d.	AB094402	BAC77411.1	Q7T2X5
GalNAc α -2,6-sialyltransferase (FtST6GalNAc)	<i>Oncorhynchus mykiss</i>	n.d.	AB087843	BAC77620.1	Q7T2X4
α -2,3-sialyltransferase ST3Gal IV QJ1217_F02.7	<i>Oryctolagus cuniculus</i>	2.4.99.-	AF121967	AAF28871.1	Q9N257
	<i>Oryza sativa</i> (japonica cultivar-group)	n.d.	AP004084	BAD07616.1	
OSJNBa0043L24.2 or OSJNBb0002J11.9	<i>Oryza sativa</i> (japonica cultivar-group)	n.d.	AL731826 AL862869	CAD41185.1 CAE04714.1	
P0683f02.18 or P0489B03.1	<i>Oryza sativa</i> (japonica cultivar-group)	n.d.	AP003288 AP003794	BAB63715.1 BAB90562.1	
α -2,6-sialyltransferase ST6GalNAc V (Siat7E) (fragment)	<i>Oryzias latipes</i>	n.d.	AJ646876	CAG26705.1	
α -2,3-sialyltransferase ST3Gal I (Siat4)	<i>Pan troglodytes</i>	n.d.	AJ744803	CAG32839.1	
α -2,3-sialyltransferase ST3Gal II (Siat5)	<i>Pan troglodytes</i>	n.d.	AJ744804	CAG32840.1	
α -2,3-sialyltransferase ST3Gal III (Siat6)	<i>Pan troglodytes</i>	n.d.	AJ626819	CAF26177.1	
α -2,3-sialyltransferase ST3Gal IV (Siat4c)	<i>Pan troglodytes</i>	n.d.	AJ626824	CAF26182.1	
α -2,3-sialyltransferase ST3Gal VI (Siat10)	<i>Pan troglodytes</i>	n.d.	AJ744808	CAG32844.1	
α -2,6-sialyltransferase (Siat7A)	<i>Pan troglodytes</i>	n.d.	AJ748740	CAG38515.1	
α -2,6-sialyltransferase (Siat7B)	<i>Pan troglodytes</i>	n.d.	AJ748741	CAG38616.1	
α -2,6-sialyltransferase ST6GalNAc III (Siat7C)	<i>Pan troglodytes</i>	n.d.	AJ634454	CAG25676.1	
α -2,6-sialyltransferase ST6GalNAc IV (Siat7D) (fragment)	<i>Pan troglodytes</i>	n.d.	AJ646870	CAG26699.1	
α -2,6-sialyltransferase ST6GalNAc V (Siat7E)	<i>Pan troglodytes</i>	n.d.	AJ646875	CAG26704.1	
α -2,6-sialyltransferase ST6GalNAc VI (Siat7F) (fragment)	<i>Pan troglodytes</i>	n.d.	AJ646882	CAG26711.1	
α -2,8-sialyltransferase 8A (Siat8A)	<i>Pan troglodytes</i>	2.4.99.8	AJ697658	CAG26896.1	
α -2,8-sialyltransferase 8B (Siat8B)	<i>Pan troglodytes</i>	n.d.	AJ697659	CAG26897.1	
α -2,8-sialyltransferase 8C (Siat8C)	<i>Pan troglodytes</i>	n.d.	AJ697660	CAG26898.1	
α -2,8-sialyltransferase 8D (Siat8D)	<i>Pan troglodytes</i>	n.d.	AJ697661	CAG26899.1	
α -2,8-sialyltransferase	<i>Pan troglodytes</i>	n.d.	AJ697662	CAG26900.1	

FIG. 2I

Protein	Organism	EC	GenBank / GenPept	SwissProt	PDB / 3D
SE (Siat8E)					
α -2,6-sialyltransferase 8F (Siat8F)	<i>Pan troglodytes</i>	n.d.	AJ697683	CAG28901.1	
β -galactosamide α -2,6-sialyltransferase I (ST6Gal I; Siat1)	<i>Pan troglodytes</i>	2.4.99.1	AJ627624	CAF28492.1	
β -galactosamide α -2,6-sialyltransferase II (ST6Gal II)	<i>Pan troglodytes</i>	n.d.	AJ627625	CAF28493.1	
GM3 synthase ST3Gal V (Siat9)	<i>Pan troglodytes</i>	n.d.	AJ744807	CAG32843.1	
S138L	<i>Rabbit fibroma virus Kesz</i>	n.d.	NC_001268	NP_052025	
α -2,3-sialyltransferase ST3Gal III	<i>Rattus norvegicus</i>	2.4.99.8	M87754 NM_031687	AAA42148.1 NP_113885.1	Q02734
α -2,3-sialyltransferase ST3Gal IV (Siat4c)	<i>Rattus norvegicus</i>	n.d.	AJ626825	CAF25183.1	
α -2,3-sialyltransferase ST3Gal VI	<i>Rattus norvegicus</i>	n.d.	AJ626743	CAF25053.1	
α -2,6-sialyltransferase ST3Gal II	<i>Rattus norvegicus</i>	2.4.99.-	X76988 NM_031685	CAA54293.1 NP_113883.1	Q11205
α -2,6-sialyltransferase ST6Gal I	<i>Rattus norvegicus</i>	2.4.99.1	M18789 M83143	AAA41198.1 AAB07233.1	P13721
α -2,6-sialyltransferase ST6GalNAc I (Siat7A)	<i>Rattus norvegicus</i>	n.d.	AJ634458	CAG25584.1	
α -2,6-sialyltransferase ST6GalNAc II (Siat7B)	<i>Rattus norvegicus</i>	n.d.	AJ634457	CAG25579.1	
α -2,6-sialyltransferase ST6GalNAc III	<i>Rattus norvegicus</i>	2.4.99.-	L29554 BC072501 NM_019123	AAC42086.1 AAH72501.1 NP_061996.1	Q04888
α -2,6-sialyltransferase ST6GalNAc IV (Siat7D) (fragment)	<i>Rattus norvegicus</i>	n.d.	AJ646871	CAG26700.1	
α -2,6-sialyltransferase ST6GalNAc V (Siat7E)	<i>Rattus norvegicus</i>	n.d.	AJ646872	CAG26701.1	
α -2,6-sialyltransferase ST6GalNAc VI (Siat7F) (fragment)	<i>Rattus norvegicus</i>	n.d.	AJ646881	CAG26710.1	
α -2,6-sialyltransferase (GD3 synthase) ST6Sia I	<i>Rattus norvegicus</i>	2.4.99.-	U53883 D45255	AAC27541.1 BAA08213.1	P70554 P97713
α -2,6-sialyltransferase (SIAT8E)	<i>Rattus norvegicus</i>	n.d.	AJ699422	CAG27684.1	
α -2,6-sialyltransferase (SIAT8F)	<i>Rattus norvegicus</i>	n.d.	AJ699423	CAG27685.1	
α -2,6-sialyltransferase ST6Sia II	<i>Rattus norvegicus</i>	2.4.99.-	L13445 NM_057156	AAA42147.1 NP_476497.1	Q07977 Q64688
α -2,6-sialyltransferase ST6Sia III	<i>Rattus norvegicus</i>	2.4.99.-	U55938 NM_013029	AAB50061.1 NP_037181.1	P97877
α -2,6-sialyltransferase ST6Sia IV	<i>Rattus norvegicus</i>	2.4.99.-	U80215	AAB49989.1	O08563
β -galactosamide α -2,6-sialyltransferase II (ST6Gal II)	<i>Rattus norvegicus</i>	n.d.	AJ627626	CAF28494.1	
GM3 synthase ST3Gal V	<i>Rattus norvegicus</i>	n.d.	AB018049 NM_031337	BAA33492.1 NP_112627.1	O88830

FIG. 2J

Protein	Organism	EC#	GenBank / GenPept	SwissProt / PDB / 3D
α -sialyltransferase ST3Gal-I (Siat4A)	<i>Rattus norvegicus</i>	n.d.	AJ748840 CAG44449.1	
α -2,3-sialyltransferase (ST3Gal-II)	<i>Sturana tropicalis</i>	n.d.	AJ585763 CAE51367.1	
α -2,6-sialyltransferase (Siat7b)	<i>Sturana tropicalis</i>	n.d.	AJ620850 CAF05846.1	
α -2,6-sialyltransferase (ST6Galnac)	<i>Strongylocentrotus purpuratus</i>	n.d.	AJ698425 CAG27887.1	
α -2,3-sialyltransferase (ST3GAL-III)	<i>Sus scrofa</i>	n.d.	AJ585765 CAE51346.1	
α -2,3-sialyltransferase (ST3GAL-IV)	<i>Sus scrofa</i>	n.d.	AJ584674 CAE48299.1	
α -2,3-sialyltransferase ST3Gal I	<i>Sus scrofa</i>	2.4.99.4	M97753 AAA31125.1	Q02745
α -2,6-sialyltransferase (fragment) ST6Gal I	<i>Sus scrofa</i>	2.4.99.1	AF136748 AAD33089.1	Q8X6G8
β -galactosamide α -2,6-sialyltransferase (ST6GalNAc-V)	<i>Sus scrofa</i>	n.d.	AJ620848 CAF06585.2	
α -sialyltransferase (fragment) ST6Gal I	<i>Sus scrofa</i>	n.d.	AF041031 AAC16833.1	Q82717
ST6GALNAC-V	<i>Sus scrofa</i>	n.d.	AJ620848 CAF06585.1	
α -2,3-sialyltransferase (Siat5-r)	<i>Takifugu rubripes</i>	n.d.	AJ744805 CAG32841.1	
α -2,3-sialyltransferase ST3Gal I (Siat4)	<i>Takifugu rubripes</i>	n.d.	AJ626816 CAF25174.1	
α -2,3-sialyltransferase ST3Gal II (Siat5) (fragment)	<i>Takifugu rubripes</i>	n.d.	AJ626817 CAF25175.1	
α -2,3-sialyltransferase ST3Gal III (Siat6)	<i>Takifugu rubripes</i>	n.d.	AJ626818 CAF25176.1	
α -2,8-sialyltransferase ST6Gal I (Siat1)	<i>Takifugu rubripes</i>	n.d.	AJ744800 CAG32838.1	
α -2,8-sialyltransferase ST6GalNAc II (Siat7B)	<i>Takifugu rubripes</i>	n.d.	AJ634480 CAG25681.1	
α -2,8-sialyltransferase ST6GalNAc II B (Siat7B-related)	<i>Takifugu rubripes</i>	n.d.	AJ634481 CAG25682.1	
α -2,8-sialyltransferase ST6GalNAc III (Siat7C) (fragment)	<i>Takifugu rubripes</i>	n.d.	AJ634456 CAG25678.1	
α -2,8-sialyltransferase ST6GalNAc IV (Siat7D) (fragment)	<i>Takifugu rubripes</i>	2.4.99.3	Y17486 CAB44338.1 AJ648869 CAG26698.1	Q9W6U6
α -2,8-sialyltransferase ST6GalNAc V (Siat7E) (fragment)	<i>Takifugu rubripes</i>	n.d.	AJ648873 CAG26702.1	
α -2,8-sialyltransferase ST6GalNAc VI (Siat7F) (fragment)	<i>Takifugu rubripes</i>	n.d.	AJ648880 CAG26709.1	
α -2,8-sialyltransferase ST6Sia I (Siat 8A) (fragment)	<i>Takifugu rubripes</i>	n.d.	AJ715534 CAG29373.1	
α -2,8-sialyltransferase ST6Sia II (Siat 8B) (fragment)	<i>Takifugu rubripes</i>	n.d.	AJ715536 CAG29377.1	
α -2,8-sialyltransferase ST6Sia III (Siat 8C) (fragment)	<i>Takifugu rubripes</i>	n.d.	AJ715541 CAG29380.1	
α -2,8-sialyltransferase ST6Sia IIIr (Siat 8Cr)	<i>Takifugu rubripes</i>	n.d.	AJ715542 CAG29381.1	
α -2,8-sialyltransferase ST6Sia V (Siat 8E)	<i>Takifugu rubripes</i>	n.d.	AJ715547 CAG29386.1	

FIG. 2K

Protein	Organism	EC#	GenBank / GenPept	SwissProt / PDB
(fragment)				
α -2,8-sialyltransferase ST8Sia VI (Siat 8F)	<i>Tadifugu rubripes</i>	n.d.	AJ715549	CAG29388.1
(fragment)				
α -2,8-sialyltransferase ST8Sia VIr (Siat 8Fr)	<i>Tadifugu rubripes</i>	n.d.	AJ715550	CAG29389.1
α -2,3-sialyltransferase (Siat5-r)	<i>Tetraodon nigroviridis</i>	n.d.	AJ744808	CAG32842.1
α -2,3-sialyltransferase ST3Gal I (Siat4)	<i>Tetraodon nigroviridis</i>	n.d.	AJ744802	CAG32838.1
α -2,3-sialyltransferase ST3Gal III (Siat6)	<i>Tetraodon nigroviridis</i>	n.d.	AJ626822	CAF25180.1
α -2,8-sialyltransferase ST6GalNAc II (Siat7B)	<i>Tetraodon nigroviridis</i>	n.d.	AJ634462	CAG25653.1
α -2,8-sialyltransferase ST6GalNAc V (Siat7E)	<i>Tetraodon nigroviridis</i>	n.d.	AJ646879	CAG28708.1
(fragment)				
α -2,8-sialyltransferase ST8Sia I (Siat 8A)	<i>Tetraodon nigroviridis</i>	n.d.	AJ715536	CAG29375.1
(fragment)				
α -2,8-sialyltransferase ST8Sia II (Siat 8B)	<i>Tetraodon nigroviridis</i>	n.d.	AJ715537	CAG29376.1
(fragment)				
α -2,8-sialyltransferase ST8Sia III (Siat 8C)	<i>Tetraodon nigroviridis</i>	n.d.	AJ715539	CAG29378.1
(fragment)				
α -2,8-sialyltransferase ST8Sia IIIr (Siat 8Cr)	<i>Tetraodon nigroviridis</i>	n.d.	AJ715540	CAG29379.1
(fragment)				
α -2,8-sialyltransferase ST8Sia V (Siat 8E)	<i>Tetraodon nigroviridis</i>	n.d.	AJ715548	CAG29387.1
(fragment)				
α -2,3-sialyltransferase (ST3Gal-II)	<i>Xenopus laevis</i>	n.d.	AJ585762	CAE51388.1
α -2,3-sialyltransferase (ST3Gal-VI)	<i>Xenopus laevis</i>	n.d.	AJ585768	CAE51390.1
α -2,3-sialyltransferase ST3Gal-III (Siat6)	<i>Xenopus laevis</i>	n.d.	AJ585784 AJ626823	CAE51388.1 CAF25181.1
α -2,8-polysialyltransferase	<i>Xenopus laevis</i>	2.4.99.-	AB007468	BAA32817.1 O83234
α -2,8-sialyltransferase ST8Sia-I (Siat8A;GD3 synthase)	<i>Xenopus laevis</i>	n.d.	AY272056 AY272057 AJ704662	AAQ16162.1 AAQ16163.1 CAG28895.1
Unknown (protein for MGC:81265)	<i>Xenopus laevis</i>	n.d.	BC068760	AAH88760.1
α -2,3-sialyltransferase (3Gal-VI)	<i>Xenopus tropicalis</i>	n.d.	AJ626744	CAF25054.1
α -2,3-sialyltransferase (Siat4c)	<i>Xenopus tropicalis</i>	n.d.	AJ622908	CAF22058.1
α -2,8-sialyltransferase ST6GalNAc V (Siat7E)	<i>Xenopus tropicalis</i>	n.d.	AJ646878	CAG28707.1
(fragment)				
α -2,8-sialyltransferase ST8Sia III (Siat 8C)	<i>Xenopus tropicalis</i>	n.d.	AJ715544	CAG29383.1
(fragment)				
β -galactosamide α -2,6-sialyltransferase II (ST6Gal II)	<i>Xenopus tropicalis</i>	n.d.	AJ627628	CAF29498.1
sialyltransferase ST8SiaI	<i>Xenopus tropicalis</i>	n.d.	AY652776	AAT67042
poly- α -2,8-sialosyl sialyltransferase (NeuS)	<i>Escherichia coli</i> K1	2.4.-.	M78370 X60598	AAA24213.1 Q57269 CAA43053.1
polysialyltransferase	<i>Escherichia coli</i> K92	2.4.-.	M88479	AAA24216.1 Q47404

FIG. 2L

Protein	Organism	EC#	GenBank / GenPept	SwissProt	PDB / 3D
α -2,8 polysialyltransferase SlaD	<i>Neisseria meningitidis</i> B1940	2.4.-.-	M85053 X78068	AAA20478.1 CAA54985.1	Q51281 Q51145
SynE	<i>Neisseria meningitidis</i> FAM18	n.d.	U75850	AAB53842.1	O08435
polysialyltransferase (SlaD)(fragment)	<i>Neisseria meningitidis</i> M1019	n.d.	AY234182	AAO85290.1	
SlaD (fragment)	<i>Neisseria meningitidis</i> M209	n.d.	AY281048	AAP34788.1	
SlaD (fragment)	<i>Neisseria meningitidis</i> M3045	n.d.	AY281044	AAP34787.1	
polysialyltransferase (SlaD)(fragment)	<i>Neisseria meningitidis</i> M3315	n.d.	AY234181	AAO85289.1	
SlaD (fragment)	<i>Neisseria meningitidis</i> M3515	n.d.	AY281047	AAP34770.1	
polysialyltransferase (SlaD)(fragment)	<i>Neisseria meningitidis</i> M4211	n.d.	AY234180	AAO85288.1	
SlaD (fragment)	<i>Neisseria meningitidis</i> M4842	n.d.	AY281048	AAP34771.1	
polysialyltransferase (SlaD)(fragment)	<i>Neisseria meningitidis</i> M5177	n.d.	AY234183	AAO85281.1	
SlaD	<i>Neisseria meningitidis</i> M5178	n.d.	AY281049	AAP34766.1	
SlaD (fragment)	<i>Neisseria meningitidis</i> M980	n.d.	AY281045	AAP34768.1	
NMB0087	<i>Neisseria meningitidis</i> MC58	n.d.	NC_003112	NP_273131	
Lst	<i>Aeromonas punctata</i> Sch3	n.d.	AF128258	AAS88824.1	
ORF2	<i>Haemophilus influenzae</i> A2	n.d.	M84855	AAA24979.1	
HI1699	<i>Haemophilus influenzae</i> Rd	n.d.	U32842 NC_000907	AAC23345.1 NP_439841.1	Q48211
α -2,3-sialyltransferase	<i>Neisseria gonorrhoeae</i> F62	2.4.99.4	U60684	AAC44539.1 AAE87205.1	P72074
α -2,3-sialyltransferase	<i>Neisseria meningitidis</i> 126E, NRCC 4010	2.4.99.4	U60682	AAC44544.2	
α -2,3-sialyltransferase	<i>Neisseria meningitidis</i> 408Y, NRCC 4030	2.4.99.4	U60681	AAC44543.1	
α -2,3-sialyltransferase (NMB0922)	<i>Neisseria meningitidis</i> MC58	2.4.99.4	U60680 AE002443 NC_003112	AAC44541.1 AAF41330.1 NP_273982.1	P72097
NMA1118	<i>Neisseria meningitidis</i> Z2491	n.d.	AL182765 NC_003118	CAB84380.1 NP_283887.1	Q9JUV6
PM0508	<i>Pasteurella multocida</i> PM70	n.d.	AE006088 NC_002883	AAK02592.1 NP_245445.1	Q9CNC4
WaaH	<i>Salmonella enterica</i> SARB25	n.d.	AF519787	AAM82850.1	Q8KS93
WaaH	<i>Salmonella enterica</i> SARB3	n.d.	AF519788	AAM82851.1	Q8KS92
WaaH	<i>Salmonella enterica</i> SARB39	n.d.	AF519789	AAM82852.1	
WaaH	<i>Salmonella enterica</i> SARB53	n.d.	AF519790	AAM82853.1	
WaaH	<i>Salmonella enterica</i> SARB57	n.d.	AF519791	AAM82854.1	Q8KS91
WaaH	<i>Salmonella enterica</i> SARB71	n.d.	AF519793	AAM82856.1	Q8KS89
WaaH	<i>Salmonella enterica</i>	n.d.	AF519792	AAM82855.1	Q8KS90

FIG. 2M

Protein	Organism	EC#	GenBank / GenPept	SwissProt	PDB / 3D
	SAR88				
WaaH	<i>Salmonella enterica</i> SARC10V	n.d.	AF519779	AAM88840.1	Q8KS99
WaaH (fragment)	<i>Salmonella enterica</i> SARC12	n.d.	AF519781	AAM88842.1	
WaaH (fragment)	<i>Salmonella enterica</i> SARC13I	n.d.	AF519782	AAM88843.1	Q8KS98
WaaH (fragment)	<i>Salmonella enterica</i> SARC14I	n.d.	AF519783	AAM88844.1	Q8K897
WaaH	<i>Salmonella enterica</i> SARC16II	n.d.	AF519784	AAM88846.1	Q8K896
WaaH	<i>Salmonella enterica</i> SARC16II	n.d.	AF519785	AAM88846.1	Q8K895
WaaH (fragment)	<i>Salmonella enterica</i> SARC3I	n.d.	AF519772	AAM88834.1	Q8KSA4
WaaH (fragment)	<i>Salmonella enterica</i> SARC4I	n.d.	AF519773	AAM88835.1	Q8KSA3
WaaH	<i>Salmonella enterica</i> SARC5IIa	n.d.	AF519774	AAM88836.1	
WaaH	<i>Salmonella enterica</i> SARC5IIa	n.d.	AF519775	AAM88837.1	Q8KSA2
WaaH	<i>Salmonella enterica</i> SARC8	n.d.	AF519777	AAM88838.1	Q8KSA1
WaaH	<i>Salmonella enterica</i> SARC9V	n.d.	AF519778	AAM88839.1	Q8KSA0
UDP-glucose : α -1,2-glucosyltransferase (WaaH)	<i>Salmonella enterica</i> subsp. <i>arizonae</i> SARC 5	2.4.1.-	AF511116	AAM46166.1	
bifunctional α -2,3/-2,8-sialyltransferase (Cat-II)	<i>Campylobacter jejuni</i> ATCC 43449	n.d.	AF401629	AAL06004.1	Q83CZ6
Cat	<i>Campylobacter jejuni</i> 81-178	n.d.	AF305571	AAL09368.1	
α -2,3-sialyltransferase (Cat-III)	<i>Campylobacter jejuni</i> ATCC 43429	2.4.99.-	AY044156	AAK73183.1	
α -2,3-sialyltransferase (Cat-III)	<i>Campylobacter jejuni</i> ATCC 43430	2.4.99.-	AF400047	AAK85419.1	
α -2,3-sialyltransferase (Cat-II)	<i>Campylobacter jejuni</i> ATCC 43432	2.4.99.-	AF215659	AAG43979.1	Q9F0M9
α -2,3/8-sialyltransferase (CatII)	<i>Campylobacter jejuni</i> ATCC 43438	n.d.	AF400048	AAK91725.1	Q93MQ0
α -2,3-sialyltransferase cat-II	<i>Campylobacter jejuni</i> ATCC 43448	2.4.99.-	AF167344	AAF34137.1	
α -2,3-sialyltransferase (Cat-II)	<i>Campylobacter jejuni</i> ATCC 43456	2.4.99.-	AF401528	AAL05990.1	Q93D06
α -2,3/- α -2,8-sialyltransferase (CatII)	<i>Campylobacter jejuni</i> ATCC 43460	2.4.99.-	AY044866	AAK96001.1	Q938X6
α -2,3/8-sialyltransferase (Cat-II)	<i>Campylobacter jejuni</i> ATCC 700297	n.d.	AF216647	AAL38462.1	
ORF	<i>Campylobacter jejuni</i> GB11	n.d.	AY422197	AAR82876.1	
α -2,3-sialyltransferase catIII	<i>Campylobacter jejuni</i> MSC57360	2.4.99.-	AF195055	AAG29922.1	
α -2,3-sialyltransferase catIII C1140	<i>Campylobacter jejuni</i> NCTC 11168	2.4.99.-	AL139077 NC_002163	CAB73395.1 NP_282268.1	Q9PNF4
α -2,3/- α -2,8-sialyltransferase II (catII)	<i>Campylobacter jejuni</i> O:10	n.d.	AX934427	AAC98889.1 CAF04167.1	
α -2,3/- α -2,8-sialyltransferase II (CatII)	<i>Campylobacter jejuni</i> O:19	n.d.	AX934431	CAF04169.1	
α -2,3/- α -2,8-sialyltransferase II (CatII)	<i>Campylobacter jejuni</i> O:36	n.d.	AX934438	CAF04171.1	
α -2,3/- α -2,8-	<i>Campylobacter</i>	n.d.	AX934434	CAF04170.1	

FIG. 2N

Protein	Organism	EC#	GenBank / GenPept	SwissProt	PDB / 3D
slalyltransferase II (CstII)	<i>Jejun</i> O:4				
α -2,3/ α -2,8-sialyltransferase II (CstII)	<i>Campylobacter jejuni</i> O:41	n.d.	-	AAO98670.1 AAT17967.1 CAF04168.1	
α -2,3-sialyltransferase cst-I	<i>Campylobacter jejuni</i> OH4384	2.4.99.-	AX934429 AF130466	AAF13486.1 AAS36281.1	Q9RGF1
bifunctional α -2,3/-2,8-sialyltransferase (Cst-II)	<i>Campylobacter jejuni</i> OH4384	2.4.99.-	AF130984 AX934425	AAF31771.1 CAF04168.1	1RO7 1RO8 C A
HI0352 (fragment)	<i>Haemophilus influenzae</i> Rd	n.d.	U32720 X57315 NC_000907	AAC22013.1 CAA40567.1 NP_438516.1	P24324
PM1174	<i>Pasteurella multocida</i> PM70	n.d.	AED06157 NC_002863	AAK03258.1 NP_248111.1	Q9CLP3
Sequence 10 from patent US 6503744	Unknown.	n.d.	-	AAO98672.1	
Sequence 10 from patent US 6699705	Unknown.	n.d.	-	AAT17969.1	
Sequence 12 from patent US 6699705	Unknown.	n.d.	-	AAT17970.1	
Sequence 2 from patent US 6709834	Unknown.	n.d.	-	AAT23232.1	
Sequence 9 from patent US 6503744	Unknown.	n.d.	-	AAO98668.1	
Sequence 3 from patent US 6699705	Unknown.	n.d.	-	AAT17965.1	
Sequence 34 from patent US 6503744	Unknown.	n.d.	-	AAO98684.1	
Sequence 35 from patent US 6503744 (fragment)	Unknown.	n.d.	-	AAO98685.1 AAS36262.1	
Sequence 48 from patent US 6699706	Unknown.	n.d.	-	AAT17988.1	
Sequence 6 from patent US 6699705	Unknown.	n.d.	-	AAT17986.1	
Sequence 9 from patent US 6503744	Unknown.	n.d.	-	AAO98671.1	

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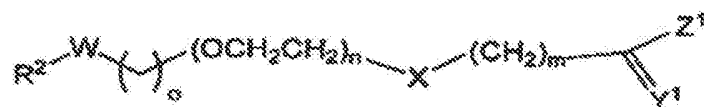
Fibroblaszt növekedési faktor (FGF) átmodellezése és glikopegilezése

Szabadalmi igénypontok

1. Fibroblaszt növekedési faktor (FGF) konjugátum, mely tartalmaz:
 egy, újonnan bevezetett N-kapcsolt vagy O-kapcsolt glikozilezési helyet tartalmazó mutáns FGF-21 peptidet, mely nincs jelen a megfelelő vad típusú fibroblaszt növekedési faktorban, ahol a megfelelő vad típusú fibroblaszt növekedési faktor a 146. számú szekvenciával legalább 95%-ban homológ aminosavszekvenciával rendelkezik;
 és ahol az újonnan bevezetett N-kapcsolt vagy O-kapcsolt glikozilezési hely a prolin gyök C- vagy N-terminális oldalán elhelyezkedő, például a 146. számú szekvencia 3, 5, 9, 50, 61, 79, 91, 116, 120, 125, 129, 134, 139, 151, 156, 166, 172, vagy 178. helyzetében található prolingyöktől 4-nél kevesebb aminosavval elválasztott; és
 egy módosító csoportot, ahol a módosító csoport kovalensen kapcsolódik a peptidhez a mutáns FGF-21 peptid előre meghatározott glikozil- vagy aminosavgyökéhez egy érintetlen glikozil kapcsoló csoporton keresztül, azaz egy olyan glikozil kapcsoló csoporton keresztül, melyben a módosító csoportot a molekula fennmaradó részéhez kapcsoló szaccharid monomer nem degradált.

2. Az 1. igénypont szerinti FGF konjugátum, ahol a mutáns FGF-21 peptid a 161-214, 220-320 és 323-337 számú szekvenciáknak megfelelő aminosavszekvenciák közül legalább egyet tartalmaz.

3. Az előző igénypontok bármelyike szerinti FGF konjugátum, ahol a módosító csoport kovalensen kapcsolódik az előre meghatározott glikozil gyöknél, adott esetben a módosító csoport egy nem-glikozidos módosító csoport; adott esetben a nem-glikozidos módosító csoport valamely egyenes láncú és elágazó láncú PEG közül választott; adott esetben az egyenes láncú PEG az alábbi:



szerkezetű, ahol

R^2 H, szubsztituált vagy szubsztituátlan alkil, szubsztituált vagy szubsztituátlan aril, szubsztituált vagy szubsztituátlan heteroaril, szubsztituált vagy szubsztituátlan heterocikloalkil, szubsztituált vagy szubsztituátlan heteroalkilcsoport, például acetál,

OHC-, $\text{H}_2\text{N-CH}_2\text{CH}_2\text{-}$, $\text{HS-CH}_2\text{CH}_2\text{-}$, és $\text{-(CH}_2\text{)}_n\text{C(Y}^1\text{)Z}^2\text{}$; -cukor-nukleotid, vagy protein közül választott;

n értéke 1 és 2500 közötti;

m, o, és q egymástól függetlenül 0 - 20;

Z OH, NH_2 , halogénatom, S-R^3 , aktivált észterek alkohol-része, $\text{-(CH}_2\text{)}_p\text{C(Y}^2\text{)V}$, $\text{-(CH}_2\text{)}_p\text{U(CH}_2\text{)}_q\text{C(Y}^2\text{)}_v$, -cukor-nukleotid, protein, és kilépő csoportok, például imidazol, p-nitrofenil, HOBT, tetrazol, halogenid közül választott;

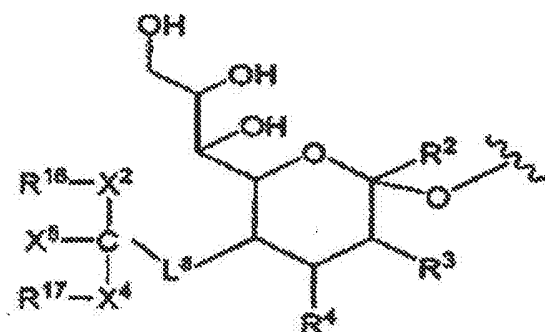
X, Y^1 , Y^2 , W és U egymástól függetlenül O, S, N-R^4 ;

V OH, NH_2 , halogénatom, S-R^5 , aktivált észterek alkohol-komponensei, aktivált amidok amin-komponensei, cukor-nukleotidok és proteinek közül választott;

p, s és v egymástól függetlenül 0 - 20; és

R^3 , R^4 és R^5 egymástól függetlenül H, szubsztituált vagy szubsztituálatlan alkil, szubsztituált vagy szubsztituálatlan heteroalkil, szubsztituált vagy szubsztituálatlan aril, szubsztituált vagy szubsztituálatlan heterocikloalkil és szubsztituált vagy szubsztituálatlan heteroarilcsoportok közül választott.

4. Az előző igénypontok bármelyike szerinti FGF konjugátum, ahol a glikozil kapcsoló csoport:



szerkezetű, ahol

R^2 H, CH_2OR^7 , COOR^7 vagy OR^7 , ahol

R^7 H, szubsztituált vagy szubsztituálatlan alkil vagy szubsztituált vagy szubsztituálatlan heteroalkilcsoport;

R^3 és R^4 egymástól függetlenül H, szubsztituált vagy szubsztituálatlan alkil, OR^8 és NHC(O)R^9 közül választott, ahol

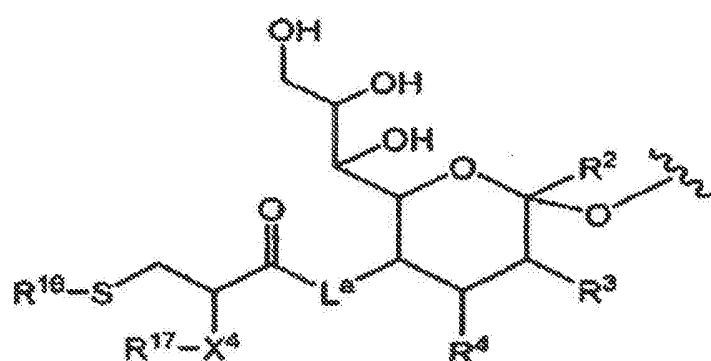
R^8 és R^9 egymástól függetlenül H, szubsztituált vagy szubsztituálatlan alkil, szubsztituált vagy szubsztituálatlan heteroalkilcsoport vagy szialinsav közül választott;

L^4 vegyértékkötés, szubsztituált vagy szubsztituálatlan alkil és szubsztituált vagy szubsztituálatlan heteroalkilcsoport közül választott kapcsoló csoport;

R^{16} és R^{17} egymástól függetlenül választott polimer kar;

X^2 és X^4 egymástól függetlenül az R^{16} és R^{17} polimer gyököket a C-hez kapcsoló kötés fragmensek közül választott, és

X⁵ egy nem-reaktív csoport; adott esetben a glikozil kapcsoló csoport szerkezete:



5. Izolált nukleinsav, mely mutáns fibroblaszt növekedési faktor-21-t kódoló polinukleotid szekvenciát tartalmaz, ahol a mutáns fibroblaszt növekedési faktor-21 egy újonnan bevezetett N-kapcsolt vagy O-kapcsolt glikozilezési hely, mely nincs jelen a megfelelő vad típusú fibroblaszt növekedési faktorban, ahol a megfelelő vad típusú fibroblaszt növekedési faktor a 146. számú szekvenciával legalább 95%-ban homológ aminosavszekvenciával rendelkezik; és ahol az újonnan bevezetett N-kapcsolt vagy O-kapcsolt glikozilezési hely a prolin gyök C- vagy N-terminális oldalán elhelyezkedő, például a 146. számú szekvencia 3, 5, 9, 50, 61, 79, 91, 116, 120, 125, 129, 134, 139, 151, 156, 166, 172, vagy 178. helyzetében található prolingyöktől 4-nél kevesebb aminosavval elválasztott.

6. Az 5. igénypont szerinti nukleinsav, ahol a mutáns FGF-21 peptid a 161-214, 220-320 és 323-337 számú szekvenciáknak megfelelő aminosavszekvenciák közül legalább egyet tartalmaz és/vagy ahol a mutáns fibroblaszt növekedési faktor-21 egynél több újonnan bevezetett glikozilezési helyet tartalmaz.

7. Expressziós kazetta vagy sejt, mely az 5. vagy 6. igénypont szerinti nukleinsavat tartalmazza.

8. Mutáns fibroblaszt növekedési faktor-21, mely egy újonnan bevezetett N-kapcsolt vagy O-kapcsolt glikozilezési helyet tartalmaz, mely nincs jelen a megfelelő vad típusú fibroblaszt növekedési faktorban, ahol a megfelelő vad típusú fibroblaszt növekedési faktor a 146. számú szekvenciával legalább 95%-ban homológ aminosavszekvenciával rendelkezik; és ahol az újonnan bevezetett N-kapcsolt vagy O-kapcsolt glikozilezési hely a prolin gyök C- vagy N-terminális oldalán elhelyezkedő, például a 146. számú szekvencia 3, 5, 9, 50, 61, 79, 91, 116, 120, 125, 129, 134, 139, 151, 156, 166, 172, vagy 178. helyzetében található prolingyöktől 4-nél kevesebb aminosavval elválasztott.

9. A 8. igénypont szerinti mutáns fibroblaszt növekedési faktor-21, ahol a mutáns FGF-21 a 161-214, 220-320 és 323-337 számú szekvenciáknak megfelelő aminosavszekvenciák közül legalább egyet tartalmaz és/vagy ahol a mutáns fibroblaszt növekedési faktor-21 egyenél több újonnan bevezetett glikozilezési helyet tartalmaz.

10. A 8. vagy 9. igénypont szerinti mutáns fibroblaszt növekedési faktor-21, mely vízzoldható, a glikozilezési helyhez egy glikozil kapcsolóval kapcsolódó polimert tartalmaz, ahol adott esetben a glikozil kapcsoló egy érintetlen glikozil kapcsoló.

11. Eljárás egy mutáns fibroblaszt növekedési faktor-21 előállítására, mely egy újonnan bevezetett N-kapcsolt vagy O-kapcsolt glikozilezési helyet tartalmaz, mely nincs jelen a megfelelő vad típusú fibroblaszt növekedési faktorban, melynek során:

- (a) rekombináns úton előállítjuk a mutáns fibroblaszt növekedési faktor-21-t; és
- (b) a mutáns fibroblaszt növekedési faktor-21-t az újonnan bevezetett glikozilezési helyen glikozilezzük, ahol a glikozilezés sejtmentes, in vitro eljárás, ahol a megfelelő vad típusú fibroblaszt növekedési faktor a 146. számú szekvenciával legalább 95%-ban homológ aminosavszekvenciával rendelkezik; és ahol az újonnan bevezetett glikozilezési hely a prolin gyök C- vagy N-terminális oldalán elhelyezkedő, például a 146. számú szekvencia 3, 5, 9, 50, 61, 79, 91, 116, 120, 125, 129, 134, 139, 151, 156, 166, 172, vagy 178. helyzetében található prolingyöktől 4-nél kevesebb aminosavval elválasztott.

12. A 11. igénypont szerinti eljárás, ahol a mutáns fibroblaszt növekedési faktor-21 a 161-214, 220-320 és 323-337 számú szekvenciáknak megfelelő aminosavszekvenciák közül legalább egyet tartalmaz és/vagy ahol a mutáns fibroblaszt növekedési faktor egyenél több újonnan bevezetett glikozilezési helyet tartalmaz.

13. Gyógyászati készítmény, mely hatásos mennyiségű, az 1-4. és 8-10. igénypontok bármelyike szerinti terméket tartalmaz.

14. Az 1-4. és 8-10. igénypontok bármelyike szerinti termék fibroblaszt növekedési faktor hiány kezelésében történő alkalmazásra.

15. Eljárás egy mutáns fibroblaszt növekedési faktor glikokonjugátumának előállítására, mely újonnan bevezetett N-kapcsolt vagy O-kapcsolt glikozilezési helyet tartalmaz, mely nincs jelen a megfelelő vad-típusú fibroblaszt növekedési faktorban, melynek során:

- a) rekombináns úton előállítjuk a mutáns fibroblaszt növekedési faktor-21-t; és

(b) a mutáns fibroblaszt növekedési faktor-21-t az újonnan bevezetett glikozilezési helyen egy módosított cukorral glikozilezzük, ahol a glikozilezés sejtmentes, in vitro eljárás, ahol a megfelelő vad típusú fibroblaszt növekedési faktor a 146. számú szekvenciával legalább 95%-ban homológ aminosavszekvenciával rendelkezik; és ahol az újonnan bevezetett glikozilezési hely a prolin gyök C- vagy N-terminális oldalán elhelyezkedő, például a 146. számú szekvencia 3, 5, 9, 50, 61, 79, 91, 116, 120, 125, 129, 134, 139, 151, 156, 166, 172, vagy 178. helyzetében található prolingyöktől 4-nél kevesebb aminosavval elválasztott, és/vagy ahol a mutáns fibroblaszt növekedési faktor-21 a 161-214, 220-320 és 323-337 számú szekvenciáknak megfelelő aminosavszekvenciák közül legalább egyet tartalmaz és/vagy ahol a mutáns fibroblaszt növekedési faktor-21 egynél több újonnan bevezetett glikozilezési helyet tartalmaz; adott esetben a módosított cukor egy vízoldható polimerrel módosított, adott esetben a vízoldható polimer poli(etilén-glikol) és m-poli(etilén-glikol) közül választott.

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