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(54) Title: GLYCOSYLATED PEPTIDE ANTAGONISTS OF THE BRADYKININ B1 RECEPTOR

(57) Abstract: A compositions of matter and pharmaceutical compositions are disclosed, which in various embodiments, comprise biologically active glycosylated peptides that can be used therapeutically or prophylactically against diseases or conditions linked to bradykinin B1 receptor as the causative agent. A method of treating, preventing, or ameliorating a disease or condition associated with B1 activity is also disclosed.

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GLYCOSYLATED PEPTIDE ANTAGONISTS OF THE BRADYKININ B1 RECEPTOR

This application claims the benefit of U. S. Provisional Application No. 60/832,464, filed July 21, 2006, which is hereby incorporated by reference.

This application incorporates by reference all subject matter contained on the compact disc, which is identified by the name of the file, A-1161 PCT SeqList072007.ST25.txt created on July 20, 2007, the size of which file is 36 KB.

Throughout this application various publications are referenced within parentheses. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the *state of the art* to which this invention pertains.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention is related to the biochemical arts, in particular to therapeutic peptides.

2. Discussion of the Related Art

More than two million people in the United States alone are incapacitated by chronic pain on any given day (T. M. Jessell & D. D. Kelly, Pain and Analgesia in PRINCIPLES OF NEURAL SCIENCE, third edition, E. R. Kandel, J. H. Schwartz, T. M. Jessell, ed., (1991)).

Pain is a perception based on signals received from the environment and transmitted and interpreted by the nervous system (for review, see Millan, M.J., The induction of pain: an integrative review. Prog Neurobiol 57:1-164 (1999)). Noxious stimuli such as heat and touch cause specialized sensory receptors in the skin to send signals to the central nervous system ("CNS"). This process is called nociception, and the peripheral sensory neurons that mediate it are nociceptors. Depending on the strength of the signal from the nociceptor(s) and the abstraction

and elaboration of that signal by the CNS, a person may or may not experience a noxious stimulus as painful. When one's perception of pain is properly calibrated to the intensity of the stimulus, pain serves its intended protective function. However, certain types of tissue damage cause a phenomenon, known as hyperalgesia or pronociception, in which relatively innocuous stimuli are perceived as intensely painful because the person's pain thresholds have been lowered. Both inflammation and nerve damage can induce hyperalgesia. Thus, persons afflicted with inflammatory conditions, such as sunburn, osteoarthritis, colitis, carditis, dermatitis, myositis, neuritis, inflammatory bowel disease, collagen vascular diseases (which include rheumatoid arthritis and lupus) and the like, often experience enhanced sensations of pain. Similarly, trauma, surgery, amputation, abscess, causalgia, collagen vascular diseases, demyelinating diseases, trigeminal neuralgia, cancer, chronic alcoholism, stroke, thalamic pain syndrome, diabetes, herpes infections, acquired immune deficiency syndrome ("AIDS"), toxins and chemotherapy cause nerve injuries that result in excessive pain.

As the mechanisms by which nociceptors transduce external signals under normal and hyperalgesic conditions become better understood, processes implicated in hyperalgesia can be targeted to inhibit the lowering of the pain threshold and thereby lessen the amount of pain experienced.

Bradykinin (BK) and the related peptide, kallidin (Lys-BK)(see Table 3) mediate the physiological actions of kinins on the cardiovascular and renal systems. However, the active peptides, BK and kallidin, are quickly degraded by peptidases in the plasma and other biological fluids and by those released from a variety of cells, so that the half-life of BK in plasma is reported to be approximately 17 seconds (McCarthy, D.A., et al., An *in vivo* estimation of the potencies and half-lives of synthetic bradykinin and kallidin. J. Pharmacol. Exp Ther. 148, 117-122 (1965)). BK and kallidin are rapidly metabolized in the body by carboxypeptidase N, which removes the carboxyterminal arginine residue to generate des-Arg-BK or des-Arg-kallidin. Des-Arg-kallidin is among the predominant kinins in man and mediates the pathphysiological actions of kinins in

man. In addition to being a very potent proinflammatory peptide, des-Arg-BK or des-Arg-kallidin is known to induce vasodilation, vascular permeability, and bronchoconstriction (for review, see Regoli and Barabe, Pharmacology of Bradykinin and Related Kinins, Pharmacological Reviews, 32(1):1-46 (1980)). In addition, des-Arg-BK and des-Arg-kallidin appear to be particularly important mediators of inflammation and inflammatory pain as well as being involved in the maintenance thereof. There is also a considerable body of evidence implicating the overproduction of des-Arg-kallidin in conditions in which pain is a prominent feature such as septic shock, arthritis, angina, and migraine.

The membrane receptors that mediate the pleiotropic actions of kinins are of two distinct classes, designated B1 and B2. Both classes of receptors have been cloned and sequenced from a variety of species, including man (Menke, et al., Expression cloning of a human b1 bradykinin receptor. J. Biol. Chem., 269:21583-21586 (1994); Hess et al., Cloning and pharmacological characterization of a human bradykinin (BK-2) receptor. Biochem. Biophys. Res. Commun. 184, 260-268 (1992)). They are typical G protein coupled receptors having seven putative membrane spanning regions. In various tissues, BK receptors are coupled to every known second messenger. B2 receptors, which have a higher affinity for BK, appear to be the most prevalent form of bradykinin receptor. Essentially all normal physiological responses and many pathophysiological responses to bradykinin are mediated by B2 receptors.

B1 receptors, on the other hand, have a higher affinity for des-Arg-BK (see Table 3) compared with BK, whereas des-Arg-BK is inactive at B2 receptors. In addition, B1 receptors are not normally expressed in most tissues. In certain instances, their expression is induced upon injury or tissue damage as well as in certain kinds of chronic inflammation or systemic insult (Marceau, F., et al., Kinin B1 receptors: a review. Immunpharmacology, 30:1-26 (1995)). Furthermore, responses mediated by B1 receptors are up-regulated from a null level following administration of bacterial lipopolysaccharide (LPS) or inflammatory cytokines in rabbits, rats, and pigs (Marceau et al., (1995)).

The pain-inducing properties of kinins coupled with the inducible expression of B1 receptors make the B1 receptor an interesting target in the development of anti-inflammatory, antinociceptive, antihyperalgesic and analgesic agents that may be directed specifically at injured tissues with minimal actions in normal tissues. While a variety of peptide antagonists targeting the B1 receptor have been identified, their development as therapeutic analgesics has been stymied by poor efficacious half-lives resulting from very rapid degradation by tissue and serum peptidases and efficient renal clearance. More recently, potent and relatively selective B1 peptide antagonists having non-natural amino acid substituents have been shown to be more resistant to peptidases in *in vitro* stability assays (for review, see Regoli et al., Bradykinin receptors and their antagonists. European Journal of Pharmacology, 348:1-10 (1998); Stewart, J.M., et al., Bradykinin antagonists: present progress and future prospects. Immunopharmacology, 43:155-161 (1999); and Stewart, J.M., et al., Metabolism-Resistant Bradykinin Antagonists: Development and Applications. Biol. Chem., 382:37-41 (2001)). While the conformational requirements for the biological activity are fulfilled by these designer peptide antagonists, they typically suffer from insufficient pharmacodynamic and pharmokinetic profiles.

A develoment in the area of pharmaceutical science has centered around efforts to increase the bioavailability of drug candidates by chemical derivatization. Glycosylation of medicinally-relevant compounds has been widely recognized as a feasible approach to explore when seeking to increase solubility and significantly extend the *in vivo* circulating half-lives of drugs or drug candidates. Early examples of peptide drugs that show improved properties when modified by simple sugar entities are presented in International Patent Publication WO 88/02756, which describes a range of peptides such as somatostatin, calcitonin, LH-RH, oxytocin, vasopressin, insulin and growth hormone releasing factor modified by sugars throughout either by Amadori or Heynes reactions.

The attachment of sugar moieties such as glucose, mannose, and galactose onto bioactive molecules to modulate pharmacokinetic and phrmacodynamic properties has been described in the literature. For example, Suzuki, et al.,

attached a glucosyl-, mannosyl- and 2-deoxyglucosy- onto Arg-vasopressin via an octamethylene linker. These molecules, when injected i.v., were selectively taken up by kidney cells presumably due to a novel transport mechanism (Suzuki, K., et al., Specific renal delivery of sugar-modified low-molecular-weight peptides. Journal of Pharmacology & Experimental Therapeutics. 288(2):888-97 (1999) and Susaki, H. et al., Renal targeting of arginine-vasopressin by modification with carbohydrates at the tyrosine side chain. Biological & Pharmaceutical Bulletin. 22(10):1094-8 (1999)). In a second example, (3-^{125}I-tyr3)-Octreotide (I-TOC) was conjugated with the sugar moieties glucose, maltose, and maltotriose. The attachment of the sugar decreased the accumulation of radiotracer in the liver and intestine, two organs that contribute to the elimination of (I-TOC). In doing so, the authors showed that higher levels of the radiotracer could be achieved in the tumors relative to the parent bioactive moiety (Schottelius, M. et al., N-terminal sugar conjugation and C-terminal Thr-for-Thr(ol) exchange in radioiodinated Tyr3-octreotide: effect on cellular ligand trafficking in vitro and tumor accumulation in vivo. *Journal of Medicinal Chemistry. 48(8):2778-89, (2005) and* Schottelius, M. et al., Improvement of pharmacokinetics of radioiodinated Tyr(3)-octreotide by conjugation with carbohydrates. Bioconjugate Chemistry 13(5):1021-30, (2002)).

Examples of sugar conjugation that have been used to improve blood-brain-barrier penetration and increase bioavailability following intraintestinal administration have also been described (Dhanasekaran, M. and Polt, R. New prospects for glycopeptide based analgesia: glycoside-induced penetration of the blood-brain barrier. Curr Drug Deliv.;2(1):59-73 (2005); Polt, R. et al., Glycosylated neuropeptides: a new vista for neuropsychopharmacology? Med. Res. Rev. 25(5):557-85 (2005); Elmagbari, N.O., et al., Antinociceptive structure-activity studies with enkephalin-based opioid glycopeptides. J. Pharmacol. Exp. Ther. 311(1):290-7 (2004); and Egleton R.D., et al., Improved blood-brain barrier penetration and enhanced analgesia of an opioid peptide by glycosylation. J. Pharmacol. Exp. Ther. 299(3):967-72 (2001)).

There is an increasing number of glycoconjugate drugs that are now proving to be superior than their non-glycosylated counterparts for the treatment of various conditions, e.g., retinamide and enkephalin glycoconjugates have shown promise as more potent agents than their non-glycosylated counterparts (H. Abou-Issa, et al. Anticancer Res. 1993, 13, 1431-1436; and T.P. Davis et al. J. Med. Chem. 2000, 43:2586-2590, respectively).

The present invention provides the benefits of glycosylated peptide antagonists of bradykinin B1 receptor.

SUMMARY OF THE INVENTION

The present invention provides glycosylated peptide conjugates which bind to and antagonize the activity of bradykinin B1 receptors (B1) and which have demonstrably superior pharmacokinetic properties *in vivo* as compared to their counterpart unglycosylated peptides.

One aspect of the invention comprises a composition of matter of the formula:

$$F\text{-}[(X^1)\text{–}(Y^1)_n] \qquad (I)$$

or a physiologically acceptable salt thereof, wherein:

X^1 and Y^1 are each independently selected from the formula $-(L^1)_a\text{-}P^1$ and $-(L^2)_b\text{-}P^2$, respectively;

F is a carbohydrate covalently bound to X^1 or Y^1;

L^1 and L^2 are each independently selected linkers;

a and b are each independently 0 or 1;

n is 0 to 1; and

P^1 and P^2 are each independently selected peptide antagonists of the bradykinin B1 receptor.

In various embodiments, the present invention encompasses compositions of matter of the formula:

$$F\text{-}(L^1\text{-}P^1) \qquad (II)$$

(i.e., in Formula I, the value of "n" is zero and the value of "a" is one) or a physiologically acceptable salt thereof, wherein:

F is a carbohydrate covalently bound to a peptidyl or non-peptidyl linker (L^1); and

P^1 is a peptide antagonist of the bradykinin B1 receptor.

Within the meaning of Formula I or Formula II, the peptide antagonist, "P" (i.e., P^1 and P^2), if more than one is present, can be independently the same or different from, any other P also present in the inventive composition. Similarly, the peptidyl or non-peptidyl linker moiety, "L" (i.e., $(L)_a$ and/or $(L)_b$), if present, can be independently the same or different from any other linker, or linkers, that may be present in the inventive composition.

Another object of the present invention is to provide a pharmaceutical composition comprising excipient carrier materials having a glycosylated B1 peptide of the invention dispersed therein.

Another object of the present invention is to provide therapeutic methods of treatment or prophylaxis which comprise administration to a mammal in need thereof a pharmaceutically effective amount of a composition comprising excipients and a glycosylated B1 peptide of the invention.

The glycosylated B1 peptides of the invention have therapeutic value for the treatment of inflammation and chronic pain states of inflammatory and neuropathic origin, and can also be used to treat other diseases mediated by B1 activation, including, but not limited to, septic shock, arthritis, angina, asthma, allergic rhinitis, and migraine.

DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS

In various embodiments, a class of peptides generally considered to be quite intolerant to substitution can be specifically glycosylated, and in various embodiments, lengthened, without significantly perturbing the pharmacology of the parent peptide pharmacophore to provide a therapeutically useful glycoconjugate with a dramatically sustained pharmacokinetic profile as compared to the unglycosylated peptide. In various embodiments, such glycoconjugates are useful for managing inflammation and pain. In various embodiments, previously described shortcomings in B1 peptide antagonists can be overcome by conjugating a glycosylated amino acid or a glycosylated linker of defined size and composition to the N-terminus, C-terminus, or another reactive functional group of the side chain of an amino acid residue comprised by a B1 receptor peptide antagonist in order to maximize preservation of antagonist activity and specificity while prolonging efficacious half-life *in vivo*. In various embodiments, the extended circulating half-lives of the glycoconjugates provide significantly greater exposure and prolonged efficacy *in vivo* when compared to the corresponding unglycosylated peptide. For example, an acetylated peptide B1 antagonist (SEQ ID NO: 37) demonstrated efficacy in relevant *in vivo* models of pain for a maximum of 4 hours following multiple dosing. Surprisingly, the same peptide conjugated to a glycosylated Cys-Gly-Gly-Gly-Gly-Gly (SEQ ID NO:68) linker in a manner disclosed herein demonstrates efficacy for greater than 24 hours after a single bolus injection. In various embodiments, glycosylated B1 receptor peptide antagonists can show marked improvement over the corresponding unglycosylated B1 peptide antagonist in terms of aqueous solubility, with only a minor loss in *in vitro* potency and no change in selectivity at bradykinin receptors. In various embodiments, the increase in hydrophilicity achieved with the glycosylated B1 receptor peptide antagonists can influence the pharmacokinetics and/or the metabolism of the peptide. Importantly, in various embodiments, such glycosylated B1 peptides can exhibit a marked increase in half-life and a decrease in systemic clearance in comparison with the corresponding unglycosylated counterpart. In various embodiments, decreases in

clearance of the glycosylated B1 peptide antagonists can be related to the reduced efficacy of hepatic or renal extraction, compared with the corresponding unglycosylated counterpart. In various embodiments, the introduction of a linker containing a sugar moiety does not produce major changes in the affinity profile of certain B1 peptide antagonists but can markedly improve its *in vivo* potency and duration of action. With these characteristics, in various embodiments, certain glycosylated peptide antagonists of the B1 receptor can be suitable candidates for clinical development and therapeutic use.

In certain embodiments, the glycosylated B1 peptide antagonists are better able to tolerate systemic exposure during treatment, by enhancing the circulating life (delayed clearance), solubility, stability, and decreasing the immunogenicity of the molecule. Increased circulating life typically results in a less frequent dosing regimen and a less frequent dosing schedule typically is more convenient to both physicians and patients, and typically is helpful to those patients involved in self-administration. In certain instances, other advantages to less frequent dosing can include less drug being introduced into patients and increased compliance. In certain instances, glycosylated peptide antagonists of the B1 receptor sufficiently antagonize B1 receptor activity. In certain instances, glycosylated peptide antagonists of B1 receptor avoid drawbacks of certain pharmaceutical forms. In certain instances, glycosylated peptide antagonists of B1 receptor are sufficiently stable *in vivo* for use to manage inflammation, pain, and/or other B1 mediated conditions including, but not limited to, asthma and allergic rhinitis.

Exemplary bradykinin B1 receptor binding peptides contemplated for conjugation with a glycosylated amino acid or a glycosylated linker include, but are not limited to, the B1-binding peptides disclosed in any one of the following publications (each of which is hereby incorporated by reference in its entirety): Regoli et al., Bradykinin receptors and their antagonists. Eur. J. of Pharma., 348:1-10 (1998); Stewart, J.M., et al., Bradykinin antagonists: present progress and future prospects. Immunopharmacology, 43:155-161 (1999); Stewart, J.M., et al., Metabolism-Resistant Bradykinin Antagonists: Development and

Applications. Biol. Chem., 382:37-41 (2001); PCT Publications WO 98/07746 and WO 2005/042027; and U.S. Patent Nos: 4,693,993, 4,801,613, 4,923,963, 5,648,336, 5,834,431, 5,849,863, 5,935,932, 5,648,333, 5,385,889, 5,444,048, and 5,541,286. In various embodiments, the bradykinin B1 receptor binding peptides contemplated for glycosylation for purposes and in the manner as described herein are the B1-binding peptides disclosed in U.S. Patent No: 5,834,431 and U.S. Patent Publication No. 2005/0215470 A1 (published on September 29, 2005).

In various embodiments, since the carbohydrate moiety and/or the target peptides can be multivalent, it is possible by the process of the invention to produce a variety of carbohydrate:peptide structures. By way of example, a univalent carbohydrate and a univalent peptide will produce a 1:1 conjugate; a bivalent peptide and a univalent carbohydrate can form conjugates wherein the peptide conjugates bear two carbohydrate moieties, whereas a bivalent carbohydrate and a univalent peptide can produce species where two peptide entities are linked to a single carbohydrate moiety; use of higher-valent carbohydrates can lead to the formation of clusters of peptide entities bound to a single carbohydrate moiety whereas higher-valent peptides can become encrusted with a plurality of carbohydrate moieties. In various embodiments, the peptide moieties are likely to have more than one reactive group which will react with the carbohydrate and the possibility of forming complex structures should be considered. In various embodiments, when it is desired to form simple structures such as 1:1 adducts of carbohydrate and peptide, or to use bivalent carbohydrates to form peptide:carbohydrate:peptide adducts, one can use predetermined ratios of activated carbohydrate and peptide material, and predetermined concentrations thereof, and one can conduct the reaction under predetermined conditions (such as duration, temperature, pH etc.) so as to form a proportion of the described product and then to separate the described product from the other reaction products. In various embodiments, the reaction conditions, proportions and concentrations of the reagents can be obtained by relatively simple trial-and-error experiments with appropriate scaling-up as necessary. In various embodiments, purification and

separation of the products is similarly achieved by conventional techniques known to those skilled in the art.

The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described. All documents or portions of documents cited in this application, including, but not limited to patents, patent applications, articles, books, and treatises, are expressly incorporated by reference herein in their entirety for any purpose. In the event that one or more of the incorporated documents defines a term that contradicts that term's definition in this application, this application controls.

In this application, the use of the singular includes the plural unless specifically stated otherwise. In this application, the use of "or" means "and/or" unless stated otherwise. Furthermore, the use of the term "including", as well as other forms, such as "includes" and "included", is not limiting. Also, terms such as "element" or "component" encompass both elements and components comprising one unit and elements and components that comprise more than one subunit unless specifically stated otherwise.

As used in the specification and the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an unglycosylated peptide antagonist" or "a glycosylated peptide antagonist" includes mixtures of such glycoconjugates and reference to "the method of treatment" includes reference to one or more methods of treatment of the type which will be known to those skilled in the art or will become known to them upon reading this specification, and so forth.

The terms used throughout this specification are defined as follows, unless otherwise limited in specific instances.

Natural amino acid residues are discussed in three ways: full name of the amino acid, standard three-letter code, or standard single-letter code in accordance with Table 1 below.

Table 1. One-letter abbreviations for the canonical amino acids. Three-letter abbreviations are in parentheses.

Alanine (Ala)	A
Glutamine (Gln)	Q
Leucine (Leu)	L
Serine (Ser)	S
Arginine (Arg)	R
Glutamic Acid (Glu)	E
Lysine (Lys)	K
Threonine (Thr)	T
Asparagine (Asn)	N
Glycine (Gly)	G
Methionine (Met)	M
Tryptophan (Trp)	W
Aspartic Acid (Asp)	D
Histidine (His)	H
Phenylalanine (Phe)	F
Tyrosine (Tyr)	Y
Cysteine (Cys)	C
Isoleucine (Ile)	I
Proline (Pro)	P
Valine (Val)	V

Unless clearly indicated otherwise, a designation herein of a natural or non-natural amino acid is intended to encompass both the D- and L- isomer of the amino acid. Abbreviations used herein for unnatural amino acids are the same as described in U.S. Patent No. 5,834,431, PCT publication WO 98/07746, and Neugebauer, W. et al., Kinin B1 receptor antagonists with multi-enzymatic resistance properties. *Canadian Journal of Physiology & Pharmacology. 80(4):287-92,* (2002), each of which is hereby incorporated by reference in its entirety. Additionally, the abbreviations "Dab" and "D-Dab" are intended to refer to the L- and D- isomer of the unnatural amino acid, D-2-aminobutyric acid,

respectively. The abbreviations "3'Pal" and "D-3'Pal" are intended to refer to the L- and D- isomer of the unnatural amino acid 3'-pyridylalanine, respectively. Also, the abbreviation "Igl" is intended to include both "Igla" and "Iglb" (α-(1-indanyl)glycine and α-(2-indanyl)glycine, respectively). Similarly, "DIgl" is intended to include both "D-Igla" and "D-Iglb" (the D-isomers of α-(1-indanyl)glycine and α-(2-indanyl)glycine, respectively). In certain embodiments, Igl is Iglb and D-Igl is D-Iglb.

Amino acid residues are commonly categorized according to different chemical and/or physical characteristics. The term "acidic amino acid" refers to amino acid residues in D- or L-form having side chains comprising acidic groups. Exemplary acidic residues include aspartatic acid and glutamatic acid residues. The term "aromatic amino acid" refers to amino acid residues in D- or L-form having side chains comprising aromatic groups. Exemplary aromatic residues include tryptophan, tyrosine, 3-(1-naphthyl)alanine, or phenylalanine residues. The term "basic amino acid" refers to amino acid residues in D- or L-form having side chains comprising basic groups. Exemplary basic amino acid residues include histidine, lysine, homolysine, ornithine, arginine, N-methyl-arginine, ω-aminoarginine, ω-methyl-arginine, 1-methyl-histidine, 3-methyl-histidine, and homoarginine residues. The term "hydrophilic amino acid" refers to amino acid residues in D- or L-form having side chains comprising polar groups. Exemplary hydrophilic residues include cysteine, serine, threonine, histidine, lysine, asparagine, aspartate, glutamate, glutamine, and citrulline (Cit) residues. The terms "lipophilic amino acid" refers to amino acid residues in D- or L-form having sidechains comprising uncharged, aliphatic or aromatic groups. Exemplary lipophilic sidechains include phenylalanine, isoleucine, leucine, methionine, valine, tryptophan, and tyrosine. Alanine (A) is amphiphilic—it is capable of acting as a hydrophilic or lipophilic residue. Alanine, therefore, is included within the definition of both "lipophilic residue" and "hydrophilic residue." The term "nonfunctional amino acid" refers to amino acid residues in D- or L-form having side chains that lack acidic, basic, or aromatic groups. Exemplary neutral amino

acid residues include methionine, glycine, alanine, valine, isoleucine, leucine, and norleucine (Nle) residues.

The term "glycosyl" and grammatical forms thereof such as "glycosylated" or "glycosylation" as used herein refers to any pyranose or furanose saccharide group, including but not limited to D- or L-glucosyl, galactosyl, mannosyl, fucosyl, N-acetyineuraminyl, glucosaminyl, galactosaminyl, etc. The term "disaccharide" as used herein refers to any pyranose or furanose saccharide group, including but not limited to D- or L-glucosyl, galactosyl, mannosyl, fucosyl, N-acetyineuraminyl, glucosaminyl, galactosaminyl, etc. linked through a glycosidic bond to another pyranose or furanose saccharide.

The term "oligosaccharide" as used herein refers to any pyranose or furanose groups including but not limited to D- or L-glucosyl, galactosyl, mannosyl, fucosyl, N acetyineuraminyl, glucosaminyl, galactosaminyl, etc., linked through glycosidic bonds; to another pyranose or furanose saccharides in which the number of saccharide groups in the oligosaccharide is no less than three.

The term "glycosyl donor" as used herein refers to any pyranose or furanose saccharide or disaccharide group capable of glycosylating an acceptor such as hydroxyl. Glycosyl donors include but are not limited to suitably protected D- or L-thiotoluyl glucopyranoside, thiotoluyl galactopyranoside, mannopyranoside, fucopyranoside, N- acetyineuraminopyranoside, glucosaminopyranoside, galactosaminopyranoside, etc. The glycosidic linkages can be alpha, beta or alpha/beta mixtures.

Monosaccharides, or simple sugars, consist of a single polyhydroxy aldehyde or ketone unit. Representative monosaccharides include, but are not limited to, hexoses such as D-glucose, D-mannose, D-xylose, D- galactose, L-fucose, and the like; pentoses such as D-ribose or D- arabinose and ketoses such as D-ribulose or D fructose. Disaccharides contain two monosaccharide units joined by a glycosidic linkage. Disaccharides include, but are not limited to, sucrose, lactose, maltose, cellobiose, and the like. Oligosaccharides typically contain from 3 to 10 monosaccharide units joined by glycosidic linkages. Polysaccharides (glycans) typically contain more than such units and include, but

are not limited to, molecules such as heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate and polysaccharide derivatives thereof.

The term "sugar" generally refers to mono-, di- or oligosaccharides. A saccharide can be substituted. Substituted sugars include, but are not limited to, glucosamine, galactosamine, acetylglucose, acetylgalactose, N-acetylglucosamine, N-acetyl- galactosamine, galactosyl-N- acetylglucosamine, N-acetyineuraminic acid (sialic acid), etc., and can contain sulfated and phosphorylated sugars. Substituted sugars. Saccharides can be either in their open or preferably in their pyranose form.

The term "amino-containing saccharide group" refers to a saccharide group having at least one amino substituent. Representative amino-containing saccharides include mycaminose, desosamine, L- vancosamine, 3-desmethyl-vancosamine, 3 epi-vancosamine, 4-epi- vancosamine, acosamine, actinosamine, daunosamine, 3 epi-daunosamine, ristosamine, N-methyl-D- glucamine and the like.

"Optionally substituted" groups can be substituted or unsubstituted. The substituents of an "optionally substituted" group can include, but are not limited to, one or more substituents independently selected from the following groups or designated t subsets thereof: alkyl, alkenyl, alkynyl, heteroalkyl, haloalkyl, haloalkenyl, t haloalkynyl, cycloalkyl, aryl, heteroaryl, arylalkyl, heteroarylalkyl, alkoxy, aryloxy, haloalkoxy, amino, alkylamino, dialkylamino, alkylthio, arylthio, heteroarylthio, oxo, carboxyesters, carboxamido, acyloxy, H. F. Cl, Br, I, CN, NO_2, NH_2, N_3, $NHCH_3$, $N(CH_3)_2$, SH, SCH_3, OH, OCH_3, OCF_3, CH_3, CF_3, $C(O)H_3$, CO_2CH_3, CO_2H, $C(O)H_2$, pyridinyl, thiophene, furanyl, indole, indazol, esters, amides, phosphonates, phosphates, phosphoramides, sulfonates, sulfates, sulfonamides, carbamates, ureas, thioureas, thioamides, thioalkyls. An optionally substituted group can be unsubstituted (e.g., $-CH_2CH_3$), fully substituted (e.g., $-CF_2CF_3$), monosubstituted (e.g., $-CH_2CH_2F$) or substituted at a level anywhere in-between fully substituted and monosubstituted (e.g., $-CH_2CF_3$).

"Aryl" is phenyl or phenyl vicinally-fused with a saturated, partially-saturated, or unsaturated 3-, 4-, or 5 membered carbon bridge, the phenyl or

bridge being substituted by 0, 1, 2 or 3 substituents selected from C_{1-8} alkyl, C_{1-4} haloalkyl or halo.

"Heteroaryl" is an unsaturated 5 , 6 or 7 membered monocyclic or partially-saturated or unsaturated 6-, 7-, 8-, 9-, 10- or 11 membered bicyclic ring, wherein at least one ring is unsaturated, the monocyclic and the bicyclic rings containing 1, 2, 3 or 4 atoms selected from N, O and S, wherein the ring is substituted by 0, 1, 2 or 3 substituents selected from C_{1-8} alkyl, C_{1-4} haloalkyl and halo.

The term "protected amino", "amine protecting group" and "protected: aminomethyl" as used herein refers to known amine protecting groups used in the synthetic organic chemistry art and include but are not limited to t-butoxycarbonyl: (BOC), benzyloxycarbonyl (Cbz), azide (N3), 2-trimethylsilylethoxycarbonyl (Teoc), allyloxycarbonyl (Alloc), 9-fluorenylmethyloxycarbonyl (Fmoc), acyl groups, such as I formyl, acetyl, trihaloacetyl, benzoyl, and nitrophenylacetyl, sulfonamide groups, imine- and cyclic imide groups. Further examples of protected amino groups are described, e.g., by Greene and Wuts in Protective Groups in Organic Synthesis, 2d edition (John Wiley & Sons, New York, 1991).

The term "modified amino" as used herein includes the terms "protected amino," "amine protecting group," "protected aminomethyl," "alkylacylamino," "acylamino" and "carboxamido".

The term "modified hydroxyl" as used herein includes the terms "protected hydroxyl", "hydroxyl protecting group", "protected hydroxymethyl," "alkoxy," "aryloxy," "acyl," "carboxy esters," and "acyloxy" The term "protected hydroxyl", "hydroxyl protecting group" and "protected hydroxymethyl" as used herein refers to known hydroxyl protecting groups used in the synthetic organic chemistry art and include but are not limited to methoxymethyl (MOM), benzyloxymethyl (BOX), benzyl (Bn), Allyl (All), p-methoxybenzyl (PMB), t butyidimethylsilyl (TBDMS), ester groups, such as acetate (Ac), chloroacetate and benzoate (Bz). Further examples of protected hydroxyl groups are described by Greene and Wuts in Protective Groups in Organic Synthesis, 2d edition (John Wiley t & Sons, New

York, 1991).

The term "conjugated" means that the peptide and the carbohydrate are covalently attached or linked to each other, either directly attached, or indirectly attached via a linker moiety. In various embodiments, conjugation of the carbohydrate to the peptide is achieved in one of three different ways. First, conjugation is achieved through a linker attached at the anomeric position of suitably protected or unprotected carbohydrate residues that terminates in a carboxylic acid function. Second, conjugation can be achieved through a linker attached at the anomeric position of such carbohydrate residues terminating in an amino function. Or third, conjugation can be achieved through a direct glycosylation reaction at the anomeric position of such a carbohydrate moiety in which the anomeric position is suitably activated.

By "glycosylated B1 peptide" is meant a peptide B1 receptor antagonist conjugate comprising at least two characteristic structural units. The two parts are (1) a B1 receptor peptide antagonist which has biological activity at the bradykinin B1 receptor and (2) either a glycosylated amino acid, or a glycosylated linker moiety conjugated to the N-terminal amino acid residue, to the C-terminal amino acid residue, or to another reactive functional group of the side chain of an amino acid residue in the primary sequence of a B1 receptor peptide antagonist of (1).

The term "comprising" in the context of an unglycosylated or glycosylated amino acid sequence (used herein interchangeably with "peptide sequence") means that the sequence can include additional amino acid residues on either, or both, of the N- and C- termini of the given amino acid sequence. In various embodiments, these additional amino acids should not significantly interfere with the biological activity of the peptide or glycosylated peptide.

As used herein, the term "native peptide" refers to an unglycosylated B1 peptide antagonist disclosed herein or known in the art, or unglycosylated analogs thereof.

As stated herein above, in accordance with the present invention, the peptide portions of the inventive composition of matter can also be chemically

derivatized at one or more amino acid residues by known organic chemistry techniques. "Chemical derivative" or "chemically derivatized" refers to a subject peptide having one or more residues chemically derivatized by reaction of a functional side group. Such derivatized molecules include, for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups can be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups can be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine can be derivatized to form N-im-benzylhistidine. Also included as chemical derivatives are those peptides which contain one or more naturally occurring amino acid derivatives of the twenty canonical amino acids, whether in L- or D- form. For example, 4-hydroxyproline can be substituted for proline; 5-hydroxylysine canbe substituted for lysine; 3-methylhistidine can be substituted for histidine; homoserine can be substituted for serine; and ornithine can be substituted for lysine.

Useful derivatizations include, in some embodiments, those in which the amino terminal of the peptide is chemically blocked so that conjugation with the vehicle will be prevented from taking place at an N-terminal free amino group. There can also be other beneficial effects of such a modification, for example a reduction in the peptide's susceptibility to enzymatic proteolysis. The N-terminus can be acylated or modified to a substituted amine, or derivatized with another functional group, such as an aromatic moiety (e.g., an indole acid, benzyl (Bzl or Bn), dibenzyl (DiBzl or Bn_2), or benzyloxycarbonyl (Cbz or Z)), *N,N*-dimethylglycine or creatine. For example, in some embodiments, an acyl moiety, such as, but not limited to, a formyl, acetyl (Ac), propanoyl, butanyl, heptanyl, hexanoyl, octanoyl, or nonanoyl, can be covalently linked to the N-terminal end of the peptide, which can prevent undesired side reactions during conjugation of the vehicle to the peptide. Other exemplary N-terminal derivative groups include -NRR^1 (other than $-NH_2$), $-NRC(O)R^1$, $-NRC(O)OR^1$, $-NRS(O)_2R^1$, $-NHC(O)NHR^1$, succinimide, or benzyloxycarbonyl-NH- (Cbz-NH-), wherein R

and R^1 are each independently hydrogen or lower alkyl and wherein the phenyl ring can be substituted with 1 to 3 substituents selected from C_1-C_4 alkyl, C_1-C_4 alkoxy, chloro, and bromo.

In some embodiments, one or more individual amino acid residues can be derivatized. Various derivatizing agents are known to react specifically with selected sidechains or terminal residues, as described in detail below by way of example.

Lysinyl residues and amino terminal residues can be reacted with succinic or other carboxylic acid anhydrides, which reverse the charge of the lysinyl residues. Other suitable reagents for derivatizing alpha-amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues can be modified by reaction with any one or combination of several conventional reagents, including phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginyl residues requires that the reaction be performed in alkaline conditions because of the high pKa of the guanidine functional group. Furthermore, these reagents can react with the groups of lysine as well as the arginine epsilon-amino group.

Specific modification of tyrosyl residues has been studied extensively, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl sidechain groups (aspartyl or glutamyl) can be selectively modified by reaction with carbodiimides (R'-N=C=N-R') such as 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues can be converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginyl residues can be deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are

deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Cysteinyl residues can be replaced by amino acid residues or other moieties either to eliminate disulfide bonding or, conversely, to stabilize cross-linking. (See, e.g., Bhatnagar et al., J. Med. Chem., 39:3814-3819 (1996)).

Other possible modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, oxidation of the sulfur atom in Cys, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains. Creighton, Proteins: Structure and Molecule Properties (W. H. Freeman & Co., San Francisco), 79-86 (1983).

The above examples of derivatizations are not intended to be an exhaustive treatment, but merely illustrative and other eamples are described herein.

The term "B1" means the bradykinin B1 receptor (see, e.g.,Judith M. Hall, A review of BK receptors. Pharmac. Ther., 56:131-190 (1992)). Unless specifically noted otherwise, B1 or bradykinin B1 receptor is intended to mean the human bradykinin B1 receptor (hB1). In certain embodiments, hB1 is the human wild-type receptor. In certain embodiments, hB1 is the bradykinin receptor described in GenBank Accession no. AJ238044.

The term "peptide" as used generally herein refers to molecules of 4 to 40 amino acid residues linked in a chain through peptide bonds, including molecules of 10 to 20 amino acid residues, and molecules of 15 to 18 amino acid residues. The terms "di-peptide" and "tri-peptide" as used herein refer to a molecule, or a portion thereof, 2 and 3 amino acid residues in length, respectively.

In certain instances, structural analysis of protein-protein interaction can also be used to suggest glycopeptides that mimic the binding activity of native protein ligands. In certain embodiments, a crystal structure can suggest the identity and relative orientation of critical residues of a protein ligand such that an analogous glycopeptide can be designed. (See, for example, Takasaki et al., Nature Biotech., Volume 15, pages 1266-1270 (1997)). Certain analytical methods can also be used to investigate the interaction between a receptor protein

and glycosylated peptides, which can suggest further modification of the glycosylated peptide to increase binding affinity.

As used herein, the terms "effective amount" and "therapeutically effective amount" when used with reference to a unglycosylated or glycosylated B1 peptide antagonist refers to an amount or dosage sufficient to produce a desired result (e.g., for therapy with the unglycosylated or glycosylated B1 peptide antagonists, the desired result can be desired reduction in inflammation and/or pain or to support an observable decrease in the level of one or more biological activities of B1). In certain embodiments, a therapeutically effective amount is an amount of the glycosylated peptide sufficient to inhibit, for some period of time, one or more of the clinically defined pathological processes associated with the condition at issue, e.g., inflammation and/or pain, in a subject treated *in vivo* with the agent. The effective amount can vary depending on the specific unglycosylated or glycosylated B1 peptide antagonist selected, and can be dependent on a variety of factors and conditions related to the subject to be treated and the severity of the disorder. For example, if the unglycosylated or glycosylated B1 peptide antagonist is to be administered *in vivo*, factors such as the age, weight and health of the patient as well as dose response curves and toxicity data obtained in preclinical animal work can be considered. If the agent is to be contacted with the cells *in vitro*, one can also design a variety of pre-clinical *in vitro* studies to assess such parameters as uptake, half-life, dose, toxicity, etc. The determination of an effective amount or a therapeutically effective amount for a given agent is within the ability of those skilled in the art.

The term "pharmacologically active" means that a substance so described is determined to have activity that affects a medical parameter or disease state (for example, pain). As used herein, this term typically refers to a B1-induced or B1-mediated disease or abnormal medical condition or disorder, and more specifically, to antagonism of inflammation or pain.

The terms "antagonist", "inhibitor", and "inverse agonist" (Rianne A. F. de Ligt, et. al, British Journal of Pharmacology 2000, 130,131.) refer to a molecule that blocks, impedes, reduces, lessens or in some way interferes with the

biological activity of the associated protein of interest, and the terms are used interchangeably herein. In various embodiments, an "antagonist", "inhibitor" or "inverse agonist" is a molecule that binds to and inhibits B1 with an IC_{50} of 500 nM or less in *in vitro* assays of B1 activity. In various embodiments, an "antagonist" or "inhibitor" is a molecule that binds to and inhibits B1 with an IC_{50} of 100 nM or less in *in vitro* assays of B1 activity. In various embodiments, an "antagonist", "inhibitor", or "inverse agonist is a molecule that binds to and inhibits B1 with an IC_{50} of 50 nM or less in *in vitro* assays of B1 activity and prevents, ameliorates or abolishes pain as measured in at least one generally accepted *in vivo* animal model of pain and/or inhibits biochemical challenges in *in vivo* animal models of edema, inflammation, or pain.

Physiologically acceptable salts of glycosylated peptides are also encompassed herein. By "physiologically acceptable salts" is meant any salts that are known or later discovered to be pharmaceutically acceptable (i.e., useful in the treatment of a warm-blooded animal). Some specific examples are: acetate; trifluoroacetate; hydrohalides, such as hydrochloride and hydrobromide; sulfate; citrate; maleate; tartrate; glycolate; gluconate; succinate; mesylate; besylate; oxalate, tannate, gallic acid ester, and cholesteryl sulfate salts.

Structure of certain glycosylated peptides

In General. In various embodiments, the inventive composition of matter is a glycosylated peptide that can be described by the following formula:

$$F\text{-}[(X^1)\text{–}(Y^1)_n] \qquad (I)$$

wherein:

X^1 and Y^1 are each independently selected peptides of the formula $\text{–}(L^1)_a\text{-}P^1$ and $\text{–}(L^2)_b\text{-}P^2$, respectively;

F is a carbohydrate covalently bound to X^1 or Y^1;

L^1 and L^2, when present (i.e., when the value of "a" and/or "b" is one), are each independently selected peptidyl or non-peptidyl linkers as described herein;

n is 0 to 1; and

P^1 and P^2 , if present (i.e., P^2 is present if the value of "n" is one), are each independently selected peptide antagonists of the bradykinin B1 receptor.

In various embodiments, glycosylated peptides of Formula I will include embodiments wherein P^1 and, if present, P^2 are each independently selected peptide antagonists of the bradykinin B1 receptor having an amino acid sequence selected from SEQ ID NOS:5-62 and analogs and/or derivatives of any of those sequences. If glycosylation is to the N-terminal of X^1, then more particularly useful are SEQ ID NOS:5-9, 11-34, 36, 38-41, 43, 45, 47, 49-51, 53, 55, 57, 59, 61, and analogs and/or derivatives of any of those sequences. Other useful embodiments wherein glycosylation is to the N-terminal of X^1, include SEQ ID NOS:5-9, 11-26, 43, 45, 47, 49-51, 53, 55, 57, 59, 61, and analogs and/or chemical derivatives of any of those sequences, further comprising a N-terminal cysteine.

In various embodiments, glycosylated peptides will include glycosylated conjugates of formula I wherein P^1 and, if present, P^2 is defined by the formula:

NH_2-$a^0a^1a^2a^3a^4a^5a^6a^7a^8a^9a^{10}a^{11}a^{12}a^{13}a^{14}$-COOH

wherein:

a^0 is a basic or neutral aromatic, aliphatic, heterocyclic, or alicyclic amino acid, basic di-peptide, or absent;

a^1, a^2, a^3, and a^4 are independently selected from basic and neutral aromatic, aliphatic, heterocyclic, and alicyclic amino acids;

a^6 is Ser;

a^5, a^7, and a^8 are independently selected from aromatic, aliphatic, heterocyclic, and alicyclic amino acids, provided that at least one of a^5, a^7, and a^8 is independently selected from Chg, Cpg, Igla, Iglb, Niga and Nigb of the D- or L-configuration; and

a^9, a^{10}, a^{11}, a^{12}, a^{13}, and a^{14} are independently selected from any amino acid or are independently absent.

By the phrase "any amino acid" is meant any natural or non-canonical amino acid residue, unless otherwise stated.

In various embodiments, P^1 and, if present, P^2 are defined by the formula:

NH_2-$a^0a^1a^2a^3a^4a^5a^6a^7a^8a^9a^{10}a^{11}a^{12}a^{13}a^{14}$-COOH

wherein:

a^0 is a basic amino acid, basic di-peptide, or absent;

a^1 is a basic amino acid;

a^2 is Pro;

a^3 is Hyp;

a^4 is Gly;

a^5 and a^8 is an Indanyl amino acid;

a^6 is Ser;

a^7 is a D-Indanyl amino acid;

a^8 is Cpg; and

a^9, a^{10}, a^{11}, a^{12}, a^{13}, and a^{14} are independently selected from any amino acid or are independently absent.

In various embodiments, P^1 and, if present, P^2 are defined by the formula:

NH_2-$a^0a^1a^2a^3a^4a^5a^6a^7a^8a^9a^{10}a^{11}a^{12}a^{13}a^{14}$-COOH

wherein:

a^0 is a basic amino acid, or a basic di-peptide, such as, but not limited to, Lys-Lys, D-Lys-Lys, D-Orn-Lys, D-Arg-Lys, or D-Orn-D-Orn, or absent;

a^1 is a basic amino acid;

a^2 is Pro;

a^3 is Hyp;

a^4 is Gly;

a^5 is Cpg;

a^6 is Ser;

a^7 is DTic;

a^8 is Cpg; and

a^9, a^{10}, a^{11}, a^{12}, a^{13}, and a^{14} are independently selected from any amino acid or are independently absent.

As mentioned herein above. The glycosylated peptides of the present invention can also be described by the following formula (II):

F-(L^1-P^1)

wherein:

F is a carbohydrate covalently bound to a linker (L^1); and

P^1 is a peptide antagonist of the bradykinin B1 receptor.

In various embodiments of the glycosylated peptides of Formula II, P^1 is selected from the peptide antagonists of the bradykinin B1 receptor having an amino acid sequence selected from SEQ ID NOS:5-62, and analogs and/or chemical derivatives of any of those sequences.

In various embodiments wherein glycosylation is directly to, or indirectly, through a linker covalently bound to, the N-terminal of P^1, then more particularly useful are SEQ ID NOS:5-9, 11-34, 36, 38-41, 43, 45, 47, 49-51, 53, 55, 57, 59, 61, and analogs and/or chemical derivatives of any of those sequences. Other useful embodiments wherein glycosylation is directly or indirectly to the N-terminal of P^1, include SEQ ID NOS:5-9, 11-26, 43, 45, 47, 49-51, 53, 55, 57, 59, 61, and analogs and/or chemical derivatives of any of those sequences, further comprising a N-terminal cysteine.

In various embodiments, glycosylated peptides include glycoconjugates of formula II wherein P^1 is defined by the formula:

NH_2-$a^0a^1a^2a^3a^4a^5a^6a^7a^8a^9a^{10}a^{11}a^{12}a^{13}a^{14}$-COOH

wherein:

a^0 is a basic or neutral aromatic, aliphatic, heterocyclic, or alicyclic amino acid, basic di-peptide, or absent;

a^1, a^2, a^3, and a^4 are basic or neutral aromatic, aliphatic, heterocyclic, or alicyclic amino acids;

a^6 is Ser;

a^5, a^7, and a^8 are aromatic, aliphatic, heterocyclic, or alicyclic amino acids, provided that at least one of a^5, a^7, and a^8 is selected from Chg, Cpg, Igla, Iglb, Niga and Nigb of the D- or L- configuration; and

a^9, a^{10}, a^{11}, a^{12}, a^{13}, and a^{14} are any amino acid or are independently absent.

In various embodiments, P^1 is defined by the formula: NH_2-$a^0a^1a^2a^3a^4a^5a^6a^7a^8a^9a^{10}a^{11}a^{12}a^{13}a^{14}$-COOH

wherein:

a^0 is a basic amino acid, basic di-peptide or absent;

a^1 is a basic amino acid;

a^2 is Pro;

a^3 is Hyp;

a^4 is Gly;

a^5 and a^8 is an Indanyl amino acid;

a^6 is Ser;

a^7 is a D-Indanyl amino acid;

a^8 is Cpg; and

a^9, a^{10}, a^{11}, a^{12}, a^{13}, and a^{14} are any amino acid or independently absent.

In various embodiments, P^1 is defined by the formula: NH_2-$a^0a^1a^2a^3a^4a^5a^6a^7a^8a^9a^{10}a^{11}a^{12}a^{13}a^{14}$-COOH

wherein:

a^0 is a basic amino acid, or a basic di-peptide, such as but not limited to, Lys-Lys, D-Orn-Lys, D-Lys-Lys, D-Arg-Lys, or D-Orn-D-Orn, or absent;

a^1 is a basic amino acid;

a^2 is Pro;

a^3 is Hyp;

a^4 is Gly;

a^5 is Cpg;

a^6 is Ser;

a^7 is DTic;

a^8 is Cpg; and

a^9, a^{10}, a^{11}, a^{12}, a^{13}, and a^{14} are any amino acid or independently absent.

In various embodiments, glycosylated peptides are provided which bind to and antagonize the activity of bradykinin B1 receptors (B1) and which have demonstrably superior pharmacokinetic properties *in vivo* as compared to unglycosylated peptide B1 antagonists.

In various embodiments, peptide conjugates having amino acid sequences that are fragments (i.e., "subsequences"), analogs, and chemical derivatives of P^1 and, if present, P^2 as defined herein are substantially equivalent with respect to *in*

vitro and/or *in vivo* anti-B1 activity as certain peptide glycoconjugates specifically disclosed herein.

The term "analog" as used herein means molecules representing one or more amino acid substitutions, deletions and/or additions to the linear array of amino acids of the native peptide (unglycosylated P^1 or P^2) or any linker (L) of the glycosylated peptides of Formulas (I) and (II), and which result in similarly glycosylated peptides which are substantially equivalent with respect to *in vitro* and/or *in vivo* anti-B1 activity as compared to at least one glycosylated peptide specifically disclosed herein.

In various embodiments, glycosylated peptide analogs have one or more amino acid substitutions, deletions and/or insertions in the sequence of P^1, P^2, L^1 or L^2. It is generally recognized that conservative amino acid changes are least likely to perturb the structure and/or function of a peptide and generally involve substitution of one amino acid with another that is similar in structure and/or function (e.g., amino acids with side chains similar in size, charge and/or shape). The nature of these substitutions are known to one skilled in the art. Exemplary amino acid substitutions are summarized in Table 1A and Table 2 below. In certain embodiments, when A, F, H, I, L, M, P, V, W, or Y is changed to C, the new cysteine remains as a free thiol.

Table 1A: Amino Acid Substitutions

Basic:
Arg; Lys; His;

Acidic:
Glu; Asp

Polar:
Glu; Asp; Gln; Asn; Ser; Thr

Hydrophilic:
Asp; Glu; Asn; Ser; Thr; Tyr

Hydrophobic:
Ala; Met; Ile; Leu; nor-Leu; Val

Aromatic:
Phe; Trp; Tyr

Small:
Gly; Ala; Ser; Thr; Met

Table 2: Exemplary Amino Acid Substitutions

Amino Acid	Preferred Substitutions	Most Preferred Substitution
Ala	Gly; Leu; Ile; Asn; Pro	Val
Arg	Ala; Asn; Gln; Ser	Lys
Asn	Arg; Gln; His; Lys; Ser; Tyr	Gln
Asp	Asn; Ser; Thr; Gln	Glu
Cys	Ala	Ser
Gln	Ala; Arg; Glu; Leu; Lys; Met; Ser; Tyr	Asn
Glu	Gln; Ser; Thr; Asn	Asp
Gly		Pro
His	Asn; Gln; Lys; Tyr; Phe	Arg
Ile	Tyr; Val; Met; Ala; Phe; nor-Leu	Leu
Leu	nor-Leu; Ile; Val; Met; Ala; Phe	Ile
Lys	Asn; Asp; Ala; Glu; Gln; Ser; Tyr	Arg
Met	Ala; Gln; Tyr; Trp; Phe	Leu
Phe	Leu; Val; Ile; Ala; Met	Leu
Pro	Ile; Val	Gly
Ser	Ala; Asn; Asp; Gly; Lys	Thr
Thr	Ala; Gly; Ile; Val; Lys	Ser
Trp	Phe; Tyr; His	Tyr
Tyr	Trp; Thr; Ser	Phe
Val	Ala; Ile; Met; Phe; Tyr; nor-Leu	Leu

In certain embodiments of the present invention, amino acid substitutions encompass, non-canonical amino acid residues, which include naturally rare (in peptides or proteins) amino acid residues or unnatural amino acid residues. Non-canonical amino acid residues can be incorporated into the peptide by chemical peptide synthesis rather than by synthesis in biological systems, such as recombinantly expressing cells, or alternatively the skilled artisan can employ known techniques of protein engineering that use recombinantly expressing cells. (See, e.g., Link et al., Non-canonical amino acids in protein engineering, Current Opinion in Biotechnology, 14(6):603-609 (2003)). The term "non-canonical amino acid residue" refers to amino acid residues in D- or L-form that are not

among the 20 canonical amino acids generally incorporated into naturally occurring proteins, for example, β-amino acids, homoamino acids, cyclic amino acids and amino acids with derivatized side chains. Examples include (in the L-form or D-form; abbreviated as in parentheses): citrulline (Cit), homocitrulline (hCit), N^{α}-methylcitrulline (NMeCit), N^{α}-methylhomocitrulline, ornithine (Orn), N^{α}-Methylornithine (N^{α}-MeOrn or NMeOrn), sarcosine (Sar), homolysine (hLys or hK), homoarginine (hArg or hR), homoglutamine (hQ), N^{α}-methylarginine (NMeR), N^{α}-methylleucine (N^{α}-MeL or NMeL), *N*-methylhomolysine (NMeHoK), N^{α}-methylglutamine (NMeQ), norleucine (Nle), norvaline (Nva), 1,2,3,4-tetrahydroisoquinoline (Tic), Octahydroindole-2-carboxylic acid (Oic), 3-(1-naphthyl)alanine (1-Nal), 3-(2-naphthyl)alanine (2-Nal), 1,2,3,4-tetrahydroisoquinoline (Tic), 2-indanylglycine (IgI), para-iodophenylalanine (pI-Phe), para-aminophenylalanine (4AmP or 4-Amino-Phe), 4-guanidino phenylalanine (Guf), nitrophenylalanine (nitrophe), aminophenylalanine (aminophe or Amino-Phe), benzylphenylalanine (benzylphe), γ-carboxyglutamic acid (γ-carboxyglu), hydroxyproline (hydroxypro), *p*-carboxyl-phenylalanine (Cpa), α-aminoadipic acid (Aad), Nα-methyl valine (NMeVal), N-α-methyl leucine (NMeLeu), Nα-methylnorleucine (NMeNle), cyclopentylglycine (Cpg), cyclohexylglycine (Chg), acetylarginine (acetylarg), α, β-diaminopropionoic acid (Dpr), α, γ-diaminobutyric acid (Dab), diaminopropionic acid (Dap), cyclohexylalanine (Cha), 4-methyl-phenylalanine (MePhe), β, β-diphenyl-alanine (BiPhA), aminobutyric acid (Abu), 4-phenyl-phenylalanine (or biphenylalanine; 4Bip), α-amino-isobutyric acid (Aib), beta-alanine, beta-aminopropionic acid, piperidinic acid, aminocaprioic acid, aminoheptanoic acid, aminopimelic acid, desmosine, diaminopimelic acid, N-ethylglycine, N-ethylaspargine, hydroxylysine, allo-hydroxylysine, isodesmosine, allo-isoleucine, N-methylglycine, N-methylisoleucine, N-methylvaline, 4-hydroxyproline (Hyp), γ-carboxyglutamate, ε-N,N,N-trimethyllysine, ε-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, ω-methylarginine, and other similar amino acids, and derivatized forms of any of these as described herein. Table 2A contains some additional exemplary non-

canonical amino acid residues that are useful in accordance with the present invention and associated abbreviations as typically used herein, although the skilled practitioner will understand that different abbreviations and nomenclatures may be applicable to the same substance and may appear interchangeably herein.

Table 2A. Useful non-canonical amino acids for amino acid addition, insertion, or substitution into amino acid sequences in accordance with the present invention. In the event an abbreviation listed in Table 2A differs from another abbreviation for the same substance disclosed elsewhere herein, both abbreviations are understood to be applicable.

Abbreviation	Amino Acid
Sar	Sarcosine
Nle	norleucine
Ile	isoleucine
1-Nal	3-(1-naphthyl)alanine
2-Nal	3-(2-naphthyl)alanine
Bip	4,4'-biphenyl alanine
Dip	3,3-diphenylalanine
Nvl	norvaline
NMe-Val	Nα-methyl valine
NMe-Leu	Nα-methyl leucine
NMe-Nle	Nα-methyl norleucine
Cpg	cyclopentyl glycine
Chg	cyclohexyl glycine
Hyp	hydroxy proline
Oic	Octahydroindole-2-Carboxylic Acid
Igl	Indanyl glycine
Aib	aminoisobutyric acid
Aic	2-aminoindane-2-carboxylic acid
Pip	pipecolic acid
BhTic	β-homo Tic
BhPro	β-homo proline
Sar	Sarcosine
Tiq	1,2,3,4-L-Tetrahydroisoquinoline-1-Carboxylic acid
Nip	Nipecotic Acid
Thz	Thiazolidine-4-carboxylic acid
Thi	3-thienyl alanine
4GuaPr	4-guanidino proline
4Pip	4-Amino-1-piperidine-4-carboxylic acid
Idc	indoline-2-carboxylic acid
Hydroxyl-Tic	1,2,3,4-Tetrahydroisoquinoline-7-hydroxy-3-carboxylic acid

Ome-Tyr	O-methyl tyrosine
I-Tyr	Iodotyrosine
Tic	1,2,3,4-L-Tetrahydroisoquinoline-3-Carboxylic acid
Igl	Indanyl glycine
BhTic	β-homo Tic
BhPhe	β-homo phenylalanine
AMeF	α-methyl Phenyalanine
BPhe	β-phenylalanine
Phg	phenylglycine
Anc	3-amino-2-naphthoic acid
Atc	2-aminotetraline-2-carboxylic acid
NMe-Phe	Nα-methyl phenylalanine
NMe-Lys	Nα-methyl lysine
Tpi	1,2,3,4-Tetrahydronorharman-3-Carboxylic acid
4Pal	4-pyridinylalanine
3Pal	3-pyridinylalanine
2Pal	2-pyridinylalanine
hPhe	homophenylalanine
BhTrp	β-homotryptophan
pI-Phe	4-iodophenylalanine
Aic	2-aminoindane-2-carboxylic acid
NMe-Lys	Nα-methyl lysine
Orn	ornithine
Dpr	2,3-Diaminopropionic acid
Dbu	2,4-Diaminobutyric acid
homoLys	homolysine
N-eMe-K	Nε-methyl-lysine
N-eEt-K	Nε-ethyl-lysine
N-eIPr-K	Nε-isopropyl-lysine
bhomoK	β-homolysine
rLys	Lys ψ(CH2NH)-reduced amide bond
rOrn	Orn ψ(CH2NH)-reduced amide bond
Acm	acetamidomethyl
Ahx	6-aminohexanoic acid
ε Ahx	6-aminohexanoic acid
K(NPeg11)	Nε-(O-(aminoethyl)-O'-(2-propanoyl)-undecaethyleneglycol)-Lysine
K(NPeg27)	Nε-(O-(aminoethyl)-O'-(2-propanoyl)-(ethyleneglycol)27-Lysine
Cit	Citrulline
hArg	homoarginine
hCit	homocitrulline
NMe-Arg	Nα-methyl arginine (NMeR)
Guf	4-guanidinyl phenylalanine
bhArg	β-homoarginine
3G-Dpr	2-amino-3-guanidinopropanoic acid
4AmP	4-amino-phenylalanine
4AmPhe	4-amidino-phenylalanine
4AmPig	2-amino-2-(1-carbamimidoylpiperidin-4-

	yl)acetic acid
4GuaPr	4-guanidino proline
N-Arg	Nα-[$(CH_2)_3NHCH(NH)NH_2$] substituted glycine
rArg	Arg ψ(CH2NH) -reduced amide bond
4PipA	4-Piperidinyl alanine
NMe-Arg	Nα-methyl arginine (or NMeR)
NMe-Thr	Nα-methyl threonine(or NMeThr)

An amino acid substitution in an amino acid sequence is typically designated herein with a one-letter abbreviation for the amino acid residue in a particular position, followed by the numerical amino acid position relative to a native sequence of interest, which is then followed by the one-letter symbol for the amino acid residue substituted in. For example, "T30D" symbolizes a substitution of a threonine residue by an aspartate residue at amino acid position 30, relative to the native sequence of interest.

Nomenclature and Symbolism for Amino Acids and Peptides by the UPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN) have been published in the following documents: Biochem. J., 1984, 219, 345-373; Eur. J. Biochem., 1984, 138, 9-37; 1985, 152, 1; 1993, 213, 2; Internat. J. Pept. Prot. Res., 1984, 24, following p 84; J. Biol. Chem., 1985, 260,14-42; Pure Appl. Chem., 1984, 56, 595-624; Amino Acids and Peptides, 1985, 16, 387-410; Biochemical Nomenclature and Related Documents, 2nd edition, Portland Press, 1992, pages 39-69.

Conservative modifications will produce analogs having functional, physical, and chemical characteristics similar to those of the peptide from which such modifications are made. Such conservatively modified forms of the peptides disclosed herein are also contemplated as being an embodiment of the present invention.

In contrast, substantial modifications in the functional and/or chemical characteristics of the peptides can be accomplished by selecting substitutions in the amino acid sequence that differ significantly in their effect on maintaining (a) the structure of the molecular backbone in the region of the substitution, for example, as an α-helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the size of the molecule.

For example, a "conservative amino acid substitution" can involve a substitution of an amino acid residue in a native peptide with an alternative residue such that there is little or no effect on the polarity or charge of the amino acid residue at that position. Furthermore, any native residue in the polypeptide can also be substituted with alanine, as has been previously described for "alanine scanning mutagenesis" (see, for example, MacLennan et al., Acta Physiol. Scand. Suppl., 643:55-67 (1998); Sasaki et al., 1998, Adv. Biophys. 35:1-24 (1998), which discuss alanine scanning mutagenesis).

Amino acid substitutions (whether conservative or non-conservative) can be determined by those skilled in the art at the time such substitutions are desired. For example, in various embodiments, amino acid substitutions can be used to identify important residues of the amino acid sequence, or to increase or decrease the affinity of the glycosylated peptide molecules described herein.

In certain embodiments, conservative or non-conservative amino acid substitutions encompass non-naturally occurring amino acid residues, which are typically incorporated by chemical peptide synthesis.

As noted above, naturally occurring residues can be divided into classes based on common side chain properties:

1) hydrophobic: norleucine (Nor), Met, Ala, Val, Leu, Ile;
2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;
3) acidic: Asp, Glu;
4) basic: His, Lys, Arg;
5) residues that influence chain orientation: Gly, Pro; and
6) aromatic: Trp, Tyr, Phe.

Conservative amino acid substitutions can involve exchange of a member of one of these classes with another member of the same class. Conservative amino acid substitutions can encompass non-naturally occurring amino acid residues, which are typically incorporated by chemical peptide synthesis rather than by synthesis in biological systems. These include peptidomimetics and other reversed or inverted forms of amino acid moieties.

Non-conservative substitutions can involve the exchange of a member of one of these classes for a member from another class. Such substituted residues can be introduced into regions of the peptide. In various embodiments, such substituted residues can be introduced into regions of the peptide that are homologous to non-human orthologs, or into the non-homologous regions of the molecule. In addition, in various embodiments, one can also make modifications using Pro or Gly for the purpose of influencing chain orientation.

In making such modifications, in various embodiments, the hydropathic index of amino acids can be considered. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics, such as: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

The importance of the hydropathic amino acid index in various instances in conferring interactive biological function on a protein is understood in the art. See, e.g., Kyte *et al.*, J. Mol. Biol., 157: 105-131 (1982). In various embodiments, certain amino acids can be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity. In various embodiments, in making changes based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within ±2 can be used, those which are within ±1 can be used, or those within ±0.5 can be used.

It is also understood in the art that, in certain instances, the substitution of like amino acids can be made effectively on the basis of hydrophilicity. In various instances, the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, i.e., with a biological property of the protein.

The following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4);

proline (-0.5 ± 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4). In various embodiments, in making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within ±2 can be used, those within ±1 can be used, and those within ±0.5 can be used. In various embodiments, one can identify epitopes from primary amino acid sequences on the basis of hydrophilicity. These regions are also referred to as "epitopic core regions."

In various embodiments, a skilled artisan will be able to determine suitable analogs of the glycosylated peptides set forth herein using well known techniques. One skilled in the art would also know, in various embodiments, that one can substitute chemically similar amino acids for residues occurring in the native peptide while retaining activity (conservative amino acid residue substitutions). Therefore, in various embodiments, even areas that can be important for biological activity and/or for structure can be subject to conservative amino acid substitutions without destroying the biological activity and/or without adversely affecting the glycosylated peptide structure.

Additionally, in various embodiments, one skilled in the art can review structure-function studies identifying residues within the (P) and/or (L) sequences that are important for activity and/or structure. In view of such a comparison, in various embodiments, one can predict the importance of amino acid residues in an amino acid sequence. In various embodiments, one skilled in the art can opt to substitute chemically similar amino acids for certain amino acid residues of (P) and/or (L) regions of the glycosylated peptides.

A number of scientific publications have been devoted to the prediction of secondary structure. See, for example, Moult J., Curr. Op. in Biotech., 7(4):422-427 (1996), Chou et al., Biochemistry, 13(2):222-245 (1974); Chou et al., Biochemistry, 113(2):211-222 (1974); Chou et al., Adv. Enzymol. Relat. Areas Mol. Biol., 47:45-148 (1978); Chou et al., Ann. Rev. Biochem., 47:251-276 and Chou et al., Biophys. J., 26:367-384 (1979). Moreover, computer programs are currently available to assist with predicting secondary structure.

Additional methods of predicting secondary structure include, but are not limited to, "threading" (Jones, D., Curr. Opin. Struct. Biol., 7(3):377-87 (1997); Sippl et al., Structure, 4(1): 15-9 (1996)), "profile analysis" (Bowie et al., Science, 253:164-170 (1991); Gribskov et al., Meth. Enzym., 183:146-159 (1990); Gribskov et al., Proc. Nat. Acad. Sci., 84(13):4355-8 (1987)), and "evolutionary linkage" (See Home, supra, and Brenner, supra).

In various embodiments, glycosylated peptide derivatives will be useful for the same purposes for which the analogous glycosylated peptides specifically disclosed herein are useful (i.e., antagonists of B1 activity *in vitro* and/or *in vivo*).

Certain Peptides. The amino acid sequences P^1 and, if present, P^2 (collectively referred to as P) within the glycosylated peptides include, as mentioned, peptides that bind to and antagonize (i.e., decrease) the activity of B1. In various embodiments, glycosylated B1 peptides comprise at least one peptide selected from the peptides as shown in SEQ ID NOS: 5-62 (see Table 3 and Table 4), or an analog and/or a chemical derivative of any one of these sequences. In some embodiments X^1 comprises a peptide (P^1 and/or P^2) having an amino acid sequence selected from SEQ ID NOS: 5-9, 11-34, 36, 38-41, 43, 45, 47, 49-51, 53, 55, 57, 59, and 61, or an analog and/or a chemical derivative of any of these. In other embodiments, X^1 comprises a peptide (P^1 and/or P^2) having an amino acid sequence selected from SEQ ID NOS: 5-9, 11-34, 36, 38-41, 43, 45, 47, 49-51, 53, 55, 57, 59, and 61, or an analog and/or a chemical derivative of any of these, or SEQ ID NOS:5-9, 11-26, 43, 45, 47, 49-51, 53, 55, 57, 59, 61 or an analog and/or a chemical derivative of any of these, which further comprises a N-terminal cysteine residue.

In various embodiments, the synthesis of peptides described herein, including preparation of appropriate amino acid chemical derivatives, their activation and coupling to form peptides and methods for purification of peptides and determination of their purity are included in the general body of knowledge of peptide chemistry. Solution phase of peptides is generally described, e.g., in Houben-Weyl "Methoden der Organischen Chemie" Vol. 16, parts I & II, (1974).

For synthesis by the solid phase method, suitable techniques are also known in the art as described in greater detail below. In various instances, a chemist skilled in the art of peptide synthesis is able to synthesize the described peptides by standard solution methods or by manual or automatic solid phase methods. In various embodiments, solid phase synthesis is used for making individual peptides because of its cost-effectiveness.

For example, solid phase peptide synthesis techniques can be used. Such techniques are well known in the art and include but are not limited to, those described in Merrifield (1973), Chem. Polypeptides, 335-361 (Katsoyannis and Panayotis eds.); Merrifield (1963), J. Am. Chem. Soc., 85:2149; Davis et al. (1985), Biochem. Intl., 10:394-414; Stewart and Young (1969), Solid Phase Peptide Synthesis; U.S. Pat. No. 3,941,763; Finn et al. (1976), The Proteins (3rd ed.) 2:105-253; and Erickson et al., The Proteins (3rd ed.) 2: 257-527 (1976). The use of protecting groups, linkers, and solid phase supports, as well as specific protection and deprotection reaction conditions, linker cleavage conditions, use of scavengers, and other aspects of solid phase peptide synthesis are well known and are also described in "Protecting Groups in Organic Synthesis," 3rd Edition, T. W. Greene and P. G. M. Wuts, Eds., John Wiley & Sons, Inc., 1999; NovaBiochem Catalog, 2000; "Synthetic Peptides, A User's Guide," G. A. Grant, Ed., W.H. Freeman & Company, New York, N.Y., 1992; "Advanced Chemtech Handbook of Combinatorial & Solid Phase Organic Chemistry," W. D. Bennet, J. W. Christensen, L. K. Hamaker, M. L. Peterson, M. R. Rhodes, and H. H. Saneii, Eds., Advanced Chemtech, 1998; "Principles of Peptide Synthesis, 2nd ed.," M. Bodanszky, Ed., Springer-Verlag, 1993; "The Practice of Peptide Synthesis, 2nd ed.," M. Bodanszky and A. Bodanszky, Eds., Springer-Verlag, 1994; "Protecting Groups," P. J. Kocienski, Ed., Georg Thieme Verlag, Stuttgart, Germany, 1994; "Fmoc Solid Phase Peptide Synthesis, A Practical Approach," W. C. Chan and P. D. White, Eds., Oxford Press, 2000, G. B. Fields et al., Synthetic Peptides: A User's Guide, 1990, 77-183, and elsewhere.

Typically, linear and cyclic peptides are synthesized using Fmoc solid-phase peptide synthesis (SPPS) methodologies on a commercially available

synthesizer, such as a *Symphony* automated synthesizer (Protein Technologies, Inc., Washington, DC) or a *Liberty* microwave assisted automated synthesizer (CEM Corporation, Matthews, NC). Protected derivatives of conical amino acids, Fmoc-Dpr(Mtt)-OH, Fmoc-Dab(Mtt)-OH, and Fmoc-Cit-OH can be purchased from EMD Biosciences, Inc. (La Jolla, CA). Fmoc-homoArg(Pmc)-OH can be purchased from Bachem California, Inc. (Torrance, CA). All other non-conical Fmoc-amino acids can be purchased from either Advanced Chemtech (Louisville, KY) or Chem-Impex International, Inc. (Wood Dale, IL). The coupling reagents 2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-Benzotriazoyloxytris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) can be purchased from Matrix Innovation, Inc. (Montreal, Quebec Canada). N-Methyl Pyrrolidone (NMP), dichloromethane (DCM), methanol (MeOH), acetonitrile (ACN), isopropanol, dimethylsulfoxide (DMSO), and anhydrous ethyl ether can be purchased from VWR International (West Chester, PA). N,N-dimethylformamide (DMF) is purchased from EMD Biosciences. Trifluoroacetic acid (TFA), N-ethylmorpholine (NEM), pyridine, piperidine, N-N-diisopropylethylamine (DIEA), triisopropylsilane (Tis), phenol, acetic anhydride, and 0.1% TFA in H_2O are purchased from Sigma-Aldrich (St. Louis, MO). All solvents and reagents are preferably ACS grade or better and can be used without further purification. Peptides are assembled on CLEAR-amide-MBHA resin (0.44 meq/g substitution), purchased from Peptides International (Louisville, KY). Typically, the syntheses are performed using 16 mL polypropylene reaction vessels fitted with course frits (Protein Technologies). Approximately 455 mg resin (0.2 mmole) is added to each reaction vessel and solvated for 10 min in DMF. The growing peptide chains are assembled on the amide-resin starting from the C-terminus using the general amino acid cycle as follows: The N^{α}-Fmoc groups are removed by addition of 5 mL 20% piperidine in DMF for 5 min, followed by a 20 min 5 mL incubation. Amino acids (3-fold molar excess) are added to the resin (3000 µL of 0.2 M amino acid solution in NMP), followed by the addition of 3-fold excess HBTU and 6-fold excess NEM (1.2 mL of 0.5 M HBTU & 1.0 M NEM in DMF). The mixture is agitated by

periodic sparging with nitrogen for 45 min, followed by emptying of the reaction vessel by positive nitrogen pressure. The resin is washed with 5 mL of DMF (4 x 30 sec). A second coupling reaction is repeated for 30 min, the reaction vessel emptied, and the N^{α}-Fmoc-protected peptide-resin is washed with 5 mL DMF (3 x 30 sec) and 5 mL DCM (2 x 30 sec). The amino acid coupling cycle is repeated with required amino acids until the desired peptide is assembled. Following N^{α}-Fmoc deprotection of the final amino acid, acetylation of the N^{α}-amine is performed by addition of 2.5 mL acetic anhydride/DIEA solution (1.0 M in DMF) to the reaction vessels and mixed for 30 min. If peptides do not require cyclization, the acetylated peptide-resin is washed with 5 mL DCM (5 x 30 sec) and dried thoroughly prior to cleavage from the resin and removal of side chain protecting groups. Deprotection of the amino acid side chains and cleavage of the acetylated-peptide from the resin is performed by incubating the peptide-resin with 15mL cleavage cocktail (92.5% TFA, 2.5% water, 2.5% Tis, 2.5% phenol) for 3 hr. The cleavage product is filtered under positive nitrogen gas pressure into tarred 50 mL polypropylene conical tubes. The resin is washed with 10 mL cleavage cocktail for 5 min, filtered, and the filtrates combined. The cleavage solutions are concentrated to approximately 5 mL total volume under a gentle stream of nitrogen. Cold (-20°C) anhydrous ethyl ether (up to 50 mL) is added to the filtrates. The flocculent peptides are pelleted by centrifugation (e.g., Eppendorf centrifuge 5702 using a swinging bucket rotor) at 3800 rpm for 5 min and the ether is decanted. The peptide pellets are washed with additional cold anhydrous ethyl ether (up to 50 mL), pelleted by centrifugation, decanted, and dried *in vacuo*. The crude peptide yields typically range from 60% to 99% of the theoretical yields. Crude peptides are dissolved in DMSO and purified by RP-HPLC using, e.g., an Agilent 1100 preparative chromatography system with a photodiode array detector (Agilent Technologies, Inc., Santa Clara , CA) and a preparative RP-HPLC bonded silica column (Phenomenex Jupiter C18(2), 300Å, 10 μM, 50 x 250 mm) and lyophilized to form amorphous solids. The purified peptides are preferably at least >95% pure as determined by the analytical RP-HPLC using a linear gradient of 2-60% B over 60 min (A=0.1% TFA in H_2O,

B=0.1% TFA in CAN, column=Phenomenex Jupiter Proteo, 90Å, 4 μM, 2.1 x 50 mm). Correct molecular mass can be confirmed by LC-MS methodologies using, e.g., a Waters Acquity UPLC equipped with a LCT Premier XE orthogonal acceleration time-of-flight (oa-TOF) benchtop mass spectrometer (Waters Corporation, Milford, MA). Peptides cyclized with a lactam bridge are prepared by selectively incorporating residues with nucleophilic side chains that will form the lactam protected with 4-methyltrityl (Mtt) and electrophilic side chains with 2-phenyl-isopropyl (2-PhiPr). Amino acids involved in the cyclizations are purchased from EMD Biosciences, Inc. All other amino acids used in the syntheses are preferably standard t-butyl, pentamethyldihydrobenzofuran-5-sulfonyl, or pentamethylchroman-6-sulfonyl side-chain protected Fmoc amino acids. Mtt and 2-PhiPr groups are selectively removed from cyclic peptide antagonists by addition of 10 mL TFA/Tis/DCM (0.3:0.5:9.2) to the peptide-resin (5 x 10 min), followed by washing the peptide-resin with 10 mL of a 2% DIEA solution (2 x 2 min) and 10 mL DCM (4 x 1 min). The lactam bridge in cyclic peptides are formed by activating electophilic carboxyl groups with 5-fold excess PyBOP and 7-fold excess DIEA in DMF. The mixtures are agitated with continuous sparging with nitrogen. Reactions are monitored by cleavage of a 1-2 mg aliquot of peptide-resin in 400 μL of cleavage cocktail, followed by filtration, concentration of the filtrate under nitrogen, and analyzing by LC-MS methodologies previously described for linear peptides. Lactam formation ranges from 70-99% after approximately 1-4 days at room temperature. The peptide-resin is washed with DMF, acetylated, and washed again with DCM following the cyclization reactions and thoroughly dried *in vacuo* prior to cleavage. Cyclic peptide-resins are cleaved, purified, and analyzed in the same manner as described for linear peptides. The preceding methods are merely illustrative, and the skilled artisan is aware of various other methods and technical variations for synthesizing the peptides.

In some embodiments, recombinant DNA- and/or RNA-mediated protein expression techniques are applicable to preparing the peptide. Non-canonical amino acid residues can be incorporated into the amino acid sequence by the

skilled artisan employing known techniques of protein engineering that use recombinantly expressing cells. (See, e.g., Link et al., Non-canonical amino acids in protein engineering, Current Opinion in Biotechnology, 14(6):603-609 (2003)). For example, the peptides can be made in transformed host cells. Briefly, a recombinant DNA molecule, or construct, coding for the peptide is prepared. Methods of preparing such DNA molecules are well known in the art. For instance, sequences encoding the peptides can be excised from DNA using suitable restriction enzymes. Any of a large number of available and well-known host cells may be used in the practice of this invention. The selection of a particular host is dependent upon a number of factors recognized by the art. These include, for example, compatibility with the chosen expression vector, toxicity of the peptides encoded by the DNA molecule, rate of transformation, ease of recovery of the peptides, expression characteristics, bio-safety and costs. A balance of these factors must be struck with the understanding that not all hosts may be equally effective for the expression of a particular DNA sequence. Within these general guidelines, useful microbial host cells in culture include bacteria (such as Escherichia coli sp.), yeast (such as Saccharomyces sp.) and other fungal cells, insect cells, plant cells, mammalian (including human) cells, e.g., CHO cells and HEK293 cells. Modifications can be made at the DNA level, as well. The peptide-encoding DNA sequence may be changed to codons more compatible with the chosen host cell. For E. coli, optimized codons are known in the art. Codons can be substituted to eliminate restriction sites or to include silent restriction sites, which may aid in processing of the DNA in the selected host cell. Next, the transformed host is cultured and purified. Host cells may be cultured under conventional fermentation conditions so that the desired compounds are expressed. Such fermentation conditions are well known in the art.

Table 3. Bradykinin Peptides

Receptor/Effect	Peptide (SEQ ID NO)				Amino acid sequence								
B2/B1 Agonist	Bradykinin, BK (SEQ ID NO:1)					Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe Arg
B2 Agonist	Kallidin, Lys-BK (SEQ ID NO:2)			Lys		Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe Arg
B2 Agonist	Met-Lys-BK (SEQ ID NO:3)		Met	Lys		Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe Arg
B1 Agonist	des-Arg-BK (SEQ ID NO:4)					Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe
B1 Antagonist	[Leu8]-Des-Arg9-BK (SEQ ID NO:5)					Arg	Pro	Pro	Gly	Phe	Ser	Pro	Leu
B1 Antagonist	DALK (SEQ ID NO:6)			Lys		Arg	Pro	Pro	Gly	Phe	Ser	Pro	Leu
B2 Antagonist	(SEQ ID NO:7)			DArg		Arg	Pro	Hyp	Gly	Thi	Ser	DTic	Oic Arg
B1/B2 Antagonist	(SEQ ID NO:8)			DArg		Arg	Pro	Hyp	Gly	Thi	Ser	DTic	Oic
B2 Antagonist	(SEQ ID NO:9)			DArg		Arg	Pro	Hyp	Gly	Thi	Ser	DHpe	Oic Arg
B1-antagonist	(SEQ ID NO:10)	Ac	Lys	Lys		Arg	Pro	Pro	Gly	Mc-Phe	Ser	D-β-Nal	Ile
B1/B2 Antagonist	(SEQ ID NO:11)			DArg		Arg	Pro	Hyp	Gly	Igl	Ser	DIgl	Oic Arg
B1 Antagonist	(SEQ ID NO:12)		Lys	Lys		Arg	Pro	Hyp	Gly	Igl	Ser	DIgl	Oic
B1 Antagonist	(SEQ ID NO:13)		Lys	Lys		Arg	Pro	Hyp	Gly	Cpg	Ser	DTic	Cpg
B1/B2 Antagonist	(SEQ ID NO:14)			DArg		Arg	Pro	Hyp	Gly	Igl	Ser	Df5f	Igl Arg
B1 Antagonist	(SEQ ID NO:15)		DOrn	Lys		Arg	Pro	Hyp	Gly	Cpg	Ser	DTic	Cpg
B1 Antagonist	(SEQ ID NO:16)		DOrn	Lys		Arg	Pro	Thz	Gly	Cpg	Ser	DTic	Cpg
B1 Antagonist	(SEQ ID NO:17)		3Pal	Lys		Arg	Pro	Hyp	Gly	Cpg	Ser	DTic	Cpg
B1 Antagonist	(SEQ ID NO:18)		4Pal	Lys		Arg	Pro	Hyp	Gly	Cpg	Ser	DTic	Cpg
B1 Antagonist	(SEQ ID NO:19)			Cha		Arg	Pro	Hyp	Gly	Cpg	Ser	DTic	Cpg
B1 Antagonist	(SEQ ID NO:20)			2-Nal		Arg	Pro	Hyp	Gly	Cpg	Ser	DTic	Cpg
B1 Antagonist	(SEQ ID NO:21)			Lys		Arg	Pro	Hyp	Gly	Cpg	Ser	DTic	Cpg
B1 Antagonist	(SEQ ID NO:22)		DLys	Lys		Arg	Pro	Hyp	Gly	Cpg	Ser	DTic	Cpg
B1 Antagonist	(SEQ ID NO:23)		Lys	DOrn		Arg	Pro	Hyp	Gly	Cpg	Ser	DTic	Cpg
B1 Antagonist	(SEQ ID NO:24)		Lys	Cha		Arg	Pro	Hyp	Gly	Cpg	Ser	DTic	Cpg
B1 Antagonist	(SEQ ID NO:25)		Lys	Abu		Arg	Pro	Hyp	Gly	Cpg	Ser	DTic	Cpg
B1 Antagonist	(SEQ ID NO:26)		Lys	2-Nal		Arg	Pro	Hyp	Gly	Cpg	Ser	DTic	Cpg
B1 Antagonist	(SEQ ID NO:43)		D-Dab	Lys		Arg	Pro	Hyp	Gly	Cpg	Ser	DTic	Cpg
B1 Antagonist	(SEQ ID NO:44)	Ac	D-Dab	Lys		Arg	Pro	Hyp	Gly	Cpg	Ser	DTic	Cpg
B1 Antagonist			DOrn	Lys		Arg	Pro	Hyp	Gly	Cpg	Ser	DTic	Cpg

	(SEQ ID NO:45)											
B1 Antagonist	(SEQ ID NO:46)	Ac	DOrn	Lys	Arg	Pro	Hyp	Gly	Cpg	Ser	DTic	Cpg
B1 Antagonist	(SEQ ID NO:47)		D-3'Pal	Lys	Arg	Pro	Hyp	Gly	Cpg	Ser	DTic	Cpg
B1 Antagonist	(SEQ ID NO:48)	Ac	D-3'Pal	Lys	Arg	Pro	Hyp	Gly	Cpg	Ser	DTic	Cpg
B1 Antagonist	(SEQ ID NO:49)		D-Lys	D-2-Nal	Arg	Pro	Hyp	Gly	Cpg	Ser	DTic	Cpg
B1 Antagonist	(SEQ ID NO:50)		Lys	D-2-Nal	Arg	Pro	Hyp	Gly	Cpg	Ser	DTic	Cpg
B1 Antagonist	(SEQ ID NO:51)			DOrn	Arg	Oic	Pro	Gly	Me-Phe	Ser	D-β-Nal	Ile
B1 Antagonist	(SEQ ID NO:52	Ac		DOrn	Arg	Oic	Pro	Gly	Me-Phe	Ser	D-β-Nal	Ile
B1 Antagonist	(SEQ ID NO:53)		DOrn	Lys	Arg	Oic	Pro	Gly	Me-Phe	Ser	D-β-Nal	Ile
B1 Antagonist	(SEQ ID NO:54)	Ac	DOrn	Lys	Arg	Oic	Pro	Gly	Me-Phe	Ser	D-β-Nal	Ile
B1 Antagonist	(SEQ ID NO:55)			Lys	Arg	Pro	Pro	Gly	Phe	Ser	D-β-Nal	Ile
B1 Antagonist	(SEQ ID NO:56)	Ac		Lys	Arg	Pro	Pro	Gly	Phe	Ser	D-β-Nal	Ile
B1 Antagonist	(SEQ ID NO:57)			Orn	Arg	Oic	Pro	Gly	Me-Phe	Ser	D-β-Nal	Ile
B1 Antagonist	(SEQ ID NO:58)	Ac		Orn	Arg	Oic	Pro	Gly	Me-Phe	Ser	D-β-Nal	Ile
B1 Antagonist	(SEQ ID NO:59)			Lys	Arg	Oic	Pro	Gly	Me-Phe	Ser	D-β-Nal	Ile
B1 Antagonist	(SEQ ID NO:60)	Ac		Lys	Arg	Oic	Pro	Gly	Me-Phe	Ser	D-β-Nal	Ile
B1 Antagonist	(SEQ ID NO:61)		D-Arg	Lys	Arg	Pro	Hyp	Gly	Cpg	Ser	DTic	Cpg
B1 Antagonist	(SEQ ID NO:62)	Ac	D-Arg	Lys	Arg	Pro	Hyp	Gly	Cpg	Ser	DTic	Cpg

Certain Vehicles. In certain embodiments, a "carbohydrate" or "sugar" prevents or reduces degradation and/or increases half-life, reduces toxicity, reduces immunogenicity, and/or increases biological activity of a therapeutic peptide or protein. In various embodiments, a B1 peptide is conjugated to an amino acid or linker that has been glycosylated by any of a wide variety of carbohydrate moieties. Certain carbohydrate starting materials that can be used to glycosylate an amino acid or linker are known in the art. Exemplary starting materials include, but are not limited to, 2,3-desoxy-2,3- dehydroglucose, 2,3-desoxy-2,3-dehydroglucose diacetate, glucoside, glucoside tetraacetate, mannoside, mannoside tetraacetate, galactoside, galactoside tetraacetate, alloside, alloside tetraacetate, guloside, guloside tetraacetate, idoside, idoside tetraacetate, taloside, taloside tetraacetate, rhamnoside, rhamnoside triacetate, maltoside, maltoside heptaacetate, 2,3-desoxy-2,3-dehydromaltoside, 2,3-desoxy-2,3-dehydromaltoside pentaacetate, 2,3-desoxymaltoside, lactoside, lactoside

tetraacetate, 2,3-desoxy-2,3-dehydrolactoside, 2,3-desoxy-2,3- dehydrolactoside pentaacetate, 2,3-desoxylactoside, glucouronate, and N- acetylglucosamine.

In various embodiments; at least one carbohydrate (F) is attached to an amino acid or a linker that is covalently fused to (P).

In various embodiments, the glycosylated molecules include peptides that are modified from a native peptide within the regions denoted by (X^1)-$(Y^1)_n$ (as defined supra.) to form an analog of the native peptide, provided that antagonism of B1 is substantially maintained.

In various embodiments, as between the unglycosylated native peptide and the glycosylated peptide version in various embodiments, no more than three non-terminal residues in the (P) region are different. In various embodiments, a molecule includes up to two amino acid substitutions, insertions, and/or deletions at any particular non-terminal locus of the (P) region of the unglycosylated or glycosylated peptide compared to the native peptide. In various embodiments, the divergence in sequence between a native peptide and a contemplated analog thereof, particularly in the specified (P) region, is in the form of one or more "conservative modifications".

Certain Linkers. Any "linker" group is optional and is absent in some embodiments (i.e., in Formula I, the value of "a" and/or "b" is zero). When present (i.e., in Formula I, the value of "a" and/or "b" is one), the linker's chemical structure is not critical, since it serves primarily as a spacer, which can be useful in optimizing pharamcologial activity of some embodiments of the inventive composition. The linker is preferably made up of amino acids linked together by peptide bonds. The linker moiety, if present, can be independently the same or different from any other linker, or linkers, that can be present in the inventive composition. The term "linker" as used herein refers to L^1 and, if present, L^2 as shown in either Formula I or II (supra.) and is collectively abbreviated herein by "(L)" or "L".

In various embodiments, (L) is peptidyl in nature (i.e., made up of amino acids linked together by peptide bonds) and comprises from 1 to about 30 amino

acids. This range and all ranges in this application include the endpoints and all values between the endpoints. The amino acids are independently selected from the twenty naturally occurring amino acids. In various embodiments, the 1 to about 30 amino acids of the linker are independently selected from cysteine, glycine, alanine, proline, asparagine, glutamine, and lysine. In various embodiments, a linker is made up of a majority of amino acids that are sterically unhindered, such as glycine and alanine. In various embodiments, a linker can contain a N-terminal cysteine, and/or other thiol, and/or other nucleophile for conjugation with a carbohydrate. In various embodiments, a linker contains an N-terminal cysteine and/or homocysteine residue, and/or other moiety for conjugation to a carbohydrate. Exemplary linkers include, but are not limited to, poly$(Gly)_{1-8}$, $(Gly)_3$ (SEQ ID NO:63), $(Gly)_5$ (SEQ ID NO:64), $(Gly)_7$ (SEQ ID NO:65), poly$(Gly\text{-}Ala)_{2-4}$ and poly$(Ala)_{1-8}$. Other specific examples of linkers include, but are not limited to, $(Gly)_5Lys$ (SEQ ID NO:66), $(Gly)_5LysArg$ (SEQ ID NO:67), $Cys(Gly)_5$ (SEQ ID NO:68), $Cys(Gly)_5Lys$ (SEQ ID NO:69). Certain other combinations of Gly and Ala can be used. To explain the above nomenclature, for example, $(Gly)_5Lys$ means Gly-Gly-Gly-Gly-Gly-Lys (SEQ ID NO:66). It is also desirable that, if present, a peptidyl linker be selected that avoids rapid proteolytic turnover in circulation *in vivo*. Some of these peptidyl linkers, amino acids can be glycosylated, as is well understood by those in the art. For example, a useful linker sequence constituting a sialylation site is $X_1X_2NX_4X_5G$ (SEQ ID NO:70), wherein X_1, X_2, X_4 and X_5 are each independently any amino acid residue.

Another useful peptidyl linker is a large, flexible linker comprising a random Gly/Ser/Thr sequence, for example: GSGSATGGSGSTASSGSGSATH (SEQ ID NO:71) or HGSGSATGGSGSTASSGSGSAT (SEQ ID NO:72), that is estimated to be about the size of a 1 kDa PEG molecule. Alternatively, a useful peptidyl linker can be comprised of amino acid sequences known in the art to form rigid helical structures (e.g., Rigid linker: -AEAAAKEAAAKEAAAKAGG-) (SEQ ID NO:73). Additionally, a peptidyl linker can also comprise a non-peptidyl segment such as a 6 carbon aliphatic

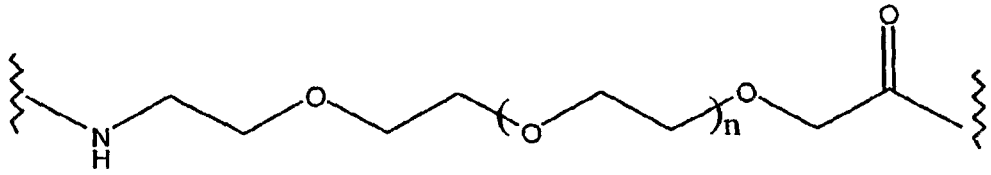
molecule of the formula -CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-. The peptidyl linkers can be altered to form chemical derivatives as described herein.

The linkers shown herein are exemplary; peptidyl linkers within the scope of this invention can be much longer and can include other residues. For example, other useful examples of peptidyl linkers include any of those disclosed in published International Patent Application No. WO 2006/116156 A2 ("Toxin Peptide Therapeutic Agents"), which is incorporated herein by reference. A peptidyl linker can contain, e.g., a N-terminal cysteine, another thiol, or nucleophile for conjugation with a vehicle. In another embodiment, the linker contains an N-terminal cysteine or homocysteine residue, or other 2-amino-ethanethiol or 3-amino-propanethiol moiety for conjugation to maleimide, iodoacetaamide or thioester, functionalized vehicles.

In various embodiments, the linker moiety, if present, comprises a non-peptide such as a polyethylene glycol (PEG), polymethylenes, or an alpha-alkoxyacetate; or $-(CH_2)_z-$, wherein z = 1 to about 30; or $-(CH_2CH_2O)_a-$ wherein a = 1 to about 9. Other useful linker groups for purposes of the invention, include aliphatic (ring or otherwise), heteroaliphatic (ring or otherwise) whose function is to creast a variable spacing element that does not adversely affect pharmacokinetic properties of the molecule or its receptor potency. For example, alkyl linkers such as -NH-$(CH_2)_s$-C(O)-, wherein s = 2-20 can be used. These alkyl linkers can further be substituted by any non-sterically hindering group such as lower alkyl (e.g., C_1-C_6) lower acyl, halogen (e.g., Cl, Br), CN, NH_2, phenyl, etc.

By "polyethylene glycol" or "PEG" is meant a polyalkylene glycol compound or a derivative thereof, with or without coupling agents or derivatization with coupling or activating moieties (e.g., with aldehyde, hydroxysuccinimidyl, hydrazide, thiol, triflate, tresylate, azirdine, oxirane, orthopyridyl disulphide, vinylsulfone, iodoacetamide or a maleimide moiety). In accordance with the present invention, useful PEG includes substantially linear, straight chain PEG, branched PEG, or dendritic PEG. (See, e.g., Merrill, US Patent No. 5,171,264; Harris et al., Multiarmed, monofunctional, polymer for

coupling to molecules and surfaces, US Patent No. 5,932,462; Shen, N-maleimidyl polymer derivatives, US Patent No. 6,602,498). An exemplary non-peptidyl linker is a PEG linker,

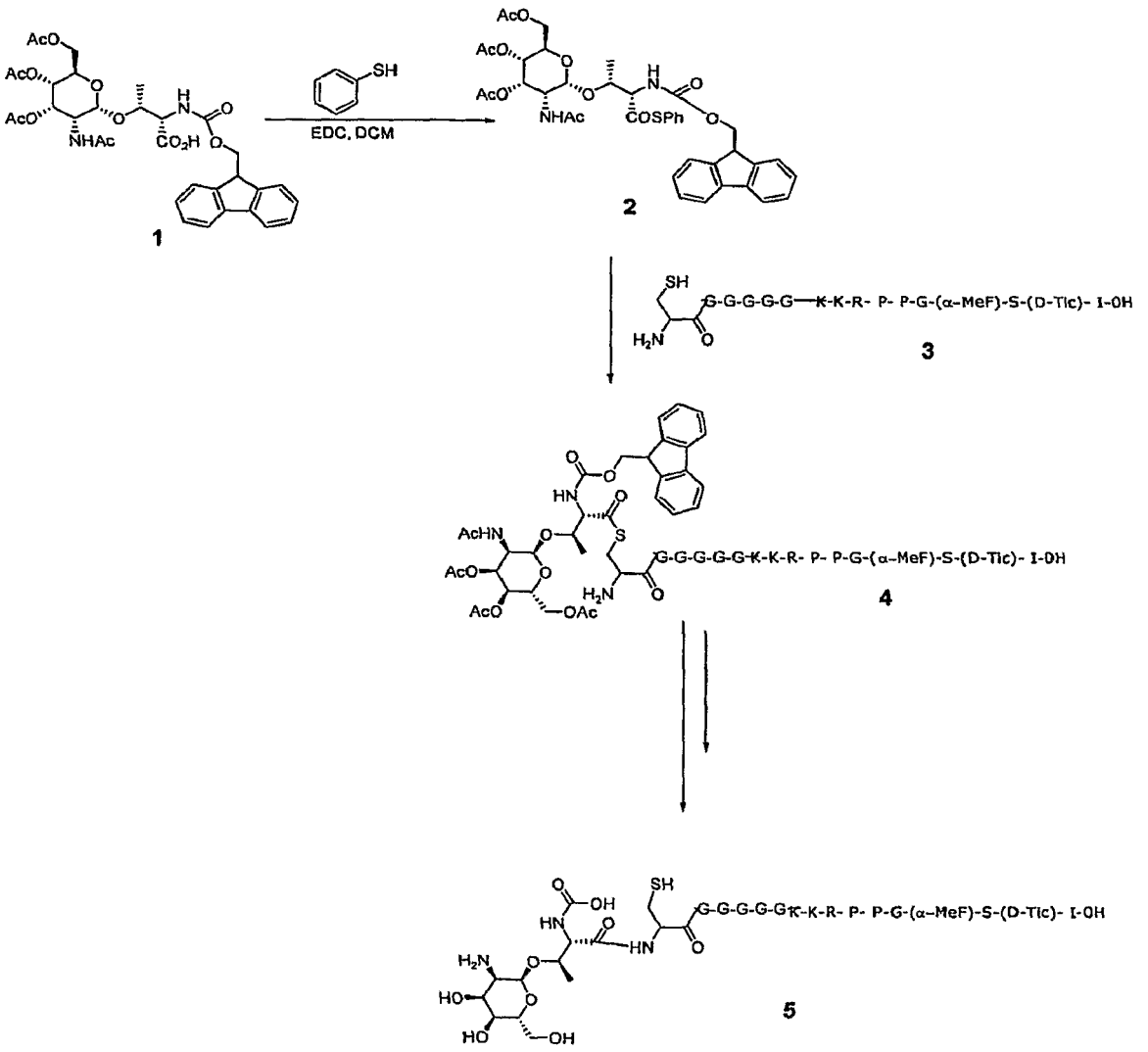


(III)

wherein n is such that the linker has a molecular weight of 100 to 5000 kDa, preferably 100 to 500 kDa. Useful linker embodiments also include aminoethyloxyethyloxy-acetyl linkers as disclosed by Chandy et al. (Chandy et al., WO 2006/042151 A2, incorporated herein by reference in its entirety).

In still other embodiments the linker moiety comprises a peptide of 1 to 30 amino acids conjugated at its N-terminus to a non-peptide such as a PEG, polymethylene, or a alpha-alkoxyacetate. As an example of a glycosylated peptide according to various embodiments, the 10 amino acid-containing B1 peptide antagonist (NH_2-K-K-R-P-P-G-(α-MeF)-S-(D-Tic)-I-OH)(SEQ ID NO:75) is conjugated to a linker NH_2-C-G-G-G-G-G-OH (SEQ ID NO:74) to form the 16 amino acid containing peptide (NH_2-C-G-G-G-G-G-K-K-R-P-P-G-(α-MeF)-S-(D-Tic)-I-OH) (SEQ ID NO:76) which is then reacted with a glycosylated amino acid or peptide thioester to form a transient intermediate thioester conjugate which rapidly rearranges to give a glyclosylated amino acid product (Scheme 1). This native ligation reaction is known in the art for assembling peptides and proteins (see, e.g., Kent, et al. Journal of the American Chemical Society, 121(50):11684-11689 (1999); Current Opinion in Chemical Biology 3(6):665-67 (1999).

Scheme 1.



Exemplary glycosylated amino acids useful in this strategy include, but are not limited to, those commercially available from vendors such as INBIOS (Seattle, WA), Chem–Impex (Wood Dale, IL), and Bachem (Torrance , CA). Examples of commercially available glycosylated amino acids include, but are not limited to, the following: -Fmoc-O-(2,3,4,6-tetra-O-acetyl-α -D-mannopyranosyl)-L-serine; N-Fmoc-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-L-serine; N-Fmoc-O-(2,3,4,6-tri-O-acetyl-β-D-xylopyranosyl)-L-serine; N-Fmoc-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-galactopyranosyl)-L-serine; N-Fmoc-O-(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)-L-thereonine; N-Fmoc-O-(2-

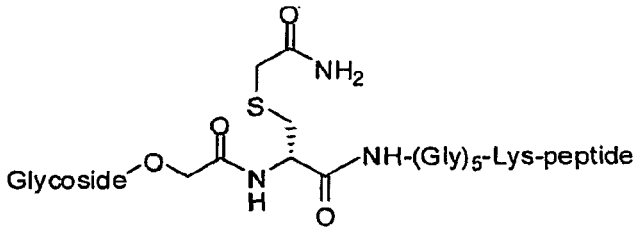
acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D- galactopyranosyl)-L-threonine; N-Fmoc-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-L-tyrosine; and N-Fmoc-N^{α}-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-glucopyranosyl)-L-asparagine. In various embodiments, other glycosylated amino acids, including C-glycosylated analogs, can be prepared by methods known in the art. Additionally, in various embodiments, di- and tri-peptide thioesters can be prepared by combining the above listed amino acids with suitably protected glycosylated or non-glycosylated amino acids. In various embodiments, these are also useful coupling partners for the native ligation strategy. In various embodiments, C-glycosylated amino acids and peptides can be used. In various embodiments, suitably protected glycosylated amino acids can be coupled onto the N-terminal residue of potent peptide ligands via known solid phase coupling protocols.

Certain exemplary methods for preparing ester- linked peptide-carbohydrate conjugates, N-linked glycoconjugates, amino acid glycosides and glycopeptides are known in the art. See, e.g., U.S. Pat. Nos. 5,470,949, 5,668,272, 5,767,254, PCT publication Nos. WO 96/28467 and WO 03/014371 (each of which is hereby incorporated by reference in their entirety).

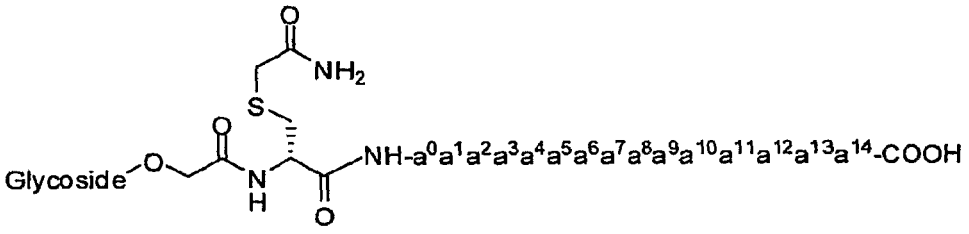
In various embodiments, the linker can be altered to form chemical derivatives in the same manner as described herein. Non-peptide portions of the inventive composition of matter, such as non-peptidyl linkers or non-peptide half-life extending moieties can be synthesized by conventional organic chemistry reactions.

In some embodiments the inventive compositions of matter have a structure, such as one of (a)-(c) shown immediately below, including a glycosylated linker moiety covalently bound to the N-terminal of the B1 antagonist peptide:

(a)



or



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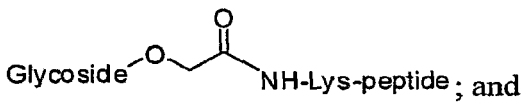
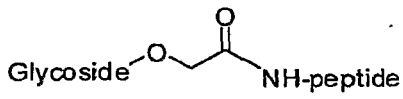
(b)

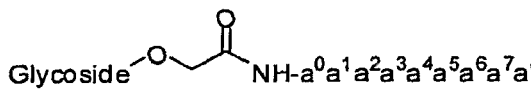


; and

(c)



or



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As shown in (a)-(c) immediately above, the "peptide" (or the peptide represented as amino acid positions a^0-a^{14} as variously described herein) is a peptide antagonist of the bradykinin B1 receptor; and "glycoside" is at least one glycoside selected from N-acetylglucoseamine, N-acetyllactoseamine, cellobiose, glucose, mannose, galactose, lactose, fucose-a-1,6-galactose, sialic acid methyl ester, gentiobiose, 2,4-diaminoglucose, 2-aminoglucose, maltotriose, maltotetrose, and

maltose, although any other suitable glycoside moiety can be used in the alternative to these, or can be included in addition to these.

The above is merely illustrative and not an exhaustive treatment of the kinds of linkers that can optionally be employed in accordance with the present invention.

Certain Chemical Derivatives. Also contemplated herein are chemical derivatives of certain glycosylated peptides. In various embodiments, such derivatives improve the solubility, absorption, and/or biological half-life of the unglycosylated or glycosylated peptides disclosed herein. In various embodiments, the added moieties can eliminate or attenuate one or more undesirable characteristic of the unglycosylated or glycosylated peptides disclosed herein. Exemplary derivatives include, but are not limited to, glycosylated peptides in which:

1. The glycosylated peptide or some portion thereof is cyclic. For example, in various embodiments, the peptide portion of a glycosylated peptide can be modified to contain two or more cysteine residues (e.g., in the peptidyl linker), which could cyclize by disulfide bond formation. See, e.g., WO 00/24782.

2. The glycosylated peptide is cross-linked or is rendered capable of cross-linking between molecules. For example, in various embodiments, the peptide portion of a glycosylated peptide can be modified to contain one or more Cys residue and thereby be able to form an intermolecular disulfide bond with a like molecule.

3. One or more peptidyl [-C(O)NR-] linkages (peptide bonds) is replaced by a non-peptidyl linkage. Exemplary non-peptidyl linkages include, but are not limited to, -CH_2-carbamate [-CH_2-OC(O)NR-], phosphonate, -CH_2-sulfonamide [-

CH_2-$S(O)_2NR$-], urea [-NHC(O)NH-], -CH_2-secondary amine, and alkylated peptide [-$C(O)NR^6$- wherein R^6 is a lower alkyl].

4. An N-terminal cysteine residue in can be substituted with a N-terminal derivative group. Typically, the N-terminus can be acylated or modified to a substituted amine. Exemplary N-terminal derivative groups include, but are not limited to, -NRR^1 (other than -NH_2), -$NRC(O)R^1$, -$NRC(O)OR^1$, -$NRS(O)_2R^1$, -$NHC(O)NHR^1$, succinimide, or benzyloxycarbonyl-NH- (CBZ-NH-), wherein R and R^1 are each independently hydrogen or lower alkyl, and wherein the phenyl ring can be substituted with 1 to 3 substituents independently selected from C_1-C_4 alkyl, C_1-C_4 alkoxy, chloro, and bromo.

In various embodiments, derivatization with bifunctional agents is useful for cross-linking the glycosylated peptides or their functional chemical derivatives to a water-insoluble support matrix or to other macromolecular carbohydrates. Exemplary cross-linking agents include, but are not limited to, 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. In various embodiments, derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]-propioimidate yield photo-activatable intermediates that are capable of forming crosslinks in the presence of light. In various embodiments, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

In various embodiments, carbohydrate (oligosaccharide) groups can be attached to sites that are known to be glycosylation sites in proteins. In various embodiments, O-linked oligosaccharides are attached to serine (Ser) or threonine (Thr) residues, while N-linked oligo-saccharides are attached to asparagine (Asn) residues when they are part of the sequence Asn-Aaa-Ser/Thr, where Aaa can be any amino acid except proline. In various embodiments, Aaa is one of the

nineteen naturally occurring amino acids other than proline. In various embodiments, the structures of N-linked and O-linked oligosaccharides and the sugar residues found in each type are different. One type of sugar that is commonly found on both is N-acetylneuraminic acid (referred to as sialic acid). Sialic acid is usually the terminal residue of both N-linked and O-linked oligosaccharides and, by virtue of its negative charge, can confer acidic properties to the glycosylated peptide. In various embodiments, such glycosylation sitessite(s) can be incorporated in the linker (L) of the peptides. In various embodiments, such sites can further be glycosylated by synthetic or semi-synthetic procedures known in the art.

Other exemplary modifications include, but are not limited to, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, oxidation of the sulfur atom in Cys, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains. See, e.g., Creighton, Proteins: Structure and Molecule Properties, W. H. Freeman & Co., San Francisco, pages 79-86 (1983).

Certain Pharmaceutical Compositions

In General. The present invention also provides pharmaceutical compositions and medicaments comprising the inventive composition of matter and a pharmaceutically acceptable carrier. Such pharmaceutical compositions can be configured for administration to a patient by a wide variety of delivery routes, e.g., an intravascular delivery route such as by injection or infusion, subcutaneous, intramuscular, intraperitoneal, epidural, or intrathecal delivery routes, or for oral, enteral, pulmonary (e.g., inhalant), intranasal, transmucosal (e.g., sublingual administration), transdermal or other delivery routes and/or forms of administration known in the art. The inventive pharmaceutical compositions can be prepared in liquid form, or can be in dried powder form, such as lyophilized form. For oral or enteral use, the pharmaceutical compositions can be configured, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsions, hard or soft capsules, syrups, elixirs or enteral formulas.

In various embodiments, methods are provided for using pharmaceutical compositions of the inventive glycosylated peptides, e.g., in the prevention or treatment of inflammation and/or inflammatory pain. Examples include, but are not limited to, inflammatory pain and associated hyperalgesia and allodynia, diabetic neuropathy pain, post-herpetic neuralgia, causalgia, sympathetically maintained pain, back pain, lower back pain, deafferentation syndromes, acute pain, tension headache, angina, migraine, general headache, cluster headache, dental pain, pain from trauma, surgery, amputation or abscess, causalgia, demyelinating diseases, diabetes, and trigeminal neuralgia. In various embodiments, the glycosylated peptides have therapeutic value for the prevention or treatment of other painful conditions associated with B1 as a causative agent of the pain. Exemplary conditions include, but are not limited to, thalamic pain syndrome, diabetes, toxins and chemotherapy, septic shock, arthritis, mixed-vascular and non-vascular syndromes, general inflammation, arthritis, rheumatic diseases, lupus, osteoarthritis, inflammatory bowel disorders, inflammatory eye disorders, inflammatory or unstable bladder disorders, psoriasis, skin complaints with inflammatory components, sunburn, carditis, inflammatory bowel disease, dermatitis, myositis, neuritis, collagen vascular diseases, chronic inflammatory conditions, epithelial tissue damage or dysfunction, herpes simplex, disturbances of visceral motility at respiratory, genitourinary, gastrointestinal or vascular regions, wounds, burns, allergic rhinitis, asthma, allergic skin reactions, pruritis, vitiligo, general gastrointestinal disorders, colitis, gastric ulceration, duodenal ulcers, or vasomotor and allergic rhinitis.

Exemplary uses of the glycosylated peptides include, but are not limited to, prevention or treatment of acute pain, dental pain, back pain, lower back pain, pain from trauma, surgical ,amputation or abscess, causalgia, demyelinating diseases, trigeminal neuralgia, cancer, chronic alcoholism, stroke, thalamic pain syndrome, diabetes, acquired immune deficiency syndrome ("AIDS"), toxins and chemotherapy, general headache, migraine, cluster headache, mixed-vascular and non-vascular syndromes, tension headache, general inflammation, arthritis, rheumatic diseases, lupus, osteoarthritis, inflammatory bowel disorders, inflammatory eye disorders, inflammatory or unstable bladder disorders, psoriasis, skin complaints with inflammatory components, sunburn, carditis, dermatitis,

myositis, neuritis, collagen vascular diseases, chronic inflammatory conditions, inflammatory pain and associated hyperalgesia and allodynia, neuropathic pain and associated hyperalgesia and allodynia, diabetic neuropathy pain, causalgia, sympathetically maintained pain, deafferentation syndromes, asthma, allergic rhinitis, epithelial tissue damage or dysfunction, herpes simplex, post-herpetic neuralgia, disturbances of visceral motility at respiratory, genitourinary, gastrointestinal or vascular regions, wounds, burns, allergic skin reactions, pruritis, vitiligo, general gastrointestinal disorders, colitis, gastric ulceration, duodenal ulcers, and bronchial disorders.

Accordingly, in various embodiments, the use of one or more glycosylated peptides can be used in the manufacture of a medicament for the treatment of a disorder. Exemplary disorders include, but are not limited to, acute pain, dental pain, back pain, lower back pain, pain from trauma, surgical pain, pain resulting from amputation or abscess, causalgia, demyelinating diseases, trigeminal neuralgia, cancer, chronic alcoholism, stroke, thalamic pain syndrome, diabetes, acquired immune deficiency syndrome ("AIDS"), toxins and chemotherapy, general headache, migraine, cluster headache, mixed-vascular and non-vascular syndromes, tension headache, general inflammation, arthritis, rheumatic diseases, lupus, osteoarthritis, inflammatory bowel disorders, inflammatory eye disorders, inflammatory or unstable bladder disorders, psoriasis, skin complaints with inflammatory components, sunburn, carditis, dermatitis, myositis, neuritis, collagen vascular diseases, chronic inflammatory conditions, inflammatory pain and associated hyperalgesia and allodynia, neuropathic pain and associated hyperalgesia and allodynia, diabetic neuropathy pain, causalgia, sympathetically maintained pain, deafferentation syndromes, asthma, allergic rhinitis, epithelial tissue damage or dysfunction, herpes simplex, post-herpetic neuralgia, disturbances of visceral motility at respiratory, genitourinary, gastrointestinal or vascular regions, wounds, burns, allergic skin reactions, pruritis, vitiligo, general gastrointestinal disorders, colitis, gastric ulceration, duodenal ulcers, and bronchial disorders.

In various embodiments, such pharmaceutical compositions or medicaments can be for administration by injection, or for oral, pulmonary, nasal, transdermal or other

forms of administration. In various embodiments, pharmaceutical compositions are provided comprising effective amounts of the inventive glycosylated peptide (in amounts effective to prevent, ameliorate, or abolish pain and/or any of the other medical conditions provided herein) together with pharmaceutically acceptable diluents, excipients, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Exemplary components of such compositions include, but are not limited to, diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (e.g., Tween 80, Polysorbate 80), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimerosol, benzyl alcohol) and bulking substances (e.g., lactose, mannitol); incorporation of the material into particulate preparations of polymeric glycosylated peptides such as polylactic acid, polyglycolic acid, etc. or into liposomes. In various embodiments, hyaluronic acid can also be used, and this can have the effect of promoting sustained duration in the circulation. In various embodiments, such compositions can further influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the glycosylated peptides. (See, for example, Remington's Pharmaceutical Sciences, 18th Edition., Mack Publishing Co., Easton, PA, pages 1435-1712 (1990), which is herein incorporated by reference). In various embodiments, the compositions can be prepared in liquid form, or as a dried powder (such as lyophilized form). In various embodiments, implantable sustained release formulations are also contemplated (see, e.g., Murthy et al., U.S. Patent No. 6,887,487, incorporated herin by reference), as are transdermal formulations.

Oral dosage forms. Contemplated for use herein according to various embodiments are oral solid dosage forms, which are described generally, e.g., in Chapter 89 of Remington's Pharmaceutical Sciences, above, which is herein incorporated by reference for any purpose. Solid dosage forms include, but are not limited to, tablets, capsules, pills, troches or lozenges, cachets or pellets. Also, in various embodiments, liposomal or proteinoid encapsulation can be used to formulate compositions (such as, for example, the proteinoid microspheres reported in U.S. Patent No. 4,925,673). In various embodiments, liposomal

encapsulation can be used, and the liposomes can be derivatized with various polymers (see, for example, U.S. Patent No. 5,013,556). A description of exemplary solid dosage forms is given, e.g., in Chapter 10 of Marshall, K., Modern Pharmaceutics, edited by G. S. Banker and C. T. Rhodes (1979), herein incorporated by reference for any purpose. In various embodiments, the formulation will include a unglycosylated peptide of the invention , as well as inert ingredients which allow for protection against the stomach environment and release of the glycosylated peptide in the intestine.

Also specifically contemplated according to various embodiments are oral dosage forms of the inventive glycosylated peptides themselves. In various embodiments, the glycosylated peptides can be chemically modified so that oral delivery is efficacious. It is also possible according to various embodiments, to use a salt of a modified aliphatic amino acid, such as sodium N-(8-[2-hydroxybenzoyl] amino) caprylate (SNAC), as a carrier to enhance absorption of the unglycosylated peptides. See, for example, U.S. Patent No. 5,792,451, entitled "Oral Drug Delivery Composition and Methods".

In various embodiments, glycosylated peptides of the invention can be included in the formulation as fine multi-particulates in the form of granules or pellets of a particle size about one millimeter. In various embodiments, the formulation of the material for capsule administration could also be as a powder, as lightly compressed plugs, or even as tablets. In various embodiments, the therapeutic could be prepared by compression.

In various embodiments, colorants and/or flavoring agents can be included. For example, in various embodiments, the glycosylated peptide or any chemical derivative thereof can be formulated (such as by liposome or microsphere encapsulation) and then further contained within an edible product, such as a refrigerated beverage containing colorants and/or flavoring agents.

In various embodiments, one can dilute or increase the volume of the glycosylated peptide with an inert material. Exemplary diluents include, but are not limited to, carbohydrates, for example, mannitol, α-lactose, anhydrous lactose, cellulose, sucrose, modified dextrans, and starch. In various embodiments, certain

inorganic salts can be used as fillers, including calcium triphosphate, magnesium carbonate and sodium chloride. Some commercially available diluents are Fast-Flo, Emdex, STA-Rx 1500, Emcompress and Avicell.

In various embodiments, disintegrants can be included in the formulation of the therapeutic into a solid dosage form. Exemplary materials used as disintegrants include, but are not limited to, starch, including, but not limited to, the commercially available disintegrant based on starch, Explotab. Sodium starch glycolate, Amberlite, sodium carboxymethylcellulose, ultramylopectin, sodium alginate, gelatin, orange peel, acid carboxymethyl cellulose, natural sponge and bentonite can also be used in various embodiments. In various embodiments, another form of the disintegrants are the insoluble cationic exchange resins. In various embodiments, powdered gums can be used as disintegrants and as binders, and these can include powdered gums such as agar, Karaya or tragacanth. Alginic acid and its sodium salt are also useful as disintegrants in various embodiments.

In various embodiments, binders can be used to hold the components of the pharmaceutical composition together to form a hard tablet. Exemplary binders include, but are not limited to, materials from natural products such as acacia, tragacanth, starch and gelatin. Other exemplary binders include, but are not limited to, methyl cellulose (MC), ethyl cellulose (EC) and carboxymethyl cellulose (CMC). In various embodiments, polyvinyl pyrrolidone (PVP) and hydroxypropylmethyl cellulose (HPMC) could both be used in alcoholic solutions to granulate the therapeutic.

In various embodiments, an anti-frictional agent can be included in the formulation to prevent sticking during the formulating process. In various embodiments, lubricants can be used as a layer between the therapeutic and the die wall, and these can include, but are not limited to: stearic acid, including its magnesium and calcium salts, polytetrafluoroethylene (PTFE), liquid paraffin, vegetable oils and waxes. In various embodiments, soluble lubricants can also be used such as sodium lauryl sulfate, magnesium lauryl sulfate, polyethylene glycol of various molecular weights, Carbowax 4000 and 6000.

In various embodiments, glidants that might improve the flow properties of the unglycosylated peptide during formulation and to aid rearrangement during compression might be added. Exemplary glidants can include, but are not limited to, starch, talc, pyrogenic silica and hydrated silicoaluminate.

In various embodiments, to aid dissolution of the glycosylated peptide into the aqueous environment, a surfactant might be added as a wetting agent. Exemplary surfactants include, but are not limited to, anionic detergents such as sodium lauryl sulfate, dioctyl sodium sulfosuccinate and dioctyl sodium sulfonate. In various embodiments, cationic detergents can be used and can include benzalkonium chloride or benzethonium chloride. Exemplary nonionic detergents that can be included in the formulation as surfactants include, but are not limited to, lauromacrogol 400, polyoxyl 40 stearate, polyoxyethylene hydrogenated castor oil 10, 50 and 60, glycerol monostearate, polysorbate 40, 60, 65 and 80, sucrose fatty acid ester, methyl cellulose and carboxymethyl cellulose. In various embodiments, these surfactants can be present in the formulation either alone or as a mixture in different ratios.

In various embodiments, additives can also be included in the formulation to enhance uptake of the unglycosylated peptide. Exemplary additives potentially having this property include, but are not limited to, various fatty acids, such as, for instance, oleic acid, linoleic acid and linolenic acid.

Controlled release formulation can be desirable. The composition of this invention can be incorporated into an inert matrix that permits release by either diffusion or leaching mechanisms e.g., gums. Slowly degenerating matrices can also be incorporated into the formulation, e.g., alginates, polysaccharides. Another form of a controlled release of the compositions of this invention is by a method based on the Oros™ therapeutic system (Alza Corp.), i.e., the drug is enclosed in a semipermeable membrane which allows water to enter and push drug out through a single small opening due to osmotic effects. Some enteric coatings also have a delayed release effect.

Other coatings can be used for the formulation. These include a variety of sugars that could be applied in a coating pan. The therapeutic agent could also be

given in a film-coated tablet and the materials used in this instance are divided into 2 groups. The first are the nonenteric materials and include methylcellulose, ethyl cellulose, hydroxyethyl cellulose, methylhydroxy-ethyl cellulose, hydroxypropyl cellulose, hydroxypropyl-methyl cellulose, sodium carboxymethyl cellulose, providone and the polyethylene glycols. The second group consists of the enteric materials that are commonly esters of phthalic acid.

A mix of materials might be used to provide the optimum film coating. Film coating can be carried out in a pan coater or in a fluidized bed or by compression coating.

Certain Pulmonary delivery forms. Pulmonary delivery of the inventive compositions of matter is also useful. The composition is delivered to the lungs of a mammal while inhaling and traverses across the lung epithelial lining to the blood stream. (Other reports of this include Adjei et al., Pharma. Res. (1990) 7: 565-9; Adjei et al. (1990), Internatl. J. Pharmaceutics 63: 135-44 (leuprolide acetate); Braquet et al. (1989), J. Cardiovasc. Pharmacol. 13 (suppl.5): s.143-146 (endothelin-1); Hubbard et al. (1989), Annals Int. Med. 3: 206-12 (α1-antitrypsin); Smith et al. (1989), J. Clin. Invest. 84: 1145-6 (α1-proteinase); Oswein et al. (March 1990), "Aerosolization of Proteins," Proc. Symp. Resp. Drug Delivery II, Keystone, Colorado (recombinant human growth hormone); Debs et al. (1988), J. Immunol. 140: 3482-8 (interferon-γ and tumor necrosis factor α) and Platz et al., U.S. Patent No. 5,284,656 (granulocyte colony stimulating factor).

Useful in the practice of this invention are a wide range of mechanical devices designed for pulmonary delivery of therapeutic products, including but not limited to nebulizers, metered dose inhalers, and powder inhalers, all of which are familiar to those skilled in the art. Some specific examples of commercially available devices suitable for the practice of this invention are the Ultravent nebulizer, manufactured by Mallinckrodt, Inc., St. Louis, Missouri; the Acorn II nebulizer, manufactured by Marquest Medical Products, Englewood, Colorado; the Ventolin metered dose inhaler, manufactured by Glaxo Inc., Research Triangle Park, North Carolina; and the Spinhaler powder inhaler, manufactured by Fisons

Corp., Bedford, Massachusetts. (See, e.g., Helgesson et al., Inhalation device, U.S. Patent No. 6,892,728; McDerment et al., Dry powder inhaler, WO 02/11801 A1; Ohki et al., Inhalant medicator, U.S. Patent No. 6,273,086).

All such devices require the use of formulations suitable for the dispensing of the inventive compound. Typically, each formulation is specific to the type of device employed and can involve the use of an appropriate propellant material, in addition to diluents, adjuvants and/or carriers useful in therapy.

The inventive compound should most advantageously be prepared in particulate form with an average particle size of less than 10 μm (or microns), most preferably 0.5 to 5 μm, for most effective delivery to the distal lung.

Pharmaceutically acceptable excipients include carbohydrates such as trehalose, mannitol, xylitol, sucrose, lactose, and sorbitol. Other ingredients for use in formulations can include DPPC, DOPE, DSPC and DOPC. Natural or synthetic surfactants can be used. PEG can be used (even apart from its use in derivatizing the protein or analog). Dextrans, such as cyclodextran, can be used. Bile salts and other related enhancers can be used. Cellulose and cellulose derivatives can be used. Amino acids can be used, such as use in a buffer formulation.

Also, the use of liposomes, microcapsules or microspheres, inclusion complexes, or other types of carriers is contemplated.

Formulations suitable for use with a nebulizer, either jet or ultrasonic, will typically comprise the inventive compound dissolved in water at a concentration of about 0.1 to 25 mg of biologically active protein per mL of solution. The formulation can also include a buffer and a simple sugar (e.g., for protein stabilization and regulation of osmotic pressure). The nebulizer formulation can also contain a surfactant, to reduce or prevent surface induced aggregation of the protein caused by atomization of the solution in forming the aerosol.

Formulations for use with a metered-dose inhaler device will generally comprise a finely divided powder containing the inventive compound suspended in a propellant with the aid of a surfactant. The propellant can be any conventional material employed for this purpose, such as a chlorofluorocarbon, a

hydrochlorofluorocarbon, a hydrofluorocarbon, or a hydrocarbon, including trichlorofluoromethane, dichlorodifluoromethane, dichlorotetrafluoroethanol, and 1,1,1,2-tetrafluoroethane, or combinations thereof. Suitable surfactants include sorbitan trioleate and soya lecithin. Oleic acid can also be useful as a surfactant. (See, e.g., Bäckström et al., Aerosol drug formulations containing hydrofluoroalkanes and alkyl saccharides, U.S. Patent No. 6,932,962).

Formulations for dispensing from a powder inhaler device will comprise a finely divided dry powder containing the inventive compound and can also include a bulking agent, such as lactose, sorbitol, sucrose, mannitol, trehalose, or xylitol in amounts which facilitate dispersal of the powder from the device, e.g., 50 to 90% by weight of the formulation.

Certain Nasal delivery forms. Nasal delivery of the glycosylated peptides is contemplated according to various embodiments. In various embodiments, nasal delivery allows the passage of the glycosylated peptides to the blood stream directly after administering the therapeutic product to the nose, without the necessity for deposition of the product in the lung. In various embodiments, the bioavailability of the glycosylated peptides after intranasal administration allows this route to be exploited as a convenient alternative to the injected administration of the glycosylated B1 peptide antagonists. In various embodiments, the difference between the bioavailability values from intranasal and sublingual administration can result in dramatic advantages for the therapeutic administration of these compounds over those previously described in the art.

Formulations suitable for intranasal administration include those with dextran or cyclodextran, and intranasal delivery devices are known. (See, e.g, Freezer, Inhaler, U.S. Patent No. 4,083,368).

In various embodiments, a new pharmaceutical composition suitable for nasal administration is provided containing:

a) a glycosylated B1 peptide antagonist;

b) a polyacrylic acid and/or its derivatives;

c) optionally, one or more excipients normally used for the preparation of pharmaceutical compositions for nasal administration (see, for example, EP1297826, which is hereby incorporated by reference in its entirety). Exemplary formulations for nasal delivery include, but are not limited to, those with dextran or cyclodextran. Delivery via transport across other mucous membranes is also contemplated according to various embodiments.

Transdermal and transmucosal (e.g., buccal) delivery forms. In some embodiments, the inventive composition is configured as a part of a pharmaceutically acceptable transdermal or transmucosal patch or a troche. Transdermal patch drug delivery systems, for example, matrix type transdermal patches, are known and useful for practicing some embodiments of the present pharmaceutical compositions. (E.g., Chien et al., Transdermal estrogen/progestin dosage unit, system and process, U.S. Patent Nos. 4,906,169 and 5,023,084; Cleary et al., Diffusion matrix for transdermal drug administration and transdermal drug delivery devices including same, U.S. Patent No. 4,911,916; Teillaud et al., EVA-based transdermal matrix system for the administration of an estrogen and/or a progestogen, U.S. Patent No. 5.605,702; Venkateshwaran et al., Transdermal drug delivery matrix for coadministering estradiol and another steroid, U.S. Patent No. 5,783,208; Ebert et al., Methods for providing testosterone and optionally estrogen replacement therapy to women, U.S. Patent No. 5,460,820). A variety of pharmaceutically acceptable systems for transmucosal delivery of therapeutic agents are also known in the art and are compatible with the practice of the present invention. (E.g., Heiber et al., Transmucosal delivery of macromolecular drugs, U.S. Patent Nos. 5,346,701 and 5,516,523; Longenecker et al., Transmembrane formulations for drug administration, U.S. Patent No. 4,994,439).

Buccal delivery of the inventive compositions is also useful. Buccal delivery formulations are known in the art for use with peptides. For example, known tablet or patch systems configured for drug delivery through the oral mucosa (e.g., sublingual mucosa), include some embodiments that comprise an

inner layer containing the drug, a permeation enhancer, such as a bile salt or fusidate, and a hydrophilic polymer, such as hydroxypropyl cellulose, hydroxypropyl methylcellulose, hydroxyethyl cellulose, dextran, pectin, polyvinyl pyrrolidone, starch, gelatin, or any number of other polymers known to be useful for this purpose. This inner layer can have one surface adapted to contact and adhere to the moist mucosal tissue of the oral cavity and can have an opposing surface adhering to an overlying non-adhesive inert layer. Optionally, such a transmucosal delivery system can be in the form of a bilayer tablet, in which the inner layer also contains additional binding agents, flavoring agents, or fillers. Some useful systems employ a non-ionic detergent along with a permeation enhancer. Transmucosal delivery devices can be in free form, such as a cream, gel, or ointment, or can comprise a determinate form such as a tablet, patch or troche. For example, delivery of the inventive composition can be via a transmucosal delivery system comprising a laminated composite of, for example, an adhesive layer, a backing layer, a permeable membrane defining a reservoir containing the inventive composition, a peel seal disc underlying the membrane, one or more heat seals, and a removable release liner. (E.g., Ebert et al., Transdermal delivery system with adhesive overlay and peel seal disc, U.S. Patent No. 5,662,925; Chang et al., Device for administering an active agent to the skin or mucosa, U.S. Patent Nos. 4,849,224 and 4,983,395). These examples are merely illustrative of available transmucosal drug delivery technology and are not limiting of the present invention.

Certain Dosages. In various embodiments, the dosage regimen involved in a method for treating the involved disease or disorder will be determined by the attending physician, considering various factors which modify the action of therapeutic agents, such as the age, condition, body weight, sex and diet of the patient, the severity of the condition being treated, time of administration, and other clinical factors. In various embodiments, the daily regimen should be in the range of 1.0-10000 micrograms (μg) of the glycosylated peptide per kilogram (kg) of body weight, or 1.0-1000 μg per kilogram of body weight, or 1.0-150 μg per kilogram of body weight.

EXAMPLES

These examples comprise embodiments and are illustrative only and not limiting.

Example 1: Synthesis and Purification of Certain B1 Receptor Peptide Antagonists.

Assorted peptide analogs of active bradykinin B1 receptor antagonists can be synthesized with different peptidyl linkers at the N-terminus, and each containing a penultimate cysteine (Table 4A) using methods well-known in the art of synthetic peptide synthesis. These peptide analogs can be conjugated to known carbohydrate structures using methods known in the art. The resultant glycoconjugates are purified by ion exchange chromatography, concentrated by lyophilization or diafiltration and dialyzed into buffer prior to *in vitro* and *in vivo* bioassay.

The glycosylated peptides can be purified by cation exchange chromatography using SP Sepharose HP columns (Amersham Biosciences) pre-equilibrated with 10 mM NaOAc, 20% EtOH, pH 4. Prior to loading, the reaction mixtures can be diluted 10-fold with 20% EtOH and the pH adjusted to 3.5 with glacial acetic acid. The diluted reaction mixtures can be loaded to an appropriate sized column such that a peptide:resin ration of 2.5 mg/ml is not exceeded.

The column is then washed with 2 column volumes (CVs) of 10 mM NaOAc, 20% EtOH, pH 4 and eluted with a linear 0-200 mM NaCl gradient in 10mM NaOAc, 20% EtOH, pH 4 over 10-20 CV. The unmodified peptide and glycoconjugate can be detected by monitoring absorbance at either 254 nm or 220 nm. Under these conditions, the excess carbohydrate and β-ME can be washed out in the unbound flow-through fraction, the glyconjugate eluted in a broad peak well resolved from the free peptide.

The eluted peak fractions can be evaluated by RP-HPLC and pooled based on homogeneity and retention times consistent with glycopeptide conjugate. The pooled conjugate peak is concentrated by drying, then reconstituted in water and

dialyzed against buffer. Alternatively diafiltration can be used to concentrate and buffer exchange the conjugate.

The final pools of glycoconjugates can be analyzed by RP-HPLC and can be typically ~98% glycoconjugate. Conjugate composition and concentrations can be determined by a combination of amino acid analyses, peptide sequencing, and absorbance spectroscopy.

Table 4A: X^1 Peptides

SEQ ID NO:	Sequence of X^1 Peptide
27	{N} CGGGKRPPGFSPL {C}
28	{N} CGGGGGKRPPGFSPL {C}
29	{N} CGGGGGKKRPGFSPL {C}
30	{N} CGGGGGKRKRPPGFSPL {C}
31	{N} CG-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-KRPPGFSPL {C}
32	{N} CGGGGGKKRPPG[AMeF]S[D-β-Nal]I {C}
33	{N}CGGGGGKKRP[Hyp]G[Cpg]S[DTic][Cpg]{C}
34	{N}CGGGGGGGKKRP[Hyp]G[Cpg]S[DTic][Cpg] {C}
35	{N} ac-CGGGGGKKRP[Hyp]G[Cpg]S[DTic][Cpg]{C}
36	{N}KKRP[Hyp]G[Cpg]S[DTic][Cpg] {C}
37	{N}acyl-KKRP[Hyp]G[Cpg]S[DTic][Cpg] {C}
38	{N} CKRPPGFSPL {C}
39	{N}CGGGGG[DOrn]KRP[Hyp]G[Cpg]S[DTic][Cpg]{C}
40	{N}CGGGGG[DOrn]KRP[Thz]G[Cpg]S[DTic][Cpg]{C}
41	{N}CGGGGGK[DOrn]RP[Hyp]G[Cpg]S[DTic][Cpg]{C}

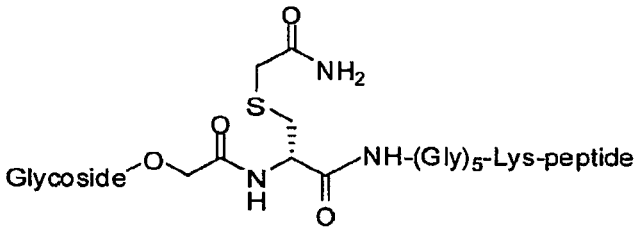
Table 4B: Y^1 Peptide

SEQ ID NO:	Sequence of Y^1 Peptide
42	{N} GGGGGKKRPPGFSPL {C}

Example 2: Additional Glycosylated B1 Peptide Antagonists

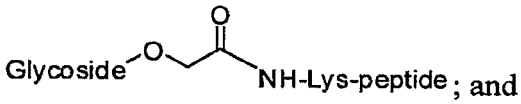
Thirty glycosylated peptides were made with three different peptides (a 9-mer, a 10-mer, and a 16-mer shown below with "peptide" in each example = NH-Lys-Arg-Pro-Hyp-Gly-Cpg-Ser-(D-Tic)-Cpg//SEQ ID NO:21) with 16 different glycosides as depicted in Table 5 below. Methods that can be used to make the thirty glycosylated peptides are set forth in the Examples below.

16-mer:



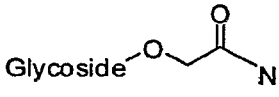
;

10-mer



; and

9-mer:



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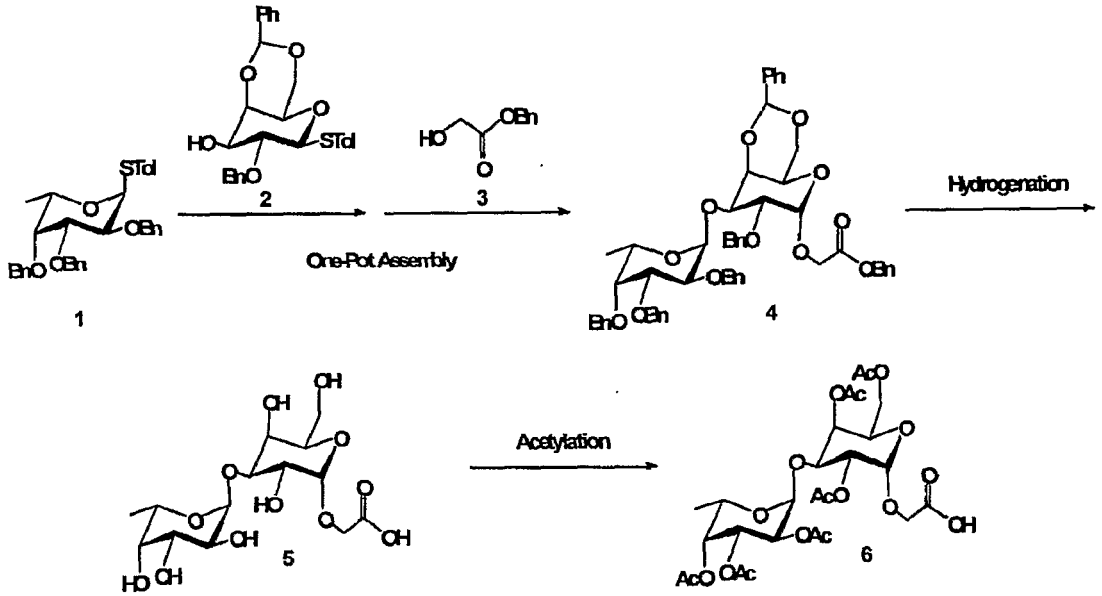
Various starting materials to make these sixteen glycosides are disclosed in U.S. Patent No. 7,019,131, International Patent Application Publication No. WO 2005/044197, and/or Zhang et al., JACS, 121:734-753 (1999), each of which is incorporated by reference herein for any purpose. Those references also disclose certain methods of making glycopeptides. Glycosylated B1 peptide antagonists other than those in Table 5 can also be prepared using the following methods and/or other methods known in the art.

The glycosylation technology below can be used to prepare a variety of sugar-glycolic acid derivatives that cover a diversity of sugar motifs; i.e., size (mono to oligosaccharide), structure (glucose, galactose, fucose, etc), functionalities (acidic or basic functional group-containing sugar) and combinations of these.

The following specifically discloses the production of O-acetyl derivative of L-fucopyranosyl-α(1→3)-D-galactopyranosyl-β(1→ glycolic acid, but as discussed above, the methods can make many different sugar-glycolic acid derivatives, including the twenty-four glycopeptides in Table 5.



This is a representative synthesis of a sugar-glycolic acid derivative. A high reactive glycosyl donor (fucose derivative, **1**) and a less reactive sugar donor (galactose derivative, **2**), in the presence of an activating reagent, formed a desired disaccharide (fucose linked to galactose), which subsequently reacted with the glycolic acid derivative **3** (non-reactive as a donor) to give a sugar-glycolic acid derivative **4**. Deprotection of **4** followed by the protection of hydroxyl groups of the sugar moiety with an easily removable acetyl group gave **6**, which can be used to modify the amino group of a peptide of interest.

<u>One-Pot Assembly</u>

To a solution of compound **1** (500 mg, 0.92 mmol), compound **2** (430 mg, 0.92 mmol) and 4 A molecular sieves (2g) in CH_2Cl_2 (DCM) (10 mL) at –20 °C was added N-iodosuccinimide (NIS) (208 mg, 0.92 mmol). The mixture was

stirred at –20 °C for 20 minutes. Subsequently, compound **3** (132 μL, 1.84 mmol) was added, followed by addition of another portion of NIS (208 mg, 0.92 mmol). The whole mixture was allowed to gradually warm up to room temperature (RT) and was stirred at RT for 1 hour. The reaction was quenched with aqueous $NaHCO_3$ (2 mL), sodium thiosulfate (2 mL), and was filtrated through a Celite. The filtrate was washed with water, dried, and concentrated. The residue was purified by a silica gel chromatography (20% EtOAc in Hexane) to give compound **4** (560 mg, 66% yield).

Hydrogenation

Compound **4** (285 mg, 0.31 mmol) was hydrogenated over 20% $Pd(OH)_2$ (45 mg) in EtOAc/EtOH/H_2O (2 mL/3 mL/1 mL) at RT for 24 hours, and the mixture was filtrated. The filtrate was concentrated to give compound **5** (112 mg, 95% yield), which was used for the next step without further purification.

Acetylation

A solution of compound **5** (119 mg, 0.31 mmol) and Ac_2O (2 mL) in pyridine (2 mL) was stirred at RT for 8 hours, and the reaction was quenched by addition of aqueous NH_4Cl (6 mL). The mixture was diluted with DCM, washed with water, dried, and concentrated. The residue was purified by a silica gel chromatography (50% EtOAc in Hexane to 1% AcOH in EtOAc) to give compound **6** (180 mg, 91% yield).

Example 3: Ligation of sugar-glycolic acid derivative to unprotected B1 peptide antagonists

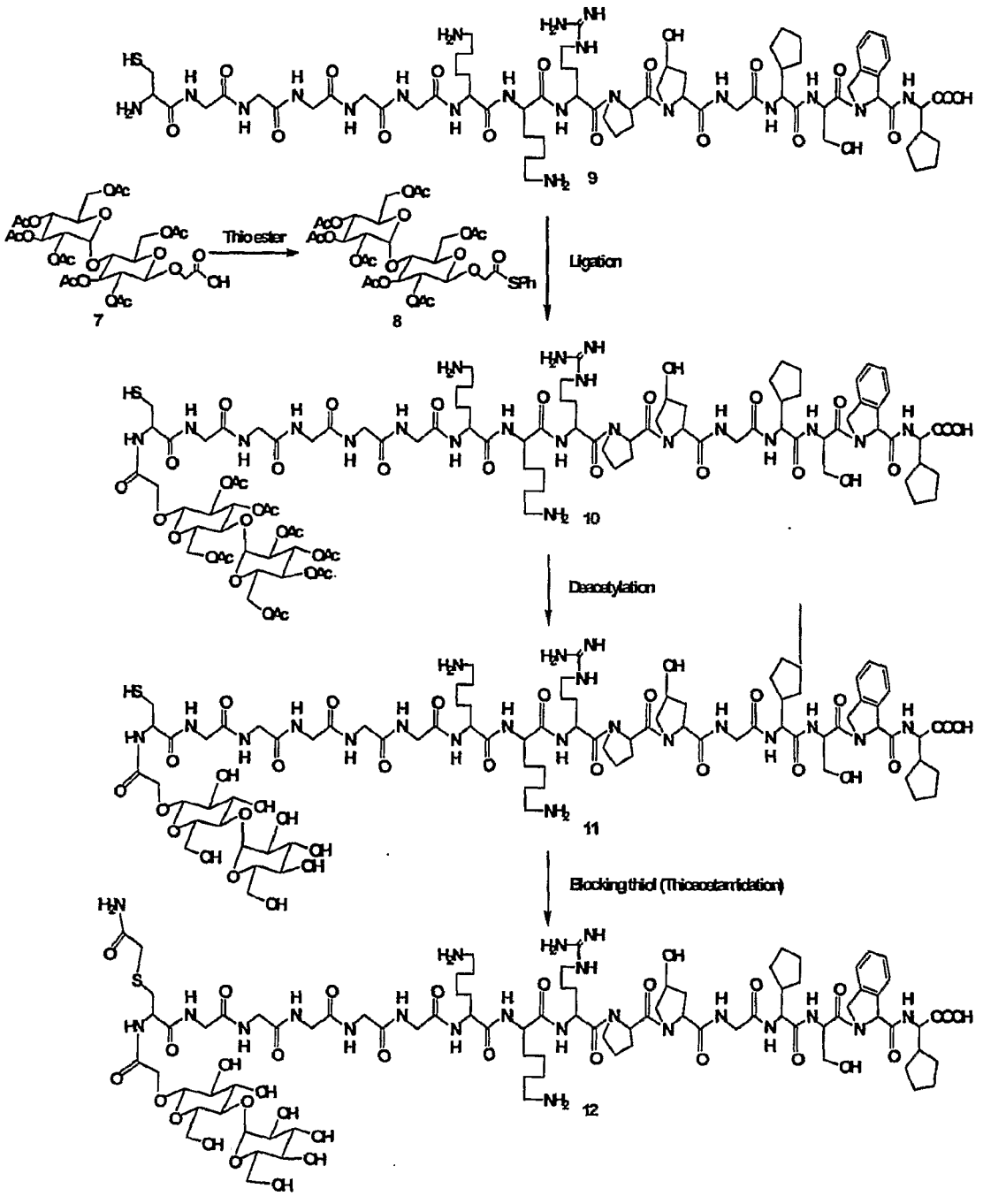
A. Ligation of sugar-glycolic acid derivative to unprotected peptide

One of the methods used for conjugating the preassembled sugar-glycolic acid portion to a peptide was a ligation method essentially as described by Kent et al. (Kent et al., Journal of the American Chemical Society, 121(50):11684-11689 (1999); Current Opinion in Chemical Biology 3(6):665-67 (1999)).

Briefly, a native B1 peptide antagonist (SEQ ID NO:13) was modified by adding a peptide linker of NH_2-Cys-$(Gly)_5$ at the N-terminus to give **9** (SEQ ID NO:33). Secondly, a sugar-glycolic acid derivative prepared as described in Example 2 above was transformed into the corresponding thio-ester **8.**

A solution of compound **7** (71 mg, 0.102 mmol) and PhSH (11.4 μL, 0.112 mmol) in the presence of 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide (EDCI) (29 mg, 0.153 mmol) in DCM (2 mL) was stirred at RT for 3 hours, and was concentrated. The residue was purified by a silica gel chromatography (50% EtOAc in hexane) to give the corresponding thio-ester **8** (60 mg, 75% yield).

Peptide **9** (with no protection groups) was ligated to the thio-ester **8** in a buffered solution. After deprotection of the sugar moiety, the remaining thio group of the product can be blocked by alkylation in order to avoid potential side reactions (disulfide formation or oxidation) at the free thiol group.


Thioester
Ligation
Deacetylation
Blocking thiol (Thioacetamidation)
7
8
9
10
11
12

Ligation

To a solution of compound **8** (32 mg, 20 μmol) and **9** (16 mg, 20 μmol) in DMF (2 mL) was added at RT a solution of thiophenol (20 μL) in phosphate buffer (pH 7.5, 1 mL) and the resulting mixture was stirred at RT for 2 hours. Formation of the product was analyzed by LCMS. The mixture was concentrated and the product was isolated from a preparative HPLC. The fractions were combined and lyophilized to give compound **10** as a white solid (17 mg, 37% yield). Analytical HPLC showed 85% purity.

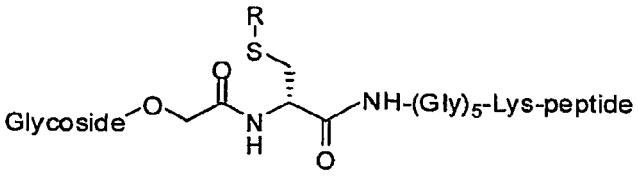
Deacetylation

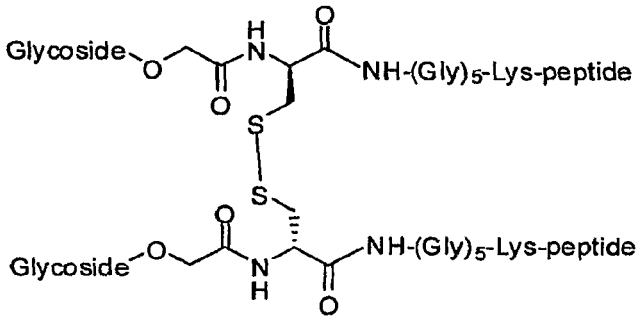
A solution of compound **10** (22.6 mg, 10 μmol) in aqueous 5% hydrazine (5 mL) in the presence of thiophenol (3 drops) was stirred at RT for 2 hours. Analytical HPLC indicated that the reaction was complete. The mixture was concentrated to white foam followed by Prep HPLC purification and lyophilization to give compound **11** as a white solid (8 mg, 41% yield).

Blocking thiol group

A solution of compound **11** (49 mg, 0.25 μmol) and iodoacetamide (84 mg, 0.45 μmol) in the presence of thiophenol (2 drops) in DMF (1 mL) and a phosphate buffer (pH 7.5, 2 mL) was stirred at RT for 2 hours. The mixture was concentrated and the product was purified by a preparative HPLC to give compound **12** (22 mg, 44%).

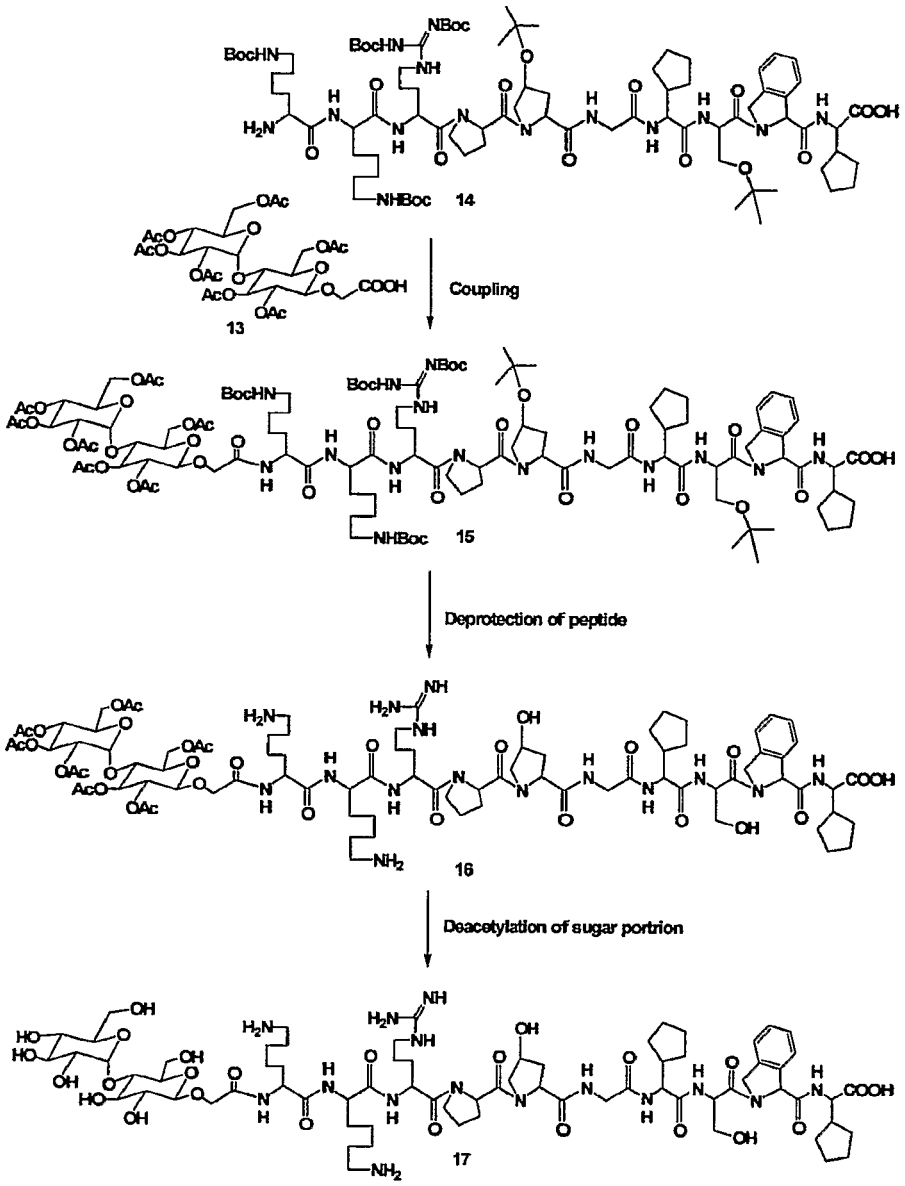
Optionally, the thiol blocking step can be omitted to provide additional glycosylated B1 peptide antagonists including, but not limited to, compounds of the following structure:





B. Coupling of sugar-glycolic acid derivative to a protected non-resin bound peptide

This is a method of coupling of a sugar-glycolic acid derivative to a free amino group of a properly protected peptide derivative.



Coupling

A solution of compound **13** (47 mg, 68 μmol), carbonyl diimidazole (CDI) (11 mg, 68 μmol) and DMAP (2 mg, catalytic amounts) in THF (2 mL) was stirred at RT for 1 hour and compound **14** (30 mg, 17 μmol) was then added to the solution. The resulting reaction mixture was stirred at RT for 10 hours. Formation was the product was analyzed by LCMS. The product was isolated by

a preparative HPLC. The fractions were combined and lyophilized to give compound **15** as a white solid (21 mg, 47% yield).

Deprotection of peptide

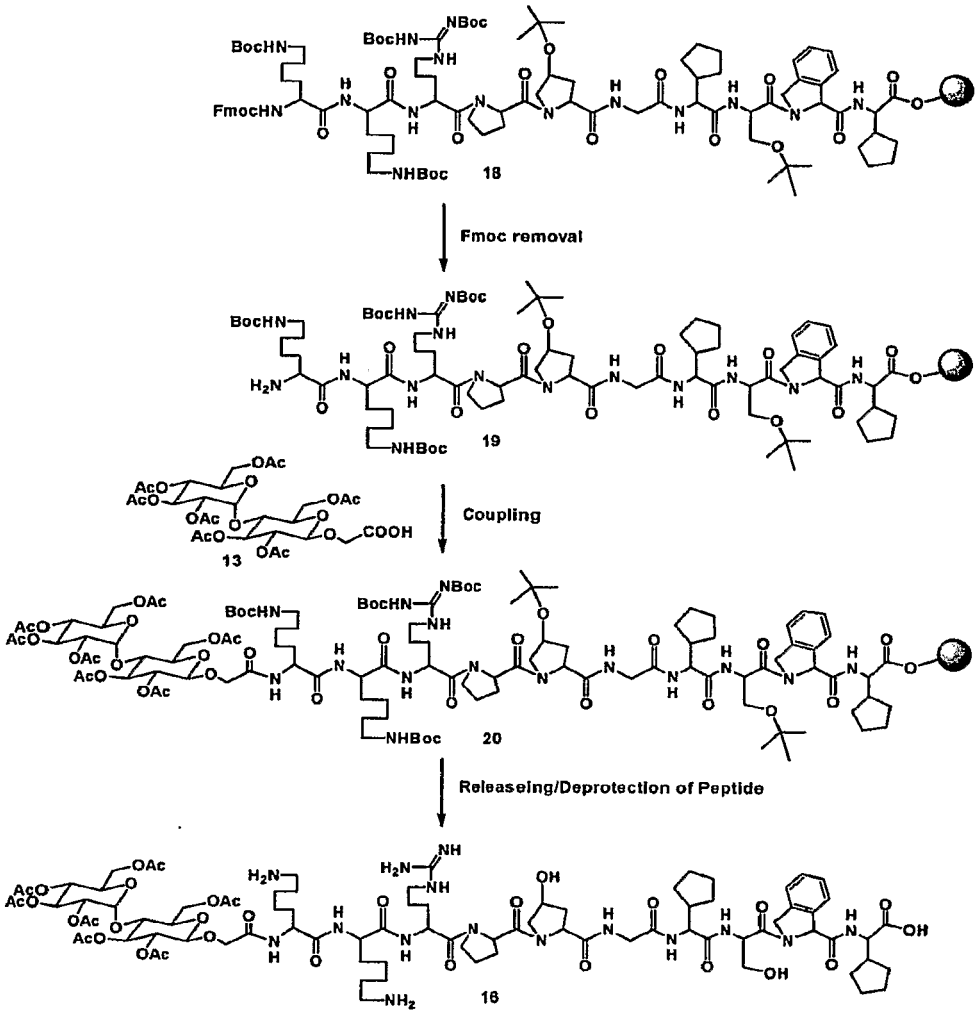
A solution of compound **15** (21.4 mg, 8 μmole) and trifluoroacetic acid (TFA) (2 mL) in DCM (2mL) in a plastic bottle was stirred at RT for 8 hours, and was concentrated. The product was purified by a preparative HPLC. The fractions were combined and lyophilized to give compound **16** (11 mg, 71%).

Deacetylation of sugar portion

Deprotection of the sugar portion of compound **16** was performed in a manner similar to the O-deacetylation conditions for the converting compound **10** to compound **11**. The product was purified by an HPLC (Phenomenx Synergi 4u Polar-RP 150 × 20 nm column eluting with a gradient of 20-45% CH_3CN/H_2O containing 0.05% TFA). The product was eluted at 22 minutes. The product **17** was obtained in 36% yield.

C. Coupling of sugar-glycolic acid derivative to a protected resin bound peptide

This method was intended to be applied for a solid-phase synthesis combined with peptide-chain elongation reactions. The N-terminal Fmoc group of a resin bound peptide was first removed and a sugar-glycolic acid derivative was attached to the newly generated amino group. The resulting sugar-glycolic acid-peptide conjugate was cleaved from the resin. The released peptide conjugate was deprotected stepwise: 1. Deprotection of the peptide portion under acidic conditions and 2. Deprotection of the sugar portion under basic conditions.



Fmoc removal

Resin bound compound **18** (200 mg, 0.07 mmol) was weighed into a fritted reaction vessel and washed with MeOH and DCM first and the washes were removed. The N-terminal Fmoc was cleaved by 2 consecutive treatments with 20% piperidine/DMF for 6 minutes each. The peptide bound resins were washed with DCM thoroughly to give compound **19**, which was used for the next step.

Coupling

Compound **13** (97 mg, 0.14 mmol) and O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) (53 mg, 0.14 mmol) were weighed into a 2-dram vial and were dissolved in DMF (2 mL). The contents of the vial were transferred to the fritted reaction vessel, containing compound **19** prepared previously. Diisopropylethylamine (DIPEA) (50 μL, 36 mg, 0.28 mmol) was added to the reaction mixture and the vessel was stirred on an oscillating stir plate for 2 hours. The reaction solution was drained and the resins were washed with DMF and DCM to yield compound **20**.

Releasing/Deprotection of peptide

Compound **20**, prepared previously, was treated with TFA/triisopropylsilane (TIS)/H_2O (2 mL, 95/2.5/2.5, v/v) for 2.5 hours to perform cleavage of the sugar-glycolic acid-peptide conjugate from the resins and deprotection of the peptide conjugate. The solution was collected and concentrated under reduced pressure. The resulting residue was triturated with Et_2O (3 times) to give compound **16** as a white solid in overall 56% yield in these 3 steps. O-Deacetylation of the sugar portion was again accomplished by treating with hydrazine as described previously.

Purification by HPLC

The crude compounds discussed above in Examples 6 A, B, and C were purified by mass directed HPLC as described below (#1). If necessary, a compound was further purified by the subsequent HPLC condition (#2) to achieve higher purity.

1. A compound (2-5 mg) was injected onto a Phenomenex Synergi 4u Polar-RP 150 × 20 mm column and eluted with a gradient of 20-45% acetonitrile:H_2O (0.05% TFA) over 22 minutes.

2. A second purification was performed on the identical column with a 20-45% acetonitrile:H_2O (NH_4HCO_3, pH 7.9) gradient. It was sometimes necessary to repeat this step to achieve 95% purity

3. Purity was evaluated by on analytical HPLC (UV detection at 214 nm) using a Waters Symmetry300 4.6 x 250 mm column and a gradient elution acetonitrile:H_2O (0.05% TFA) 15-40% over 30 minutes.

Example 4: *In vitro* B1-Inhibition Activity of glycosylated peptides

Glycosylated peptide antagonists of the bradykinin B1 receptor can be synthesized with N-terminal extensions containing cysteine as the conjugation site for a carbohydrate. The analogs can contain a variety of peptidyl linkers between the peptide and the cysteine conjugation site that can be useful for thiolselective conjugation approaches (e.g., see Scheme 1). The conjugates can be then purified by IEX-FPLC, characterized by RP-HPLC and profiled *in vitro* for B1 receptor binding and functional antagonism.

A. *In vitro* Assay of human B1 Receptor Function using Calcium Flux:

Activation of the G_q linked B1 receptor results in an increase in intracellular calcium. The calcium sensitive photoprotein aequorin can, therefore, be used as an indicator of B1 receptor activation. Aequorin is a 21-kDa photoprotein that forms a bioluminescent complex when linked to the chromophore cofactor coelenterazine. Following the binding of calcium to this complex, an oxidation reaction of coelenterazine results in the production of apoaequorin, coelenteramide, CO_2, and light that can be detected by conventional luminometry

A CHO D- cell line (e.g., ATCC CRL 9096) stably transfected with human B1 receptor (e.g., GenBank Accession no. AJ238044) and Aequorin was established and maintained in suspension in spinner bottles containing a 1:1 ratio of DMEM and HAM F12 (Gibco 11765-047), high glucose (Gibco 11965-084), 10% Heat Inactivated Dialyzed serum (Gibco 26300-061), 1X Non-Essential

Amino Acids (Gibco 11140-050), 1X Glutamine-Pen-Strep (Gibco 10378-016), and Hygromycin, 300 μg/ml (Roche 843555). Fifteen to twenty four hours prior to the luminometer assay, 25,000 cells/well (2.5E6 cells/10 ml/plate) are plated in 96-well black-sided clear bottom assay plates (Costar #3904).

Media is removed from the wells and replaced with 60 μl of serum free HAM's F12 with 30 mM HEPES (pH 7.5) and 15 μM coelenterazine (Coelenterazine h Luciferin #90608 from Assay Designs). The plates are then incubated for 1.5-2 hours. Ten point IC_{50} compound plates containing 1:3 or 1:5 dilutions of antagonist compounds and an agonist activator plate (20 nM des-Arg10-Kallidin final concentration, EC_{80}) are prepared using Ham's F12 with 30 mM HEPES, pH 7.5. Following coelenterazine incubation, an automated flash-luminometer platform is used to dispense the B1 antagonist compounds to the cell plate, a CCD camera situated underneath the cell plate takes 12 images of the cell plate at 5 second intervals to determine if there is any agonist activity with the compounds. The hB1 agonist, des-Arg_{10}-Kallidin, is then added to the cell plate and another 12 images are recorded to determine the IC_{50} of the antagonist(s).

B. *In vitro* Assay of hB2 Receptor Function using Calcium Flux:

The intracellular calcium flux induced by hB2 receptor activation is analyzed using a hB2 recombinant cell line (CHO-K1) purchased from PerkinElmer (Catalog Number: RBHB2C000EA) on a fluorometric imaging plate reader (FLIPR). The cells are cultured in T225 flask containing Ham's F12 Nutrient Mixture (Invitrogen Corp., Cat # 11765-047), 10% Fetal Clone II Bovine Serum (HyClone, Cat # SH3006603), 1 mM Sodium pyruvate (100 mM stock, Invitrogen Corp., Cat# 12454-013), and 0.4 mg/ml Geneticin (G418; 50 mg/ml active geneticin, Invitrogen, Cat# 10131-207). Culture medium is changed every other day. 24 hours prior to the FLIPR assay, the hB2/CHO cells are washed once with PBS (Invitrogen, Cat.#) and 10 ml of Versene (1:5000, Invitrogen, Cat# 15040-066) is added to each flask. After 5 minutes incubation at 37°C, Versene is removed and cells are detached from the flask and resuspended in culture medium. Cells are counted and 25,000cells/well are plated in 96-well black-sided

clear bottom assay plates (Costar #3904). Cells are incubated in a 37°C CO_2 incubator overnight.

The medium is aspirated from the cells and replaced with 65 µL of dye-loading buffer. The loading buffer is prepared by diluting a stock solution of 0.5 mM Fluo-4 AM (Molecular Probes, dissolved in DMSO containing 10% [w/v] pluronic acid) to a concentration of 1 µM in Clear Dulbecco's Modified Eagle Medium (DMEM) containing 0.1% BSA, 20 mM HEPES, and 2.5 mM probenecid (probenecid inhibits activity of the anion transport protein, and thus improves dye loading in the cells). The cells are dye-loaded for 1 hour at room temperature. The excess dye is removed by washing the cells two times with assay buffer. The assay buffer consists of Hank's Balanced Salt Solution (HBSS) containing 20 mM HEPES, 0.1% BSA, and 2.5 mM probenecid. After the wash cycles, a volume of 100 µL is left in each well, and the plate is ready to be assayed in the FLIPR System. Single point (10 µM final concentration) POC antagonist compound plates or ten point IC_{50} compound plates containing 1:3 or 1:5 dilutions of antagonist compounds and an agonist activator plate (0.3 nM bradykinin final concentration, EC_{80}) are prepared using assay buffer. The cell plate and the compound plates are loaded onto the FLIPR and during the assay, fluorescence readings are taken simultaneously from all 96 wells of the cell plate. Ten 1-second readings are taken to establish a stable baseline for each well, then 25 µL from the B1 antagonist plate is rapidly (50 µL/sec.) added. The fluorescence signal is measured in 1-second (for 1 minute) followed by 6-second (for 2 minutes) intervals for a total of 3 minutes to determine if there is any agonist activity with the compounds. The B2 agonist, bradykinin, is then added to the cell plate and another 3 minutes are recorded to determine the percent inhibition at 10 µM (POC plates) or the IC_{50} of the antagonist.

The IC_{50} values for unglycosylated peptides tested in the hB1 aequorin assay can be on average slightly reduced *in vitro* activity compared to peptides conjugated to carbohydrate. For example, the native peptide represented by SEQ ID NO:24 and its acetylated form represented by SEQ ID NO:25, resulted in an IC_{50} of 3.0 nM (+/- 5 nM, n=8) and 3.2 nM (+/- 3.2 nM, n=9), respectively at the

hB1 receptor. However, the same glycosylated peptide as described herein demonstrated approximately a 10-fold increase in IC_{50}. The native, acetylated, and glycosylated forms of the peptide can be inactive up to 10 μM in the hB2 FLIPR assay. None of the compounds showed agonist activity at either the hB1 or hB2 receptor.

C. Cell and Tissue based *In Vitro* Assays of hB1 Receptor Binding Peptides:

The aim of these studies is to investigate the antagonist activity of several unglycosylated peptides at the bradykinin B1 receptors in *in vitro* cell-based and isolated organ assays.

1. Rabbit endothelial cell B1-specific PGI2 secretion assay: This assay can be conducted as described by Galizzi et al. (Galizzi, JP et al., Up-regulation of [^{3}H]-des-Arg10-kallidin binding to the bradykinin B1 receptor by interleukin-1 beta in isolated smooth muscle cells: correlation with B1 agonist-induced PGI2 production, Br J Pharmacol. 1994 Oct;113(2):389-94 (1994)).

2. B1 and B2 umblical vein Assay: The antagonist activity and selectivity for bradykinin B1 over B2 receptor of the peptides and/or vehicle-conjugated peptides of the present invention are determined with the *in vitro* human umbilical Vein (HUV) contractility assay described below:

Endothelium-denuded vessels are suspended in 20-ml organ baths containing an oxygenated (95 % O_2 and 5 % CO_2) and pre-warmed (37°C) standard physiological salt solution of the following composition (in mM): NaCl 118.0, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 11.0 (pH 7.4). High K+ solutions (80 mM KCl) are prepared by equimolar replacement of NaCl with KCl. Hoe 140 (1 μM), mergetpa (1 μM) and captopril (10 μM) is also present throughout the experiments to block the B2 receptors and to prevent peptide degradation, respectively. The tissues are connected to force

transducers for isometric tension recordings then allowed to equilibrate for a sufficient time under an optimal resting tension. The experiments are typically carried out using semi-automated isolated organ systems possessing eight organ baths each, with multichannel data acquisition.

The tissues are exposed first to a high K^+ solution (80 mM KCl) to obtain a control contraction. Following washings and a subsequent 60-min equilibration period, the tissues are exposed to cumulative increasing concentrations of the reference agonist Lys-desArg9-BK to obtain concentration-response curves in the absence (control preparations) or presence of various concentrations of the test compounds or the reference antagonist Lys-desArg9[Leu8]-BK (test preparations), which are added 15 min before the exposure to Lys-desArg9-BK. A concentration-response curve to Lys-desArg9-BK is generated in each preparation.

The parameter measured is the maximal change in tension induced by each agonist concentration and the results expressed as a percent of the control responses to KCl. The EC_{50} values of the agonist (concentration producing a half-maximum response) are calculated by linear regression analysis of its concentration-response curves. The antagonist potencies of the test compounds and Lys-desArg9[Leu8]-BK are evaluated in terms of pA2 values (-log concentration producing a two-fold rightward shift of the agonist concentration-response curve), which are calculated according to Van Rossum (Van Rossum, J.M., Cumulative dose-response curves. II. Technique for the making of dose-response curves in isolated organs and the evaluation of drug parameters. Arch. Int. Pharmacodyn. Ther., 143:299-330 (1963)). The pA2 values are calculated using only antagonist concentrations that caused a significant rightward shift of the agonist concentration-response curve. Typically, the pA2 values are given as the mean ± S.E.M. of three determinations. Statistical significance of the differences are determined using Student's t test and p values < 0.05 are considered statistically significant.

D. *In vitro* B1-Inhibition Activity of Conjugated Peptides

The effectiveness of the glycosylated peptides prepared according to Example 1 as inhibitors of B1 activity (i.e., B1 "neutralization") can be evaluated by measuring the ability of each glycosylated peptide to block B1 stimulated CGRP and substance P release and calcium signaling in Dorsal Root Ganglion (DRG) neuronal cultures.

Dorsal Root Ganglion Neuronal Cultures. Dorsal root ganglia are dissected one by one under aseptic conditions from all spinal segments of embryonic 19-day old (E19) rats that are surgically removed from the uterus of timed-pregnant, terminally anesthetized Sprague-Dawley rats (Charles River, Wilmington, MA). DRG are collected in ice-cold L-15 media (GibcoBRL, Grand Island, NY) containing 5% heat inactivated horse serum (GibcoBRL), and any loose connective tissue and blood vessels are removed. The DRG are rinsed twice in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffered saline (DPBS), pH 7.4 (GibcoBRL). The DRG are then dissociated into single cell suspension using a papain dissociation system (Worthington Biochemical Corp., Freehold, NJ). Briefly, DRG are incubated in a digestion solution containing 20 U/ml of papain in Earle's Balanced Salt Solution (EBSS) at 37°C for fifty minutes. Cells are dissociated by trituration through fire-polished Pasteur pipettes in a dissociation medium consisting of MEM/Ham's F12, 1:1, 1 mg/ml ovomucoid inhibitor and 1 mg/ml ovalbumin, and 0.005% deoxyribonuclease I (DNase). The dissociated cells are pelleted at 200 x g for five minutes and re-suspended in EBSS containing 1 mg/ml ovomucoid inhibitor, 1 mg/ml ovalbumin and 0.005% DNase. Cell suspension is centrifuged through a gradient solution containing 10 mg/ml ovomucoid inhibitor, 10 mg/ml ovalbumin at 200 x g for six minutes to remove cell debris, and then filtered through a 88-μm nylon mesh (Fisher Scientific, Pittsburgh, PA) to remove any clumps. Cell number is determined with a hemocytometer, and cells are seeded into poly-ornithine 100 μg/ml (Sigma, St. Louis, MO) and mouse laminin 1 μg/ml (GibcoBRL)-coated 96-well plates at 10 x 10^3 cells/well in complete medium. The complete medium consists of minimal

essential medium (MEM) and Ham's F12, 1:1, penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% heat inactivated horse serum (GibcoBRL). The cultures are kept at 37°C, 5% CO_2 and 100% humidity. For controlling the growth of non-neuronal cells, 5-fluoro-2'-deoxyuridine (75 µM) and uridine (180 µM) are included in the medium.

Treatment with B1 and anti-B1 peptides. Two hours after plating, cells are treated with recombinant human β-B1 or recombinant rat β-B1 at a concentration of 10 ng/ml (0.38 nM). Positive controls comprising serial-diluted anti-B1 antibody (R&D Systems, Minneapolis, MN) are applied to each culture plate. Glycosylated peptides (according to Example 1) are added at ten concentrations using 3.16-fold serial dilutions. All samples are diluted in complete medium before being added to the cultures. Incubation time is generally around 40 hours prior to measurement of VR1 expression.

Measurement of VR1 Expression in DRG Neurons. Cultures are fixed with 4% paraformaldehyde in Hanks' balanced salt solution for fifteen minutes, blocked with Superblock (Pierce, Rockford, IL), and permeabilized with 0.25% Nonidet P-40 (Sigma) in Tris.HCl (Sigma)-buffered saline (TBS) for one hour at room temperature. Cultures are rinsed once with TBS containing 0.1% Tween 20 (Sigma) and incubated with rabbit anti-VR1 IgG (prepared at Amgen) for one and one-half hours at room temperature, followed by incubation of Eu-labeled anti-rabbit second antibody (Wallac Oy, Turku, Finland) for one hour at room temperature. Washes with TBS (3 x five minutes with slow shaking) are applied after each antibody incubation. Enhance solution (150 µl/well, Wallac Oy) is added to the cultures. The fluorescence signal is then measured in a time-resolved fluorometer (Wallac Oy). VR1 expression in samples treated with the unglycosylated peptides is determined by comparing to a standard curve of B1 titration from 0–1000 ng/ml. Percent inhibition (compared to maximum possible inhibition) of B1 effect on VR1 expression in DRG neurons is determined by comparing to controls that are not B1-treated.

Example 5: *In vivo* antinociceptive activity of anti-B1 glycosylated peptides in rat and monkey pain models

The therapeutic or prophylactic efficacy of the inventive composition is tested preclinically using any appropriate animal model known to those skilled in the art related to a particular condition of interest, such as chronic pain, for example, but not limited to, the exemplary animal pain models described herein below. Animal models of migraine are also known. (See, e.g., Akerman, S and Goadsby PJ, The role of dopamine in a model of trigeminovascular nociception, Pharmacol. Exp. Ther. 314(1):162-169 (2005).

A. Rat Neuropathic Pain Model. Male Sprague-Dawley rats (200 g) are anesthetized with isoflurane inhalant anesthesia and the left lumbar spinal nerves at the level of L5 and L6 are tightly ligated (4-0 silk suture) distal to the dorsal root ganglion and prior to entrance into the sciatic nerve, as first described by Kim and Chung (Kim, S.H.; Chung, J.M. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain 50:355-363, (1992)). The incisions are closed and the rats are allowed to recover. This procedure results in mechanical (tactile) allodynia in the left hind paw as assessed by recording the pressure at which the affected paw (ipsilateral to the site of nerve injury) is withdrawn from graded stimuli (von Frey filaments ranging from 4.0 to 148.1 mN) applied perpendicularly to the plantar surface of the paw (between the footpads) through wire-mesh observation cages. A paw withdrawal threshold (PWT) is determined by sequentially increasing and decreasing the stimulus strength and analyzing withdrawal data using a Dixon non-parametric test, as described by Chaplan et al. (Chaplan, S.R. et al., Quantitative assessment of tactile allodynia in the rat paw. J. Neurosci. Meth, 53:55-63 (1994)).

Normal rats and sham surgery rats (nerves isolated but not ligated) withstand at least 148.1 mN (equivalent to 15 g) of pressure without responding. Spinal nerve ligated rats respond to as little as 4.0 mN (equivalent to 0.41 g) of pressure on the affected paw. Rats are included in the study only if they do not

exhibit motor dysfunction (e.g., paw dragging or dropping) and their PWT is below 39.2 mN (equivalent to 4.0 g). At least seven days after surgery rats are treated with glycosylated peptides (usually a screening dose of 60 mg/kg) or control diluent (PBS) once by s.c. injection and PWT is determined each day thereafter for 7 days.

B. Rat CFA Inflammatory Pain Model. Male Sprague-Dawley rats (200 g) are lightly anesthetized with isoflurane inhalant anesthesia and the left hindpaw is injected with complete Freund's adjuvant (CFA), 0.15 ml. This procedure results in mechanical (tactile) allodynia in the left hind paw as assessed by recording the pressure at which the affected paw is withdrawn from graded stimuli (von Frey filaments ranging from 4.0 to 148.1 mN) applied perpendicularly to the plantar surface of the paw (between the footpads) through wire-mesh observation cages. PWT is determined by sequentially increasing and decreasing the stimulus strength and analyzing withdrawal data using a Dixon non-parametric test, as described by Chaplan et al. (1994). Rats are included in the study only if they do not exhibit motor dysfunction (e.g., paw dragging or dropping) or broken skin and their PWT is below 39.2 mN (equivalent to 4.0 g). At least seven days after CFA injection rats are treated with glycosylated peptides (usually a screening dose of 60 mg/kg) or control solution (PBS) once by s.c. injection and PWT is determined each day thereafter for 7 days. Average paw withdrawal threshold (PWT) is converted to percent of maximum possible effect (%MPE) using the following formula: %MPE = 100 * (PWT of treated rats – PWT of control rats)/(15-PWT of control rats). Thus, the cutoff value of 15 g (148.1 mN) is equivalent to 100% of the MPE and the control response is equivalent to 0% MPE.

At the screening dose of 60 mg/kg, glycosylated peptides are expected to produce an antinociceptive effect with a PD relationship.

C. Green Monkey LPS Inflammation Model. The effectiveness of the glycosylated peptides according to Example 1 as inhibitors of B1 activity can be evaluated in Male green monkeys (*Cercopithaecus aethiops St Kitts*) challenged

locally with B1 agonists essentially as described by deBlois and Horlick (British Journal of Pharmacology. 132:327-335 (2002), which is hereby incorporated by reference in its entirety).

In order to determine whether glycosylated peptide antagonists inhibit B1 induced oedema the studies described below can be conducted on male green monkeys (*Cercopithaecus aethiops St Kitts*) at the Caribbean Primates Ltd. experimental farm (St Kitts, West Indies). Procedures can be reviewed and accepted by the Animal Care Committees of the CR-CHUM (Montreal, Canada) and of Caribbean Primates Ltd. (St Kitts, West Indies). Animals weighing 6.0±0.5 kg (n=67) can be anaesthetized (50 mg ketamine kg^{-1}) and pretreated with a single intravenous injection of LPS (90 μg kg^{-1}) or saline (1 ml) *via* the saphenous vein.

Inflammation studies

Kinin-induced oedema can be evaluated by the ventral skin fold assay (Sciberras *et al.*, 1987). Briefly, anaesthetized monkeys are injected with captopril (1 mg kg^{-1} 30 min before assay). A single subcutaneous injection of des-Arg-kallidin, bradykinin or the carbohydrate (2 mM amastatin in 100 μl Ringer's lactate) is given in the ventral area and the increase in thickness of skin folds is monitored for 30–45 min using a calibrated caliper. The results can be expressed as the difference between the skin fold thickness before and after the subcutaneous injection. Captopril and amastatin can be used to reduce degradation of kinins at the carboxyl- and amino-terminus, respectively.

Antagonist Schild Analysis: The dose-response relationship for des-Arg-kallidin (1–100 nmol)-induced oedema is determined at 24 hours post-LPS in the absence or presence of different concentrations of glycosylated-peptide antagonist. BK (30 nmol) is used as a positive control.

Antagonist Time Course. The time course of inhibition by antagonist is determined at 4, 24 and 48 hours, 72 and/or 96 hours after single bolus administration. BK (30 nmol) is used as a positive control.

Drugs. Ketamine hydrochloride, LPS, amastatin and captopril can be purchased from Sigma (MO, U.S.A.). All peptides can be purchased from Phoenix Pharmaceuticals (CA, U.S.A.).

Statistics. Values are presented as mean ± standard error of the mean (s.e. mean). In edema studies, the pre-injection thickness of the skin folds is subtracted from the values after subcutaneous challenge. Curve fitting and EC_{50} calculations can be obtained using the Delta Graph 4.0 software for Apple Computers. Data can be compared by two-way analysis of variance followed by unpaired, one tail Student's *t*-test with Bonferroni correction. $P<0.05$ is considered statistically significant.

LPS administration to green monkeys increased from a null level their sensitivity to a B_1 receptor agonist in an edema formation assay. Comparatively, responses to the B_2 receptor agonist BK were not affected.

Surprisingly, a single subcutaneous dose at 10 mg/kg of a representative glycosylated peptide can be sufficient to relieve a pre-established B1 agonist induced inflammatory response and suppress successive daily agonist challenges for 24 and 48 hours, respectively. Furthermore, the glycoconjugate can inhibit edema in response to des-Arg-kallidin challenge longer than the unglycosylated (i.e., native peptide) peptide although rapid onset and efficacy can be comparable for both molecules up to 1.25 hours.

As indicated below in Table 5, certain glycosylated peptides showed significant improvement over a reference nonglycosylated B1 peptide antagonist (SEQ ID NO: 15) with respect to *in vitro* potency and/or the PK measurements. For example, the *in vitro* potency and the PK were surprisingly better for the N-acetyllactoseamine conjugated 16-mer (SEQ ID NO:33) than the *in vitro* potency activity and PK of the reference nonglycosylated peptide (SEQ ID NO:15). More specifically, the N-acetyllactoseamine conjugated 16-mer (SEQ ID NO:33) exhibited an hB1 Ki = 0.20 nM, an hB1 IC_{50} = 0.3 nM, and a hB2 Ki - 1500 nM). Following I.V. administration to rats using a rat study essentially as described in Example 7, the N-acetyllactoseamine conjugated 16-mer (SEQ ID NO:33) exhibited a 4-fold improvement in exposure relative to the reference

nonglycosylated peptide (10mg/kg i.v., $AUC_{(0\ to\ \infty)}$ = 7720 ng·h/mL, Cl = 1300 mL/h/kg, Vdss = 421 mL/kg). In fact, PK exposure of the N-acetyllactoseamine conjugated 16-mer (relative to the reference nonglycosylated B1 peptide) was significantly improved when the 16-mer was conjugated to either cellobiose, N-acteyllactoseamine, glucose, mannose, galactose, or lactose. On the other hand, the 10-mer B1 peptide (SEQ ID NO:13) resulted in a greater than 2-fold improvement in exposure relative to the reference nonglycosylated peptide (SEQ ID NO:15) only when conjugated to mannose. The 9-mer B1 peptide (SEQ ID NO:21) resulted in significant improvements in exposure relative to the reference nonglycosylated B1 peptide (SEQ ID NO:15) when conjugated to N-acetylglycoseamine, cellobiose, and glucose (4.15 fold, 3.38 fold, and 3.30 fold, respectively). The data described here was generated essentially as described in Example 2.

Table 5

Glycoside	Fold-improvement over Reference B1 Peptide Antagonist (SEQ ID No: 15)		
	16-mer	10-mer	9-mer
N-acetylglucoseamine	3.72	1.53	4.15
Cellobiose	3.50	0.83	3.38
N-Acetyllactoseamine	3.24	.	.
Glucose	3.14	.	3.30
Mannose	3.01	2.363	.
Galactose	2.88	1.13	.
Lactose	2.77	0.893	.
fucose-a-1,6-galactose	2.05	.	.
Sialic acid methyl ester	1.63	.	.
Gentiobiose	1.52	.	1.57
2,4-diaminoglucose	.	1.713	.
2-aminoglucose	.	1.67	.

SEQ ID NO:15	.	1	.
Maltotriose	.	0.01	.
Gentiobiose	.	0.89	.
Maltose	.	0.11	.
Maltotetrose	.	.	.

Fold improvement refers to the ratio of a compound's dose-normalized $AUC_{(0 \text{ to } \infty)}$ to $AUC_{(0 \text{ to } \infty)}$ of 772 ng·h/mL obtained for unglycosylated B1 peptide reference (SEQ ID NO:15). Compounds were dosed at either 3 mg/kg or 10 mg/kg i.v.

Example 6: *In vitro* bradykinin receptor binding assays

A. Preparation of cell membranes expressing human B1 receptor: Cell membranes were prepared from the CHO-D$^-$/human B1 receptor/aequorin cells described above in Example 4A. For large-scale production of membranes, cells were grown in 100 L suspension culture to $1x10^8$ cells/mL, and then harvested using the Viafuge at continuous centrifugation of 1000x g. For pilot studies, cells were grown in 2 L spinner culture and harvested by centrifugation in a J-6M centrifuge using a JS 4.2 rotor at 1900x g for 10 min at 4°C. The cell pellet was washed with PBS, centrifuged again, and the pellet was resuspended in lysis buffer [25 mM HEPES pH 7.4, 5 mM EDTA, 5 mM EGTA, 3 mM $MgCl_2$, 10% (w/v) sucrose, and Complete Protease Inhibitor tablet (EDTA-free) 1 tablet per 50 mL buffer] to a density of 14% (w/v) for passage 3x through Microfluidics microfluidizer at 6000 psi. The cell lysate was centrifuged in a J-6M centrifuge using a JS 4.2 rotor at 1900x g for 10 min at 4°C. The supernatant was collected and centrifuged in an L8-70 centrifuge using a Ti45 rotor at 142,000x g for 1 hr at 4°C to obtain the crude particulate fraction. The resulting pellet was homogenized in lysis buffer in 1/3 the original volume and centrifuged again in the L8-70 centrifuge using the Ti45 rotor at 142,000x g for 1 hr at 4°C. The particulate fraction was suspended by homogenization in buffer [25 mM HEPES pH 7.4, 3mM $MgCl_2$, 10% (w/v) sucrose, and Complete Protease Inhibitor tablet (EDTA-free) 1 tablet per 50 mL buffer]. Single–use aliquots were prepared and frozen in liquid N_2 prior to storage at –80°C.

B. Human B1 receptor binding assay: Human B1 receptor filtration binding assays were performed using [^{3}H]-des-Arg^{10}-Kallidin ([^{3}H]-DAK, New England Nuclear) and cell membranes prepared from hB1R/CHO-D$^-$/aequorin stable cell line. Aliquots of cell membranes suspended in binding buffer (24 mM TES/NH_4OH pH 6.8, 1 mM phenanthroline, 0.5 mM pefabloc, 2 μg/mL Aprotinin, 5 μg/mL Leupeptin, 0.7 μg/mL Pepstatin, and 0.3% BSA) were incubated with 0.4 nM [^{3}H]-DAK in a final volume of 200 μL in 96-well plates at room temperature for 90 min. Competition binding and IC_{50} of B1 antagonists were determined by incubating various concentrations of compound (10,000 – 0.005 nM) with the membranes and [^{3}H]-DAK. The binding reaction was terminated by rapid filtration through GF/C filter, presoaked in 0.5% PEI for 1 hr, and immediately rinsed 6 times with ice-cold Tris-HCl buffer (50 mM pH 7.5). After the filters were dried, 40 μL of Microscint-20 was added per well and the plates were sealed. The filter plates were read on a Packard TopCount NXT to determine the radioactive counts. Nonspecific binding was determined in the presence of 2 μM des-Arg^{10}-Leu^9-Kallidin.

C. Preparation of cell membranes expressing human B2 receptor: Cell membranes containing hB2R were purchased from Receptor Biology (now known as PerkinElmer Life Science). These membranes were derived from a CHO-K1 cell line stably expressing the hB2R. The hB2R/CHO stable cell line was subsequently purchased from PerkinElmer and cell membranes were prepared in-house using the method described for hB1R, except cells were grown in roller bottles and harvested using Cellmate.

D. Human B2 receptor binding assay: The human B2 receptor filtration binding assay was performed similar to the hB1R binding assay, except cell membranes were prepared from a hB2R-transfected CHO stable cell line purchased from PerkinElmer and incubated with B1 antagonists at concentrations ranging between 50,000 and 0.026 nM. Radioligand [^{3}H]-bradykinin (0.3 nM final concentration, New England Nuclear) was used in this hB2R binding assay. Nonspecific binding was determined in the presence of 2 μM bradykinin.

Example 7: Rat Pharmacokinetic Studies

Various peptides or conjugated peptides (in an aqueous medium) are dosed as a bolus to male Sprague-Dawley rats via an intravenous (iv) or subcutaneous (sc) route. Blood samples are collected at various time points (e.g., 0, 15, 30 min. and/or 1, 2, 4, 6, 8, 10, 12, 18, 24, 30, 36, 42, 48, 60, 72, 84, 96, 120, 240, and/or 320 hours after the injection) into heparized tubes. Plasma is removed from pelleted cells upon centrifugation and either frozen or immediately processed. The compound of interest in the plasma is quantitated by an analyte-specific LC-MS/MS or an ELISA method. Various standard pharmacokinetic parameters such as clearance (CL), apparent clearance (CL/F), volume of distribution (Vss), mean residence time (MRT), area under the curve (AUC), and terminal half-life ($t_{1/2}$) can be calculated by non-compartmental method.

CLAIMS

What is claimed is:

1. A composition of matter of the formula:
$F-[(X^1)-(Y^1)_n]$ or a physiologically acceptable salt thereof, wherein:

X^1 and Y^1 are each independently selected peptides of the formula $-(L^1)_a-P^1$ and $-(L^2)_b-P^2$, respectively;

F is a carbohydrate covalently bound to X^1 or Y^1;

L^1 and L^2 are each independently selected linkers;

a and b are each independently 0 or 1;

n is 0 to 1; and

P^1 and P^2 are each independently selected peptide antagonists of the bradykinin B1 receptor.

2. The composition of matter of claim 1 wherein P^1 and P^2 are each an independently selected peptide antagonist of the bradykinin B1 receptor comprising an amino acid sequence selected from SEQ ID NOS: 5-62, or an analog and/or a chemical derivative of any one of these sequences.

3. The composition of matter of claim 1 or claim 2 wherein X^1 comprises a peptide having an amino acid sequence selected from SEQ ID NOS: 5-9, 11-34, 36, 38-41, 43, 45, 47, 49-51, 53, 55, 57, 59, and 61, or an analog and/or a chemical derivative of any of these sequences.

4. The composition of matter of claim 1 or claim 2 wherein X^1 is a peptide having an amino acid sequence selected from SEQ ID NOS:5-9, 11-26, 43, 45, 47, 49-51, 53, 55, 57, 59, and 61, further comprising a N-terminal cysteine.

5. The composition of matter of claim 1 or claim 2 wherein n is 0.

6. The composition of matter of claim 1 or claim 2, wherein a is 1.

7. A composition of matter of the formula:

F-[(X^1)-$(Y^1)_n$] or a physiologically acceptable salt thereof, wherein:

X^1 and Y^1 are each independently selected peptides of the formula $-(L^1)_a$-P^1 and $-(L^2)_b$-P^2, respectively;

F is a carbohydrate covalently bound to X^1 or Y^1;

L^1 and L^2 are each independently selected linkers;

a and b are each independently 0 or 1;

n is 0 to 1; and

P^1 and P^2 are each independently selected peptide antagonists from the group of peptides defined by the formula:

$$NH_2\text{-}a^0a^1a^2a^3a^4a^5a^6a^7a^8a^9a^{10}a^{11}a^{12}a^{13}a^{14}\text{-}COOH$$

wherein:

a^0 is a basic or neutral aromatic, aliphatic, heterocyclic, or alicyclic amino acid, a basic di-peptide, or absent;

a^1, a^2, a^3, and a^4 are independently selected from basic and neutral aromatic, aliphatic, heterocyclic, and alicyclic amino acids;

a^6 is Ser;

a^5, a^7, and a^8 are independently selected from aromatic, aliphatic, heterocyclic, and alicyclic amino acids, provided that at least one of a^5, a^7, and a^8 is independently selected from Chg, Cpg, Igla, Iglb, Niga and Nigb of the D- or L-configuration; and

a^9, a^{10}, a^{11}, a^{12}, a^{13}, and a^{14} are independently selected from any amino acid, or are independently absent.

8. The composition of matter of claim 7 wherein:

a^0 is a basic amino acid or basic di-peptide or absent;

a^1 is a basic amino acid;

a^2 is Pro;

a^3 is Hyp;

a^4 is Gly;

a^5 is an Indanyl amino acid;

a^6 is Ser;

a^7 is a D-Indanyl amino acid;

a^8 is Cpg; and

a^9, a^{10}, a^{11}, a^{12}, a^{13}, and a^{14} are independently selected from any amino acid, or are independently absent.

9. The composition of matter of claim 7 wherein:

a^0 is Lys-Lys, D-Orn-Lys, D-Lys-Lys, D-Arg-Lys, or D-Orn-D-Orn;

a^1 is Arg;

a^2 is Pro;

a^3 is Hyp;

a^4 is Gly;

a^5 is Cpg;

a^6 is Ser;

a^7 is DTic; and

a^8 is Cpg.

10. The composition of matter of claim 7 wherein:

a^0 is Lys-Lys, D-Orn-Lys, D-Lys-Lys, D-Arg-Lys, or D-Orn-D-Orn;

a^1 is Arg;

a^2 is Pro;

a^3 is Hyp;

a^4 is Gly;

a^5 is Iglb;

a^6 is Ser;

a^7 is DIglb; and

a^8 is Oic.

11. The composition of matter of claim 7 wherein:

a^0 is DArg;

a^1 is Arg;

a^2 is Pro;

a^3 is Hyp;

a^4 is Gly;

a^5 is Igl;

a^6 is Ser;

a^7 is DIgl; and

a^8 is Oic.

12. A composition of matter of the formula:

$F-[(X^1)-(Y^1)_n]$ or a physiologically acceptable salt thereof, wherein:

X^1 and Y^1 are each independently selected peptides of the formula $-(L^1)_a-P^1$ and $-(L^2)_b-P^2$, respectively;

F is a carbohydrate covalently bound to X^1 or Y^1;

L^1 and L^2 are each independently selected linkers;

a and b are each independently 0 or 1;

n is 0 to 1; and

P^1 and P^2 are each independently selected peptide antagonists from the group of peptides defined by the formula:

$$NH_2-a^0a^1a^2a^3a^4a^5a^6a^7a^8a^9a^{10}a^{11}a^{12}a^{13}a^{14}-COOH$$

wherein:

a^0 is Lys-Lys, D-Orn-Lys, D-Lys-Lys, D-Arg-Lys, or D-Orn-D-Orn;

a^1 is Arg;

a^2 is Pro;

a^3 is Pro;

a^4 is Gly;

a^5 is AMeF;

a^6 is Ser;

a^7 is D-β-Nal; and

a^8 is Ile; and

a^9, a^{10}, a^{11}, a^{12}, a^{13}, and a^{14} are independently selected from any amino acid, or are independently absent.

13. The composition of matter of claim 7, 8, 9, 10, 11, or 12 wherein a is 1 and L^1 is a peptidyl linker comprising an amino acid sequence of 1 to about 30 amino acid residues.

14. The composition of matter of Claim 13 wherein L^1 is selected from SEQ ID NOS:63-75.

15. The composition of matter of claim 1, 2, 7, 8, 9, 10, 11, or 12, wherein said composition of matter is capable of antagonizing B1 receptor activity *in vitro* and has a therapeutically acceptable half-life *in vivo* in mammals.

16. A method of treating, preventing, or ameliorating a disease or condition associated with B1 activity comprising administering to a human or animal subject a therapeutically effective amount of the composition of matter according to any one of Claims 1, 2, 7, 8, 9, 10, 11, or 12.

17. The method according to claim 16 wherein the disease or condition is selected from inflammation, inflammatory pain, acute pain, dental pain, back pain, lower back pain, pain from trauma, post-herpetic neuralgia, and surgical pain.

18. The method according to claim 16 wherein the disease or condition is selected from inflammatory bowel disorders, asthma, and allergic rhinitis.

19. A pharmaceutical composition comprising a composition of matter according to Claim 1, 2, 7, 8, 9, 10, 11, or 12, and at least one pharmaceutically-acceptable diluent, excipient, or carrier.

20. The use of a composition of matter according to Claim 1, 2, 7, 8, 9, 10, 11, or 12 in the manufacture of a medicament for the treatment of a disease or condition selected from inflammation, inflammatory pain, acute pain, dental pain, back pain, lower back pain, pain from trauma, post-herpetic neuralgia, surgical pain, inflammatory bowel disorders, asthma, and allergic rhinitis.

21. A composition of matter of the formula:

$F\text{-}[(X^1)\text{–}(Y^1)_n]$ or a physiologically acceptable salt thereof, wherein:

X^1 and Y^1 are each independently selected peptides of the formula $\text{–}(L^1)_a\text{-}P^1$ and $\text{–}(L^2)_b\text{-}P^2$, respectively;

F is a carbohydrate covalently bound to X^1;

L^1 and L^2 are each independently selected linkers;

a and b are each independently 0 or 1;

n is 0 to 1; and

P^1 and P^2 are each independently selected peptide antagonists from the group of peptides defined by the formula:

$$NH_2\text{-}a^0a^1a^2a^3a^4a^5a^6a^7a^8a^9a^{10}a^{11}a^{12}a^{13}a^{14}\text{-}COOH$$

wherein:

a^0 is selected from basic and neutral aromatic, aliphatic, heterocyclic, and alicyclic amino acids, a basic di-peptide, and absent;

a^1, a^2, a^3, and a^4 are independently selected from basic and neutral aromatic, aliphatic, heterocyclic, and alicyclic amino acids;

a^6 is Ser;

a^5, a^7, and a^8 are independently selected from aromatic, aliphatic, heterocyclic, and alicyclic amino acids, provided that at least one of a^5, a^7, and a^8 is independently selected from Chg, Cpg, Igla, Iglb, Niga and Nigb, of the D- or L- configuration; and

a^9, a^{10}, a^{11}, a^{12}, a^{13}, and a^{14} are independently selected from any amino acid, or are independently absent.

22. The composition of matter of claim 21 wherein:
a^0 is a basic amino acid or basic di-peptide or absent;
a^1 is a basic amino acid;
a^2 is Pro;
a^3 is Hyp;
a^4 is Gly;
a^5 is an Indanyl amino acid;
a^6 is Ser;
a^7 is a D-Indanyl amino acid;
a^8 is Cpg.

23. The composition of matter of claim 21 wherein:
a^0 is Lys-Lys, D-Lys-Lys, D-Orn-Lys, D-Arg-Lys, or D-Orn-D-Orn;
a^1 is Arg;
a^2 is Pro;
a^3 is Hyp;
a^4 is Gly;
a^5 is Cpg;
a^6 is Ser;
a^7 is DTic; and
a^8 is Cpg.

24. The composition of matter of claim 21 wherein:
a^0 is Lys-Lys, D-Lys-Lys, D-Orn-Lys, D-Arg-Lys, or D-Orn-D-Orn;
a^1 is Arg;
a^2 is Pro;
a^3 is Hyp;
a^4 is Gly;
a^5 is Iglb;
a^6 is Ser;

a^7 is DIglb; and

a^8 is Oic.

25. The composition of matter of claim 21 wherein:

a^0 is DArg;

a^1 is Arg;

a^2 is Pro;

a^3 is Hyp;

a^4 is Gly;

a^5 is Igl;

a^6 is Ser;

a^7 is DIgl; and

a^8 is Oic.

26. A composition of matter of the formula:

F-[(X^1)–$(Y^1)_n$]or a physiologically acceptable salt thereof, wherein:

X^1 and Y^1 are each independently selected peptides of the formula –$(L^1)_a$-P^1 and –$(L^2)_b$-P^2, respectively;

F is a carbohydrate covalently bound to X^1;

L^1 and L^2 are each independently selected linkers;

a and b are each independently 0 or 1;

n is 0 to 1; and

P^1 and P^2 are each independently selected peptide antagonists from the group of peptides defined by the formula:

$$NH_2\text{-}a^0a^1a^2a^3a^4a^5a^6a^7a^8a^9a^{10}a^{11}a^{12}a^{13}a^{14}\text{-}COOH$$

wherein:

a^0 is Lys-Lys, D-Lys-Lys, D-Orn-Lys, D-Arg-Lys, or D-Orn-D-Orn;

a^1 is Arg;

a^2 is Pro;

a^3 is Pro;

a^4 is Gly;

a^5 is AMeF;

a^6 is Ser;

a^7 is D-β-Nal; and

a^8 is Ile; and

a^9, a^{10}, a^{11}, a^{12}, a^{13}, and a^{14} are independently selected from any amino acid, or are independently absent.

27. The composition of matter of any of Claims 21, 22, 23, 24, 25, or 26 wherein a is 1 and L^1 is a peptidyl linker comprising an amino acid sequence of 1 to about 30 amino acid residues.

28. The composition of matter of Claim 27 wherein L^1 is selected from SEQ ID NOS:63-75.

29. The composition of matter of any one of claims 1, 2, 7, 12, 21, or 26, wherein the composition of matter is capable of antagonizing B1 receptor activity *in vitro* and has a therapeutically acceptable half-life *in vivo* in mammals.

30. A method of treating, preventing, or ameliorating a disease or condition associated with B1 activity comprising administering to a human or animal subject a therapeutically effective amount of the composition of matter according to any one of Claims 1, 2, 7, 12, 21, or 26.

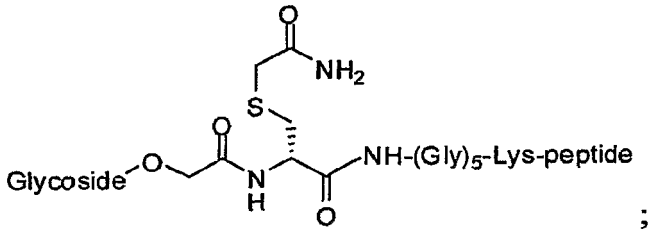
31. The method according to claim 30, wherein the disease or condition is selected from inflammation, inflammatory pain, acute pain, dental pain, back pain, lower back pain, pain from trauma, post-herpetic neuralgia, surgical pain, inflammatory bowel disorders, asthma, and allergic rhinitis.

32. A pharmaceutical composition comprising a composition of matter according to Claim 21 or Claim 26, and at least one pharmaceutically-acceptable diluent, excipient, or carrier.

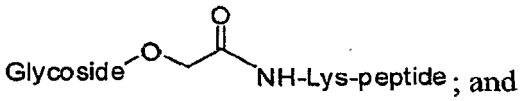
33. The use of a composition of matter according to Claim 21 or 26 in the manufacture of a medicament for the treatment of a disease or condition selected from inflammation, inflammatory pain, acute pain, dental pain, back pain, lower back pain, pain from trauma, post-herpetic neuralgia, surgical pain, inflammatory bowel disorders, asthma, and allergic rhinitis.

34. The composition of matter of Claim 1, having a structure selected from:

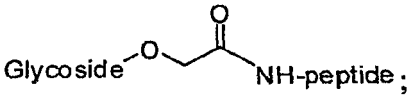
(a)



;

(b) 

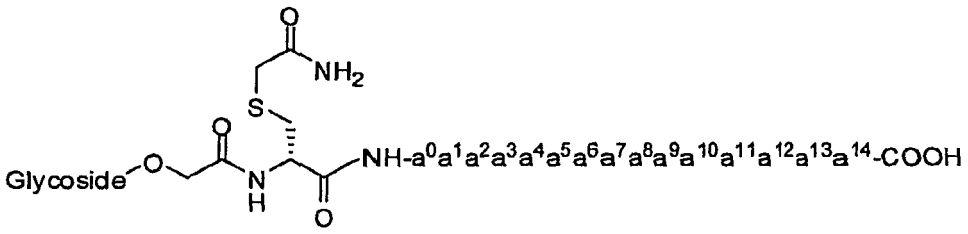
; and

(c) 

;

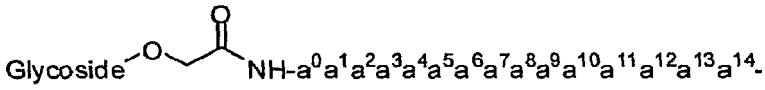
wherein the peptide is a peptide antagonist of the bradykinin B1 receptor; and wherein glycoside is at least one glycoside selected from N-acetylglucoseamine, N-acetyllactoseamine, cellobiose, glucose, mannose, galactose, lactose, fucose-a-1,6-galactose, sialic acid methyl ester, gentiobiose, 2,4-diaminoglucose, 2-aminoglucose, maltotriose, maltotetrose, and maltose.

35. The composition of matter of Claim 21 or Claim 26, wherein the composition has the structure:



36. The composition of matter of Claim 35, wherein the glycoside is at least one glycoside selected from N-acetylglucoseamine, N-acetyllactoseamine, cellobiose, glucose, mannose, galactose, lactose, fucose-a-1,6-galactose, sialic acid methyl ester, gentiobiose, 2,4-diaminoglucose, 2-aminoglucose, maltotriose, maltotetrose, and maltose.

37. The composition of matter of Claim 21 or Claim 26, wherein a is 1; and wherein the composition has the structure:



38. The composition of matter of Claim 37, wherein the glycoside is at least one glycoside selected from N-acetylglucoseamine, N-acetyllactoseamine, cellobiose, glucose, mannose, galactose, lactose, fucose-a-1,6-galactose, sialic acid methyl ester, gentiobiose, 2,4-diaminoglucose, 2-aminoglucose, maltotriose, maltotetrose, and maltose.

39. The composition of matter of any one of Claims 34, 35, or 37, wherein the composition of matter is capable of antagonizing B1 receptor activity *in vitro* and has a therapeutically acceptable half-life *in vivo* in mammals.

40. A method of treating, preventing, or ameliorating a disease or condition associated with B1 activity comprising administering to a human or animal subject a therapeutically effective amount of the composition of matter according to any one of Claims 34, 35, or 37.

41. The method according to claim 40, wherein the disease or condition is selected from inflammation, inflammatory pain, acute pain, dental pain, back pain, lower back pain, pain from trauma, post-herpetic neuralgia, surgical pain, inflammatory bowel disorders, asthma, and allergic rhinitis.

42. A pharmaceutical composition comprising a composition of matter according to any one of Claims 34, 35, or 37, and at least one pharmaceutically-acceptable diluent, excipient, or carrier.

43. The use of a composition of matter according to any one of Claims 34, 35, or 37 in the manufacture of a medicament for the treatment of a disease or condition selected from inflammation, inflammatory pain, acute pain, dental pain, back pain, lower back pain, pain from trauma, post-herpetic neuralgia, surgical pain, inflammatory bowel disorders, asthma, and allergic rhinitis.

44. The composition of matter according to any of Claims 1, 7, 12, 21, or 26, wherein the carbohydrate comprises at least one glycoside selected from N-acetylglucoseamine, N-acetyllactoseamine, cellobiose, glucose, mannose, galactose, lactose, fucose-a-1,6-galactose, sialic acid methyl ester, gentiobiose, 2,4-diaminoglucose, 2-aminoglucose, maltotriose, maltotetrose, and maltose.