

METHODS FOR SITE-SPECIFIC PEGYLATION

BACKGROUND OF THE INVENTION

5 The present invention relates to methods for the chemo-selective pegylation of the cysteine residue having an unoxidized sulfhydryl side-chain and a free α -amino group in proteins, peptides and other molecules.

 Unlike small molecule drugs which are usually administered by oral route, protein- and peptide-based therapeutic agents are typically administered by injection due
10 to their extremely low oral bioavailability. After injection most proteins and peptides are rapidly cleaved by enzymes and cleared from the body, resulting in short *in vivo* circulating half-life. The short circulating half-life is responsible for lower efficacy, more frequent administration, reduced patient compliance, and higher cost of protein and peptide therapeutics. Thus, there is a strong need to develop methods to prolong the
15 duration of action of protein and peptide drugs.

 Covalent attachment of proteins or peptides to polyethylene glycol (PEG) has proven to be a useful method to increase the circulating half-lives of proteins and peptides in the body (Abuchowski, A. *et al.*, *Cancer Biochem. Biophys.*, 1984, 7:175-186; Hershfield, M.S. *et al.*, *N. Engl. J. Medicine* 316:589-596; and Meyers, F. J. *et al.*,
20 *Clin. Pharmacol. Ther.*, 1991, 49:307-313). Covalent attachment of PEG to proteins and peptides not only protects the molecules against enzymatic degradation, but also reduces their clearance rate from the body. The size of PEG attached to a protein has significant impact on the circulating half-life of the protein. Usually the larger the PEG is, the longer the *in vivo* half-life of the attached protein is. Several sizes of PEGs are
25 commercially available (*Nektar Advanced PEGylation Catalog* 2005-2006; and *NOF DDS Catalogue Ver 7.1*), which are suitable for producing proteins and peptides with targeted circulating half-lives. PEG moiety also increases water solubility and decreases immunogenicity of proteins, peptides and other molecules (Katre, N.V. *et al.*, *Proc. Natl. Aced. Sci. USA*, 1998, 84:1487-1491; and Katre N.V. *et al.*, *J. Immunology*, 1990,
30 144:209-213).

 Several methods of pegylating proteins have been reported in the literature. For example, N-hydroxy succinimide (NHS)-PEG was used to pegylate the free amine groups of lysine residues and N-terminus of proteins. Because proteins usually contain

multiple lysine residues and terminal amine group, multiple sites of a protein are pegylated by using this method. Such non-selective pegylation results in decreasing the potency of the pegylated proteins because multiple PEG moieties usually disturb the interaction between the proteins and their biological target molecules (Teh, L.-C. and
5 Chapman, G.E., *Biochem. Biophys. Res. Comm.*, 1988, 150:391-398; and Clark, R. *et al.*,
J. Biol. Chem. 1996, 271:21969-21977). Multiple-site, non-selective pegylation also generates heterogeneous mixtures of final products. Many of these heterogeneous pegylated proteins are not suitable for medical use because of low specific activities. It is difficult to purify and characterize heterogeneous pegylated proteins. The variation of
10 contents between different product batches of heterogeneous pegylated proteins is usually high and quality control on these mixtures is difficult.

Although PEGs bearing aldehyde groups have been used to pegylate the amino-termini of proteins in the presence of a reducing reagent, such a method does not generate exclusive N-terminal pegylated proteins and the lysine residues of the proteins
15 are also pegylated. Thus, the resulting proteins are also heterogeneous mixtures (Kinstler O. B. *et al.*, U.S. Application No. 09/817,725). This method also suffers the drawback of using harsh reduction reaction conditions. The reducing reagents such as cyanoborohydride could harm the proteins and give lower reaction yields.

PEGs with maleimide functional groups were used for selectively pegylating the
20 free thiol groups of cysteine residues in proteins. Such method often requires point mutation with new cysteine. Because most proteins contain one or more cysteine residues, to selectively keep the thiol group of the new, "unnatural" cysteine residue from forming a disulfide bridge with other cysteine residues and then to selectively pegylate that particular new cysteine requires complicated reaction conditions (U.S.
25 Patent No. 6,753,165, issued June 22, 2004; and U.S. Patent No. 6,608,183, issued August 19, 2003). Even under the controlled reaction conditions, other cysteine residues can be pegylated and heterogeneous materials are obtained.

Site-specific pegylation of acetyl-phenylalanine residue of growth hormone analogs were reported. Such method requires point mutation with unnatural amino acid
30 acetyl-phenylalanine (U.S. Application No. 11/046,432, filed January 28, 2005). One of the drawbacks of this method is that pegylation of proteins bearing unnatural amino acids, such as acetyl-phenylalanine, can only been done in bacteria but not in mammalian cells.

The free thiol and amine groups generated from the reaction of an amine

thiolactone with free amine group of interleukin-2 have been used to pegylate the protein. However, in this method, the amine thiolactone used reacts with any amine functional groups of lysine residues and N-terminus in proteins and the method is not site-selective (U.S. Patent Number 6,310,180, issued October 30, 2001).

5 Therefore, despite the previous efforts from different groups, there is still a strong need to develop easy and practical methods for site-specific pegylation of proteins, peptides and other molecules.

SUMMARY OF THE INVENTION

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The present invention generally relates to new methods for site-specific pegylation of proteins, peptides and other molecules. It was discovered that PEG containing an aldehyde functional group (PEG-aldehyde) reacts spontaneously with cysteine bearing an unoxidized sulfhydryl side-chain and a free α -amino group in aqueous solution in a wide range of pHs to generate thiazolidine allowing for PEG-aldehyde to react with a peptide fragment containing variety of functional groups which was not certain due to the hydrophilic nature and large size (e.g., 30 kDa) of PEG. We also discovered that only the cysteine residue having an unoxidized sulfhydryl side-chain and a free α -amino group reacts with PEG-aldehyde. The other functional groups in other residues (e.g., thiol group of cysteine without a free α -amino group, guanidinyll group of Arg, amino group of Lys, side-chain carboxylic acid group of Asp, side-chain carboxylic acid group of Glu, hydroxyl group of Tyr, and hydroxyl group of Ser) do not react with PEG-aldehyde.

20 By using the present methods, only cysteine residues having an unoxidized sulfhydryl side-chain and a free α -amino group, but not any other amino acids in proteins, peptides and other molecules, are pegylated. Thus, the present methods are highly site-selective. The site-specific nature of the present pegylation methods results in more homogeneous products which are easy to characterize, purify and manufacture and have less content variation between different batches. The PEG attached at a specific site (i.e., N-terminal cysteine) of proteins and peptides should have less chance to interact with the biological targets and should therefore yield more potent therapeutic agents.

In the present invention, the aldehyde functional group of PEG spontaneously

reacts with the amine and thiol functional groups of cysteine residue at the N-terminus of protein or peptide in aqueous solution in a range of pH (e.g., pH2-8) and at different temperatures (e.g., room temperature). The newly generated functional group between PEG and protein or peptide is a 1,3-thiazolidine. The carboxy groups of glutamic and aspartic acid residues and the C-terminus carboxy group, the amine groups of lysine residues, guanidiny groups of arginine residues, thiol groups of middle cysteine residues, and hydroxy groups of serine, threonine and tyrosine residues do not react with the aldehyde functional group of PEG under such pegylation conditions. Thus, the present invention provides site-specific pegylation of the N-terminal cysteine residue. To prevent disulfide bridge formation during the pegylation, reducing agents such as tris(carboxyethyl)phosphine (TCEP) can be used and the reactions can be done under nitrogen and argon. 1-4 equivalents of PEG-aldehyde can be used. Reactions usually complete in 2 to 72 hours depending on the pH of the solution and the equivalents of PEG-aldehyde used. If the pegylation happens on unfolded proteins, the protein products can be refolded after pegylation. If the pegylation is done on correctly folded proteins, refolding step is omitted.

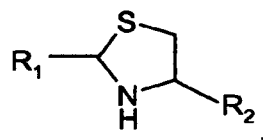
PEGs used in the present invention can have different molecular weights (e.g., 2-40 kDa), have linear, branched and multi-arm structures and contain one or more than one aldehyde functional group. When PEG containing two aldehyde functional groups is used, the final product will be protein or peptide dimer and the linker in between is the PEG. PEG with multiple aldehyde functional groups will generate multimer of pegylated proteins or peptides.

To control the pH of the reaction solution, buffered solution systems such as PBS can be used. The reaction solutions can also contain other agents such as EDTA to facilitate the reactions.

The final pegylated proteins and peptides can be purified by different purification methods such as reversed phase high performance liquid chromatography (RP-HPLC), size-exclusive chromatography, and ion-exchange chromatography, and characterized by MALDI-MS, chromatography methods, electrophoresis, amino acid analysis, and protein and peptide sequencing technologies.

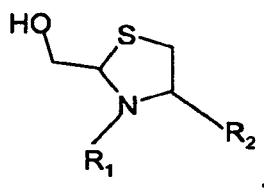
In a first embodiment, the invention is directed to a method of chemically conjugating PEG containing a free aldehyde group to the unoxidized sulfhydryl side-chain and the free α -amino group of a cysteine residue of a molecule, said method

comprising reacting the free aldehyde group of said PEG with the unoxidized sulfhydryl side-chain and the free α -amino group of said cysteine residue to generate a 1,3-thiazolidine group in a product, wherein said product has the structure of

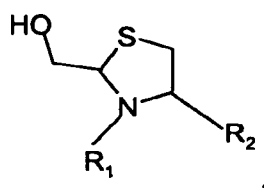


5 wherein R_1 is said PEG, and R_2 is said molecule.

In a second embodiment, the invention is directed to a method of chemically conjugating PEG containing a free aldehyde group to the unoxidized sulfhydryl side-chain and the free α -amino group of a cysteine residue of a molecule, said method comprising reacting the free aldehyde group of said PEG with the unoxidized sulfhydryl side-chain and the free α -amino group of said cysteine residue in a reaction solution to generate a 1,3-thiazolidine group in an intermediate, and adjusting the pH of the reaction solution to about 7, whereby said intermediate rearranges to form a final product, wherein said intermediate has the structure of

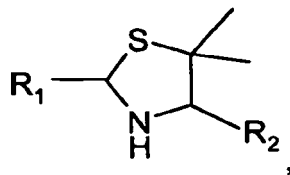


15 and said final product has the structure of



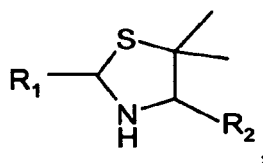
wherein R_1 is said PEG, and R_2 is said molecule. Here, the term "about" means $\pm 10\%$.

In a third embodiment, the invention is directed to a method of chemically conjugating PEG containing a free aldehyde group to the unoxidized sulfhydryl side-chain and the free α -amino group of a penicillamine residue of a molecule, said method comprising reacting the free aldehyde group of said PEG with the unoxidized sulfhydryl side-chain and the free α -amino group of said penicillamine residue to generate a 5,5-dimethyl-1,3-thiazolidine group in a product, wherein said product has the structure of

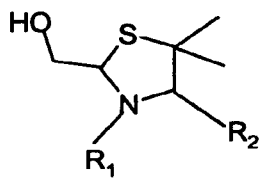


wherein R₁ is said PEG, and R₂ is said molecule.

In a fourth embodiment, the invention is directed to a method of chemically conjugating PEG containing a free aldehyde group to the unoxidized sulfhydryl side-chain and the free α -amino group of a penicillamine residue of a molecule, said method comprising reacting the free aldehyde group of said PEG with the unoxidized sulfhydryl side-chain and the free α -amino group of said penicillamine residue in a reaction solution to generate a 5,5-dimethyl-1,3-thiazolidine group in an intermediate, and adjusting the pH of the reaction solution to about 7, whereby said intermediate rearranges to form a final product, wherein said intermediate has the structure of

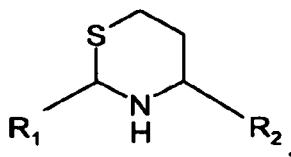


and said final product has the structure of



wherein R₁ is said PEG, and R₂ is said molecule. Here, the term "about" means $\pm 10\%$.

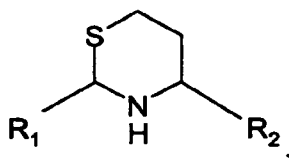
In a fifth embodiment, the invention is directed to a method of chemically conjugating PEG containing a free aldehyde group to the unoxidized sulfhydryl side-chain and the free α -amino group of a homocysteine residue of a molecule, said method comprising reacting the free aldehyde group of said PEG with the unoxidized sulfhydryl side-chain and the free α -amino group of said homocysteine residue to generate a six-membered ring system in a product, wherein said product has the structure of



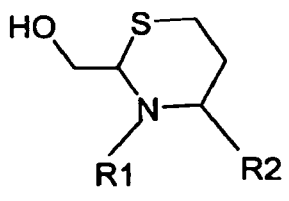
wherein R₁ is said PEG, and R₂ is said molecule.

In a sixth embodiment, the invention is directed to a method of chemically

conjugating PEG containing a free aldehyde group to the unoxidized sulfhydryl side-chain and the free α -amino group of a homocysteine residue of a molecule, said method comprising reacting the free aldehyde group of said PEG with the unoxidized sulfhydryl side-chain and the free α -amino group of said homocysteine residue in a reaction solution to generate a six-membered ring system in an intermediate, and adjusting the pH of the reaction solution to about 7, whereby said intermediate rearranges to form a final product, wherein said intermediate has the structure of

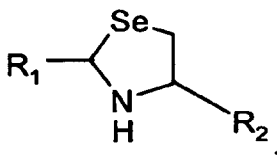


and said final product has the structure of



wherein R_1 is said PEG, and R_2 is said molecule. Here, the term "about" means $\pm 10\%$.

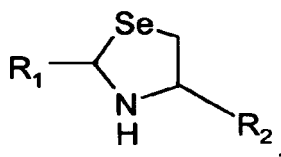
In a seventh embodiment, the invention is directed to a method of chemically conjugating PEG containing a free aldehyde group to the unoxidized free seleno group and the free α -amino group of a selenocysteine residue of a molecule, said method comprising reacting the free aldehyde group of said PEG with the unoxidized free seleno group and the free α -amino group of said selenocysteine residue to generate a five-membered ring system in a product, wherein said product has the structure of



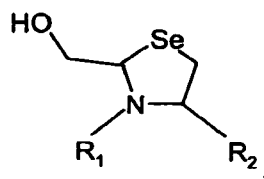
wherein R_1 is said PEG, and R_2 is said molecule.

In an eighth embodiment, the invention is directed to a method of chemically conjugating PEG containing a free aldehyde group to the unoxidized free seleno group and the free α -amino group of a selenocysteine residue of a molecule, said method comprising reacting the free aldehyde group of said PEG with the unoxidized free seleno group and the free α -amino group of said selenocysteine residue in a reaction solution to generate a five-membered ring system in an intermediate, and adjusting the pH of the

reaction solution to about 7, whereby said intermediate rearranges to form a final product, wherein said intermediate has the structure of



and said final product has the structure of

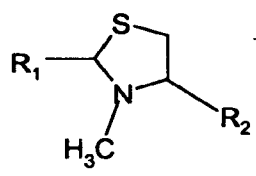


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wherein R₁ is said PEG, and R₂ is said molecule. Here, the term “about” means $\pm 10\%$.

In a ninth embodiment, the invention is directed to a method of chemically conjugating PEG containing a free aldehyde group to the unoxidized sulfhydryl side-chain and the free α -methyl-amino group of an N-methyl-cysteine residue of a molecule, said method comprising reacting the free aldehyde group of said PEG with the unoxidized sulfhydryl side-chain and the free α -methyl-amino group of said N-methyl-cysteine residue to generate a 3-methyl-1,3-thiazolidine group in a product, wherein said product has the structure of

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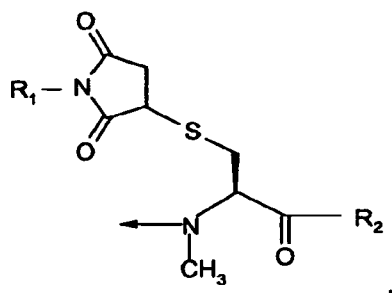
wherein R₁ is said PEG, and R₂ is said molecule.

In each of the foregoing embodiments of the invention – *i.e.*, the first through ninth embodiments of the invention – the free aldehyde group is attached to said PEG through a linker that may contain amide, ester, sulfonamide, sulfonyl, thiol, oxy, alkyl, alkenyl, alkynyl, aryl, maleimide, or amine functional group, or any combination thereof.

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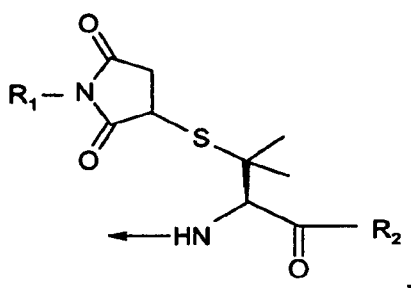
In a tenth embodiment, the invention is directed to a method of chemically conjugating PEG containing a free maleimide group to the unoxidized sulfhydryl side-chain of an N-methyl-cysteine residue of a molecule, said method comprising reacting the free maleimide group of said PEG with the unoxidized sulfhydryl side-chain of said N-methyl-cysteine to generate a conjugate product, wherein said conjugate product has the structure of

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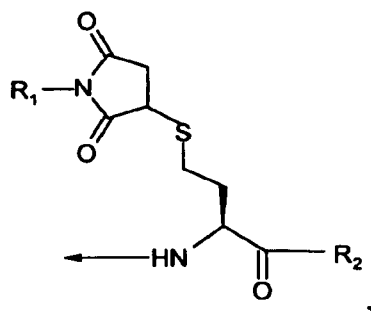
wherein R_1 is said PEG, and R_2 is said molecule.

In an eleventh embodiment, the invention is directed to a method of chemically conjugating PEG containing a free maleimide group to the unoxidized sulfhydryl side-chain of a penicillamine residue of a molecule, said method comprising reacting the free maleimide group of said PEG with the unoxidized sulfhydryl side-chain of said penicillamine residue to generate a conjugate product, wherein said conjugate product has the structure of



wherein R_1 is said PEG, and R_2 is said molecule.

In a twelfth embodiment, the invention is directed to a method of chemically conjugating PEG containing a free maleimide group to the unoxidized sulfhydryl side-chain of a homocysteine residue of a molecule, said method comprising reacting the free maleimide group of said PEG with the unoxidized sulfhydryl side-chain of said homocysteine residue to generate a conjugate product, wherein said conjugate product has the structure of

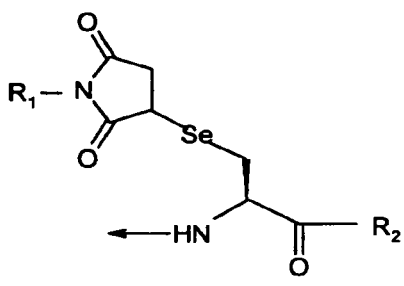


wherein R_1 is said PEG, and R_2 is said molecule.

In a thirteenth embodiment, the invention is directed to a method of chemically

conjugating PEG containing a free maleimide group to the unoxidized seleno side-chain of a selenocysteine residue of a molecule, said method comprising reacting the free maleimide group of said PEG with the unoxidized seleno side-chain of said selenocysteine residue to generate a conjugate product, wherein said conjugate product

5 has the structure of



wherein R_1 is said PEG, and R_2 is said molecule.

In each of the foregoing embodiments of the invention – *i.e.*, the tenth through thirteenth embodiments of the invention – the free maleimide group is attached to said PEG through a linker that may contain amide, ester, sulfonamide, sulfonyl, thiol, oxy,

10 alkyl, alkenyl, alkynyl, aryl, maleimide, or amine functional group, or any combination thereof.

In all of the foregoing embodiments of the invention, the PEG may have a linear structure, a branched structure, or a multi-arm structure.

15 In all of the foregoing embodiments of the invention, the PEG has average molecular weight of about 100 Da to about 500,000 Da, and more preferably has average molecular weight of about 1,000 Da to about 50,000 Da.

DETAILED DESCRIPTION OF THE INVENTION

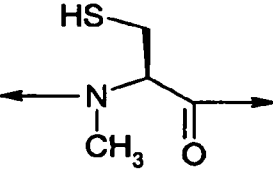
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It is believed that one skilled in the art can, based on the description herein, utilize the present invention to its fullest extent. The following specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

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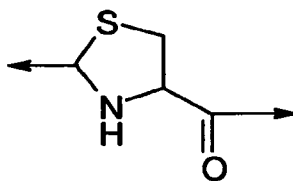
Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Also, all publications, patent applications, patents and other references mentioned herein are incorporated by reference, each in its entirety.

Nomenclature and Abbreviations

	Symbol	Meaning
	Ala or A	alanine
	Arg or R	arginine
	Asn or N	asparagine
5	Asp or D	aspartic acid
	Cys or C	cysteine
	hCys	homocysteine
	Gln or Q	glutamine
	Glu or E	glutamic acid
10	Gly or G	glycine
	His or H	histidine
	Ile or I	isoleucine
	Leu or L	leucine
	Lys or K	lysine
15	Met or M	methionine
	Nle	norleucine
	N-Me-Cys or NMeCys	N-methyl-cysteine, which has the structure of
		
	PEG	polyethylene glycol
20	Pen	penicillamine
	Phe or F	phenylalanine
	Pro or P	proline
	Ser or S	serine
	selenoCys	selenocysteine
25	Thr or T	threonine
	Trp or W	tryptophan
	Tyr or Y	tyrosine
	Val or V	valine

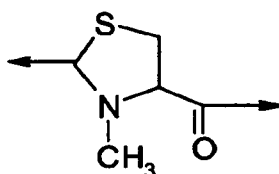
Certain other abbreviations used herein are defined as follows:

	Boc	<i>tert</i> -butoxycarbonyl
	Bzl	benzyl
	DCM	dichloromethane
5	DIC	N, N-diisopropylcarbodiimide
	DIEA	diisopropylethyl amine
	Dmab	4-{N-(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl)-amino} benzyl
	DMAP	4-(dimethylamino)pyridine
10	DMF	dimethylformamide
	DNP	2,4-dinitrophenyl
	DTT	dithiothreitol
	EDTA	ethylenediaminetetraacetic acid
	Fmoc	Fluorenylmethoxycarbonyl
15	HBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
	cHex	cyclohexyl
	HOAT	O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
20	HOBt	1-hydroxy-benzotriazole
	Me	methyl
	Mmt	4-methoxytrityl
	NMP	N-methylpyrrolidone
	Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
25	tBu	<i>tert</i> -butyl
	TCEP	tris(carboxyethyl)phosphine
	TIS	triisopropylsilane
	TOS	tosyl
	trt	trityl
30	TFA	trifluoro acetic acid
	TFFH	tetramethylfluoroformidinium hexafluorophosphate
	Z	benzyloxycarbonyl
	Tha	1,3-thiazolidine-4-carboxylic acid, which has the structure of:



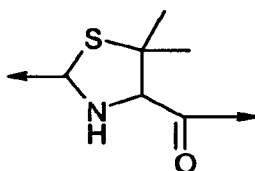
Tmc

1,3-thiazolidine-3-methyl-4-carboxylic acid, which has the structure of:



5 Dma

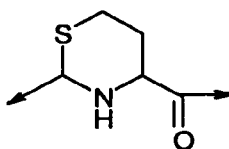
5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid, which has the structure of:



Thc

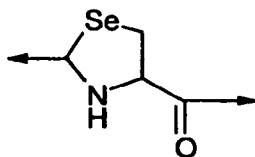
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1,3-thiazinane-4-carboxylic acid, which has the structure of:



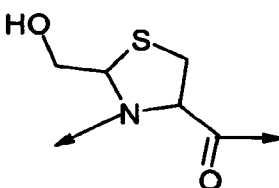
Sez

1,3-selenazolidine-4-carboxylic acid, which has the structure of:



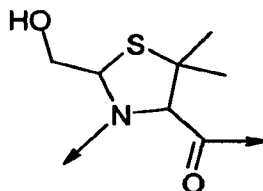
15 Hth

2-hydroxymethyl-1,3-thiazolidine-4-carboxylic acid, which has the structure of:



Hdm

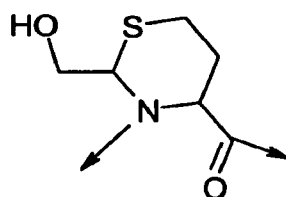
2-hydroxymethyl-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid, which has the structure of:



Haz

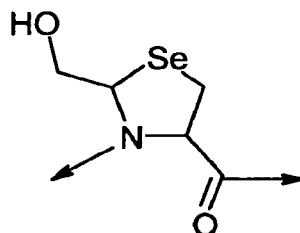
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2-hydroxymethyl-1,3-thiazinane-4-carboxylic acid, which has the structure of:

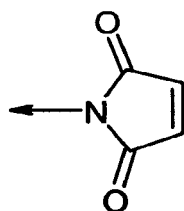


Hsz

2-hydroxymethyl-1,3-selenazolidine-4-carboxylic acid, which has the structure of:

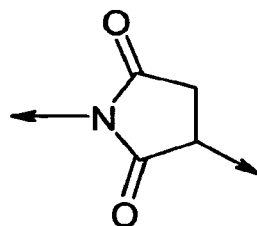


10 Maleimide has the structure of:

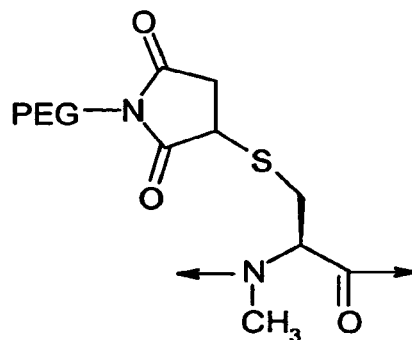


Prd

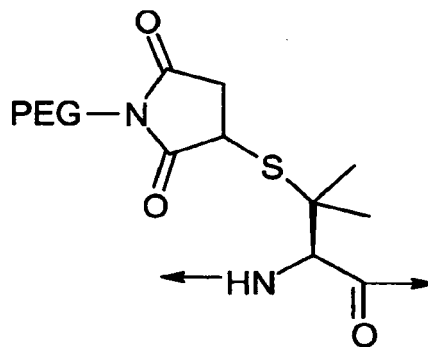
pyrrolidine-2,5-dione, which has the structure of:



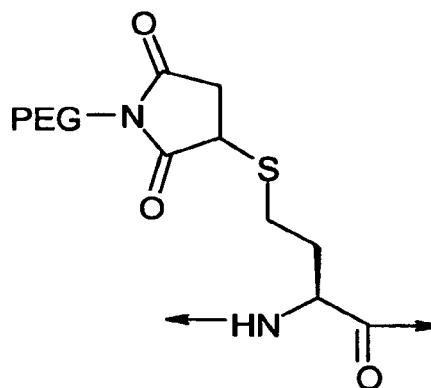
NMeCys(Prd-PEG) has the structure of :



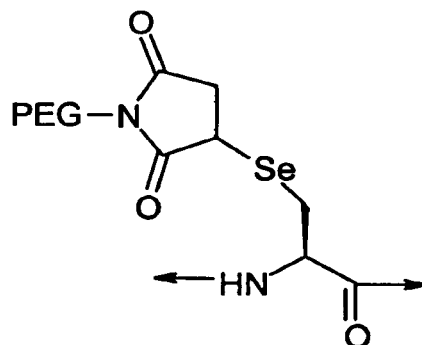
Pen(Prd-PEG) has the structure of :



5 hCys(Prd-PEG) has the structure of :



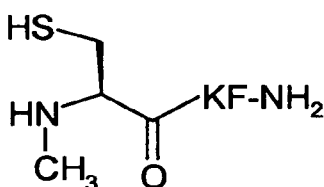
selenoCys(Prd-PEG) has the structure of:



PEG is a well-known, water soluble polymer that is commercially available or can be prepared by ring-opening polymerization of ethylene glycol according to methods known in the art (Sandler and Karo, Polymer Synthesis, Academic Press, New York, VO13, pages 138-161). The term "PEG" is used broadly to encompass any polyethylene glycol molecule, without regard to size or to modification at end of the PEG. PEG may have linear, branched or multi-armed structure.

EXAMPLES

10 Example 1) Preparation of H-NMeCys-Lys-Phe-NH₂



Rink amide MBHA resin (211mg, 0.152mmole) (Novabiochem, San Diego, Calif.) was swollen in dichloromethane (DCM) and washed with dimethylformamide (DMF). The resin was deblocked by treatment with a 25% piperidine/DMF (10mL) solution for 2 x 10 min. The resin was washed with DMF (10mL) three times. The first amino acid was coupled to the resin by treatment with a solution of Fmoc-Phe-OH (Novabiochem, San Diego, Calif.) (235mg, 0.606mmole), 1-hydroxybenzotriazole (HOBt) (92.3mg, 0.606mmole), and diisopropylcarbodiimide (DIC) (77mg, 0.606mmole) in N-methylpyrrolidone (NMP) (2mL) for one hour. The resin was filtered and washed with DMF (10mL) three times.

The Fmoc protecting group was removed by treatment with a 25% piperidine/DMF (10mL) solution for 2 x 10 min and the resin was washed with DMF (10mL) three times. Fmoc-Lys(Boc)-OH (Novabiochem, San Diego, Calif.) (285mg 0.606mmole) was coupled to the resulting free amine resin in the presence of HOBt (0.606mmole) and DIC (0.606mmole) in NMP (2mL) for one hour.

The deblocking and washing procedures were repeated as above. Fmoc-N-Me-Cys(Trt)-OH (Timen Chemicals, Lodz, Poland.) (100mg, 0.167mmole) was coupled to the resulting peptide-resin by using HOBt (51mg, 0.33mmole) and DIC (83.8mg, 0.66mmole) in NMP (2mL) for 12 hours. The coupling of Fmoc-N-Me-Cys(Trt)-OH (45mg, 0.075mmole) was repeated by using

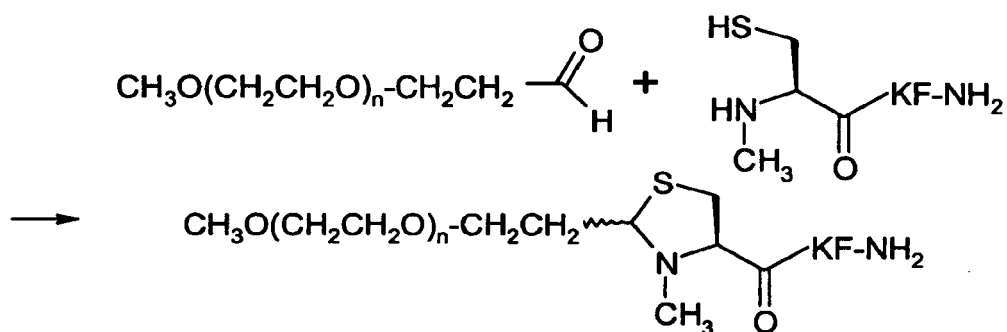
tetramethylfluoroformamidiniumpentafluorophosphate (TFFH) (20mg, 0.075mmole) and diisopropylethylamine (DIEA) (19.4mg, 0.150mmole) in NMP (2mL) for one hour. The deblocking and washing procedures were repeated as above. The resin was washed with DCM three times then with methanol three times. The resin was dried under vacuum.

5 The peptide was cleaved off from the resin by shaking the resin with 8% trispropylsilane/trifluoroacetic acid (TFA) (2mL) for two hours. The resin was filtered and washed with DCM (2mL). The filtrates were combined and concentrated to 1mL. Diethyl ether (35mL) was added to precipitate the peptide. The precipitated peptide was collected after centrifuging. The pellet was dissolved in water and acetonitrile and then
10 was lyophilized.

The resulting crude product was purified on a reverse phase HPLC system (Luna 5micron C8 (2) 100X20mm column), eluted from 100% buffer A (0.1% TFA in water) and 0% buffer B (0.1% TFA in acetonitrile) to 80% buffer A and 20% buffer B over 30 minutes monitoring at 235nm. After the lyophilization, 51.2 mg of the final product was
15 obtained. An M+1 ion at 410.3 Da was detected by ESI mass spectroscopy, which is consistent with the calculated molecular weight of 409.6 Da.

Example 2) Preparation of mPEG-Tmc-Lys-Phe-NH₂

mPEG herein has the structure of $\text{CH}_3\text{O}(\text{CH}_2\text{CH}_2\text{O})_n-(\text{CH}_2)_2-$, wherein n is a
20 positive integer.

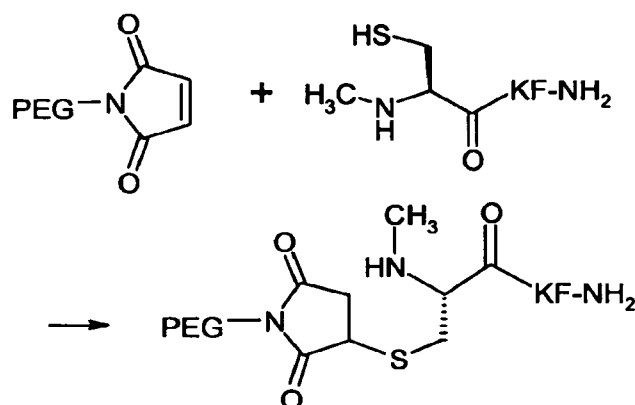


The peptide product of Example 1 (0.5mg 1.22micromole) was dissolved in
25 1.0mL of a pH 4 buffer (20mmolar NaOAc, 150mmolar NaCl, and 1mmolar EDTA). To the resulting solution was added mPEG-aldehyde (1.5 equivalents, the average molecular weight is 31378 Da, NOF Corp., Tokyo, Japan). The reaction was approximately 90%

complete after 27 hours at room temperature based on the analysis done by using a reverse-phase analytical HPLC system (Vydac C₁₈ 5 μ peptide/protein column, 4.6 x 250mm). The reaction mixture was applied to a 5mL Zeba™ desalt spin column (Pierce Biotechnology, Rockford, IL). A white foam was obtained after lyophilization (36.7mg).

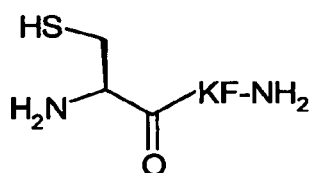
5

Example 3) Preparation of H-NMeCys(Prd-PEG)-Lys-Phe-NH₂



The peptide product of Example 1 (0.5mg 1.22micromole) was dissolved in 1.0mL of a pH 7 buffer (20mmolar NaOAc). To the resulting solution was added α -(3-(3-maleimido-1-oxopropyl)amino)propyl- ω -methoxy-polyoxyethylene (1.5 equivalents, the average molecular weight is 11962 Da, NOF Corp., Tokyo, Japan) and 2 equivalents of Tris(2-carboxyethyl)phosphine hydrochloride (TCEP). The reaction was complete after one hour at room temperature based on the analysis done by using a reverse-phase analytical HPLC system (Vydac C₁₈ 5 μ peptide/protein column, 4.6 x 250mm). The reaction mixture was applied to a 5mL Zeba™ desalt spin column (Pierce Biotechnology, Rockford, IL). A white foam was obtained after lyophilization (15.1mg). The product was further purified on High Trap™ SPXL cation exchange column (GE Healthcare, Piscataway, NJ). The molecular weight distribution of the purified product was determined by using MALDI-TOF mass spectroscopy. The obtained experimental result was consistent with the calculated molecular weight distribution.

Example 4) Preparation of H-Cys-Lys-Phe-NH₂



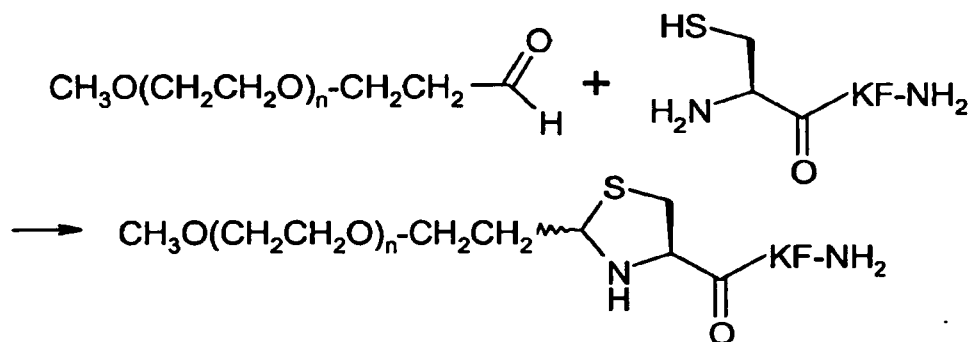
The title peptide was synthesized on a Liberty™ model microwave peptide synthesizer (CEM Corp., Matthews, NC) using Rink amide MBHA resin (347mg 0.25 mmole) (Novabiochem, San Diego, Calif.). The amino acids Fmoc-Phe-OH, Fmoc Lys(Boc)-OH, and Fmoc-Cys(Trt)-OH (Novabiochem, San Diego, CA) were used in four fold excess using HBTU activation and each coupling was repeated.

The peptide was cleaved from the resin by shaking resin with 8% trispropylsilane/trifluoroacetic acid (TFA) with 1% dithiothreitol (10mL) for three hours. The resin was filtered and washed with DCM (5mL). The filtrates were combined and concentrated to 3mL. Diethyl ether (35mL) was added to precipitate the peptide. The precipitated peptide was collected after centrifuging. The pellet was dissolved in water and acetonitrile and then was lyophilized.

The resulting crude product was purified on a reverse phase HPLC system (Luna 5micron C8 (2) 100X20mm column), eluted from 100% buffer A (0.1% TFA in water) and 0% buffer B (0.1% TFA in acetonitrile) to 70% buffer A and 30% buffer B over 35 minutes monitoring at 235nm. After the lyophilization, 89.1 mg of the final product was obtained. An M+1 ion at 396.5 Da was detected by ESI mass spectroscopy, which is consistent with the calculated molecular weight 395.5 Da.

Example 5) Preparation of mPEG-Tha-Lys-Phe-NH₂

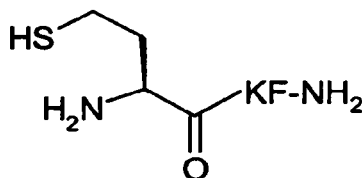
mPEG herein has the structure of CH₃O(CH₂CH₂O)_n-(CH₂)₂-, wherein n is a positive integer.



The peptide product of Example 4 (0.5mg 1.26 micromole) was dissolved in 1.0mL of a pH 4 buffer (20mmolar NaOAc). To the resulting solution was added mPEG-aldehyde (1.5 equivalents, the average molecular weight is 20644 Da, NOF Corp., Tokyo, Japan) and TCEP (2.0 equivalents). The reaction was approximately 85% complete after three hours at room temperature based on the analysis done by using a

reverse-phase analytical HPLC system (Vydac C₁₈ 5 μ peptide/protein column, 4.6 x 250mm). The reaction mixture was applied to a 10mL Zeba™ desalt spin column (Pierce Biotechnology, Rockford, IL). A white foam was obtained after lyophilization.

Example 6) Preparation of H-hCys- Lys-Phe-NH₂



5

The title peptide was synthesized on a Liberty™ model microwave peptide synthesizer (CEM Corp., Matthews, NC) using Rink amide MBHA resin (347mg 0.25 mmole) (Novabiochem, San Diego, Calif.). The amino acids Fmoc-Phe-OH, Fmoc Lys(Boc)-OH, and Fmoc-hCys(Trt)-OH (Novabiochem, San Diego, CA) were used in four fold excess using HBTU activation and each coupling was repeated.

10

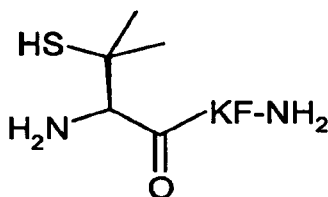
The peptide was cleaved from the resin by shaking resin with 8% trispropylsilane/trifluoroacetic acid (TFA) with 1% dithiothreitol (10mL) for three hours. The resin was filtered and washed with DCM (5mL). The filtrates were combined and concentrated to 3mL. Diethyl ether (35mL) was added to precipitate the peptide. The precipitated peptide was collected after centrifuging. The pellet was dissolved in water and acetonitrile and then was lyophilized.

15

The resulting crude product was purified on a reverse phase HPLC system (Luna 5micron C8 (2) 100X20mm column), eluted from 100% buffer A (0.1% TFA in water) and 0% buffer B (0.1% TFA in acetonitrile) to 75% buffer A and 25% buffer B over 35 minutes monitoring at 235nm. After the lyophilization, 85.7 mg of the final product was obtained. An M+1 ion at 410.5 Da was detected by ESI mass spectroscopy, which is consistent with the calculated molecular weight 409.6 Da.

20

Example 7) Preparation of H-Pen- Lys-Phe-NH₂



25

The title peptide was synthesized on a Liberty™ model microwave peptide

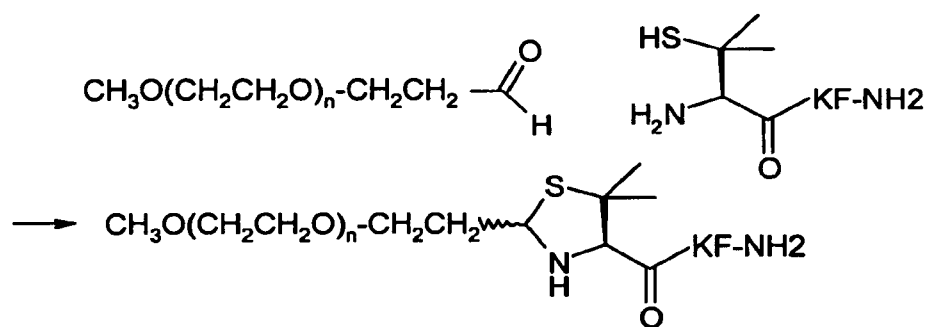
synthesizer (CEM Corp., Matthews, NC) using Rink amide MBHA resin (347mg 0.25 mmole) (Novabiochem, San Diego, Calif.). The amino acids Fmoc-Phe-OH, Fmoc Lys(Boc)-OH, and Fmoc-Pen(Trt)-OH (Novabiochem, San Diego, CA) were used in four fold excess using HBTU activation and each coupling was repeated.

5 The peptide was cleaved from the resin by shaking resin with 8% trispropylsilane/trifluoroacetic acid (TFA) with 1% dithiothreitol (10mL) for three hours. The resin was filtered and washed with DCM (5mL). The filtrates were combined and concentrated to 3mL. Diethyl ether (35mL) was added to precipitate the peptide. The precipitated peptide was collected after centrifuging. The pellet was dissolved in water
10 and acetonitrile and then was lyophilized.

The resulting crude product was purified on a reverse phase HPLC system (Luna 5micron C8 (2) 100X20mm column), eluted from 100% buffer A (0.1% TFA in water) and 0% buffer B (0.1% TFA in acetonitrile) to 80% buffer A and 20% buffer B over 35 minutes monitoring at 235nm. After the lyophilization, 83.9 mg of the final product was
15 obtained. An M+1 ion at 424.5 Da was detected by ESI mass spectroscopy, which is consistent with the calculated molecular weight 423.6 Da.

Example 8) Preparation of mPEG-Dma- Lys-Phe-NH₂

mPEG herein has the structure of CH₃O(CH₂CH₂O)_n-(CH₂)₂-, wherein n is a
20 positive integer.

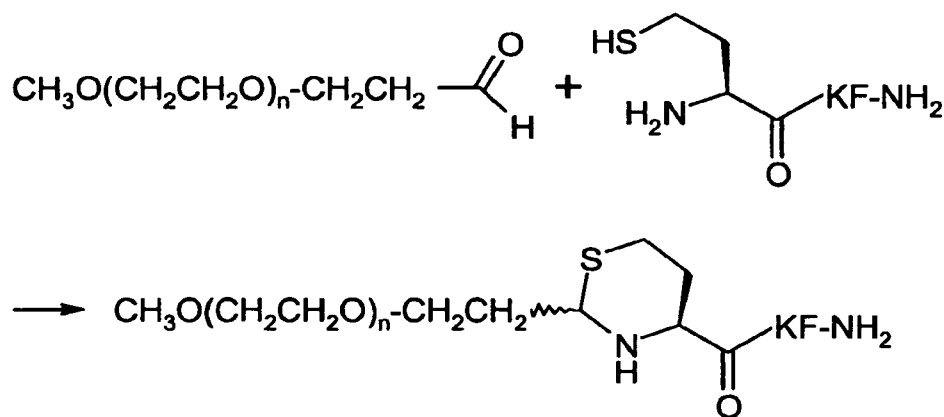


The peptide product of Example 7 (0.5mg 1.18 micromole) was dissolved in 1.0mL of a pH 4 buffer (20mmolar NaOAc). To the resulting solution was added mPEG-aldehyde (1.5 equivalents, the average molecular weight is 20644 Da, NOF
25 Corp., Tokyo, Japan) and TCEP (2.0 equivalents). The reaction was approximately 80% complete after three hours at room temperature based on the analysis done by using a reverse-phase analytical HPLC system (Vydac C₁₈ 5μ peptide/protein column, 4.6 x

250mm). The reaction mixture was applied to a 10mL Zeba™ desalt spin column (Pierce Biotechnology, Rockford, IL). A white foam was obtained after lyophilization.

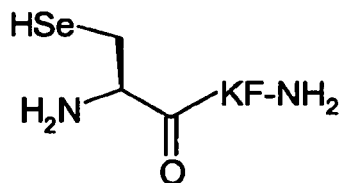
Example 9) Preparation of mPEG-Thc-Lys-Phe-NH₂

- 5 mPEG herein has the structure of CH₃O(CH₂CH₂O)_n-(CH₂)₂-, wherein n is a positive integer.



- The peptide product of Example 6 (0.5mg 1.22 micromole) was dissolved in 1.0mL of a pH 4 buffer (20mmolar NaOAc). To the resulting solution was added mPEG-aldehyde (1.5 equivalents, the average molecular weight is 20644 Da, NOF Corp., Tokyo, Japan) and TCEP (2.0 equivalents). The reaction was approximately 90% complete after three hours at room temperature based on the analysis done by using a reverse-phase analytical HPLC system (Vydac C₁₈ 5μ peptide/protein column, 4.6 x 250mm). The reaction mixture was applied to a 10mL Zeba™ desalt spin column (Pierce Biotechnology, Rockford, IL). A white foam was obtained after lyophilization

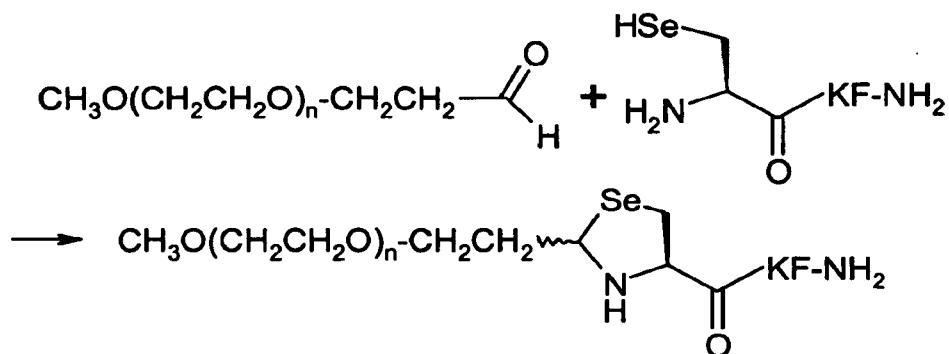
Example 10) Preparation of selenoCys- Lys-Phe-NH₂



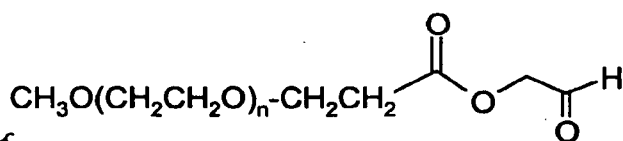
- The title peptide is synthesized substantially according to the procedure described in Example 1. Fmoc-selenoCys(4-MeOBzl)-OH (Novabiochem, San Diego, CA) is used for the incorporation of selenocysteine residue at the N-terminus.

Example 11) Preparation of mPEG-Sez-Lys-Phe-NH₂

mPEG herein has the structure of $\text{CH}_3\text{O}(\text{CH}_2\text{CH}_2\text{O})_n-(\text{CH}_2)_2-$, wherein n is a positive integer.



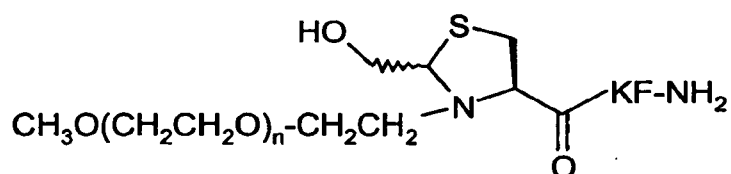
- 5 The title peptide is synthesized substantially according to the procedure described in Example 2. The product obtained from Example 10 is the peptide starting material.

Example 12) Preparation of

- 10 mPEG herein has the structure of $\text{CH}_3\text{O}(\text{CH}_2\text{CH}_2\text{O})_n-(\text{CH}_2)_2-$, wherein n is a positive integer.

mPEG-C(O)OH cesium salt reacts with bromoacetaldehyde dimethyl acetal in DMF at 60 °C for 2 days. After removing the solvent, the product is treated with 40% TFA in DCM with small amount of water at 0 °C for about 30 min.

- 15 Example 13) Preparation of mPEG-Hth-Lys-Phe-NH₂

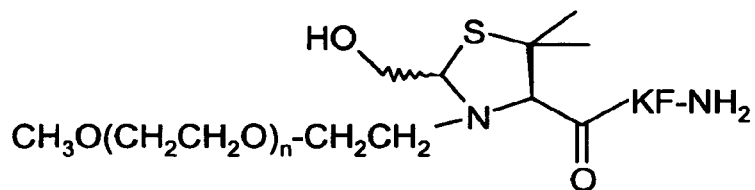


The mPEG herein has the structure of $\text{CH}_3\text{O}(\text{CH}_2\text{CH}_2\text{O})_n-(\text{CH}_2)_2-$, wherein n is a positive integer.

- 20 The title peptide is synthesized substantially according to the procedure described in Example 2. The peptide starting material is the product obtained from Example 4. The PEG-aldehyde starting material is the product obtained in Example 12. There is an

additional step of adjusting pH of the buffer solution: after standing at room temperature for 2 hours at pH4, the pH of the reaction solution is adjusted to 7 and stands at room temperature for 3 days before purification.

5 Example 14) Preparation of mPEG-Hdm-Lys-Phe-NH₂

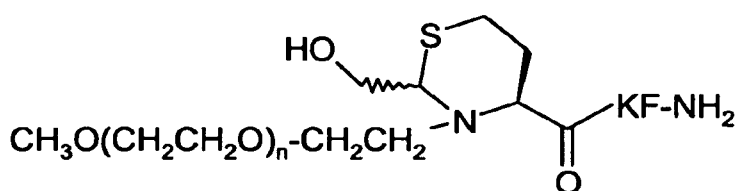


The mPEG herein has the structure of CH₃O(CH₂CH₂O)_n-(CH₂)₂-, wherein n is a positive integer.

10 The title peptide is synthesized substantially according to the procedure described for Example 8. The peptide starting material is the product obtained from Example 7. The PEG-aldehyde starting material is the product obtained in Example 12. There is an additional step of adjusting pH of the buffer solution: after standing at room temperature overnight, the pH of the reaction solution is adjusted to 7 and the solution stands at room temperature for 3 days before purification.

15

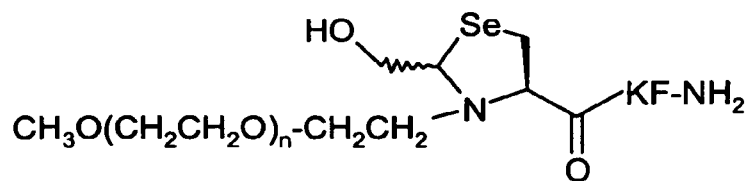
Example 15) Preparation of mPEG-Haz-Lys-Phe-NH₂



The mPEG herein has the structure of CH₃O(CH₂CH₂O)_n-(CH₂)₂-, wherein n is a positive integer.

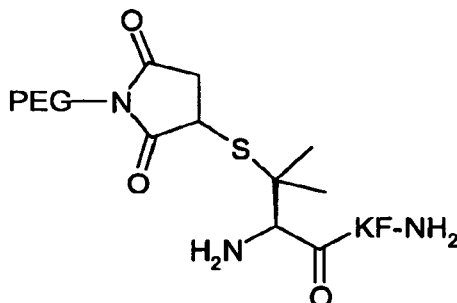
20 The title peptide is synthesized substantially according to the procedure described for Example 9. The peptide starting material is the product obtained from Example 6. The PEG-aldehyde starting material is the product obtained in Example 12. There is an additional step of adjusting pH of the buffer solution: after standing at room temperature overnight at pH4, the pH of the reaction solution is adjusted to 7 and the solution stands at room temperature for 3 days before purification.

25

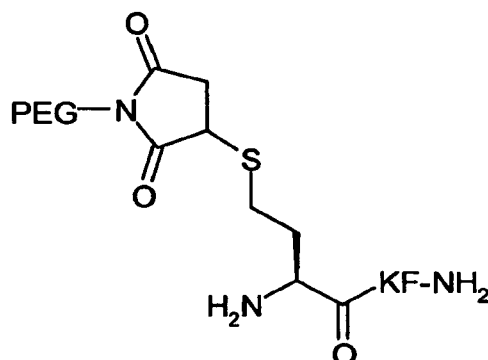
Example 16) Preparation of mPEG-Hsz-Lys-Phe-NH₂

The mPEG herein has the structure of $\text{CH}_3\text{O}(\text{CH}_2\text{CH}_2\text{O})_n-(\text{CH}_2)_2-$, wherein n is a positive integer.

- 5 The title peptide is synthesized substantially according to the procedure described for Example 11. The peptide starting material is the product obtained from Example 10. The PEG-aldehyde starting material is the product obtained in Example 12. There is an additional step of adjusting pH of the buffer solution: after standing at room temperature for 2 hours at pH4, the pH of the reaction solution is adjusted to 7 and the solution stands
- 10 at room temperature for 3 days before purification.

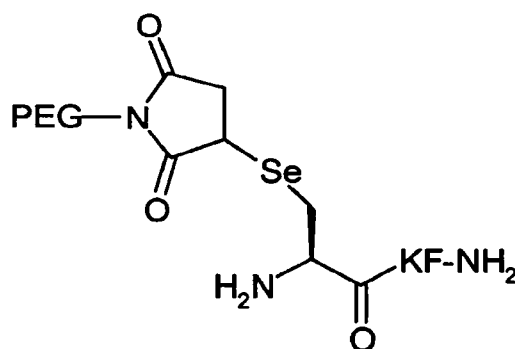
Example 17) Preparation of H-Pen(Prd-PEG)-Lys-Phe-NH₂

- 15 The title peptide is synthesized substantially according to the procedure described in Example 3. The peptide starting material is the product obtained from Example 7.

Example 18) Preparation of H-hCys(Prd-PEG)-Lys-Phe-NH₂

The peptide product of Example 6 (1.0mg 2.44micromole) was dissolved in 1.0mL of a pH 7 buffer (20mmolar NaOAc). To the resulting solution was added α -(3-(3-maleimido-1-oxopropyl)amino)propyl- ω -methoxy-polyoxyethylene (1.5 equivalents, the average molecular weight is 11962 Da, NOF Corp., Tokyo, Japan) and 2 equivalents of Tris(2-carboxyethyl)phosphine hydrochloride (TCEP). The reaction was complete after one hour at room temperature based on the analysis done by using a reverse-phase analytical HPLC system (Vydac C₁₈ 5 μ peptide/protein column, 4.6 x 250mm). The reaction mixture was applied to a 10mL Zeba™ desalt spin column (Pierce Biotechnology, Rockford, IL). A white foam was obtained after lyophilization.

Example 19) Preparation of H-selenoCys(Prd-PEG)-Lys-Phe-NH₂

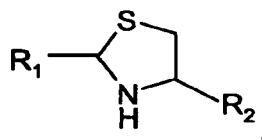


The title peptide is synthesized substantially according to the procedure described in Example 3. The peptide starting material is the product obtained from Example 10.

CLAIMS

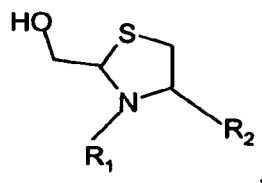
What is claimed is:

1. A method of chemically conjugating PEG containing a free aldehyde group to the unoxidized sulfhydryl side-chain and the free α -amino group of a cysteine residue of a molecule, said method comprising reacting the free aldehyde group of said PEG with the unoxidized sulfhydryl side-chain and the free α -amino group of said cysteine residue to generate a 1,3-thiazolidine group in a product, wherein said product has the structure of

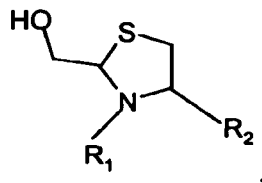


wherein R_1 is said PEG, and R_2 is said molecule.

2. A method of chemically conjugating PEG containing a free aldehyde group to the unoxidized sulfhydryl side-chain and the free α -amino group of a cysteine residue of a molecule, said method comprising reacting the free aldehyde group of said PEG with the unoxidized sulfhydryl side-chain and the free α -amino group of said cysteine residue in a reaction solution to generate a 1,3-thiazolidine group in an intermediate, and adjusting the pH of the reaction solution to about 7, whereby said intermediate rearranges to form a final product, wherein said intermediate has the structure of



and said final product has the structure of



wherein R_1 is said PEG, and R_2 is said molecule.

3. The method according to claim 1 or claim 2, wherein the free aldehyde group is attached to said PEG through a linker that may contain amide, ester, sulfonamide, sulfonyl, thiol, oxy, alkyl, alkenyl, alkynyl, aryl, maleimide, or amine functional group, or any combination thereof.

5

4. The method according to claim 1 or claim 2, wherein said PEG has a linear structure.

5. The method according to claim 1 or claim 2, wherein said PEG has a
10 branched structure.

6. The method according to claim 1 or claim 2, wherein said PEG has a multi-arm structure.

7. The method according to claim 1 or claim 2, wherein one or more free
15 aldehyde groups are attached to said PEG.

8. The method according to claim 1 or claim 2, wherein said PEG has
20 average molecular weight of about 100 Da to about 500,000 Da.

9. The method according to claim 8, wherein said PEG has average
molecular weight of about 1,000 Da to about 50,000 Da.

10. The method according to claim 1 or claim 2, wherein said cysteine residue
25 is in L-form.

11. The method according to claim 1 or claim 2, wherein said cysteine residue
is in D-form.

12. The method according to claim 1 or claim 2, wherein said cysteine residue
30 is in a protein.

13. The method according to claim 1 or claim 2, wherein said cysteine residue
is in a peptide.

14. The method according to claim 1 or claim 2, wherein said cysteine residue is in an organic molecule.

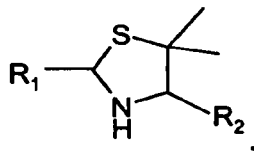
15. The method according to claim 1 or claim 2, wherein said product or said final product contains one or more protein, peptide, or organic molecule moieties, or any combination thereof.

16. The method according to claim 2, wherein said intermediate contains one or more protein, peptide, or organic molecule moieties, or any combination thereof.

17. The method according to claim 1 or claim 2, wherein a reducing agent is used in the reaction.

18. The method according to claim 16, wherein said reducing agent is selected from the group consisting of TCEP and compounds containing unoxidized sulfhydryl group.

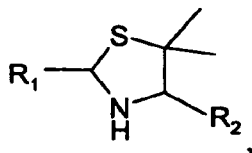
19. A method of chemically conjugating PEG containing a free aldehyde group to the unoxidized sulfhydryl side-chain and the free α -amino group of a penicillamine residue of a molecule, said method comprising reacting the free aldehyde group of said PEG with the unoxidized sulfhydryl side-chain and the free α -amino group of said penicillamine residue to generate a 5,5-dimethyl-1,3-thiazolidine group in a product, wherein said product has the structure of



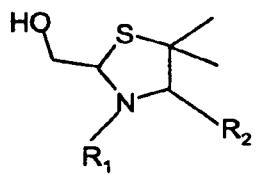
20. wherein R_1 is said PEG, and R_2 is said molecule.

21. A method of chemically conjugating PEG containing a free aldehyde group to the unoxidized sulfhydryl side-chain and the free α -amino group of a penicillamine residue of a molecule, said method comprising reacting the free aldehyde group of said PEG with the unoxidized sulfhydryl side-chain and the free α -amino group of said penicillamine residue in a reaction solution to generate a 5,5-dimethyl-1,3-

thiazolidine group in an intermediate, and adjusting the pH of the reaction solution to about 7, whereby said intermediate rearranges to form a final product, wherein said intermediate has the structure of



5 and said final product has the structure of



wherein R₁ is said PEG, and R₂ is said molecule.

21. The method according to claim 19 or claim 20, wherein the free aldehyde
10 group is attached to said PEG through a linker that may contain amide, ester, sulfonamide, sulfonyl, thiol, oxy, alkyl, alkenyl, alkynyl, aryl, maleimide, or amine functional group, or any combination thereof.

22. The method according to claim 19 or claim 20, wherein said PEG has a
15 linear structure.

23. The method according to claim 19 or claim 20, wherein said PEG has a branched structure.

20 24. The method according to claim 19 or claim 20, wherein said PEG has a multi-arm structure.

25. The method according to claim 19 or claim 20, wherein one or more free aldehyde groups are attached to PEG.

25

26. The method according to claim 19 or claim 20, wherein said PEG has average molecular weight of about 100 Da to about 500,000 Da.

27. The method according claim 26, wherein said PEG has average molecular weight of about 1,000 Da to about 50,000 Da.

28. The method according to claim 19 or claim 20, wherein said
5 penicillamine residue is in L-form.

29. The method according to claim 19 or claim 20, wherein said penicillamine residue is in D-form.

10 30. The method according to claim 19 or claim 20, wherein said penicillamine residue is in a protein.

31. The method according to claim 19 or claim 20, wherein said penicillamine residue is in a peptide.

15 32. The method according to claim 19 or claim 20, wherein said penicillamine residue is in an organic molecule.

33. The method according to claim 19 or claim 20, wherein said product or
20 said final product contains one or more protein, peptide, or organic molecule moieties, or any combination thereof.

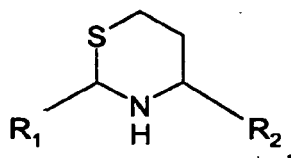
34. The method according to claim 19 or claim 20, wherein said intermediate
25 contains one or more protein, peptide, or organic molecule moieties, or any combination thereof.

35. The method according to claim 19 or claim 20, wherein a reducing agent is used in the reaction.

30 36. The method according to claim 35, wherein said reducing agent is selected from the group consisting of TCEP and compounds containing unoxidized sulfhydryl group.

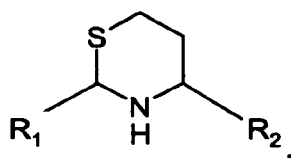
37. A method of chemically conjugating PEG containing a free aldehyde

group to the unoxidized sulfhydryl side-chain and the free α -amino group of a homocysteine residue of a molecule, said method comprising reacting the free aldehyde group of said PEG with the unoxidized sulfhydryl side-chain and the free α -amino group of said homocysteine residue to generate a six-membered ring system in a product,
 5 wherein said product has the structure of

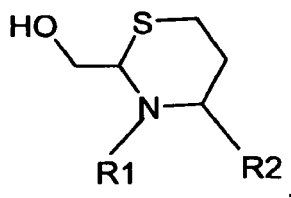


wherein R₁ is said PEG, and R₂ is said molecule.

38. A method of chemically conjugating PEG containing a free aldehyde
 10 group to the unoxidized sulfhydryl side-chain and the free α -amino group of a homocysteine residue of a molecule, said method comprising reacting the free aldehyde group of said PEG with the unoxidized sulfhydryl side-chain and the free α -amino group of said homocysteine residue in a reaction solution to generate a six-membered ring system in an intermediate, and adjusting the pH of the reaction solution to about 7,
 15 whereby said intermediate rearranges to form a final product, wherein said intermediate has the structure of



and said final product has the structure of



20 wherein R₁ is said PEG, and R₂ is said molecule.

39. The method according to claim 37 or claim 38, wherein the free aldehyde group is attached to said PEG through a linker that may contain amide, ester, sulfonamide, sulfonyl, thiol, oxy, alkyl, alkenyl, alkynyl, aryl, maleimide, or amine
 25 functional group, or any combination thereof.

40. The method according to claim 37 or claim 38, wherein said PEG has a linear structure.

5 41. The method according to claim 37 or claim 38, wherein said PEG has a branched structure.

42. The method according to claim 37 or claim 38, wherein said PEG has a multi-arm structure.

10

43. The method according to claim 37 or claim 38, wherein one or more free aldehyde groups are attached to said PEG.

44. The method according to claim 37 or claim 38, wherein said PEG has average molecular weight of about 100 Da to about 500,000 Da.

15

45. The method according to claim 44, wherein said PEG has average molecular weight of about 1,000 Da to about 50,000 Da.

20 46. The method according to claim 37 or claim 38, wherein said homocysteine residue is in L-form.

47. The method according to claim 37 or claim 38, wherein said homocysteine residue is in D-form.

25

48. The method according to claim 37 or claim 38, wherein said homocysteine residue is in a protein.

49. The method according to claim 37 or claim 38, wherein said homocysteine residue is in a peptide.

30

50. The method according to claim 37 or claim 38, wherein said homocysteine residue is in an organic molecule.

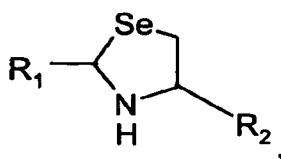
51. The method according to claim 37 or claim 38, wherein said product or final product contains one or more protein, peptide, or organic molecule moieties, or any combination thereof.

52. The method according to claim 37 or claim 38, wherein said intermediate contains one or more protein, peptide, or organic molecule moieties, or any combination thereof.

53. The method according to claim 37 or claim 38, wherein a reducing agent is used in the reaction.

54. The method according to claim 53, wherein said reducing agent is selected from the group consisting of TCEP and compounds containing unoxidized sulfhydryl group.

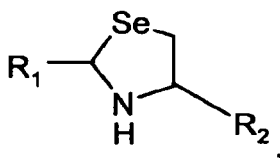
55. A method of chemically conjugating PEG containing a free aldehyde group to the unoxidized free seleno group and the free α -amino group of a selenocysteine residue of a molecule, said method comprising reacting the free aldehyde group of said PEG with the unoxidized free seleno group and the free α -amino group of said selenocysteine residue to generate a five-membered ring system in a product, wherein said product has the structure of



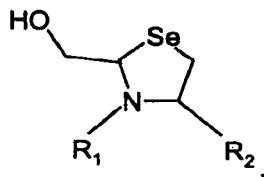
wherein R₁ is said PEG, and R₂ is said molecule.

56. A method of chemically conjugating PEG containing a free aldehyde group to the unoxidized free seleno group and the free α -amino group of a selenocysteine residue of a molecule, said method comprising reacting the free aldehyde group of said PEG with the unoxidized free seleno group and the free α -amino group of said selenocysteine residue in a reaction solution to generate a five-membered ring system in an intermediate, and adjusting the pH of the reaction solution to about 7, whereby said intermediate rearranges to form a final product, wherein said intermediate

has the structure of



and said final product has the structure of



5 wherein R₁ is said PEG, and R₂ is said molecule.

57. The method according to claim 55 or claim 56, wherein the free aldehyde group is attached to said PEG through a linker that may contain amide, ester, sulfonamide, sulfonyl, thiol, oxy, alkyl, alkenyl, alkynyl, aryl, maleimide, or amine functional group, or any combination thereof.

58. The method according to claim 55 or claim 56, wherein said PEG has a linear structure.

15 59. The method according to claim 55 or claim 56, wherein said PEG has a branched structure.

60. The method according to claim 55 or claim 56, wherein said PEG has a multi-arm structure.

20

61. The method according to claim 55 or claim 56, wherein one or more free aldehyde groups are attached to said PEG.

62. The method according to claim claim 55 or claim 56, wherein said PEG has average molecular weight of about 100 Da to about 500,000 Da.

25

63. The method according to claim 62, wherein said PEG has average molecular weight of about 1,000 Da to about 50,000 Da.

64. The method according to claim 55 or claim 56, wherein said selenocysteine residue is in L-form.

5 65. The method according to claim 55 or claim 56, wherein said selenocysteine residue is in D-form.

66. The method according to claim 55 or claim 56, wherein said selenocysteine residue is in a protein.

10

67. The method according to claim 55 or claim 56, wherein said selenocysteine residue is in a peptide.

68. The method according to claim 55 or claim 56, wherein said selenocysteine residue is in an organic molecule.

15

69. The method according to claim 55 or claim 56, wherein said product or said final product contains one or more protein, peptide, or organic molecule moieties, or any combination thereof.

20

70. The method according to claim 56, wherein said intermediate contains one or more protein, peptide, or organic molecule moieties, or any combination thereof.

71. The method according to claim 55 or claim 56, wherein a reducing agent is used in the reaction.

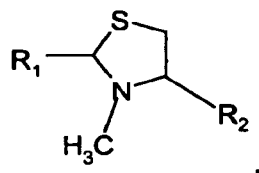
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72. The method according to claim 71, wherein said reducing agent is selected from the group consisting of TCEP and compounds containing unoxidized sulfhydryl or unoxidized free seleno group.

30

73. A method of chemically conjugating PEG containing a free aldehyde group to the unoxidized sulfhydryl side-chain and the free α -methyl-amino group of an N-methyl-cysteine residue of a molecule, said method comprising reacting the free

aldehyde group of said PEG with the unoxidized sulfhydryl side-chain and the free α -methyl-amino group of said N-methyl-cysteine residue to generate a 3-methyl-1,3-thiazolidine group in a product, wherein said product has the structure of



5 wherein R_1 is said PEG, and R_2 is said molecule.

74. The method according to claim 73, wherein the free aldehyde group is attached to said PEG through a linker that may contain amide, ester, sulfonamide, sulfonyl, thiol, oxy, alkyl, alkenyl, alkynyl, aryl, maleimide, or amine functional group,
10 or any combination thereof.

75. The method according to claim 73, wherein said PEG has a linear structure.

15 76. The method according to claim 73, wherein said PEG has a branched structure.

77. The method according to claim 73, wherein said PEG has a multi-arm structure.

20

78. The method according to claim 73, wherein one or more free aldehyde groups are attached to said PEG.

79. The method according to claim 73, wherein said PEG has average
25 molecular weight of about 100 Da to about 500,000 Da.

80. The method according to claim 79, wherein said PEG has average molecular weight of about 1,000 Da to about 50,000 Da.

30 81. The method according to claim 73, wherein said N-methyl-cysteine residue is in L-form.

82. The method according to claim 73, wherein said N-methyl-cysteine residue is in D-form.

83. The method according to claim 73, wherein said N-methyl-cysteine
5 residue is in a protein.

84. The method according to claim 73, wherein said N-methyl-cysteine residue is in a peptide.

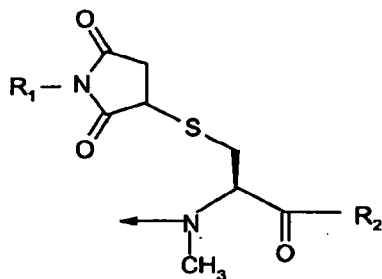
10 85. The method according to claim 73, wherein said N-methyl-cysteine residue is in an organic molecule.

86. The method according to claim 73, wherein said product contains one or more protein, peptide, or organic molecule moieties, or any combination thereof.
15

87. The method according to claim 73, wherein a reducing agent is used in the reaction.

88. The method according to claim 87, wherein said reducing agent is
20 selected from the group consisting of TCEP and compounds containing unoxidized sulfhydryl group.

89. A method of chemically conjugating PEG containing a free maleimide group to the unoxidized sulfhydryl side-chain of an N-methyl-cysteine residue of a
25 molecule, said method comprising reacting the free maleimide group of said PEG with the unoxidized sulfhydryl side-chain of said N-methyl-cysteine to generate a conjugate product, wherein said conjugate product has the structure of



wherein R₁ is said PEG, and R₂ is said molecule.

90. The method according to claim 89, wherein the free maleimide group is attached to said PEG through a linker that may contain amide, ester, sulfonamide, sulfonyl, thiol, oxy, alkyl, alkenyl, alkynyl, aryl, maleimide, or amine functional group, or any combination thereof.

5

91. The method according to claim 89, wherein said PEG has a linear structure.

92. The method according to claim 89, wherein said PEG has a branched structure.

10

93. The method according to claim 89, wherein said PEG has a multi-arm structure.

94. The method according to claim 89, wherein one or more free maleimide groups are attached to said PEG.

15

95. The method according to claim 89, wherein said PEG has average molecular weight of about 100 Da to about 500,000 Da.

20

96. The method according to claim 95, wherein said PEG has average molecular weight of about 1,000 Da to about 50,000 Da.

97. The method according to claim 89, wherein said N-methyl-cysteine residue is in L-form.

25

98. The method according to claim 89, wherein said N-methyl-cysteine residue is in D-form.

99. The method according to claim 89, wherein said N-methyl-cysteine residue is in a protein.

30

100. The method according to claim 89, wherein said N-methyl-cysteine residue is in a peptide.

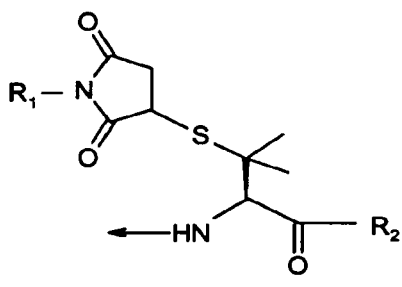
101. The method according to claim 89, wherein said N-methyl-cysteine residue is in an organic molecule.

5 102. The method according to claim 89, wherein said conjugate product contains one or more protein, peptide, or organic molecule moieties, or any combination thereof.

10 103. The method according to claim 89, wherein a reducing agent is used in the reaction.

104. The method according to claim 103, wherein said reducing agent is TCEP.

15 105. A method of chemically conjugating PEG containing a free maleimide group to the unoxidized sulfhydryl side-chain of a penicillamine residue of a molecule, said method comprising reacting the free maleimide group of said PEG with the unoxidized sulfhydryl side-chain of said penicillamine residue to generate a conjugate product, wherein said conjugate product has the structure of



20 wherein R_1 is said PEG, and R_2 is said molecule.

25 106. The method according to claim 105, wherein said free maleimide group is attached to said PEG through a linker that may contain amide, ester, sulfonamide, sulfonyl, thiol, oxy, alkyl, alkenyl, alkynyl, aryl, maleimide, or amine functional group, or any combination thereof.

107. The method according to claim 105, wherein said PEG has a linear structure.

108. The method according to claim 105, wherein said PEG has a branched structure.

5 109. The method according to claim 105, wherein said PEG has a multi-arm structure.

110. The method according to claim 105, wherein one or more free maleimide groups are attached to said PEG.

10

111. The method according to claim 105, wherein said PEG has average molecular weight of about 100 Da to about 500,000 Da.

112. The method according to claim 111, wherein said PEG has average
15 molecular weight of about 1,000 Da to about 50,000 Da.

113. The method according to claim 105, wherein said penicillamine residue is in L-form.

20 114. The method according to claim 105, wherein said penicillamine residue is in D-form.

115. The method according to claim 105, wherein said penicillamine residue is in a protein.

25

116. The method according to claim 105, wherein said penicillamine residue is in a peptide.

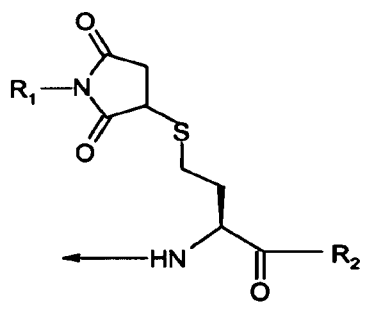
117. The method according to claim 105, wherein said penicillamine residue is
30 in an organic molecule.

118. The method according to claim 105, wherein said conjugate product contains one or more protein, peptide, or organic molecule moieties, or any combination thereof.

119. The method according to claim 105, wherein a reducing agent is used in the reaction.

5 120. The method according to claim 119, wherein said reducing agent is TCEP.

121. A method of chemically conjugating PEG containing a free maleimide group to the unoxidized sulfhydryl side-chain of a homocysteine residue of a molecule, said method comprising reacting the free maleimide group of said PEG with the unoxidized sulfhydryl side-chain of said homocysteine residue to generate a conjugate product, wherein said conjugate product has the structure of



wherein R_1 is said PEG, and R_2 is said molecule.

15

122. The method according to claim 121, wherein said free maleimide group is attached to said PEG through a linker that may contain amide, ester, sulfonamide, sulfonyl, thiol, oxy, alkyl, alkenyl, alkynyl, aryl, maleimide, or amine functional group, or any combination thereof.

20

123. The method according to claim 121, wherein said PEG has a linear structure.

124. The method according to claim 121, wherein said PEG has a branched structure.

25

125. The method according to claim 121, wherein said PEG has a multi-arm structure.

126. The method according to claim 121, wherein one or more maleimide groups are attached to said PEG.

5 127. The method according to claim 121, wherein said PEG has average molecular weight of about 100 Da to about 500,000 Da.

128. The method according to claim 127 wherein said PEG has average molecular weight of about 1,000 Da to about 50,000 Da.

10

129. The method according to claim 121, wherein said homocysteine residue is in L-form.

15

130. The method according to claim 121, wherein said homocysteine residue is in D-form.

131. The method according to claim 121, wherein said homocysteine residue is in a protein.

20

132. The method according to claim 121, wherein said homocysteine residue is in a peptide.

25

133. The method according to claim 121, wherein said homocysteine residue is in an organic molecule.

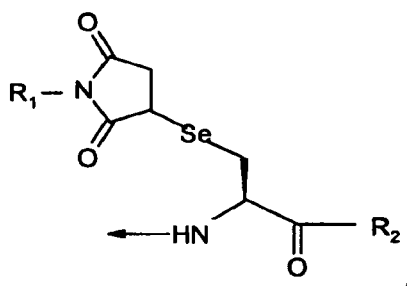
134. The method according to claim 121, wherein said conjugate product contains one or more protein, peptide, or organic molecule moieties, or any combination thereof.

30

135. The method according to claim 121, wherein a reducing agent is used in the reaction.

136. The method according to claim 135, wherein said reducing agent is TCEP.

137. A method of chemically conjugating PEG containing a free maleimide group to the unoxidized seleno side-chain of a selenocysteine residue of a molecule, said method comprising reacting the free maleimide group of said PEG with the unoxidized seleno side-chain of said selenocysteine residue to generate a conjugate product, wherein
 5 said conjugate product has the structure of



wherein R₁ is said PEG, and R₂ is said molecule.

138. The method according to claim 137, wherein the free maleimide group is attached to said PEG through a linker that may contain amide, ester, sulfonamide, sulfonyl, thiol, oxy, alkyl, alkenyl, alkynyl, aryl, maleimide, or amine functional group, or any combination thereof.
139. The method according to claim 137, wherein said PEG has a linear structure.
140. The method according to claim 137, wherein said PEG has a branched structure.
141. The method according to claim 137, wherein said PEG has a multi-arm structure.
142. The method according to claim 137, wherein one or more free maleimide groups are attached to said PEG.
143. The method according to claim 137, wherein said PEG has average molecular weight of about 100 Da to about 500,000 Da.

144. The method according to claim 143, wherein said PEG has average molecular weight of about 1,000 Da to about 50,000 Da.

5 145. The method according to claim 137, wherein said selenocysteine residue is in L-form.

146. The method according to claim 137, wherein said selenocysteine residue is in D-form.

10 147. The method according to claim 137, wherein said selenocysteine residue is in a protein.

148. The method according to claim 137, wherein said selenocysteine residue is in a peptide.

15 149. The method according to claim 137, wherein said selenocysteine residue is in an organic molecule.

20 150. The method according to claim 137, wherein said conjugate product contains one or more protein, peptide, or organic molecule moieties, or any combination thereof.

25 151. The method according to claim 137, wherein a reducing agent is used in the reaction.

152. The method according to claim 151, wherein said reducing agent is TCEP.