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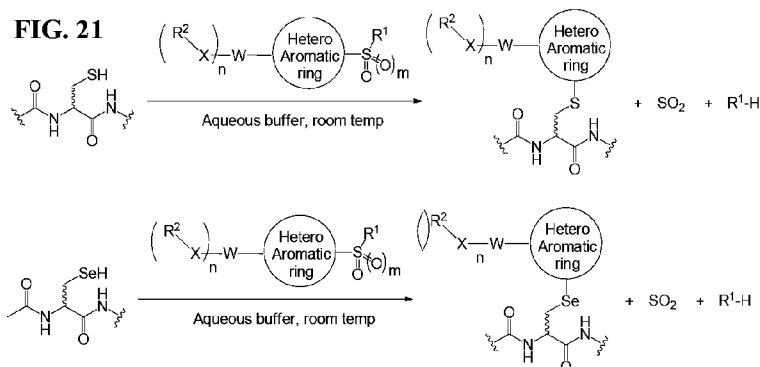
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(54) Title: NOVEL THIOL & AMINO MODIFYING REAGENTS FOR PROTEIN CHEMISTRY AND METHODS OF USE THEREOF



(57) Abstract: Provided herein are aryl and heteroaryl sulfone and sulfoxide modifying agents for facile and selective chemical alteration of proteins at thiol in cysteine residues, thiol- and selenol-containing moieties. Also provided herein are TAK242 derivatives for selective reaction with amino-containing compounds. The modifying agents disclosed herein can be used under mild reaction conditions for a variety of conjugation applications in molecules that possess amino, thiol or selenol functionalities. Also provided herein are methods of chemoselectively modifying a moiety containing the amino acids cysteine or lysine. Provided herein are CCR5 and CXCR antagonist derivatives modified for bioconjugation to macromolecules.

NOVEL THIOL & AMINO MODIFYING REAGENTS FOR PROTEIN CHEMISTRY AND METHODS OF USE THEREOF

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

[0001] The present disclosure relates generally to bioconjugation of biomolecules and, more specifically, to reagents and methods useful for the selective modification of thiol groups in cysteine residues, or amino groups in lysine residues, to form stable linkages for protein alteration.

BACKGROUND INFORMATION

[0002] Bioconjugation is the process of coupling two biomolecules together in a covalent linkage. Methods for the mild and site-specific derivatization of proteins, DNA, RNA, and carbohydrates have been developed for applications such as ligand discovery, disease diagnosis, and high-throughput screening. For instance, fluorescent or affinity tagging allows otherwise difficult analysis and tracking of proteins both *in vitro* and *in vivo*. Therapeutic protein conjugates have also gained prominence in the fight against HIV, cancer, malaria, and pathogenic bacteria. These powerful methods owe their existence to the discovery of chemoselective reactions that enable bioconjugation under physiological conditions.

[0003] HIV-1 infection is typically managed by a treatment regimen known as highly active antiretroviral therapy or HAART, which commonly involves the administration of combinations of reverse transcriptase and protease inhibitors. This approach has significant problems, for example, the emergence of drug-resistant escape variants demanding the need for improved therapies for the targeting of viral proteins. A promising approach to the viral escape issues is to target host cell proteins required for viral entry and propagation because such components would not mutate in the face of drug pressure. The host proteins, are not under selective pressure to evolve to escape the therapeutic agent. As a consequence, the cellular receptors in HIV-1 entry are receiving attention as a target to blocking viral entry.

[0004] CCR5 and CXCR4 are G-protein coupled 7-transmembrane chemokine co-receptors that are involved in fusion of the viral protein and host cell. The most advanced approaches in this area inhibit the CCR5 receptor. Maraviroc, is currently the only FDA

approved CCR5 receptor antagonist that has been developed and a major breakthrough that inhibits an early step in the viral life cycle. A small molecule, GSK812397, was discovered to be a potent CXCR4 receptor antagonist, and demonstrates effective pharmacokinetic properties and bioavailability across species.

[0005] CCR5 and CXCR4 are considered to be the major co-receptors operated by all HIV-1 strains. CXCR4 co-receptor is utilized by T-tropic HIV strains to gain entry into T-cells and are considered to be more pathogenic than M-tropic strains. The abundance of CXCR4 utilizing strains of HIV is associated with a decrease in the number of T-cells and accelerates disease progression. The accepted mechanism of HIV infection involves initial attachment of the virus to the host cell receptor CD4 via interaction with the viral gp120 envelope protein. This binding event then triggers a conformational change in the envelope protein that provides for binding to chemokine co-receptors CCR5 or CXCR4 and finally membrane fusion after viral gp41 insertion into the target cell. Blockade of chemokine receptor engagement by the virus therefore blocks infection.

[0006] To date the only approved chemokine receptor targeted inhibitor is Maraviroc, a potent CCR5 antagonist that received FDA approval in 2007. Maraviroc treatment regimens, however, require twice daily dosing with as much as 1.2 grams of drug per day making patient compliance an issue. Significantly, the biology of CCR5 is not limited to HIV-1 and recent studies have demonstrated beneficial activity of Maraviroc therapy in graft-versus-host disease and *Staphylococcus aureus* pathogenesis. Thus, the development of Maraviroc derivatives with extended pharmacokinetic profiles would be an invaluable contribution to various therapeutic regimens. Key to the development of effective chemically programmed antibodies and PEGylated small molecules, is the discovery of a linkage chemistry that minimally impacts activity of the parental drug.

[0007] Chemical modification of proteins is a rapidly expanding area in chemical biology and selective installation of biochemical probes has led to a better understanding of natural protein modification and macromolecular function. In other cases such chemical alterations have changed the protein function entirely. Additionally, tethering therapeutic cargo to proteins has proven invaluable in campaigns against disease.

[0008] Bioconjugates have found utility in the discovery of biological interactions. Proteins and other biopolymers regulate and perform biological functions by binding to

ligands. Accordingly, discovering and characterizing the natural ligands of biopolymers is crucial to understanding biological processes. A promising approach for ligand discovery involves appending biomolecules of interest with synthetic small molecules that can function as probes that report on ligand binding. Such probes include fluorescent molecules, biotin, and NMR probes. The ability to screen large numbers of potential ligands rapidly is highly desirable. One “high-throughput” approach involves the introduction of non-natural functional groups into biomolecules, followed by site-specific immobilization on surfaces via a chemoselective reaction that occurs exclusively at the nascent appendage. The immobilized biomolecule can be exposed subsequently to various molecules to identify ligands. DNA microarrays and protein microarrays are important examples of this approach.

[0009] Bioconjugates also find use in the realm of biochemical assays. Small molecules appended to biomolecules can serve as probes for rigorous biochemical analyses. For example, Förster resonance energy transfer (FRET) can be used to generate signals that are sensitive to molecular conformational changes in the 1–10 nm range. A typical FRET experiment entails attachment of a pair of fluorescent molecules to different regions of a biomolecule. One of these fluorophores serves as a “donor” by transferring energy non-radiatively to the other fluorophore, which serves as an “acceptor”. Subsequently, the acceptor emits radiation at its characteristic emission frequency, thereby reporting on the distance between the donor and acceptor. FRET has been used to characterize protein folding, RNA folding, and biochemical reactions. Modern single-molecule fluorescence approaches have elevated FRET-based approaches to an unprecedented level of specificity.

[0010] Non-fluorescent small molecules are also employed as mechanistic probes. For example, biotin has been attached to a K⁺-ion channel, enabling the conformational changes accompanying channel opening to be mapped by measuring accessibility of the biotin to exogenous avidin. A nitrile group has also been introduced into an enzyme as a vibrational probe, and its stretching frequency was a sensitive reporter of the electrostatic environment within the enzymic active site.

[0011] Qualitative and quantitative detection of analytes in clinical samples is crucial for the early diagnosis of disease and bioconjugates lend themselves readily to diagnostic applications. The complexity and heterogeneity of clinical samples presents a challenging environment for the detection of individual molecules. Chromatographic purification of

analytes prior to analysis is time-consuming and labor-intensive, and hence impractical. Accordingly, chemical and immunological methods have become favored for medical diagnoses. Clinical chemistry exploits an intrinsic physicochemical property of the analyte to generate a unique signal, thus circumventing analyte purification. Examples of this approach include spectrophotometric detection of metal ions and chromogenic and fluorogenic substrate-based assays for characterizing enzymes of interest. Clinical chemistry approaches are limited to special cases because many analytes lack a unique signal-generating property. Moreover, clinical chemistry approaches are often not sensitive enough to be useful in clinical regimes.

[0012] In comparison to chemical methods, immunological approaches are often more sensitive. The high specificity of antibody–antigen interactions avoids sample purification. Moreover, since antibodies can be generated against almost any analyte, this method is widely applicable.

[0013] Traditional diagnostic methods require significant biochemical experimental protocols that are time-consuming and require specialized laboratory equipment, limiting their applicability. There is an urgent need to develop reusable biosensors for economical and rapid detection of analytes that would be usable in locations far removed from a laboratory setting, such as in the office of a medical doctor or in a remote geographical location. Most biosensors consist of biomolecules attached to surfaces via robust bioconjugation linkages. For example, a commercially available glucose sensor has been developed in which glucose oxidase is immobilized to an electrode surface. The immobilized enzyme converts glucose into hydrogen peroxide, which is recorded as a digital signal. This device is used to monitor glucose levels in diabetes patients. Some biosensor applications employ optical techniques such as surface plasmon resonance (SPR) to detect binding of analytes to biomolecules immobilized on a surface. SPR is used to measure binding of ligands, and yields accurate binding constant values. SPR-detection requires expensive instrumentation. A more practical and still highly sensitive detection method based on the orientational behavior of liquid crystals on nanostructured surfaces has also been demonstrated.

[0014] The diagnostic methods discussed above are limited to cases wherein the nature of the disease allows for the preparation of clinical samples. In many cases, sample preparation is unfeasible, and the diagnosis needs to be performed directly inside the body. Thus, *in vivo*

imaging using bioconjugates is desirable. Methods such as magnetic resonance imaging (MRI) and radioimaging are employed in such situations.

[0015] Contrast agents are used to improve signal-sensitivity in MRI. Gd(III) complexes are effective contrast agents. Antibodies conjugated to Gd(III) complexes have been used for *in vivo* targeting. Other contrast agents such as magnetite have also been conjugated to antibodies for similar applications.

[0016] Radioimaging is another powerful method for *in vivo* imaging. Isotopes of iodine (i.e., ^{123}I and ^{131}I) are commonly used radionuclides. The iodo group is especially convenient because it can be introduced readily into the tyrosine residues of proteins, but the observation of *in vivo* deiodination raises concerns. Metal nuclides such as $^{99\text{m}}\text{Tc}$ and ^{111}In are useful alternatives, and can be attached to proteins via organic chelating agents such as EDTA.

[0017] Positron emission tomography (PET) continues to grow as an imaging tool. PET is used often in clinical oncology, as well as for the clinical diagnosis of certain diffuse brain diseases such as those causing various types of dementias. PET is also an important research tool to map normal human brain and heart function. PET relies on gamma rays emitted indirectly by a positron-emitting radionuclide, usually an [^{18}F]fluoro group attached to glucose. The conjugation of ^{18}F to proteins is a promising area for future development.

[0018] The conjugation of polyethyleneglycol (PEG) molecules to proteins is a well-established technique. Commonly referred to as “PEGylation”, attachment of PEGs can endow proteins with many desirable attributes, such as enhanced water solubility, reduced immunogenicity, improved circulating half-life *in vivo*, enhanced proteolytic resistance, reduced toxicity, and improved thermal and mechanical stability.

[0019] Traditional strategies for covalent bioconjugation preclude control over the regiochemistry of reactions, producing heterogeneous reaction products. Poor control over the site of modification often results in loss of the biological function of the target biomolecule. In contrast, more recent methods of bioconjugation are highly site-specific and cause minimal perturbation to the active form of the biomolecule. Moreover, biomolecules immobilized site-specifically can possess higher ligand binding ability and display stronger spectral polarization. Thus, site-specific bioconjugation is preferable to random bioconjugation.

[0020] The opportunity to study, understand, and influence biological processes with modified proteins prompts the development of selective reactions that yield well-defined protein constructs. The chemical reactions amenable to selective protein modification must satisfy a number of challenging criteria.

[0021] For a reaction to be of general use in protein modification, it must selectively modify a residue of interest in the presence of hundreds of competing side chains of the unprotected polypeptide. This selectivity must also be achieved in conditions required to prevent protein denaturation: aqueous media, low to ambient temperature, and at or near neutral pH. Additionally, the reaction must tolerate salts and surfactants often needed for protein stability. Finally, since proteins are often only available in low concentrations, the reaction must be rapid to achieve full conversion. These requirements present a considerable chemical challenge. The reaction will be most useful if it is specific for the residue of interest.

[0022] Common linkages for site-specific bioconjugation rely on cysteine or lysine residues, both of which bear nucleophilic functionalities. Of the proteogenic amino acids, cysteine is perhaps the most convenient target for selective modification owing to the strongly nucleophilic side chain sulfhydryl. Furthermore, cysteine's relatively low natural abundance, combined with standard site-directed mutagenesis, allows access to protein constructs with a single cysteine at a predetermined site.

[0023] Protein-drug conjugates offer a number of advantages compared to the small molecule drug alone. These include extension of a half-life, localization to target tissue, minimization of drug-drug interactions, reduction of dosage frequency, and reduced drug side effects. Two types of protein-drug conjugates are known, which include formation prior to patient treatment (e.g., antibody-drug conjugates (ADC)) and *in vivo* through a specific conjugation reaction with a targeted protein.

[0024] Human serum albumin (HSA) is the most abundant protein in human blood. HSA transports hormones, fatty acids, and other compounds and buffers pH, and maintains osmotic pressure. The half-life of HSA in the blood is approximately 20 days. Because of this long half-life, HSA is a suitable protein for *in vivo* labeling.

[0025] Presynthesized conjugates of HSA with peptides or small molecules by cysteine-maleimide conjugation have been prepared. Thus, HSA has been employed as a delivery vehicle for some drugs. However, cysteine-maleimide adducts, which are commonly used

for the preparation of protein conjugates, have been reported to exhibit instability in the blood, where hydrolysis of the succinimide ring and exchange reaction with reactive free thiol in the blood have been observed. Therefore, there is a compelling need for an alternative to cysteine conjugation by cysteine-maleimide chemistry.

[0026] Reagents that react with lysine residues have also been reported; however, lysine-reactive compounds, such as antibiotics of β -lactam type, do not react specifically with HSA and thus are not suitable for HSA labeling in the blood. TAK-242, which is a potent toll-like receptor 4 (TLR4) inhibitor, has been reported to react with a lysine residue residing in HSA in human plasma.

[0027] Typical thiol-reactive functional groups include iodoacetamides, maleimides, and disulfides (Figure 1). Iodoacetamides (Figure 1A) were used in classic experiments for determining the presence of free cysteines in proteins. More recently, iodoacetamido groups have been used extensively for labeling proteins with fluorophores, PEGylation, and protein immobilization. Chloroacetamides appear to exhibit even greater specificity than iodoacetamides for cysteine residues.

[0028] Like iodoacetamides, maleimides are commonly used electrophiles for thiol-mediated bioconjugation. Thiols undergo a Michael addition reaction with maleimides to form succinimidyl thioethers (Figure 1B). However, an undesirable and underappreciated aspect of maleimide conjugates is the susceptibility of their imido groups to undergo spontaneous hydrolysis, resulting in undesirable heterogeneity. Both molybdate and chromate have been shown to catalyze the hydrolysis of an imido group near neutral pH, providing a means to decrease the heterogeneity of bioconjugates derived from maleimides. Additionally, the resulting succinimide thioethers formed by a Michael type addition of a thiol to maleimides, such as N-ethylmaleimide (NEM), once generally accepted as stable, have been reported to undergo retro-Michael and exchange reactions in the presence of other thiol compounds at physiological pH and temperature.

[0029] Moreover, the thiol-selectivity of iodoacetamides and maleimides is compromised at high concentrations of the reagents, as nucleophilic side chains of amino acid residues such as histidines and lysines can also be modified covalently. While disulfide reagents react selectively with thiols, as (Figure 1C), they are susceptible to reduction by biological

reducing agents, like glutathione. Hence, the use of disulfides is limited to *in vitro* applications, such as the crosslinking and immobilization of peptides and proteins.

[0030] The utility of cysteine in protein modification for biological and therapeutic applications cannot be overstated. The versatile reactivity of this residue has enabled access to a range of modified proteins that have allowed insight into complex biological problems. However, there exists a need for chemical methodology and reaction engineering to provide mild, selective reaction at cysteine residues, cysteine derivatives, and sensitive thiol functionalities, in general.

[0031] Disclosed herein are methods for the selective modification of thiol groups, selenol groups and cysteine at physiological pH and temperature. The methods provided herein circumvent problems associated with common thiol blocking groups, such as maleimide exchange and succinimide hydrolysis, plaguing current synthetic methodology, thereby allowing for the precise alteration of proteins under mild conditions.

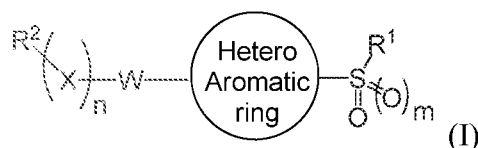
SUMMARY OF THE INVENTION

[0032] The present invention is based on the seminal discovery that aryl and heteroaryl sulfones and sulfoxides react with selenol and thiol functionalities in cysteine residues with a high degree of chemoselectivity under reaction conditions that mimic physiological parameters. The resulting adducts are stable across all pH ranges and do not form degradation or exchange products resulting from, for instance, hydrolysis or reversible reactions.

[0033] The present invention is also based on the discovery that the CCR5 antagonist Maraviroc and the CXCR4 antagonist GSK812397 can be derivatized for linkage to macromolecules without loss of activity.

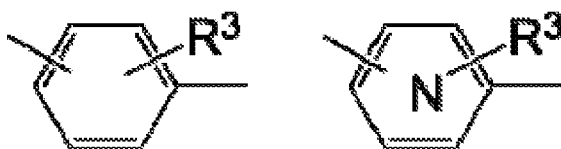
[0034] TAK-242 derivatives have been found to selectively react with a lysine moiety in HSA *in vivo* and *in vitro* to form HSA bioconjugates.

[0035] Provided herein are compounds of structural Formula I:



[0036] or an optically pure stereoisomer or a salt thereof, wherein:

[0037] W is selected from bond, hydrogen, and



;

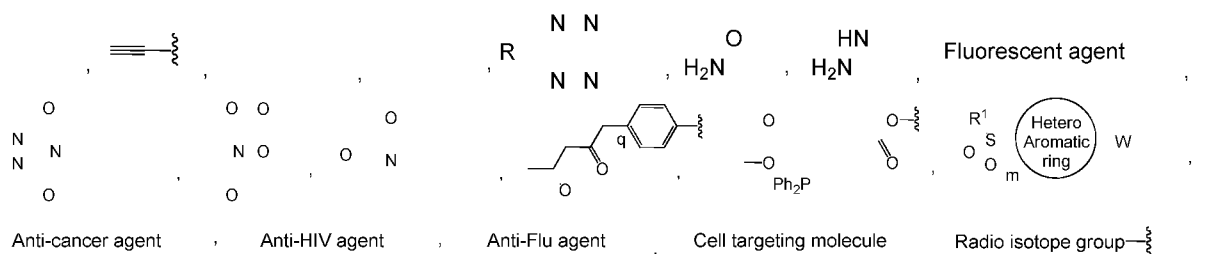
[0038] X is a linear or branched connecting chain of atoms comprising any of C, H, O, N, P, S, Si, F, Cl, Br, or I, or a salt thereof;

[0039] m is 0 or 1;

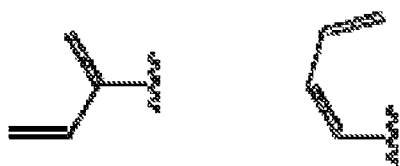
[0040] n is an integer from 0 to 5;

[0041] R^1 is selected from substituted or unsubstituted alkyl or substituted and unsubstituted aryl, wherein the substituents include, but are not limited to perhaloalkyl, cyano and carboxyl;

[0042] R^2 is selected from unsubstituted alkyl, substituted or unsubstituted aryl, $(-OCH_2CH_2)_p$, $-CO_2H$, $-NH_2$, OH , $-N_3$,



Peptide—, Protein—, Antibody—, siRNA—, miRNA—, DNA—, PEG chain (> 5k Da)—, toxin—

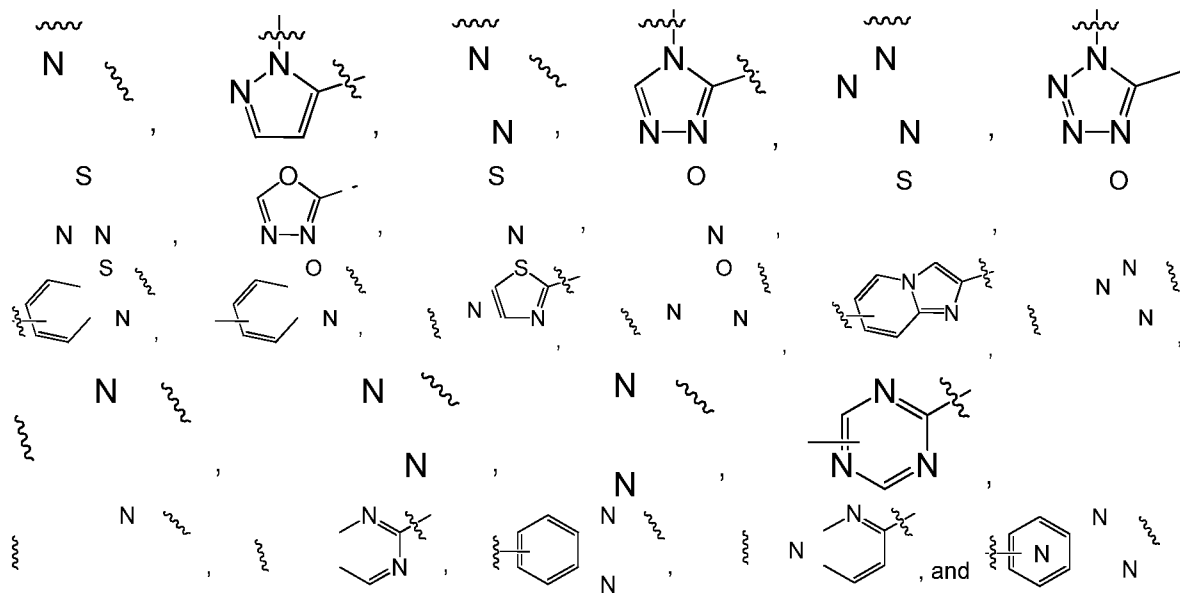


, and ;

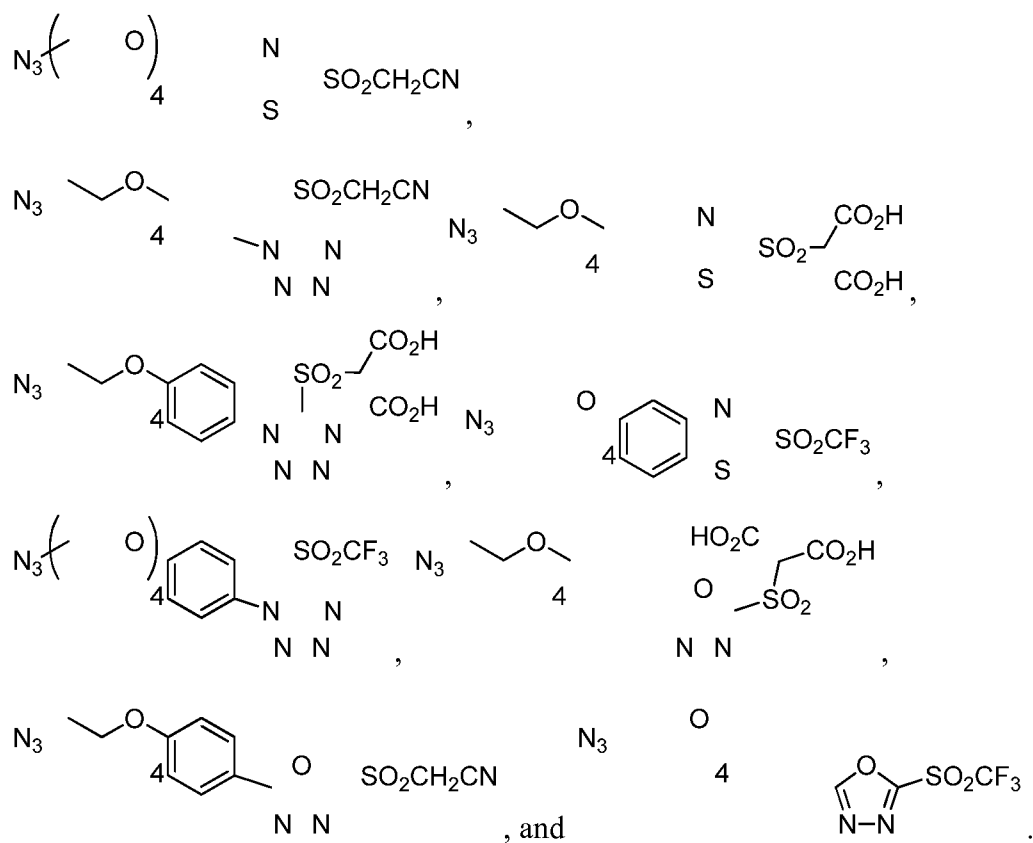
[0043] p is an integer from 0 to 1000;

[0044] R^3 is selected from hydrogen, halogen, $-CO_2H$, $-NH_2$, OH , $-N_3$, and $-alkyne$; and

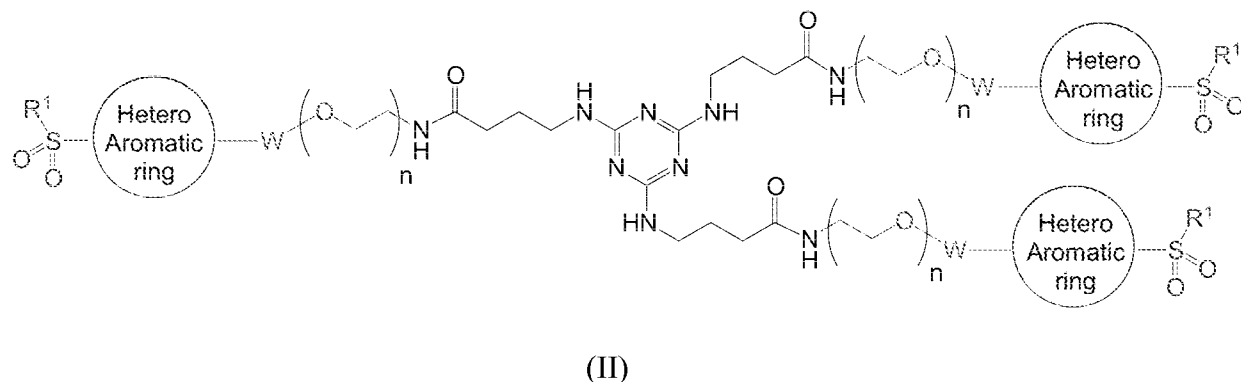
[0045] Heteroaromatic ring is selected from



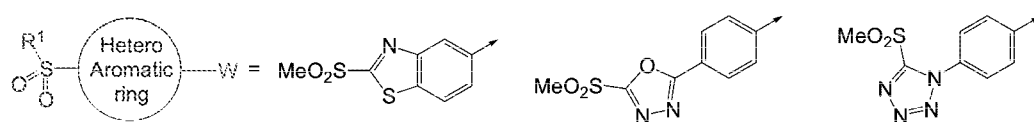
[0046] In certain embodiments, compounds of Formula I have structural formulas selected from



[0047] Compounds of structural Formula II, or an optically pure stereoisomer or a salt thereof, are provided herein:



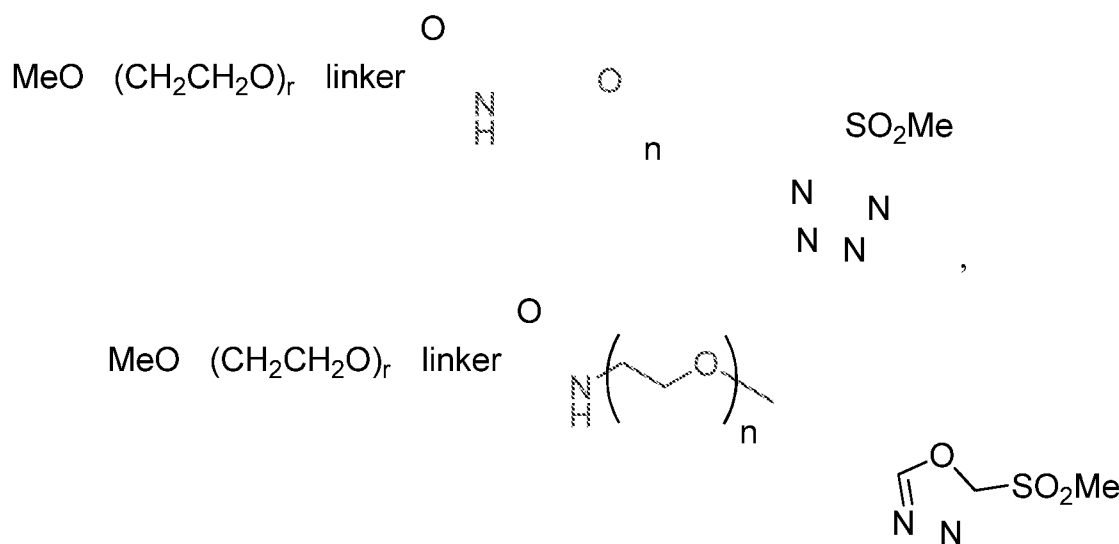
[0048] wherein:

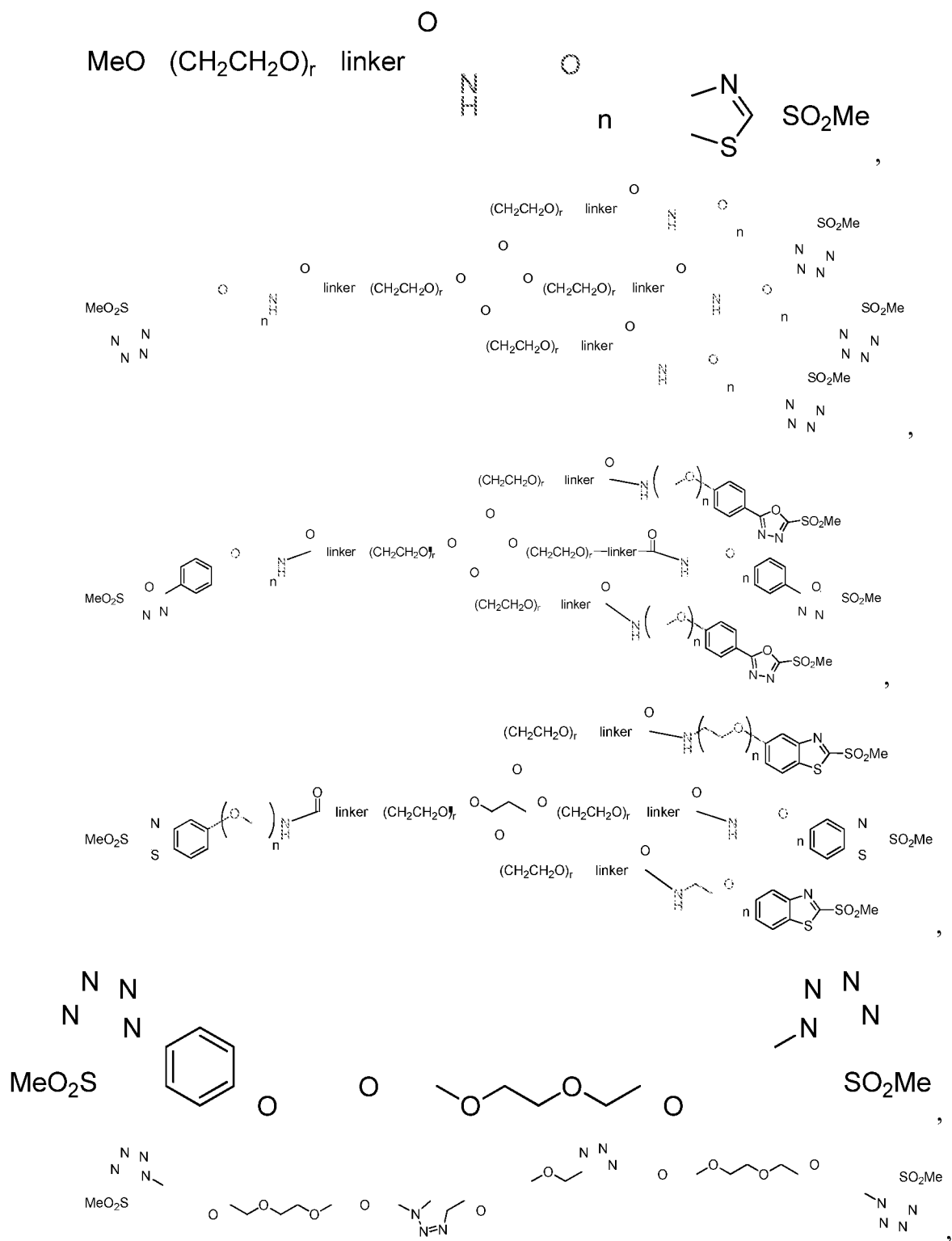


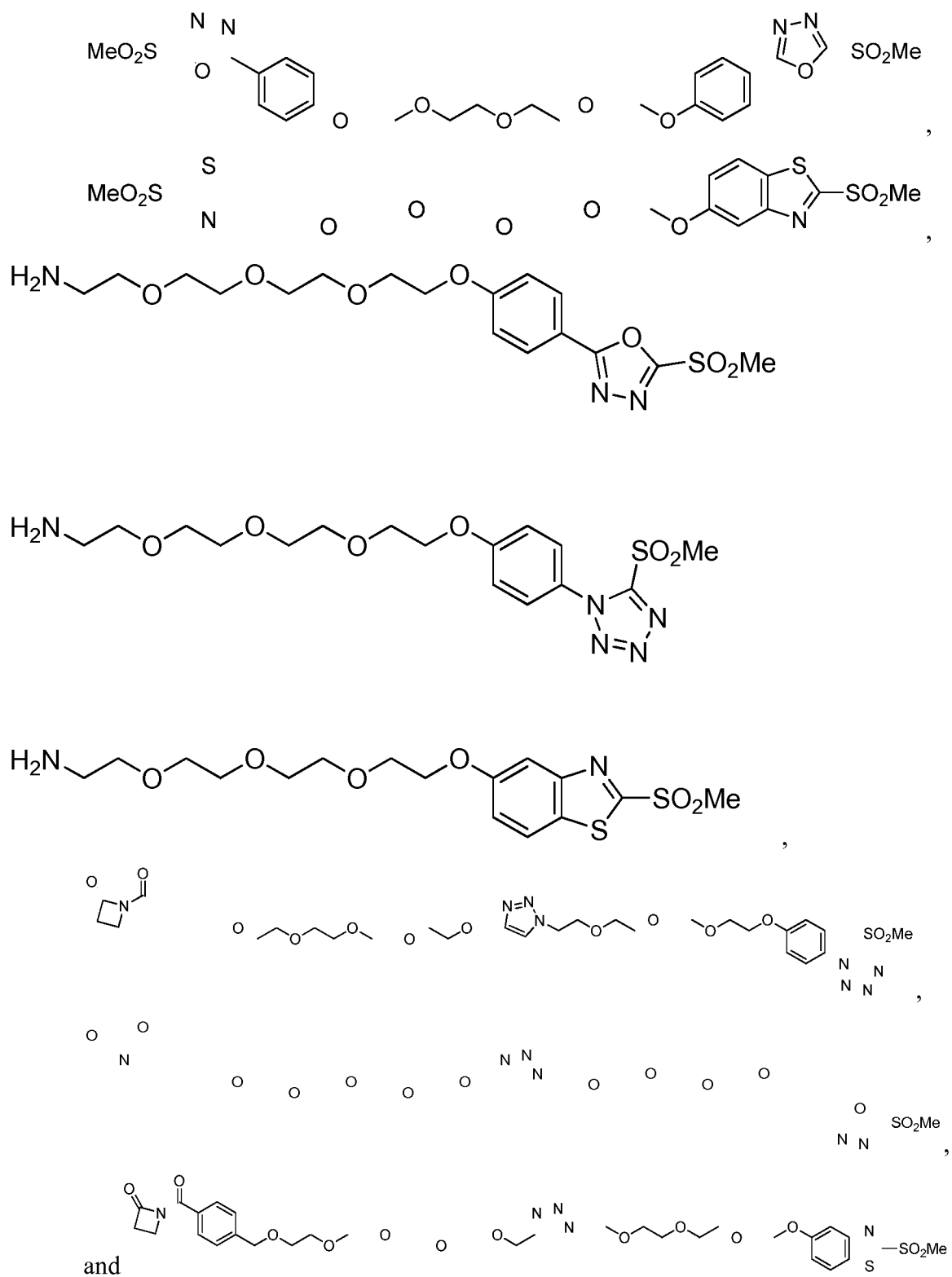
$n = 1-1000$

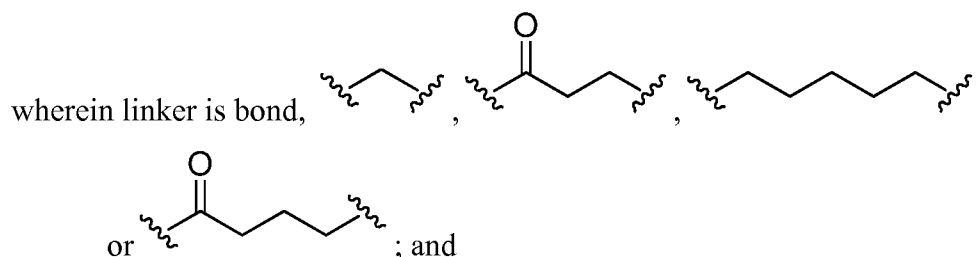
[0049] Also provided herein are compounds formed by reaction of a compound of Formula I with a thiol- or selenol- containing compound, wherein the reaction is performed a) outside the body by admixture of a thiol- or selenol- containing compound or b) within a living organism, wherein the thiol- or selenol- containing compound is found in the organism.

[0050] Provided herein are polyethylene glycol linked sulfones or sulfoxides selected from:









[0051] r and n are each independently an integer from 0 to 1000.

[0052] Compounds formed by reaction of the polyethylene glycol linked sulfones or sulfoxides above with a thiol- or selenol-containing compound are provided herein. Compounds formed by the reaction of compounds of Formula I with a thiol or selenol containing second molecule are also provided herein.

[0053] Provided herein are methods for conjugating two thiol containing compounds, two selenol containing compounds, or a selenol containing compound and a thiol containing compound. The method includes: (i) reacting a first compound containing a thiol or selenol group with a compound of Formula I or Formula II; and (ii) adding the product of the reaction of (i) to a medium containing a second compound containing a thiol or selenol group. In certain aspects, the second step is performed outside the body. In other aspects, the second step is performed inside the body and the second molecule is a protein. In one embodiment, the second step is performed inside the body and the second molecule is albumin.

[0054] In some aspects, the first and/or second thiol or selenol-containing compounds are each independently selected from the group consisting of a) antibodies, aldolase antibodies, zybodies, or antibody fragments, antibody Fc, antibodies engineered for increased half-life or effector function, scFvs, domain antibodies, diabodies, and immunoglobulin domains or variants therein engineered to possess a free thiol(s) (cysteine) or free selenol(s) (selenocysteine) residue; b) albumin or albumin fragments comprising a free thiol (cysteine) or free selenol (selenocysteine) or engineered variants of albumins or muteins with extended half-lives; c) an affibody or an engineered ankrin repeat protein; d) a nucleic acid; e) a peptide; f) an organic molecule of mw at least 200 Daltons; g) a protein; h) a polyethylene glycol chain; i) a toxin; j) FGF21 and known muteins; k) GLP-1; l) doxorubicin; m) aptamer;

n) Aib2 C24 chimera lactam; o) biotin; p) a fluorescent molecule; q) aurastatin and derivatives; and r) maytansinoid and derivatives.

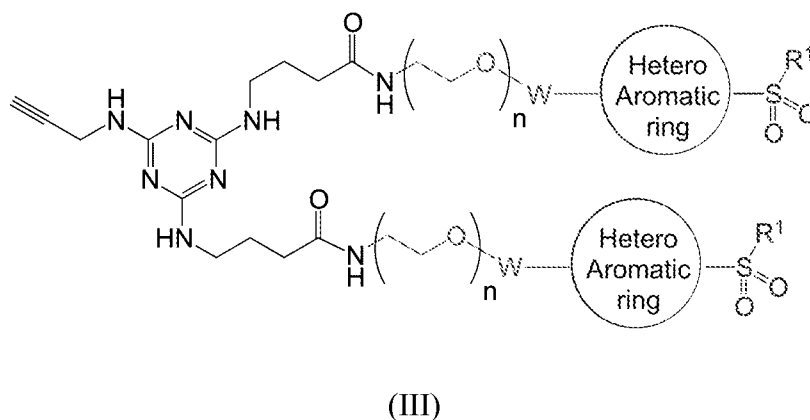
[0055] Provided herein is an antibody or antibody fragment or immunoglobulin domain drug conjugate prepared by the methods for conjugating two thiol containing compounds, two selenol containing compounds, or a selenol containing compound and a thiol containing compound.

[0056] Also provided herein are bi-, tri-, tetra-, penta-, or hexa- specific antibodies prepared by the methods for conjugating two thiol containing compounds, two selenol containing compounds, or a selenol containing compound and a thiol containing compound.

[0057] A modified albumin, albumin mutein, or albumin fragment linked to one or more molecules prepared by the methods for conjugating two thiol containing compounds, two selenol containing compounds, or a selenol containing compound and a thiol containing compound are provided herein.

[0058] A molecule synthesized to replace a maleimide or substituted maleimide within a known structure with thiol- or selenol reactive a heteroaromatic sulfone or sulfoxide of Formula I are provided herein.

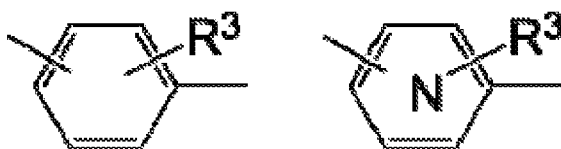
[0059] Provided herein is an intermediate of structural Formula III:



[0060] or salt thereof, wherein:

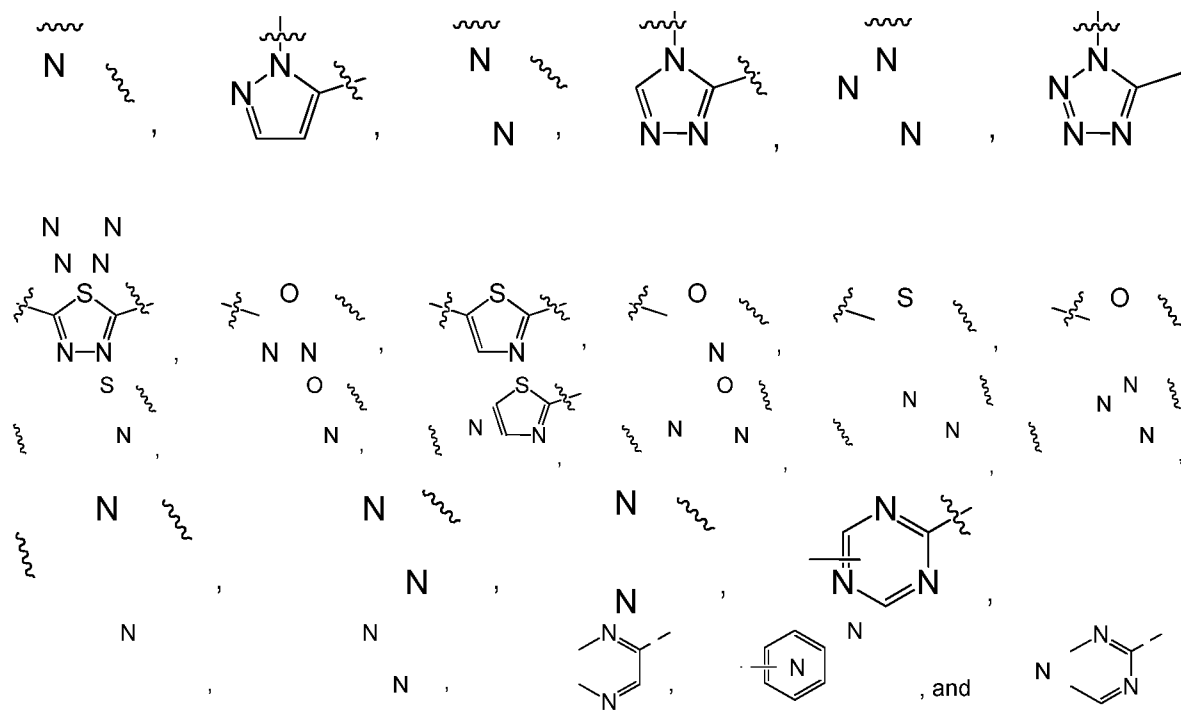
[0061] n is an integer from 0 to 1000;

[0062] W is selected from bond, hydrogen, and

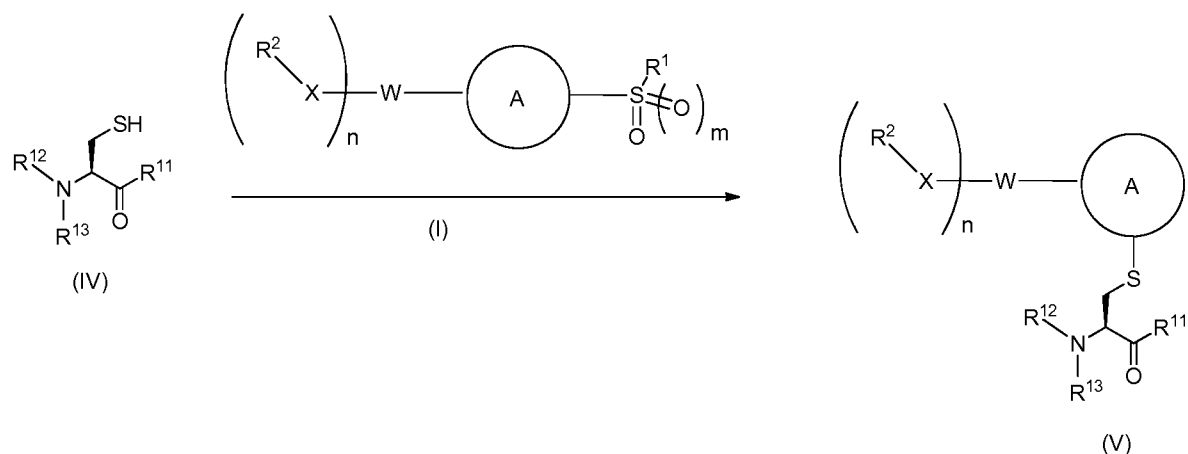


; and

[0063] Heteroaromatic ring is selected from

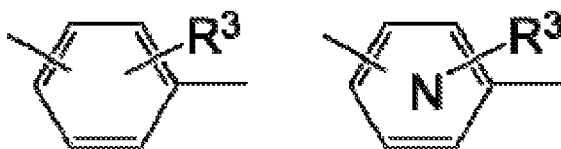


[0064] A method of chemoselectively modifying a moiety containing the amino acid cysteine is provided herein. The method comprises reacting a compound of Formula I with a compound of Formula IV to produce a compound of Formula V, thereby modifying the moiety containing the amino acid cysteine:



[0065] wherein:

[0066] W is selected from bond, hydrogen, and



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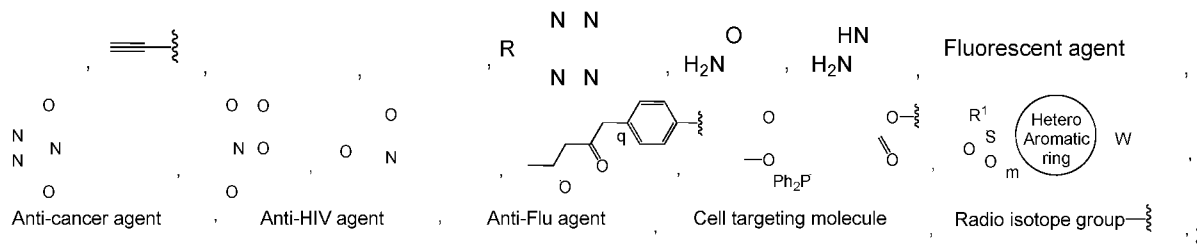
[0067] X is a linear or branched connecting chain of atoms comprising any of C, H, O, N, P, S, Si, F, Cl, Br, or I, or a salt thereof;

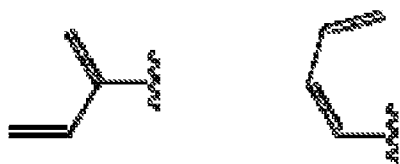
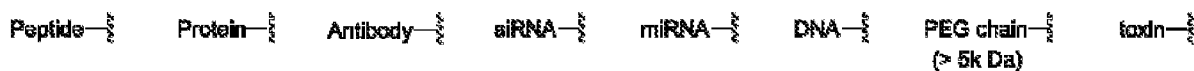
[0068] m is 0 or 1;

[0069] n is an integer from 0 to 5;

[0070] R¹ is selected from substituted or unsubstituted alkyl or substituted and unsubstituted aryl, wherein the substituents include but are not limited to perhaloalkyl, cyano and carboxyl;

[0071] R² is selected from unsubstituted alkyl, substituted or unsubstituted aryl, (-OCH₂CH₂)_p, -CO₂H, -NH₂, OH, -N₃,



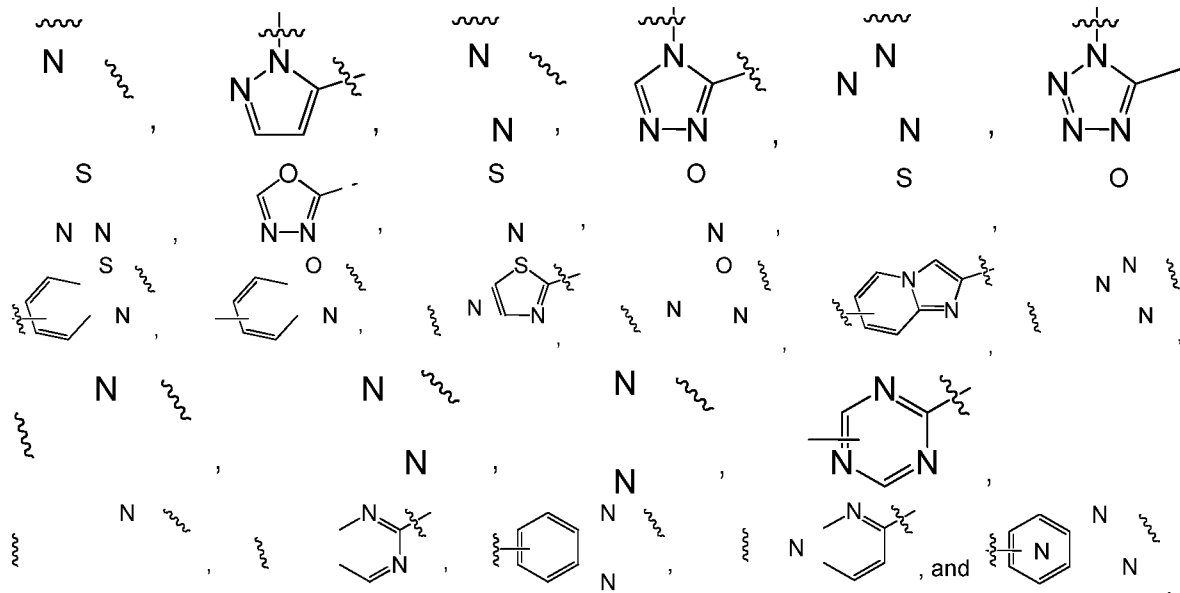


, and ;

[0072] p is an integer from 1 to 1000;

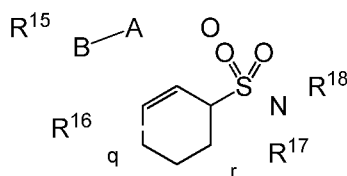
[0073] R^3 is selected from hydrogen, halogen, $-\text{CO}_2\text{H}$, $-\text{NH}_2$, OH , $-\text{N}_3$, and $-\text{alkyne}$; and

[0074] A is selected from



[0075] R^{12} , R^{13} , and R^{14} are each independently hydrogen, hydroxyl, amino, substituted or unsubstituted alkyl, substituted or unsubstituted thioalkyl, perfluoroalkyl, substituted or unsubstituted alkoxy, substituted or unsubstituted aryl, substituted or unsubstituted aryloxy, substituted or unsubstituted heteroaryl, substituted or unsubstituted heteroaryloxy, substituted or unsubstituted alkylaryl, substituted or unsubstituted alkylheteroaryl, or R^{12} , R^{13} , and R^{14} are in a cysteine residue of a peptide or a protein or a thiol group on an organic molecule. In certain aspects, the reaction occurs in an aqueous media at a pH between 2 and 10. In one embodiment, the aqueous media is a phosphate buffer at about a pH of 7.4. In other aspects the reaction occurs in a mixed organic/aqueous media.

[0076] Provided herein are compounds of structural formula IV:



(IV)

or salt thereof, wherein:

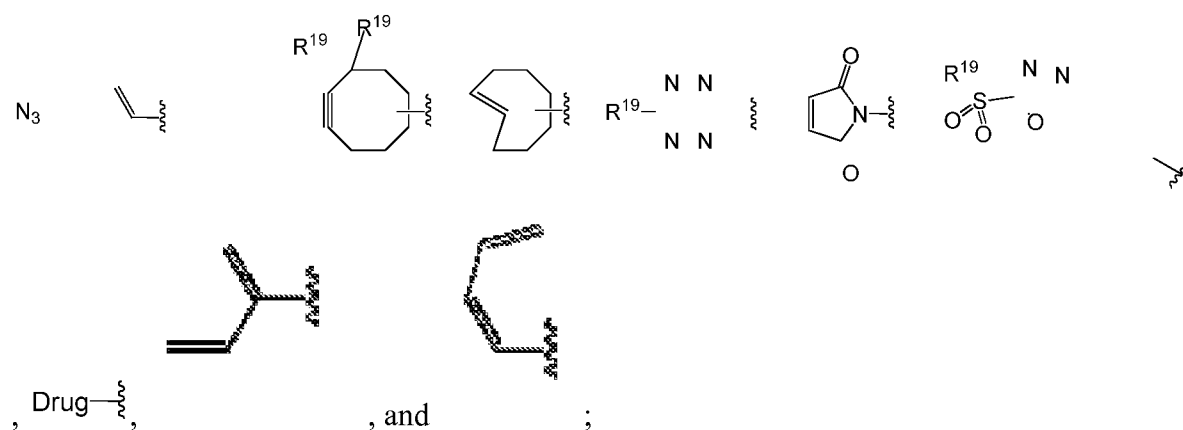
[0077] q is an integer from 0 to 5;

[0078] r is an integer from 0 to 3;

[0079] A is O or CH₂;

[0080] B is aryl, heteroaryl, or a linear or branched connecting chain of atoms comprising any of C, H, O, N, P, S, Si, F, Cl, Br, and I or a salt thereof;

[0081] R¹⁵ is selected from substituted or unsubstituted alkyl, substituted or unsubstituted aryl, (-OCH₂CH₂)_p, (-OCH₂CH₂)_p-N₃, -CO₂H, -NH₂, OH, -N₃,



[0082] p is an integer from 1 to 1000;

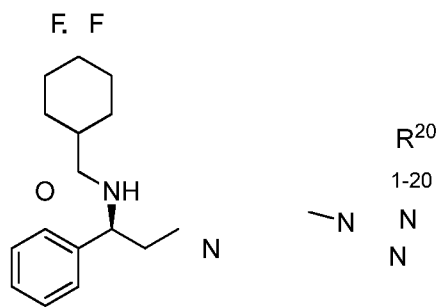
[0083] R¹⁶ is H, C₁₋₅alkyl or F;

[0084] R¹⁷ is H or C₁₋₅alkyl;

[0085] R¹⁸ is substituted or unsubstituted aryl and heteroaryl; and

[0086] R¹⁹ is H, F, substituted or unsubstituted C₁₋₅alkyl, aryl or heteroaryl.

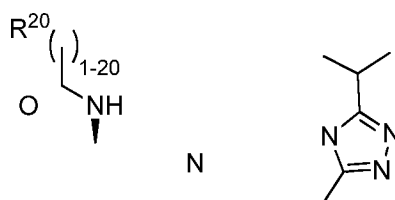
[0087] Provided herein are compounds of structural formula V:



V

or salt thereof.

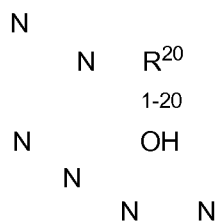
[0088] Provided herein are compounds of structural formula VI:



VI

or salt thereof.

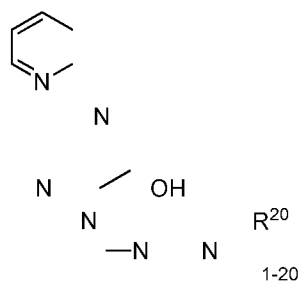
[0089] Provided herein are compounds of structural formula VII:



VII

or salt thereof.

[0090] Provided herein are compounds of structural formula VIII:

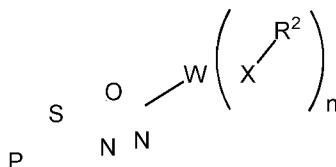


VIII

or salt thereof.

[0091] In certain aspects, R^{20} is $(CH_2)_{10}N_3$, $(CH_2)_{10}N_3$, 4,4-difluoro-cycloHx, $-(OCH_2CH_2)_p-$, $-N_3$, $-(OCH_2CH_2)_p-N_3$, a compound of formula I, a compound of formula IV, an antibody, or a protein. In other aspects, p is an integer from 0 to 1000.

[0092] Provided herein are compounds of structural Formula IX:

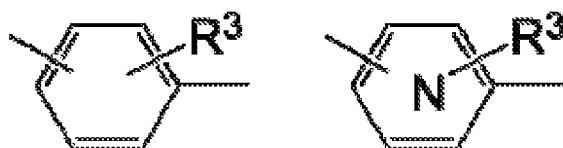


IX

[0093] or salt thereof, wherein:

[0094] P is protein, peptide, nucleic acid, or other molecule;

[0095] W is selected from bond, hydrogen, and



;

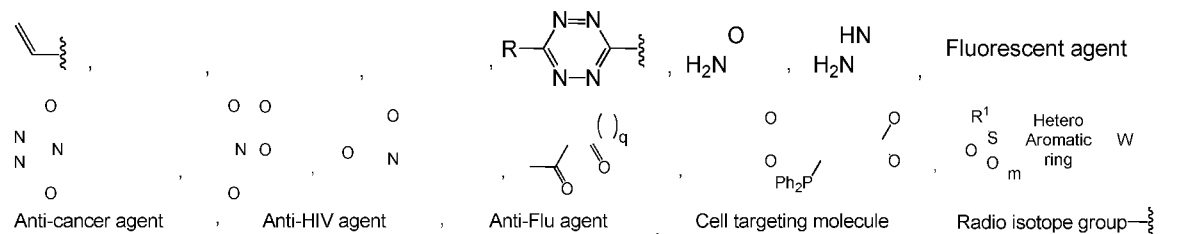
[0096] X is a linear or branched connecting chain of atoms comprising any of C, H, O, N, P, S, Si, F, Cl, Br, or I, or a salt thereof;

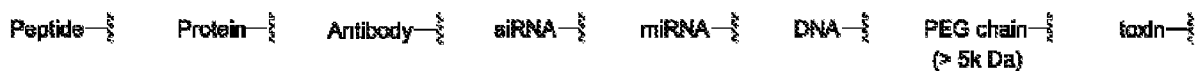
[0097] m is 0 or 1;

[0098] n is an integer from 0 to 5;

[0099] R^1 is selected from substituted or unsubstituted alkyl or substituted and unsubstituted aryl;

[0100] R^2 is selected from substituted or unsubstituted alkyl, substituted or unsubstituted aryl, $-(OCH_2CH_2)_p-$, $-CO_2H$, $-NH_2$, OH , $-N_3$,



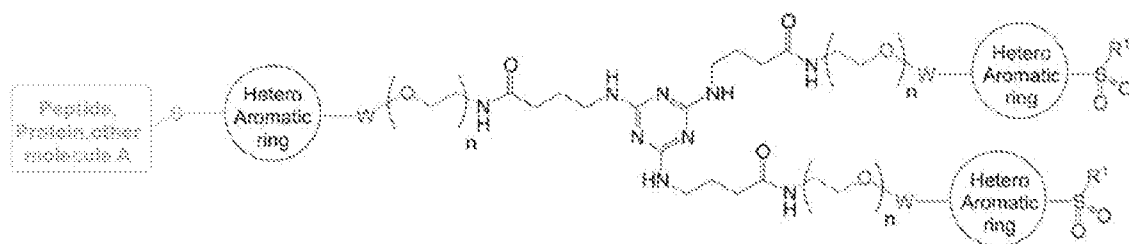


, and ;

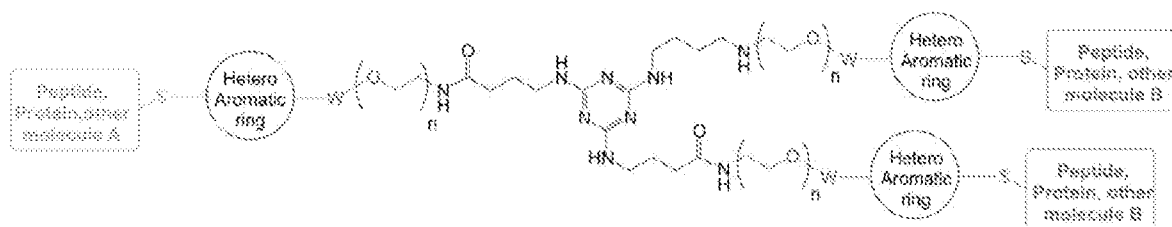
[0101] p is an integer from 1 to 1000; and

[0102] R³ is selected from hydrogen, halogen, -CO₂H, -NH₂, OH, -N₃, and -alkyne.

[0103] Provided herein are compounds of structural Formula X and XI:

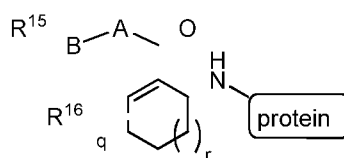


X

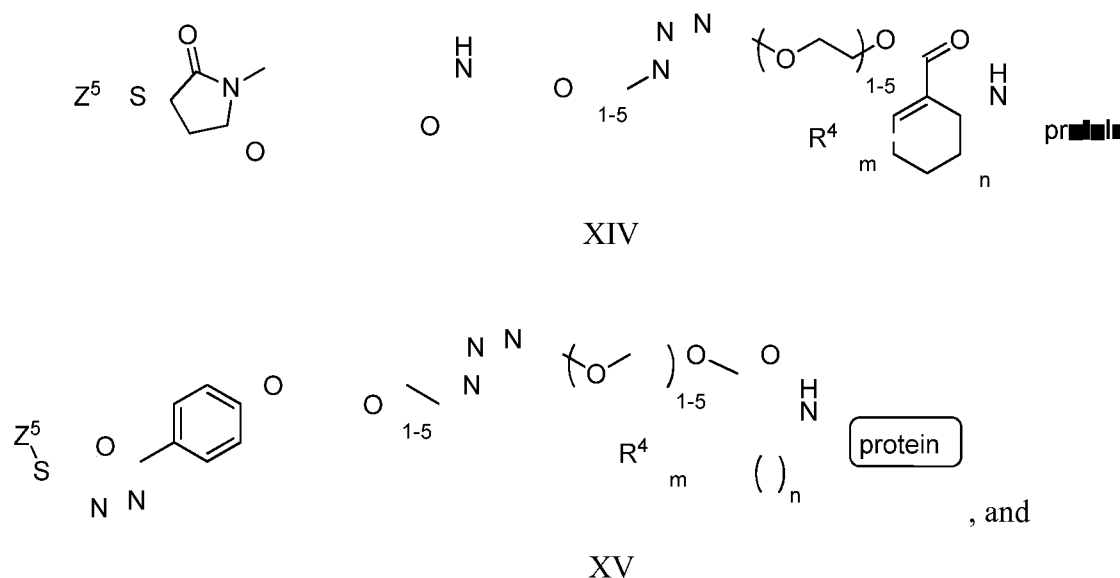


XI

[0104] Also provided herein are compounds of structural Formula XII:



XII



[0115] or salt thereof, wherein:

[0116] Z is protein, peptide, nucleic acid, or other molecule.

[0117] Also provided herein are compounds formed by reaction of a compound of Formula IV with an amino group- containing compound, wherein the reaction is performed a) outside the body by admixture of an amino group- containing compound or b) within a living organism, wherein the amino group- containing compound is found in the organism. In one aspect, the amino group- containing compound is a protein including, but not limited to, human serum albumin (HSA).

[0118] In certain aspects, the anti-HIV agent of the compounds disclosed herein is a CCR5 or CXCR4 antagonist. In one embodiment, the CCR5 antagonist is Maraviroc. In another embodiment, the CXCR4 antagonist is GSK812397.

BRIEF DESCRIPTION OF THE DRAWINGS

[0119] Figure 1A-C is a schematic representation of the reaction of typical thiol-reactive functional groups (A) iodoacetamides; (B) maleimides; (C) and disulfides.

[0120] Figure 2 shows the HPLC chromatograms of the products resulting from reaction of 2-(methylsulfonyl)-5-phenyl-1,3,4-oxadiazole with cysteine and deprotection of (*R*)-methyl 2-((tert-butoxycarbonyl)amino)-3-((5-phenyl-1,3,4-oxadiazol-2-yl)thio)propanoate with trifluoroacetic acid (TFA).

[0121] Figure 3 is a graphic representation of the relative stability of maleimide, benzothiazole and oxadiazole adducts.

[0122] Figure 4 shows the HPLC chromatograms of maleimide and oxadiazole adducts in human plasma stability assays.

[0123] Figure 5 depicts the ESI-MS chromatogram of modified and unmodified human albumin.

[0124] Figure 6 depicts the ESI-MS chromatogram of modified and unmodified maltose binding protein (MBP).

[0125] Figure 7 is a graphical representation of the stability of heteroaryl-cysteine conjugates in human plasma.

[0126] Figures 8 & 9 are schematic representations of thiol modifying agents for use in conjugation reactions.

[0127] Figure 10 depicts reaction schemes to form various thiol-modifying agents and subsequent reaction with a thiol functionality.

[0128] Figures 11-14, 16, and 17 show reaction schemes to form various thiol-modifying agents for use in conjugation reactions.

[0129] Figure 15 is a schematic representation of selective thiol-modifying agents employed in conjugation reactions with cysteine residues in proteins.

[0130] Figure 18 illustrates *in vitro* conjugation reaction of thiol-modifying agents.

[0131] Figure 19 shows bioconjugation reactions of thiol-modifying agents.

[0132] Figure 20 depicts various thiol-modifying agents for linking to cysteine.

[0133] Figure 21 is a schematic representation of the reaction between aryl and heteroaryl sulfone modifying agents with sulfur and selenium.

[0134] Figures 22 & 23 are schematic representations of modifying agents.

[0135] Figure 24 is a reaction scheme depicting formation of thiol-modifying agents and subsequent reaction with thiol-containing moieties.

[0136] Figures 25-30 are schematic representations of practical applications for thiol-modifying agents.

[0137] Figure 31 schematically depicts *in vivo* and *ex vivo* conjugation procedures with thiol-modifying agents.

[0138] Figures 32-36 are schematic representations of amino-modifying agents and subsequent reaction with amino-containing moieties.

[0139] Figures 37-50 are schematic representations of derivitization and bioconjugation of CCR5 and CXCR4 antagonists.

[0140] Figures 51-52 are schematic representations of practical applications for amino-modifying agents.

DETAILED DESCRIPTION OF THE INVENTION

[0141] Thiol-based Michael-type addition reactions have emerged as a widely employed strategy for covalent conjugation of proteins, peptides, and drugs (to various polymers and other molecules) by the reaction of free cysteine or thiols with acrylamides, acrylates, vinyl sulfones and maleimides. Specificity to thiols, fast aqueous reaction kinetics, lack of byproducts, and the stability of the thioether addition product crosslinkers, heterobifunctional crosslinkers, fluorescent labels, as well as in PEGylation reagents¹⁶ and crosslinking of hydrogels. Maleimides have thus been utilized in homobifunctional crosslinkers, heterobifunctional crosslinkers, fluorescent labels, as well as in PEGylation reagents and crosslinking of hydrogels.

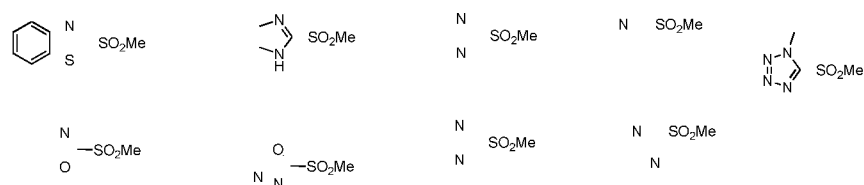
[0142] While the wide use of maleimide-thiol conjugation reactions have been motivated by the product stability, there have been limited reports indicating that select succinimide thioethers can undergo retro reactions at high temperatures (>300°C) and in some aqueous environments although the mechanisms and exact solution conditions for these retro reactions were not elucidated in great detail.

[0143] The advantage of maleimide chemistry is selective reactivity toward cysteine residues in the protein. The disadvantages of this method are instability of formed succinimide linkage and maleimide exchange with reactive thiol such as albumin, free cysteine or glutathione through the retro-Michael reaction. As a result, heterogeneous mixture of conjugate can be formed *in vivo* leading to different pharmacokinetics, efficacy and toxicity. New conjugation methods are disclosed herein, which result in stable linkages without thiol exchange ultimately allowing for better control of heterogeneous conjugates. The thiol-specific modifying reagents disclosed herein provide stable linkages that are suitable for protein conjugates such as antibody-drug conjugates. Other applications include, but are not limited to, albumin conjugates and applications in peptide chemistry.

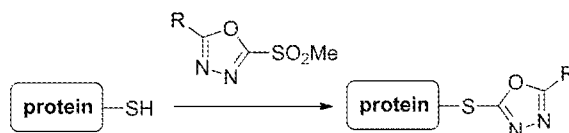
[0144] New thiol-modifying reagents have been developed, for example as shown below in Scheme 1, that are selective toward cysteine residues and afford stable adducts (as shown in

Scheme 2) not susceptible to thiol exchange and/or succinimide hydrolysis. These modifying agents contain sulfone or sulfoxide functional groups and also react selectively with selenol containing moieties.

Check these reactivity to thiol



Scheme 1



Key profiles

- Selective reactivity toward cysteine
- Stability (no thiol exchange and hydrolysis)

Scheme 2

[0145] The thiol-modifying (and selenol-modifying) agents described herein have applicability and may be used in bioconjugation reactions and covalent conjugation of proteins, peptides, and drugs (to various polymers and other molecules). Accordingly, the reagents and methods described herein are contemplated for use in a fashion analogous to maleimide-thiol conjugation agents and reactions, including but not limited to use as homobifunctional crosslinkers, heterobifunctional crosslinkers, fluorescent labels, as well as in PEGylation reagents and crosslinking of hydrogels. Examples for uses of the thiol-linking agents are described in Bioconjugate Chem. 2008, 19, 759–765, Bioconjugate Chem. 2005, 16, 1282-1290, Bioconjugate Chem. 2012, 23, 2007–2013, Clin Cancer Res 2010;16:4769-4778, Drug Discovery Today, Volume 10, Number 21, November 2005, Journal of Biological Chemistry, Volume 287, Number 34, August, 2012, “Site-Specific PEGylation of Human Thyroid Stimulating Hormone to Prolong Duration of Action,” Bioconjugate Chem. January 27, 2013, WO 2012/059873 A2, all of which are incorporated herein by reference.

[0146] Disclosed herein are compounds related to Julia-type olefination reagents that have been observed to react rapidly with thiols and selenols to provide a new type of "thiol-click" reaction. The reaction occurs rapidly between a thiol or selenol containing molecule and

heteroaromatic sulfone linked to a functional molecule. The resulting molecules are then linked.

[0147] The heteroaromatic sulfones described herein are contemplated to replace maleimide type reactive groups that are widely used in protein and thiol chemistry. The advantage of the heteroaromatic sulfones agents provided herein include rapid reactions that can be performed in a wide variety of biologically relevant buffers, which includes the blood of a living organism or in serum, over a wide pH range. The resulting linkages are more stable to thiol exchange as well as pH changes as compared to maleimide based linkages.

[0148] Uses for the reactions and reagents provided herein include linking or binding drugs, peptides, domains, proteins, aptamers, nucleic acids or small molecules to antibodies, antibody fragments or engineered variants of thereof as well, in addition to albumins or engineered variants of albumin that have improved pharmacokinetic profiles.

[0149] Chemically programmed antibodies (cpAbs), which link a catalytic antibody to a small molecule drug, peptide, or aptamer dramatically extend the pharmacokinetic profile of the attached molecule. Chemically programmed antibodies are based on monoclonal antibody (mAb) 38C2, an aldolase antibody generated by reactive immunization using a 1,3-diketone hapten. The antibody is comprised of a low pK_a lysine in its binding site that is essential for the catalytic mechanism of the antibody that can be selectively treated with β -lactam to form an amide and generate chemically programmed antibodies. Based on this strategy, several mAb conjugates have been prepared, which show promising activity against cancer models, CCR5 blocking mAb that inhibit HIV-1 entry and a potent inhibitor of influenza neuraminidase. Moreover, it is expected that the conjugate between mAb 38C2 and small molecule inhibitors against CXCR4 will be directed to bind to CXCR4 and block HIV-1 T-tropic strains from entering and infecting the cell.

[0150] Chemical programming of monoclonal antibody (mAb) 38C2 is facilitated by a low pK_a lysine residue in its binding site that is key to its aldolase activity. This lysine can be site-selectively labelled with N-acyl- β -lactams to produce a chemically programmed antibody. The cpAb approach has demonstrated efficacy in a number of disease models including anti-infectives. For example with a derivative of Zanamivir, a neuraminidase inhibitor, the cpAb approach provided long-term systemic exposure without loss of neuraminidase inhibitory activity.

[0151] Based on the combination of structure activity relationship and synthetic feasibility, two different linkage points of conjugating the GSK812397 compound to mAb 38C2 were explored by tethering the chemically programmed antibody to the piperazine and the central amine moieties. The ensuing conjugates possessed the long half-life and effector function of an antibody and the therapeutic activity of a small molecule drug.

[0152] Additionally, two routes towards linked variants of Maraviroc were undertaken. The first westerly linkage point, at the benzoic acid moiety, was chosen based on ease of synthesis and known structural tolerance at the cyclohexyl position. Introduction of a linker onto the triazole ring of Maraviroc (easterly connection) was also achieved. Linear and branch PEGylated compounds were prepared by simple amidation of N-hydroxysuccinimide (NHS) esters. N-Acyl- β -lactam derivatives were prepared through a standard click reaction and the lactams used for conjugation to mAb 38C2.

[0153] Another approach to extending the pharmacokinetic profiles of drugs involves their conjugation to polyethylene glycol (PEG), a process known as PEGylation. PEGylation often imparts other significant pharmacological advantages, such as improved solubility, minimized proteolytic cleavage, reduced dosage frequency, increased serum half-life, and reduced immunogenicity and antigenicity. PEGINTRON[®], an α -interferon derivative, is the first FDA-approved, PEG-modified drug. The plasma circulating half-life of PEGINTRON, which is used for treatment of hepatitis C, is about 10 times that of native IFN α -2b, allows weekly subcutaneous dosing. PEGylation also imparts desired properties on small molecule drugs.

[0154] The structure activity relationships of linked Maraviroc and GSK812397 derivatives with mAb 38C2, polyethylene glycol variants, HSA and thiol functionalities designed to create potent long-lived CCR5 and CXCR4 antagonists are also described herein.

[0155] TAK-242-based compounds have been designed and synthesized and the specificity of their reaction with HSA in vitro and in vivo was investigated. TAK-242 derivatives for HSA specific labeling are also contemplated herein. Fluorescein-attached TAK-242 derivative was prepared, and showed HSA specificity with no TLR4 inhibitory activity. Moreover, the HSA conjugate showed long half-life in human plasma (153 hours). Additionally, a labeling position in HSA was investigated by proteomics, and the results

suggested Lys64 was a target amino acid for TAK-242 analog. The amino acids targeted by the TAK-242-based molecules were determined using blocking experiments and proteomics.

[0156] Maraviroc-attached TAK derivative was also prepared, and the conjugates of this compound with recombinant HSA or human plasma showed strong neutralization activity. The technology described herein would be useful for both *in vitro* and *in vivo* labeling against HSA to create novel drugs.

[0157] TAK-242 binds to Cys747 in the intracellular region of TLR4 to block protein-protein interactions between TLR4 and its adaptor proteins. The compound forms adducts with reactive amino acids, such as lysine and cysteine, via Michael addition to the β -carbon of the ester moiety of TAK-242 and elimination of a sulfonamide such as SO₂ or 2-chloro-4-fluoroaniline. Analysis of a TAK-242 analogue showed that the alkyl group of the ester moiety had to be short and hydrophilic for the TLR4 inhibition. Based on this information, a polyethylene glycol (PEG) TAK-242 analogue with fluorobenzene sulfonamide as a leaving group was designed to retain labeling of HSA and reduce TLR-4 inhibitory activity.

[0158] As used herein, the terms below have the meanings indicated.

[0159] When ranges of values are disclosed, and the notation “from n1 ... to n2” or “between n1 ... and n2” is used, where n1 and n2 are the numbers, then unless otherwise specified, this notation is intended to include the numbers themselves and the range between them. This range may be integral or continuous between and including the end values. By way of example, the range “from 2 to 6 carbons” is intended to include two, three, four, five, and six carbons, since carbons come in integer units. Compare, by way of example, the range “from 1 to 3 μ M (micromolar),” which is intended to include 1 μ M, 3 μ M, and everything in between to any number of significant figures (e.g., 1.255 μ M, 2.1 μ M, 2.9999 μ M, etc.). When n is set at 0 in the context of “0 carbon atoms”, it is intended to indicate a bond or null.

[0160] The term “about,” as used herein, is intended to qualify the numerical values which it modifies, denoting such a value as variable within a margin of error. When no particular margin of error, such as a standard deviation to a mean value given in a chart or table of data, is recited, the term “about” should be understood to mean that range which would encompass the recited value and the range which would be included by rounding up or down to that figure as well, taking into account significant figures.

[0161] The term “acyl,” as used herein, alone or in combination, refers to a carbonyl attached to an alkenyl, alkyl, aryl, cycloalkyl, heteroaryl, heterocycle, or any other moiety where the atom attached to the carbonyl is carbon. An “acetyl” group refers to a -C(O)CH_3 group. An “alkylcarbonyl” or “alkanoyl” group refers to an alkyl group attached to the parent molecular moiety through a carbonyl group. Examples of such groups include methylcarbonyl and ethylcarbonyl. Examples of acyl groups include formyl, alkanoyl and aroyl.

[0162] The term “alkenyl,” as used herein, alone or in combination, refers to a straight-chain or branched-chain hydrocarbon group having one or more double bonds and containing from 2 to 20 carbon atoms. In certain embodiments, said alkenyl will comprise from 2 to 6 carbon atoms. The term “alkenylene” refers to a carbon-carbon double bond system attached at two or more positions such as ethenylene [(-CH=CH-) , (-C::C-)]. Examples of suitable alkenyl groups include ethenyl, propenyl, 2-methylpropenyl, 1,4-butadienyl and the like. Unless otherwise specified, the term “alkenyl” may include “alkenylene” groups.

[0163] The term “alkoxy,” as used herein, alone or in combination, refers to an alkyl ether group, wherein the term alkyl is as defined below. Examples of suitable alkyl ether groups include methoxy, ethoxy, n-propoxy, isopropoxy, n-butoxy, iso-butoxy, sec-butoxy, tert-butoxy, and the like.

[0164] The term “alkyl,” as used herein, alone or in combination, refers to a straight-chain or branched-chain alkyl group containing from 1 to 20 carbon atoms. In certain embodiments, said alkyl will comprise from 1 to 10 carbon atoms. In further embodiments, said alkyl will comprise from 1 to 6 carbon atoms. Alkyl groups may be optionally substituted as defined herein. Examples of alkyl groups include methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, pentyl, iso-amyl, hexyl, octyl, noyl and the like. The term “alkylene,” as used herein, alone or in combination, refers to a saturated aliphatic group derived from a straight or branched chain saturated hydrocarbon attached at two or more positions, such as methylene ($\text{-CH}_2\text{-}$). Unless otherwise specified, the term “alkyl” may include “alkylene” groups.

[0165] The term “alkylamino,” as used herein, alone or in combination, refers to an alkyl group attached to the parent molecular moiety through an amino group. Suitable alkylamino groups may be mono- or dialkylated, forming groups such as, for example, N-methylamino, N-ethylamino, N,N-dimethylamino, N,N-ethylmethylamino and the like.

[0166] The term “alkylidene,” as used herein, alone or in combination, refers to an alkenyl group in which one carbon atom of the carbon-carbon double bond belongs to the moiety to which the alkenyl group is attached.

[0167] The term “alkylthio,” as used herein, alone or in combination, refers to an alkyl thioether (R-S-) group wherein the term alkyl is as defined above and wherein the sulfur may be singly or doubly oxidized. Examples of suitable alkyl thioether groups include methylthio, ethylthio, n-propylthio, isopropylthio, n-butylthio, iso-butylthio, sec-butylthio, tert-butylthio, methanesulfonyl, ethanesulfinyl, and the like.

[0168] The term “alkynyl,” as used herein, alone or in combination, refers to a straight-chain or branched-chain hydrocarbon group having one or more triple bonds and containing from 2 to 20 carbon atoms. In certain embodiments, said alkynyl comprises from 2 to 6 carbon atoms. In further embodiments, said alkynyl comprises from 2 to 4 carbon atoms. The term “alkynylene” refers to a carbon-carbon triple bond attached at two positions such as ethynylene ($-C\equiv C-$, $-C\equiv C-$). Examples of alkynyl groups include ethynyl, propynyl, hydroxypropynyl, butyn-1-yl, butyn-2-yl, pentyn-1-yl, 3-methylbutyn-1-yl, hexyn-2-yl, and the like. Unless otherwise specified, the term “alkynyl” may include “alkynylene” groups.

[0169] The terms “amido” and “carbamoyl,” as used herein, alone or in combination, refer to an amino group as described below attached to the parent molecular moiety through a carbonyl group, or vice versa. The term “C amido” as used herein, alone or in combination, refers to a $C(=O)NR_2$ group with R as defined herein. The term “N amido” as used herein, alone or in combination, refers to a $RC(=O)NH$ group, with R as defined herein. The term “acylamino” as used herein, alone or in combination, embraces an acyl group attached to the parent moiety through an amino group. An example of an “acylamino” group is acetylamino ($CH_3C(O)NH-$).

[0170] The term “amino,” as used herein, alone or in combination, refers to $-NRR'$, wherein R and R' are independently selected from the group consisting of hydrogen, alkyl, acyl, heteroalkyl, aryl, cycloalkyl, heteroaryl, and heterocycloalkyl, any of which may themselves be optionally substituted. Additionally, R and R' may combine to form heterocycloalkyl, either of which may be optionally substituted.

[0171] The term “aryl,” as used herein, alone or in combination, means a carbocyclic aromatic system containing one, two or three rings wherein such polycyclic ring systems are

fused together. The term "aryl" embraces aromatic groups such as phenyl, naphthyl, anthracenyl, and phenanthryl.

[0172] The term "arylalkenyl" or "aralkenyl," as used herein, alone or in combination, refers to an aryl group attached to the parent molecular moiety through an alkenyl group.

[0173] The term "arylalkoxy" or "aralkoxy," as used herein, alone or in combination, refers to an aryl group attached to the parent molecular moiety through an alkoxy group.

[0174] The term "arylalkyl" or "aralkyl," as used herein, alone or in combination, refers to an aryl group attached to the parent molecular moiety through an alkyl group.

[0175] The term "arylalkynyl" or "aralkynyl," as used herein, alone or in combination, refers to an aryl group attached to the parent molecular moiety through an alkynyl group.

[0176] The term "arylalkanoyl" or "aralkanoyl" or "aroyl," as used herein, alone or in combination, refers to an acyl group derived from an aryl-substituted alkanecarboxylic acid such as benzoyl, naphthoyl, phenylacetyl, 3-phenylpropionyl (hydrocinnamoyl), 4-phenylbutyryl, (2-naphthyl)acetyl, 4-chlorohydrocinnamoyl, and the like.

[0177] The term aryloxy as used herein, alone or in combination, refers to an aryl group attached to the parent molecular moiety through an oxy.

[0178] The terms "benzo" and "benz," as used herein, alone or in combination, refer to the divalent group $C_6H_4=$ derived from benzene. Examples include benzothiophene and benzimidazole.

[0179] The term "carbamate," as used herein, alone or in combination, refers to an ester of carbamic acid ($-NHCOO-$) which may be attached to the parent molecular moiety from either the nitrogen or acid end, and which may be optionally substituted as defined herein.

[0180] The term "O carbamyl" as used herein, alone or in combination, refers to a $OC(O)NRR'$ group with R and R' as defined herein.

[0181] The term "N carbamyl" as used herein, alone or in combination, refers to a $ROC(O)NR'$ group, with R and R' as defined herein.

[0182] The term "carbonyl," as used herein, when alone includes formyl [$-C(O)H$] and in combination is a $-C(O)-$ group.

[0183] The term "carboxyl" or "carboxy," as used herein, refers to $-C(O)OH$ or the corresponding "carboxylate" anion, such as is in a carboxylic acid salt. An "O carboxy"

group refers to a RC(O)O- group, where R is as defined herein. A “C carboxy” group refers to a -C(O)OR groups where R is as defined herein.

[0184] The term “cyano,” as used herein, alone or in combination, refers to -CN .

[0185] The term “cycloalkyl,” or, alternatively, “carbocycle,” as used herein, alone or in combination, refers to a saturated or partially saturated monocyclic, bicyclic or tricyclic alkyl group wherein each cyclic moiety contains from 3 to 12 carbon atom ring members and which may optionally be a benzo fused ring system which is optionally substituted as defined herein. In certain embodiments, said cycloalkyl will comprise from 5 to 7 carbon atoms. Examples of such cycloalkyl groups include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, tetrahydronaphthyl, indanyl, octahydronaphthyl, 2,3-dihydro-1H-indenyl, adamantyl and the like. “Bicyclic” and “tricyclic” as used herein are intended to include both fused ring systems, such as decahydronaphthalene, octahydronaphthalene as well as the multicyclic (multicentered) saturated or partially unsaturated type. The latter type of isomer is exemplified in general by, bicyclo[1,1,1]pentane, camphor, adamantane, and bicyclo[3,2,1]octane.

[0186] The term “ester,” as used herein, alone or in combination, refers to a carboxy group bridging two moieties linked at carbon atoms.

[0187] The term “ether,” as used herein, alone or in combination, refers to an oxy group bridging two moieties linked at carbon atoms.

[0188] The term “halo,” or “halogen,” as used herein, alone or in combination, refers to fluorine, chlorine, bromine, or iodine.

[0189] The term “haloalkoxy,” as used herein, alone or in combination, refers to a haloalkyl group attached to the parent molecular moiety through an oxygen atom.

[0190] The term “haloalkyl,” as used herein, alone or in combination, refers to an alkyl group having the meaning as defined above wherein one or more hydrogens are replaced with a halogen. Specifically embraced are monohaloalkyl, dihaloalkyl and polyhaloalkyl groups. A monohaloalkyl group, for one example, may have an iodo, bromo, chloro or fluoro atom within the group. Dihalo and polyhaloalkyl groups may have two or more of the same halo atoms or a combination of different halo groups. Examples of haloalkyl groups include fluoromethyl, difluoromethyl, trifluoromethyl, chloromethyl, dichloromethyl, trichloromethyl, pentafluoroethyl, heptafluoropropyl, difluorochloromethyl,

dichlorofluoromethyl, difluoroethyl, difluoropropyl, dichloroethyl and dichloropropyl. "Haloalkylene" refers to a haloalkyl group attached at two or more positions. Examples include fluoromethylene ($-\text{CFH}-$), difluoromethylene ($-\text{CF}_2-$), chloromethylene ($-\text{CHCl}-$) and the like.

[0191] The term "heteroalkyl," as used herein, alone or in combination, refers to a stable straight or branched chain, or cyclic hydrocarbon group, or combinations thereof, fully saturated or containing from 1 to 3 degrees of unsaturation, consisting of the stated number of carbon atoms and from one to three heteroatoms selected from the group consisting of O, N, and S, and wherein the nitrogen and sulfur atoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized. The heteroatom(s) O, N and S may be placed at any interior position of the heteroalkyl group. Up to two heteroatoms may be consecutive, such as, for example, $-\text{CH}_2\text{-NH-OCH}_3$.

[0192] The term "heteroaryl," as used herein, alone or in combination, refers to a 3 to 7 membered unsaturated heteromonocyclic ring, or a fused monocyclic, bicyclic, or tricyclic ring system in which at least one of the fused rings is aromatic, which contains at least one atom selected from the group consisting of O, S, and N. In certain embodiments, said heteroaryl will comprise from 5 to 7 carbon atoms. The term also embraces fused polycyclic groups wherein heterocyclic rings are fused with aryl rings, wherein heteroaryl rings are fused with other heteroaryl rings, wherein heteroaryl rings are fused with heterocycloalkyl rings, or wherein heteroaryl rings are fused with cycloalkyl rings. Examples of heteroaryl groups include pyrrolyl, pyrrolinyl, imidazolyl, pyrazolyl, pyridyl, pyrimidinyl, pyrazinyl, pyridazinyl, triazolyl, pyranyl, furyl, thienyl, oxazolyl, isoxazolyl, oxadiazolyl, thiazolyl, thiadiazolyl, isothiazolyl, indolyl, isoindolyl, indolizinyl, benzimidazolyl, quinolyl, isoquinolyl, quinoxalinyl, quinazolinyl, indazolyl, benzotriazolyl, benzodioxolyl, benzopyranyl, benzoxazolyl, benzoxadiazolyl, benzothiazolyl, benzothiadiazolyl, benzofuryl, benzothienyl, chromonyl, coumarinyl, benzopyranyl, tetrahydroquinolinyl, tetrazolopyridazinyl, tetrahydroisoquinolinyl, thienopyridinyl, furopyridinyl, pyrrolopyridinyl and the like. Exemplary tricyclic heterocyclic groups include carbazolyl, benzidolyl, phenanthrolinyl, dibenzofuranlyl, acridinyl, phenanthridinyl, xanthenyl and the like.

[0193] The terms "heterocycloalkyl" and, interchangeably, "heterocycle," as used herein, alone or in combination, each refer to a saturated, partially unsaturated, or fully unsaturated

monocyclic, bicyclic, or tricyclic heterocyclic group containing at least one heteroatom as a ring member, wherein each said heteroatom may be independently selected from the group consisting of nitrogen, oxygen, and sulfur. In certain embodiments, said heterocycloalkyl will comprise from 1 to 4 heteroatoms as ring members. In further embodiments, said heterocycloalkyl will comprise from 1 to 2 heteroatoms as ring members. In certain embodiments, said heterocycloalkyl will comprise from 3 to 8 ring members in each ring. In further embodiments, said heterocycloalkyl will comprise from 3 to 7 ring members in each ring. In yet further embodiments, said heterocycloalkyl will comprise from 5 to 6 ring members in each ring. "Heterocycloalkyl" and "heterocycle" are intended to include sulfones, sulfoxides, N-oxides of tertiary nitrogen ring members, and carbocyclic fused and benzo fused ring systems; additionally, both terms also include systems where a heterocycle ring is fused to an aryl group, as defined herein, or an additional heterocycle group. Examples of heterocycle groups include aziridinyl, azetidiny, 1,3-benzodioxolyl, dihydroisoindolyl, dihydroisoquinoliny, dihydrocinnoliny, dihydrobenzodioxiny, dihydro[1,3]oxazolo[4,5-b]pyridiny, benzothiazolyl, dihydroindolyl, dihydropyridiny, 1,3-dioxanyl, 1,4-dioxanyl, 1,3-dioxolanyl, isoindoliny, morpholiny, piperaziny, pyrrolidiny, tetrahydropyridiny, piperidiny, thiomorpholiny, and the like. The heterocycle groups may be optionally substituted unless specifically prohibited.

[0194] The term "hydraziny" as used herein, alone or in combination, refers to two amino groups joined by a single bond, i.e., --N--N-- .

[0195] The term "hydroxy," as used herein, alone or in combination, refers to --OH .

[0196] The term "hydroxyalkyl," as used herein, alone or in combination, refers to a hydroxy group attached to the parent molecular moiety through an alkyl group.

[0197] The term "imino," as used herein, alone or in combination, refers to =N-- .

[0198] The term "iminohydroxy," as used herein, alone or in combination, refers to =N(OH) and =N--O-- .

[0199] The phrase "in the main chain" refers to the longest contiguous or adjacent chain of carbon atoms starting at the point of attachment of a group to the compounds of any one of the formulas disclosed herein.

[0200] The term "isocyanato" refers to a --NCO group.

[0201] The term "isothiocyanato" refers to a --NCS group.

[0202] The phrase “linear chain of atoms” refers to the longest straight chain of atoms independently selected from carbon, nitrogen, oxygen and sulfur.

[0203] The term “lower,” as used herein, alone or in a combination, where not otherwise specifically defined, means containing from 1 to and including 6 carbon atoms.

[0204] The term “lower aryl,” as used herein, alone or in combination, means phenyl or naphthyl, which may be optionally substituted as provided.

[0205] The term “lower heteroaryl,” as used herein, alone or in combination, means either: 1) monocyclic heteroaryl comprising five or six ring members, of which between one and four said members may be heteroatoms selected from the group consisting of O, S, and N; or 2) bicyclic heteroaryl, wherein each of the fused rings comprises five or six ring members, comprising between them one to four heteroatoms selected from the group consisting of O, S, and N.

[0206] The term “lower cycloalkyl,” as used herein, alone or in combination, means a monocyclic cycloalkyl having between three and six ring members. Lower cycloalkyls may be unsaturated. Examples of lower cycloalkyl include cyclopropyl, cyclobutyl, cyclopentyl, and cyclohexyl.

[0207] The term “lower heterocycloalkyl,” as used herein, alone or in combination, means a monocyclic heterocycloalkyl having between three and six ring members, of which between one and four may be heteroatoms selected from the group consisting of O, S, and N. Examples of lower heterocycloalkyls include pyrrolidinyl, imidazolidinyl, pyrazolidinyl, piperidinyl, piperazinyl, and morpholinyl. Lower heterocycloalkyls may be unsaturated.

[0208] The term “lower amino,” as used herein, alone or in combination, refers to —NRR' , wherein R and R' are independently selected from the group consisting of hydrogen, lower alkyl, and lower heteroalkyl, any of which may be optionally substituted. Additionally, the R and R' of a lower amino group may combine to form a five- or six-membered heterocycloalkyl, either of which may be optionally substituted.

[0209] The term “mercaptyl” as used herein, alone or in combination, refers to an RS— group, where R is as defined herein.

[0210] The term “nitro,” as used herein, alone or in combination, refers to —NO_2 .

[0211] The terms “oxy” or “oxa,” as used herein, alone or in combination, refer to —O— .

[0212] The term “oxo,” as used herein, alone or in combination, refers to =O .

[0213] The term “perhaloalkoxy” refers to an alkoxy group where all of the hydrogen atoms are replaced by halogen atoms.

[0214] The term “perhaloalkyl” as used herein, alone or in combination, refers to an alkyl group where all of the hydrogen atoms are replaced by halogen atoms.

[0215] The terms “sulfonate,” “sulfonic acid,” and “sulfonic,” as used herein, alone or in combination, refer to the SO_3H group and its anion as the sulfonic acid is used in salt formation.

[0216] The term “sulfanyl,” as used herein, alone or in combination, refers to $\text{S}-$.

[0217] The term “sulfinyl,” as used herein, alone or in combination, refers to $\text{S(O)}-$.

[0218] The term “sulfonyl,” as used herein, alone or in combination, refers to S(O)_2- .

[0219] The term “N sulfonamido” refers to a $\text{RS(=O)}_2\text{NR}'$ group with R and R' as defined herein.

[0220] The term “S sulfonamido” refers to a $\text{S(=O)}_2\text{NRR}'$, group, with R and R' as defined herein.

[0221] The terms “thia” and “thio,” as used herein, alone or in combination, refer to a $\text{S}-$ group or an ether wherein the oxygen is replaced with sulfur. The oxidized derivatives of the thio group, namely sulfinyl and sulfonyl, are included in the definition of thia and thio.

[0222] The term “thiol,” as used herein, alone or in combination, refers to an SH group.

[0223] The term “thiocarbonyl,” as used herein, when alone includes thioformyl C(S)H and in combination is a $\text{C(S)}-$ group.

[0224] The term “N thiocarbamyl” refers to an $\text{ROC(S)NR}'-$ group, with R and R' as defined herein.

[0225] The term “O thiocarbamyl” refers to a $\text{OC(S)NRR}'$, group with R and R' as defined herein.

[0226] The term “thiocyanato” refers to a CNS group.

[0227] The term “trihalomethanesulfonamido” refers to a $\text{X}_3\text{CS(O)}_2\text{NR}-$ group with X is a halogen and R as defined herein.

[0228] The term “trihalomethanesulfonyl” refers to a $\text{X}_3\text{CS(O)}_2-$ group where X is a halogen.

[0229] The term “trihalomethoxy” refers to a $\text{X}_3\text{CO}-$ group where X is a halogen.

[0230] The term “trisubstituted silyl,” as used herein, alone or in combination, refers to a silicone group substituted at its three free valences with groups as listed herein under the

definition of substituted amino. Examples include trimethylsilyl, tert-butyldimethylsilyl, triphenylsilyl and the like.

[0231] Any definition herein may be used in combination with any other definition to describe a composite structural group. By convention, the trailing element of any such definition is that which attaches to the parent moiety. For example, the composite group alkylamido would represent an alkyl group attached to the parent molecule through an amido group, and the term alkoxyalkyl would represent an alkoxy group attached to the parent molecule through an alkyl group.

[0232] When a group is defined to be “null,” what is meant is that said group is absent.

[0233] The term “optionally substituted” means the antecedent group may be substituted or unsubstituted. When substituted, the substituents of an “optionally substituted” group may include, without limitation, one or more substituents independently selected from the following groups or a particular designated set of groups, alone or in combination: lower alkyl, lower alkenyl, lower alkynyl, lower alkanoyl, lower heteroalkyl, lower heterocycloalkyl, lower haloalkyl, lower haloalkenyl, lower haloalkynyl, lower perhaloalkyl, lower perhaloalkoxy, lower cycloalkyl, phenyl, aryl, aryloxy, lower alkoxy, lower haloalkoxy, oxo, lower acyloxy, carbonyl, carboxyl, lower alkylcarbonyl, lower carboxyester, lower carboxamido, cyano, hydrogen, halogen, hydroxy, amino, lower alkylamino, arylamino, amido, nitro, thiol, lower alkylthio, lower haloalkylthio, lower perhaloalkylthio, arylthio, sulfonate, sulfonic acid, trisubstituted silyl, N₃, SH, SCH₃, C(O)CH₃, CO₂CH₃, CO₂H, pyridinyl, thiophene, furanyl, lower carbamate, and lower urea. Two substituents may be joined together to form a fused five-, six-, or seven-membered carbocyclic or heterocyclic ring consisting of zero to three heteroatoms, for example forming methylenedioxy or ethylenedioxy. An optionally substituted group may be unsubstituted (e.g., -CH₂CH₃), fully substituted (e.g., -CF₂CF₃), monosubstituted (e.g., -CH₂CH₂F) or substituted at a level anywhere in-between fully substituted and monosubstituted (e.g., -CH₂CF₃). Where substituents are recited without qualification as to substitution, both substituted and unsubstituted forms are encompassed. Where a substituent is qualified as “substituted,” the substituted form is specifically intended. Additionally, different sets of optional substituents to a particular moiety may be defined as needed; in these cases, the optional substitution will be as defined, often immediately following the phrase, “optionally substituted with.”

[0234] The term R or the term R', appearing by itself and without a number designation, unless otherwise defined, refers to a moiety selected from the group consisting of hydrogen, alkyl, cycloalkyl, heteroalkyl, aryl, heteroaryl and heterocycloalkyl, any of which may be optionally substituted. Such R and R' groups should be understood to be optionally substituted as defined herein. Whether an R group has a number designation or not, every R group, including R, R' and R_n where n=(1, 2, 3, ...n), every substituent, and every term should be understood to be independent of every other in terms of selection from a group. Should any variable, substituent, or term (e.g. aryl, heterocycle, R, etc.) occur more than one time in a formula or generic structure, its definition at each occurrence is independent of the definition at every other occurrence. Those of skill in the art will further recognize that certain groups may be attached to a parent molecule or may occupy a position in a chain of elements from either end as written. Thus, by way of example only, an unsymmetrical group such as -C(O)N(R)- may be attached to the parent moiety at either the carbon or the nitrogen.

[0235] Asymmetric centers exist in the compounds disclosed herein. These centers are designated by the symbols "R" or "S," depending on the configuration of substituents around the chiral carbon atom. It should be understood that the invention encompasses all stereochemical isomeric forms, including diastereomeric, enantiomeric, and epimeric forms, as well as d-isomers and l-isomers, and mixtures thereof. Individual stereoisomers of compounds can be prepared synthetically from commercially available starting materials which contain chiral centers or by preparation of mixtures of enantiomeric products followed by separation such as conversion to a mixture of diastereomers followed by separation or recrystallization, chromatographic techniques, direct separation of enantiomers on chiral chromatographic columns, or any other appropriate method known in the art. Starting compounds of particular stereochemistry are either commercially available or can be made and resolved by techniques known in the art. Additionally, the compounds disclosed herein may exist as geometric isomers. The present invention includes all cis, trans, syn, anti, entgegen (E), and zusammen (Z) isomers as well as the appropriate mixtures thereof. Additionally, compounds may exist as tautomers; all tautomeric isomers are provided by this invention. Additionally, the compounds disclosed herein can exist in unsolvated as well as

solvated forms with pharmaceutically acceptable solvents such as water, ethanol, and the like. In general, the solvated forms are considered equivalent to the unsolvated forms.

[0236] The term “bond” refers to a covalent linkage between two atoms, or two moieties when the atoms joined by the bond are considered to be part of larger substructure. A bond may be single, double, or triple unless otherwise specified. A dashed line between two atoms in a drawing of a molecule indicates that an additional bond may be present or absent at that position.

[0237] The term “optically pure stereoisomer” refers to stereoisomeric, such as enantiomeric or diastereomeric excess or the absolute difference between the mole fraction of each enantiomer or diastereomer.

[0238] In one aspect, antibodies are included as proteins in the compositions and methods of the disclosure, including functional fragments thereof. "Antibodies" as used herein includes polyclonal and monoclonal antibodies, chimeric, single chain, and humanized antibodies, as well as Fab fragments, including the products of an Fab or other immunoglobulin expression library. Antibodies which consists essentially of pooled monoclonal antibodies with different epitopic specificities, as well as distinct monoclonal antibody preparations are provided. Monoclonal antibodies are made by methods well known to those skilled in the art. The term antibody as used in this disclosure is meant to include intact molecules as well as fragments thereof, such as Fab and F(ab')₂, Fv and SCA fragments which are capable of binding an epitopic determinant on a protein of interest. An Fab fragment consists of a mono-valent antigen-binding fragment of an antibody molecule, and can be produced by digestion of a whole antibody molecule with the enzyme papain, to yield a fragment consisting of an intact light chain and a portion of a heavy chain. An Fab' fragment of an antibody molecule can be obtained by treating a whole antibody molecule with pepsin, followed by reduction, to yield a molecule consisting of an intact light chain and a portion of a heavy chain. Two Fab' fragments are obtained per antibody molecule treated in this manner. An (Fab')₂ fragment of an antibody can be obtained by treating a whole antibody molecule with the enzyme pepsin, without subsequent reduction. A (Fab')₂ fragment is a dimer of two Fab' fragments, held together by two disulfide bonds. An Fv fragment is defined as a genetically engineered fragment containing the variable region of a light chain and the variable region of a heavy chain expressed as two chains. (5) A single chain antibody ("SCA") is a genetically

engineered single chain molecule containing the variable region of a light chain and the variable region of a heavy chain, linked by a suitable, flexible polypeptide linker.

[0239] As used herein, a “monoclonal antibody” may be from any origin, such as mouse or human, including a chimeric antibody thereof. Additionally, the antibody may be humanized.

[0240] Examples of monoclonal antibodies (as named by the World Health Organization in International Nonproprietary Names (INN) for Biological and Biotechnological Substances publications; the number corresponding to the INN List including the antibody) include those of mouse origin including: abagovomab (95), afelimomab (80), altumomab (80), anatumomab mafenatox, (86) arcitumomab (74), bectumomab (81), besilesomab (92), biciromab (66), capromab (80), detumomab (80), dorlimomab aritox (66), edobacomab (80), edrecolomab (74), elsilimomab (89), enlimomab (80), enlimomab pegol (77), epitumomab (82), epitumomab cituxetan (89), faralimomab (81), gavilimomab (84), ibritumomab tiuxetan (86), igovomab (86), imciromab (66), inolimomab (80), lemalesomab (86), maslimomab (66), minretumomab (80), mitumomab (82), nacolomab tafenatox (80), nerelimomab (81), odulimomab (81), oregovomab (86), satumomab (81), sulesomab (86), taplitumomab paptox (84), technetium (^{99m}Tc) fanolesomab (86), technetium (^{99m}Tc) nofetumomab merpentan (81), technetium, (^{99m}Tc) pintumomab (86), telimomab aritox (66), tositumomab (80), vepalimomab (80), zolimomab aritox (80); those of human origin including: adalimumab (85), adecatumumab (90), atorolimumab (80), belimumab (89), bertilimumab (88), denosumab (94), efungumab(95), exbivirumab (91), golimumab (91), ipilimumab (94), iratumumab (94), lerdelimomab (86), lexatumumab (95), libivirumab (91), mapatumumab (93), metelimomab (88), morolimumab (79), nebacomab (66), ofatumumab (93), panitumumab (91), pritumumab (89), raxibacomab (92), regavirumab (80), sevirumab (66), stamulumab (95), ticilimumab (95), tuvirumab (66), votumumab (80), zalutumumab (93), zanolimumab (92), ziralimumab (84); those of chimeric origin including: abciximab (80), basiliximab (81), bavituximab (95), cetuximab (82), clenoliximab (77), ecromeximab (87), galiximab (89), infliximab (77), keliximab (81), lumiliximab (90), pagibaximab (93), priliximab (80), rituximab (77), teneliximab (87), vapaliximab (87), volociximab (93); and those of humanized origin including: alemtuzumab (83), apolizumab (87), aselizumab (88), bapineuzumab (93), bevacizumab (86), bivatuzumab (86), cantuzumab mertansine (89), cedelizumab (81), certolizumab pegol (90), daclizumab (78), eculizumab (87), efalizumab

(85), epratuzumab (82), erlizumab (84), felvizumab (77), fontolizumab (87), gemtuzumab (83), inotuzumab ozogamicin (92), labetuzumab (85), lintuzumab (86), matuzumab (88), mepolizumab (81), motavizumab (95), natalizumab (79), nimotuzumab (94), ocrelizumab (95), omalizumab (84), palivizumab (79), pascolizumab (87), pertuzumab (89), pexelizumab (86), ranibizumab (90), reslizumab (85), rovelizumab (81), ruplizumab (83), sibrotuzumab (86), sipilizumab (87), sontuzumab (94), tadocizumab (94), talizumab (89), tefibazumab (92), tocilizumab (90), toralizumab (87), trastuzumab (78), tucotuzumab celmoleukin (95), urtoxazumab (90), visilizumab (84), yttrium ⁹⁰Y tacatuzumab tetraxetan (93).

[0241] The terms peptide, polypeptide and protein may be used interchangeably herein, or a peptide, polypeptide or variant thereof. As used herein, the term "polypeptide" is interpreted to mean a polymer composed of amino acid residues, e.g. Ala, Arg, Asn, Asp, Cys, Glu, Gln, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof linked via peptide bonds, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof. Synthetic polypeptides can be synthesized, for example, using an automated polypeptide synthesizer. The term "protein" typically refers to large polypeptides. The term "peptide" typically refers to short polypeptides. "Polypeptide(s)" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds. "Polypeptide(s)" refers to both short chains, commonly referred to as peptides, oligopeptides and oligomers and to longer chains generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene encoded amino acids. "Polypeptide(s)" include those modified either by natural processes, such as processing and other post-translational modifications, but also by chemical modification techniques. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature, and they are well-known to those of skill in the art. It will be appreciated that the same type of modification may be present in the same or varying degree at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains, and the amino or carboxyl termini. Modifications include, for example, acetylation, acylation, AD Pribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of

a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-link formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins, such as arginylation, and ubiquitination. See, for instance, PROTEINS-STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York (1993) and Wold, F., Posttranslational Protein Modifications: Perspectives and Prospects, pgs. 1 12 in POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, New York (1983); Seifter et al., Meth. Enzymol. 182:626 646 (1990) and Rattan et al., Protein Synthesis: Posttranslational Modifications and Aging, Ann. N.Y. Acad. Sci. 663: 48 62 (1992). Polypeptides may be branched or cyclic, with or without branching. Cyclic, branched and branched circular polypeptides may result from post-translational natural processes and may be made by entirely synthetic methods, as well.

[0242] As used herein, the term "agent" is interpreted to mean a chemical compound, a mixture of chemical compounds, a sample of undetermined composition, a combinatorial small molecule array, a biological macromolecule, a bacteriophage peptide display library, a bacteriophage antibody (e.g., scFv) display library, a polysome peptide display library, or an extract made from biological materials such as bacteria, plants, fungi, or animal cells or tissues. Suitable techniques involve selection of libraries of recombinant antibodies in phage or similar vectors. See, Huse et al. (1989) Science 246: 1275 1281; and Ward et al. (1989) Nature 341: 544 546. The protocol described by Huse is rendered more efficient in combination with phage display technology. See, e.g., Dower et al., WO 91/17271 and McCafferty et al., WO 92/01047.

[0243] As used herein, the term "isolated" is interpreted to mean altered "by the hand of man" from its natural state, i.e., if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or a polypeptide naturally

present in a living organism is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein.

[0244] As used herein, the term "variant" is interpreted to mean a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide respectively, but retains essential properties. A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of a polynucleotide or polypeptide may be a naturally occurring such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques, by direct synthesis, and by other recombinant methods known to skilled artisans.

[0245] The term "disease" as used herein is intended to be generally synonymous, and is used interchangeably with, the terms "disorder" and "condition" (as in medical condition), in that all reflect an abnormal condition of the body or of one of its parts that impairs normal functioning and is typically manifested by distinguishing signs and symptoms.

[0246] The term "combination therapy" means the administration of two or more therapeutic agents to treat a therapeutic condition or disorder described in the present disclosure. Such administration encompasses co-administration of these therapeutic agents in a substantially simultaneous manner, such as in a single capsule having a fixed ratio of active ingredients or in multiple, separate capsules for each active ingredient. In addition, such administration also encompasses use of each type of therapeutic agent in a sequential manner. In either case, the

treatment regimen will provide beneficial effects of the drug combination in treating the conditions or disorders described herein.

[0247] The phrase "therapeutically effective" is intended to qualify the amount of active ingredients used in the treatment of a disease or disorder. This amount will achieve the goal of reducing or eliminating the said disease or disorder.

[0248] As used herein, reference to "treatment" of a patient is intended to include prophylaxis. The term "patient" means all mammals including humans. Examples of patients include humans, cows, dogs, cats, goats, sheep, pigs, and rabbits. Preferably, the patient is a human.

[0249] The term "prodrug" refers to a compound that is made more active in vivo. Certain of the present compounds can also exist as prodrugs, as described in Hydrolysis in Drug and Prodrug Metabolism: Chemistry, Biochemistry, and Enzymology (Testa, Bernard and Mayer, Joachim M. Wiley-VHCA, Zurich, Switzerland 2003). Prodrugs of the compounds described herein are structurally modified forms of the compound that readily undergo chemical changes under physiological conditions to provide the compound. Additionally, prodrugs can be converted to the compound by chemical or biochemical methods in an ex vivo environment. For example, prodrugs can be slowly converted to a compound when placed in a transdermal patch reservoir with a suitable enzyme or chemical reagent. Prodrugs are often useful because, in some situations, they may be easier to administer than the compound, or parent drug. They may, for instance, be bioavailable by oral administration whereas the parent drug is not. The prodrug may also have improved solubility in pharmaceutical compositions over the parent drug. A wide variety of prodrug derivatives are known in the art, such as those that rely on hydrolytic cleavage or oxidative activation of the prodrug. An example, without limitation, of a prodrug would be a compound which is administered as an ester (the "prodrug"), but then is metabolically hydrolyzed to the carboxylic acid, the active entity. Additional examples include peptidyl derivatives of a compound. The term "therapeutically acceptable prodrug," refers to those prodrugs or zwitterions which are suitable for use in contact with the tissues of patients without undue toxicity, irritation, and allergic response, are commensurate with a reasonable benefit/risk ratio, and are effective for their intended use.

[0250] The compounds of the present invention can exist as therapeutically acceptable salts. The present invention includes compounds listed above in the form of salts, including acid addition salts. Suitable salts include those formed with both organic and inorganic acids. Such acid addition salts will normally be pharmaceutically acceptable. However, salts of non-pharmaceutically acceptable salts may be of utility in the preparation and purification of the compound in question. Basic addition salts may also be formed and be pharmaceutically acceptable. For a more complete discussion of the preparation and selection of salts, refer to *Pharmaceutical Salts: Properties, Selection, and Use* (Stahl, P. Heinrich. Wiley-VCHA, Zurich, Switzerland, 2002).

[0251] The term "therapeutically acceptable salt," as used herein, represents salts or zwitterionic forms of the compounds of the present invention which are water or oil-soluble or dispersible and therapeutically acceptable as defined herein. The salts can be prepared during the final isolation and purification of the compounds or separately by reacting the appropriate compound in the form of the free base with a suitable acid. Representative acid addition salts include acetate, adipate, alginate, L-ascorbate, aspartate, benzoate, benzenesulfonate (besylate), bisulfate, butyrate, camphorate, camphorsulfonate, citrate, digluconate, formate, fumarate, gentisate, glutarate, glycerophosphate, glycolate, hemisulfate, heptanoate, hexanoate, hippurate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethansulfonate (isethionate), lactate, maleate, malonate, DL-mandelate, mesitylenesulfonate, methanesulfonate, naphthylenesulfonate, nicotinate, 2-naphthalenesulfonate, oxalate, pamoate, pectinate, persulfate, 3-phenylpropionate, phosphonate, picrate, pivalate, propionate, pyroglutamate, succinate, sulfonate, tartrate, L-tartrate, trichloroacetate, trifluoroacetate, phosphate, glutamate, bicarbonate, para-toluenesulfonate (p-tosylate), and undecanoate. Also, basic groups in the compounds of the present invention can be quaternized with methyl, ethyl, propyl, and butyl chlorides, bromides, and iodides; dimethyl, diethyl, dibutyl, and diamyl sulfates; decyl, lauryl, myristyl, and steryl chlorides, bromides, and iodides; and benzyl and phenethyl bromides. Examples of acids which can be employed to form therapeutically acceptable addition salts include inorganic acids such as hydrochloric, hydrobromic, sulfuric, and phosphoric, and organic acids such as oxalic, maleic, succinic, and citric. Salts can also be formed by coordination of the compounds with an alkali metal or alkaline earth ion. Hence, the present invention

contemplates sodium, potassium, magnesium, and calcium salts of the compounds disclosed herein, and the like.

[0252] Basic addition salts can be prepared during the final isolation and purification of the compounds by reacting a carboxyl group with a suitable base such as the hydroxide, carbonate, or bicarbonate of a metal cation or with ammonia or an organic primary, secondary, or tertiary amine. The cations of therapeutically acceptable salts include lithium, sodium, potassium, calcium, magnesium, and aluminum, as well as nontoxic quaternary amine cations such as ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, diethylamine, ethylamine, tributylamine, pyridine, N,N-dimethylaniline, N-methylpiperidine, N-methylmorpholine, dicyclohexylamine, procaine, dibenzylamine, N,N-dibenzylphenethylamine, 1-phenamine, and N,N'-dibenzylethylenediamine. Other representative organic amines useful for the formation of base addition salts include ethylenediamine, ethanolamine, diethanolamine, piperidine, and piperazine.

[0253] While it may be possible for the compounds of the subject invention to be administered as the raw chemical, it is also possible to present them as a pharmaceutical formulation. Accordingly, provided herein are pharmaceutical formulations which comprise one or more of certain compounds of the present invention, or one or more pharmaceutically acceptable salts, esters, prodrugs, amides, or solvates thereof, together with one or more pharmaceutically acceptable carriers thereof and optionally one or more other therapeutic ingredients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof. Proper formulation is dependent upon the route of administration chosen. Any of the well-known techniques, carriers, and excipients may be used as suitable and as understood in the art; e.g., in Remington's Pharmaceutical Sciences. The pharmaceutical compositions disclosed herein may be manufactured in any manner known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or compression processes.

[0254] The formulations include those suitable for oral, parenteral (including subcutaneous, intradermal, intramuscular, intravenous, intraarticular, and intramedullary), intraperitoneal, transmucosal, transdermal, rectal and topical (including dermal, buccal, sublingual and

intraocular) administration although the most suitable route may depend upon for example the condition and disorder of the recipient. The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Typically, these methods include the step of bringing into association a compound of the subject invention or a pharmaceutically acceptable salt, ester, amide, prodrug or solvate thereof ("active ingredient") with the carrier which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both and then, if necessary, shaping the product into the desired formulation.

[0255] Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

[0256] Pharmaceutical preparations which can be used orally include tablets, push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. Tablets may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with binders, inert diluents, or lubricating, surface active or dispersing agents. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein. All formulations for oral administration should be in dosages suitable for such administration. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may

optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

[0257] The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in powder form or in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, saline or sterile pyrogen-free water, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

[0258] Formulations for parenteral administration include aqueous and non-aqueous (oily) sterile injection solutions of the active compounds which may contain antioxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

[0259] In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

[0260] For buccal or sublingual administration, the compositions may take the form of tablets, lozenges, pastilles, or gels formulated in conventional manner. Such compositions may comprise the active ingredient in a flavored basis such as sucrose and acacia or tragacanth.

[0261] The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter, polyethylene glycol, or other glycerides.

[0262] Certain compounds of the present invention may be administered topically, that is by non-systemic administration. This includes the application of a compound of the present invention externally to the epidermis or the buccal cavity and the instillation of such a compound into the ear, eye and nose, such that the compound does not significantly enter the blood stream. In contrast, systemic administration refers to oral, intravenous, intraperitoneal and intramuscular administration.

[0263] Formulations suitable for topical administration include liquid or semi-liquid preparations suitable for penetration through the skin to the site of inflammation such as gels, liniments, lotions, creams, ointments or pastes, and drops suitable for administration to the eye, ear or nose. The active ingredient for topical administration may comprise, for example, from 0.001% to 10% w/w (by weight) of the formulation. In certain embodiments, the active ingredient may comprise as much as 10% w/w. In other embodiments, it may comprise less than 5% w/w. In certain embodiments, the active ingredient may comprise from 2% w/w to 5% w/w. In other embodiments, it may comprise from 0.1% to 1% w/w of the formulation.

[0264] Gels for topical or transdermal administration may comprise, generally, a mixture of volatile solvents, nonvolatile solvents, and water. In certain embodiments, the volatile solvent component of the buffered solvent system may include lower (C1-C6) alkyl alcohols, lower alkyl glycols and lower glycol polymers. In further embodiments, the volatile solvent is ethanol. The volatile solvent component is thought to act as a penetration enhancer, while also producing a cooling effect on the skin as it evaporates. The nonvolatile solvent portion of the buffered solvent system is selected from lower alkylene glycols and lower glycol polymers. In certain embodiments, propylene glycol is used. The nonvolatile solvent slows the evaporation of the volatile solvent and reduces the vapor pressure of the buffered solvent system. The amount of this nonvolatile solvent component, as with the volatile solvent, is

determined by the pharmaceutical compound or drug being used. When too little of the nonvolatile solvent is in the system, the pharmaceutical compound may crystallize due to evaporation of volatile solvent, while an excess may result in a lack of bioavailability due to poor release of drug from solvent mixture. The buffer component of the buffered solvent system may be selected from any buffer commonly used in the art; in certain embodiments, water is used. A common ratio of ingredients is about 20% of the nonvolatile solvent, about 40% of the volatile solvent, and about 40% water. There are several optional ingredients which can be added to the topical composition. These include, but are not limited to, chelators and gelling agents. Appropriate gelling agents can include, but are not limited to, semisynthetic cellulose derivatives (such as hydroxypropylmethylcellulose) and synthetic polymers, and cosmetic agents.

[0265] Lotions include those suitable for application to the skin or eye. An eye lotion may comprise a sterile aqueous solution optionally containing a bactericide and may be prepared by methods similar to those for the preparation of drops. Lotions or liniments for application to the skin may also include an agent to hasten drying and to cool the skin, such as an alcohol or acetone, and/or a moisturizer such as glycerol or an oil such as castor oil or arachis oil.

[0266] Creams, ointments or pastes are semi-solid formulations of the active ingredient for external application. They may be made by mixing the active ingredient in finely-divided or powdered form, alone or in solution or suspension in an aqueous or non-aqueous fluid, with the aid of suitable machinery, with a greasy or non-greasy base. The base may comprise hydrocarbons such as hard, soft or liquid paraffin, glycerol, beeswax, a metallic soap; a mucilage; an oil of natural origin such as almond, corn, arachis, castor or olive oil; wool fat or its derivatives or a fatty acid such as stearic or oleic acid together with an alcohol such as propylene glycol or a macrogel. The formulation may incorporate any suitable surface active agent such as an anionic, cationic or non-ionic surfactant such as a sorbitan ester or a polyoxyethylene derivative thereof. Suspending agents such as natural gums, cellulose derivatives or inorganic materials such as siliceous silicas, and other ingredients such as lanolin, may also be included.

[0267] Drops may comprise sterile aqueous or oily solutions or suspensions and may be prepared by dissolving the active ingredient in a suitable aqueous solution of a bactericidal and/or fungicidal agent and/or any other suitable preservative, and, in certain embodiments,

including a surface active agent. The resulting solution may then be clarified by filtration, transferred to a suitable container which is then sealed and sterilized by autoclaving or maintaining at 98-100 °C for half an hour. Alternatively, the solution may be sterilized by filtration and transferred to the container by an aseptic technique. Examples of bactericidal and fungicidal agents suitable for inclusion in the drops are phenylmercuric nitrate or acetate (0.002%), benzalkonium chloride (0.01%) and chlorhexidine acetate (0.01%). Suitable solvents for the preparation of an oily solution include glycerol, diluted alcohol and propylene glycol.

[0268] Formulations for topical administration in the mouth, for example buccally or sublingually, include lozenges comprising the active ingredient in a flavored basis such as sucrose and acacia or tragacanth, and pastilles comprising the active ingredient in a basis such as gelatin and glycerin or sucrose and acacia.

[0269] For administration by inhalation, compounds may be conveniently delivered from an insufflator, nebulizer pressurized packs or other convenient means of delivering an aerosol spray. Pressurized packs may comprise a suitable propellant such as dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Alternatively, for administration by inhalation or insufflation, the compounds according to the invention may take the form of a dry powder composition, for example a powder mix of the compound and a suitable powder base such as lactose or starch. The powder composition may be presented in unit dosage form, in for example, capsules, cartridges, gelatin or blister packs from which the powder may be administered with the aid of an inhalator or insufflator.

[0270] Preferred unit dosage formulations are those containing an effective dose, as herein below recited, or an appropriate fraction thereof, of the active ingredient.

[0271] It should be understood that in addition to the ingredients particularly mentioned above, the formulations described above may include other agents conventional in the art having regard to the type of formulation in question, for example those suitable for oral administration may include flavoring agents.

[0272] Compounds may be administered orally or via injection at a dose of from 0.1 to 500 mg/kg per day. The dose range for adult humans is generally from 5 mg to 2 g/day. Tablets or

other forms of presentation provided in discrete units may conveniently contain an amount of one or more compounds which is effective at such dosage or as a multiple of the same, for instance, units containing 5 mg to 500 mg, usually around 10 mg to 200 mg.

[0273] The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration.

[0274] The compounds can be administered in various modes, e.g., orally, topically, or by injection. The precise amount of compound administered to a patient will be the responsibility of the attendant physician. The specific dose level for any particular patient will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diets, time of administration, route of administration, rate of excretion, drug combination, the precise disorder being treated, and the severity of the indication or condition being treated. Also, the route of administration may vary depending on the condition and its severity.

[0275] In certain instances, it may be appropriate to administer at least one of the compounds described herein (or a pharmaceutically acceptable salt, ester, or prodrug thereof) in combination with another therapeutic agent. By way of example only, if one of the side effects experienced by a patient upon receiving one of the compounds herein is hypertension, then it may be appropriate to administer an anti-hypertensive agent in combination with the initial therapeutic agent. Or, by way of example only, the therapeutic effectiveness of one of the compounds described herein may be enhanced by administration of an adjuvant (i.e., by itself the adjuvant may only have minimal therapeutic benefit, but in combination with another therapeutic agent, the overall therapeutic benefit to the patient is enhanced). Or, by way of example only, the benefit of experienced by a patient may be increased by administering one of the compounds described herein with another therapeutic agent (which also includes a therapeutic regimen) that also has therapeutic benefit. By way of example only, in a treatment for diabetes involving administration of one of the compounds described herein, increased therapeutic benefit may result by also providing the patient with another therapeutic agent for diabetes. In any case, regardless of the disease, disorder or condition being treated, the overall benefit experienced by the patient may simply be additive of the two therapeutic agents or the patient may experience a synergistic benefit.

[0276] In any case, the multiple therapeutic agents (at least one of which is a compound of the present invention) may be administered in any order or even simultaneously. If simultaneously, the multiple therapeutic agents may be provided in a single, unified form, or in multiple forms (by way of example only, either as a single pill or as two separate pills). One of the therapeutic agents may be given in multiple doses, or both may be given as multiple doses. If not simultaneous, the timing between the multiple doses may be any duration of time ranging from a few minutes to four weeks.

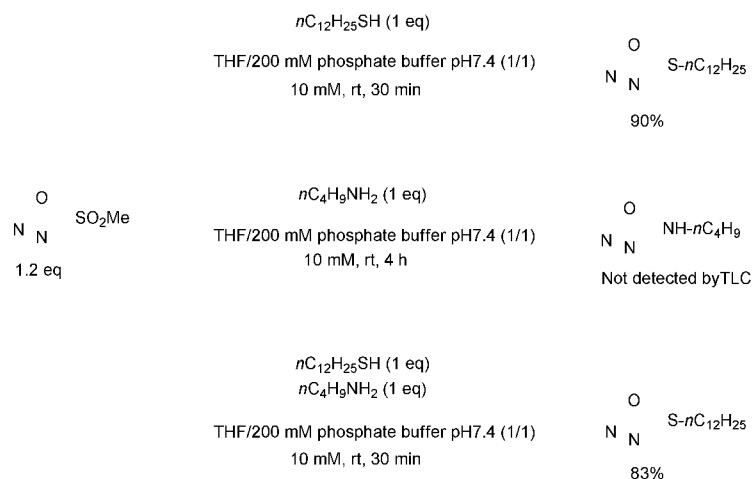
[0277] The following examples are intended to illustrate but not limit the invention.

EXAMPLE 1

SELECTIVE REACTIVITY TO THIOL AND CYSTEINE

[0278] This example illustrates the chemoselectivity of the modifying reagents toward the thiol group in cysteine and thiol group in the presence of other nucleophiles.

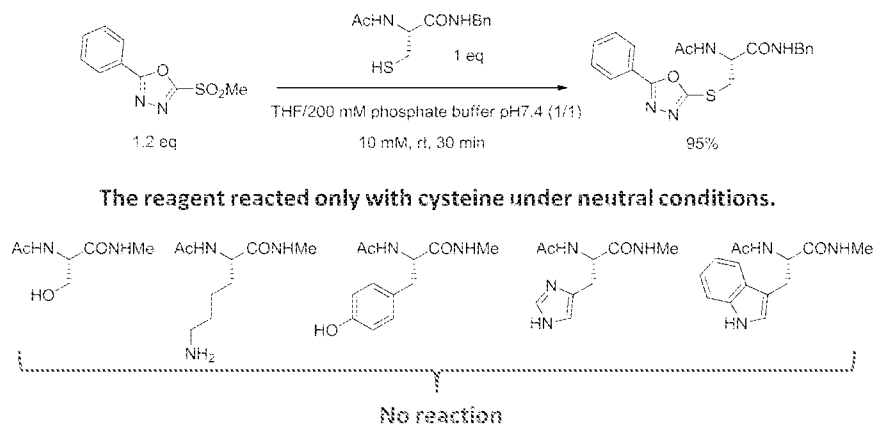
[0279] First, the reactivity of 2-(methylsulfonyl)-5-phenyl-1,3,4-oxadiazole with 1-dodecanethiol compared to 1-butylamine was investigated. The reagent reacted with dodecanethiol, but did not react with butylamine under neutral aqueous conditions after 30 minutes (Scheme 3) demonstrating that the modifying reagent was selective for thiol in the presence of an amino group.



Scheme 3

[0280] Next addressed was the selectivity to various amino acids with the oxadiazole modifying agent. To a solution of amino acid (0.02 mmol, 1 eq) in THF (1 mL) and phosphate buffer (200 mM, pH=7.4, 1 mL) was added 2-(methylsulfonyl)-5-phenyl-1,3,4-

oxadiazole (5.4 mg, 0.024 mmol, 1.2 eq) at room temperature. The reaction mixture was stirred for 30 minutes and checked the conversion of reaction by HPLC. Only cysteine-oxadiazole adduct was observed in these experiments. Thus, the oxadiazole reagent showed good selectivity toward cysteine over other amino acids as depicted in Scheme 4.



Scheme 4

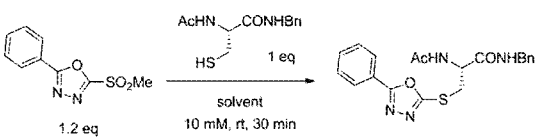
EXAMPLE 2**REACTION PARAMETERS**

[0281] This example demonstrates the effect of pH on the reaction.

[0282] The effects of buffer concentration and pH dependence in the reaction were analyzed. Ionic strength was observed to be an important factor with pH affecting the product yield.

[0283] To a solution of (*R*)-2-acetamido-*N*-benzyl-3-mercaptopropanamide (2.5 mg, 0.010 mmol) in solvent systems according to Tables 1 and 2 (total volume 1 mL) was added 2-(methylsulfonyl)-5-phenyl-1,3,4-oxadiazole (2.7 mg, 0.012 mmol) at room temperature. The reaction mixture was stirred for 30 minutes and the reaction progress monitored by HPLC. As shown in Tables 1 and 2, ionic strength and pH were important factors and influenced substrate to product conversion.

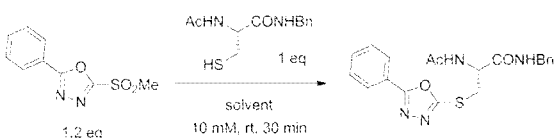
Table 1



	Solvent	pH	Conversion
1	THF	-	0%
2	THF/H ₂ O (1/1)	-	20%
3	THF/1 mM phosphate buffer (1/1)	7.4	24%
4	THF/10 mM phosphate buffer (1/1)	7.4	75% (94% 16 h)
5	THF/50 mM phosphate buffer (1/1)	7.4	>99%
6	THF/100 mM phosphate buffer (1/1)	7.4	>99%
7	THF/200 mM phosphate buffer (1/1)	7.4	>99%

Conversion was measured by HPLC (220nm).

Table 2



	Solvent	pH	Conversion
1	THF/100 mM phosphate buffer (1/1)	5.8	70%
2	THF/100 mM phosphate buffer (1/1)	7.0	>99%
3	THF/100 mM phosphate buffer (1/1)	7.4	>99%
4	THF/100 mM phosphate buffer (1/1)	8.0	>99%
5	THF/100 mM HEPES buffer (1/1)	7.4	>99%
6	THF/100 mM Tris buffer (1/1)	7.4	99%

Conversion was measured by HPLC (220nm).

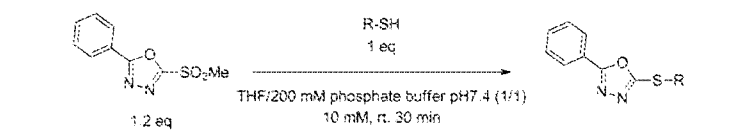
EXAMPLE 3

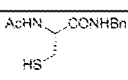
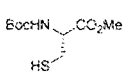
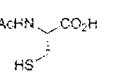
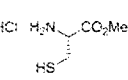
SUBSTRATE SCOPE

[0284] This example illustrates the applicability of the thiol-modifying reagents to a variety of substrates.

[0285] A variety of substrates were reacted with the thiol-modifying reagent, 2-(methylsulfonyl)-5-phenyl-1,3,4-oxadiazole, as shown in Table 3.

Table 3



	RSH	yield		RSH	yield
1	 <chem>CC(C(=O)N[C@@H](CS)C(=O)OCC1=CC=CC=C1)C(=O)OCC1=CC=CC=C1</chem>	95%	4	<chem>CCCCCCCCCCCCCS</chem>	90%
2	 <chem>CC(C(=O)N[C@@H](CS)C(=O)OCC1=CC=CC=C1)C(=O)OCC1=CC=CC=C1</chem>	99%	5	 <chem>C(C(=O)O)[C@@H](CS)C(=O)N</chem>	83%
3	 <chem>CC(C(=O)N[C@@H](CS)C(=O)OCC1=CC=CC=C1)C(=O)OCC1=CC=CC=C1.[Cl-]</chem>	78%*			

*Reaction mixture was stirred for 12 h.

[0286] If cysteine has free amine, the ring opening product could potentially be formed as depicted in Figure 2. To investigate this possibility, the HPLC retention times of compound 1 and 2 prepared by different methods were compared. The results indicate that ring opening of oxadiazole did not occur.

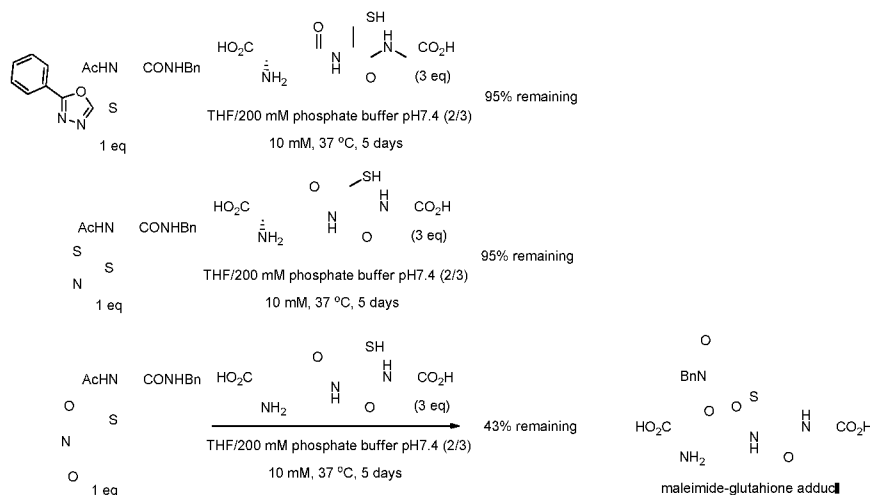
EXAMPLE 4

ADDUCT STABILITY

[0287] This example highlights that the thiol-modifying agents described herein afford products that are stable across a wide breadth of conditions.

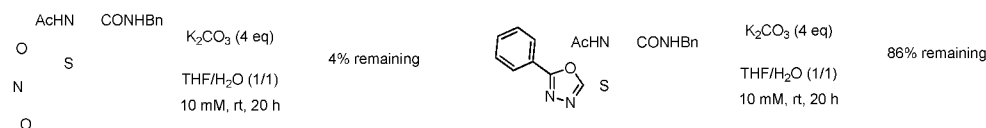
[0288] Product stability was analyzed under neutral conditions with glutathione as follows. The solution of the substrate (0.005 mmol, 1 eq) and glutathione (4.6 mg, 0.015 mmol, 3 eq) in THF (0.2 mL) and phosphate buffer (200 mM, pH=7.4, 0.3 mL) was incubated for 5 days at 37 °C. Remaining substrate (expressed as a percentage) was determined by HPLC at 0 hours, 24 hours, and 120 hours.

[0289] Oxadiazole and benzothiazole adducts demonstrated increased stability compared to the maleimide adduct under neutral conditions. It is noteworthy that thiol exchange products were not detected for oxadiazole and benzothiazole adducts. In contrast, maleimide-glutathione adduct was detected (Scheme 5).



Scheme 5

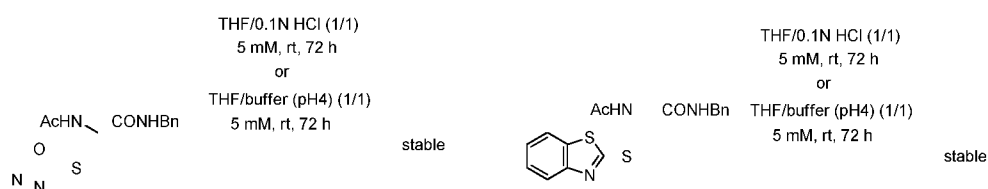
[0290] The product stability was analyzed under basic conditions. The solution of the substrate (0.01 mmol, 1 eq) and K₂CO₃ (5.5 mg, 0.04 mmol, 4 eq) in THF (0.5 mL) and water (0.5 mL) was stirred for 20 hours at room temperature. % Remaining of substrate was checked by HPLC at 0 hours and 20 hours. The succinimide ring opening was main degradation product of the maleimide-thiol adduct (Scheme 6).



Scheme 6

[0291] The product stability was analyzed under acidic conditions. The solution of the substrate (0.0025 mmol) in THF (0.25 mL) and 0.1N HCl (0.25 mL) or phosphate buffer (100 mM, pH = 4, 0.25 mL) was stirred for 72 hours at room temperature. % Remaining of substrate was checked by HPLC at 0 hours, 24 hours, 48 hours, and 72 hours (Scheme 7).

[0292] The new linkages formed by reaction of cysteine with the oxadiazole and benzothiazole modifying reagents demonstrated stability up to 72 hours under both acidic and basic conditions.



Scheme 7

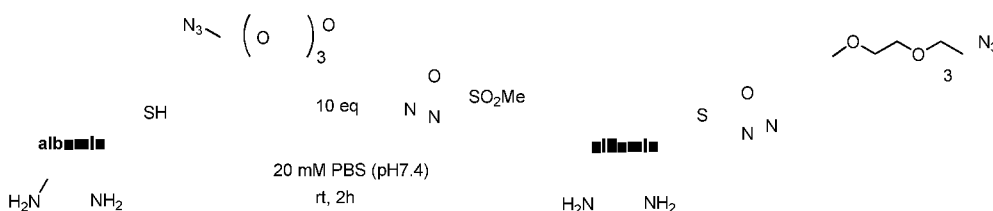
[0293] Human plasma stability tests were also conducted to investigate stability of the adducts under physiological conditions. The substrate (50 μ L, 2 mg/mL in DMSO) was added to human plasma (950 μ L) at 0 °C. Final concentration was 100 μ g/mL. The mixture was incubated for 72 h at 37 °C. Samples for HPLC analysis (100 μ L) were collected from the mixture at each time point shown in figure. To the collected sample was added CH₃CN (600 μ L) and centrifuged for 1 min at 10000 rpm. The supernatant (600 μ L) was transferred to 1.5 mL tube and the solvent was removed by centrifuge evaporator. The residue was diluted with CH₃CN containing methyl 3-hydroxybenzoate (internal standard) and analyzed by HPLC. As shown in Figures 3 & 4, both benzothiazole and oxadiazole adducts were found to be more stable than the corresponding maleimide adduct.

EXAMPLE 5

CYSTEINE CONJUGATION IN PROTEINS

[0294] This example illustrates the selectivity of the thiol-modifying agents to cysteine residues in proteins.

[0295] The selective cysteine modification in proteins was investigated. Human albumin, which has reactive free cysteine 34, was first used (Scheme 8).



Scheme 8

[0296] To the solution of HSA (60 μ L, 2 mg/mL in 20 mM PBS pH=7.4, Recombinant Human Serum Albumin purchased from eEnzyme) was added the solution of ODA-PEG-azide (2 μ L, 4 mg/mL in DMSO, 10 eq) at room temperature.

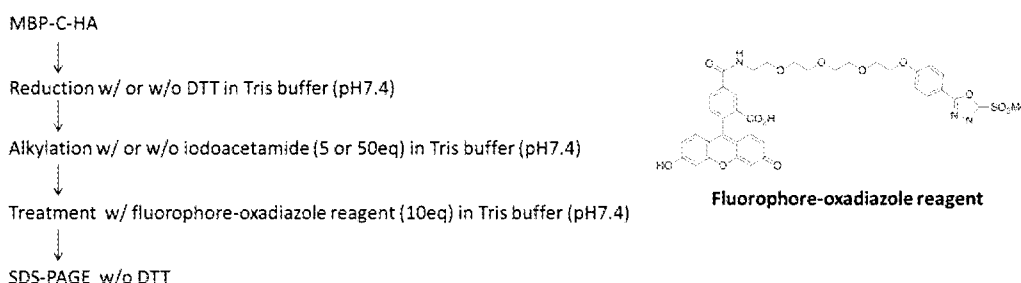
[0297] The reaction mixture was maintained for 2 hours at room temperature. Unreacted small molecules were removed by gel filtration using BioRad Micro Spin 6 column (6000 molecular weight cut-off) to exchange PBS buffer to water. One modification to albumin was confirmed by ESI-MS of unmodified and modified albumin as shown in Figure 5.

[0298] While confirmation of site modification by proteomics with or without DTT were attempted, cysteine modification was not observed.

[0299] MBP-C-HA protein instead of albumin was next employed for the modification of the oxadiazole reagent. MBP-C-HA has only one cysteine, 36 lysine, and the mixture of disulfide dimer and monomer.

[0300] The protein conjugation was analyzed by SDS-PAGE and ESI-MS analysis after the reaction with the fluorophore-oxadiazole reagent.

[0301] The procedure for the conjugation is shown in Scheme 9.



Scheme 9

[0302] For non-reduced maltose binding protein (MBP):

[0303] 1. 1.1mg/mL non-reduced MBP (18 μ g, 18 μ L, 0.45nmol) was incubated with H₂O (1.1 μ L) or 5eq iodoacetamide (1.1 μ L, 2 mM in H₂O) or 50 eq iodoacetamide (1.1 μ L, 20 mM in H₂O) for 1 h at room temperature.

[0304] 2. 10 eq of ODA-fluorescein (1.8 μ L, 2 mg/mL, 2.59 mM in DMSO) was added to MBP.

[0305] 3. Incubation for 1 h at room temperature.

[0306] 4. Small molecules were removed by BioRad Micro Spin 6 column using Tris buffer (50 mM, pH=7.4).

[0307] 5. SDS-PAGE without DTT (10 μ g protein loading)

[0308] For reduced MBP:

[0309] 1. 1.1 mg/mL MBP (90 μ L) in Tris buffer was reduced by 10 mM DTT (10 μ L, 100 mM) for 30 min at room temperature, and then desalted by Tris buffer (50 mM, pH=7.4) twice by BioRad Micro Spin 6 column.

[0310] 2. The reduced MBP (20 μ g, ca 20 μ L, 0.45 nmol) was incubated with H₂O (1.1 μ L) or 5 eq iodoacetamide (1.1 μ L, 2 mM in H₂O) or 50 eq iodoacetamide (1.1 μ L, 20 mM in H₂O) for 1 h at room temperature.

- [0311] 3. 10eq of ODA-fluorescein (1.8 μ L, 2 mg/mL, 2.59 mM in DMSO) was added to MBP.
- [0312] 4. Incubation for 1 h at room temperature.
- [0313] 5. Small molecules were removed by BioRad Micro Spin 6 column using Tris buffer (50 mM, pH=7.4).
- [0314] 6. SDS-PAGE without DTT (10 μ g protein loading)
- [0315] Fluorescence was not observed in MBP-C-HA disulfide dimer but in monomer and pretreatment with iodoacetamide blocked the conjugation of fluorophore reagent. These results clearly indicate that the oxadiazole reagent reacted selectively with cysteine in MBP-C-HA protein. Furthermore, ESI-MS data showed one modification of protein. Fluorescence was not observed in MBP-C-HA disulfide dimer but in monomer and pretreatment with iodoacetamide blocked the conjugation of fluorophore reagent. These results clearly indicate that the oxadiazole reagent reacted selectively with cysteine in MBP-C-HA protein. Furthermore, ESI-MS data showed one modification of protein (Figure 6).

EXAMPLE 6

STABILITY OF MODIFIED MBP-C-HA PROTEINS

- [0316] The stability study of modified MBP-C-HA proteins was conducted according to the following protocol.
- [0317] 1. 1.1 mg/mL MBP (63 μ L) in Tris buffer was reduced by 10 mM DTT (7.0 μ L, 100 mM) for 30 min at room temperature, and then desalted by Tris buffer (50 mM, pH=7.4) twice by BioRad Micro Spin 6 column.
- [0318] 2. The reduced MBP (70 μ g, ca 70 μ L, 1.58 nmol) was diluted with H₂O (7.0 μ L).
- [0319] 3. 5eq of ODA-fluorescein (3.0 μ L, 2 mg/mL, 2.59 mM in DMSO) was added to MBP.
- [0320] 4. Incubation for 1 h at room temperature.
- [0321] 5. Small molecules were removed by BioRad Micro Spin 6 column using PBS buffer (10 mM, pH=7.4).
- [0322] 6. Modified MBP protein (50 μ L, 1.0mg/mL) was added to human plasma (50 μ L) on the ice.
- [0323] 7. Incubation for 72 h at 37 °C.

[0324] 8. Samples (20 μ L) were collected from the mixture at 0 h (before incubation), 24 h, 48 h, and 72 h. Collected samples were stored at -20 °C until SDS-PAGE analysis.

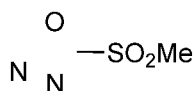
[0325] 9. SDS-PAGE without DTT.

[0326] Fluorescent reagent was not transferred to albumin or other proteins because fluorescence was found only in MBP protein. 83% of fluorescent intensity was kept up to 72 h. The oxadiazole linkage appears very stable in human plasma.

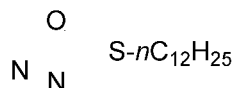
EXAMPLE 7

COMPOUNDS & SYNTHETIC PROCEDURES

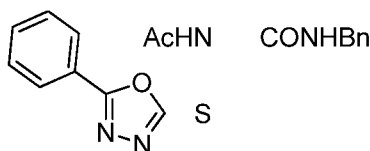
[0327] 2-(methylsulfonyl)-5-phenyl-1,3,4-oxadiazole



[0328] To a solution of 5-phenyl-1,3,4-oxadiazole-2-thiol (1.78 g, 9.99 mmol) in THF (25 mL) was added MeI (658 μ L, 10.6 mmol) and Et₃N (1.65 mL, 11.8 mmol) at 0 °C, and stirred for 3 h at room temperature. Insoluble material was removed by filtration and the filtrate was evaporated. The residue was dissolved with EtOAc. Organic layer was washed with water, 1N HCl and brine, dried over MgSO₄, concentrated *in vacuo* to afford 2-(methylthio)-5-phenyl-1,3,4-oxadiazole (1.76 g) as a colorless oil, which was used in the next step without further purification. To a solution of 2-(methylthio)-5-phenyl-1,3,4-oxadiazole (880 mg, 4.58 mmol) in CH₃CN (20 mL) was added *m*CPBA (70wt%, 3.40 g, 13.8 mmol) at 0 °C and stirred for 14 h at room temperature. Another portion of *m*CPBA (70wt%, 1.00 g, 4.06 mmol) was added to the reaction mixture. The mixture was stirred for 6 h at room temperature. Insoluble material was removed by filtration and the filtrate was evaporated. The residue was dissolved with CH₂Cl₂. The organic layer was washed with saturated aqueous solution of NaHCO₃ and water, dried over MgSO₄, filtered and evaporated. Purification by silica gel column chromatography (hexane to hexane/EtOAc = 2/1) gave 2-(methylsulfonyl)-5-phenyl-1,3,4-oxadiazole (746 mg, 2 steps 67%) as a white solid. ¹H NMR (500 MHz, CDCl₃) δ 8.15-8.13 (m, 2H), 7.67-7.62 (m, 1H), 7.55-7.59 (m, 2H), 3.53 (s, 3H); ¹³C NMR (126 MHz, CDCl₃) δ 166.61, 162.10, 133.28, 129.36, 127.73, 122.03, 42.97; HRMS calcd for C₉H₈N₄O₅ (M+H)⁺ 225.0328, found 225.0328.

[0329] 2-(dodecylthio)-5-phenyl-1,3,4-oxadiazole

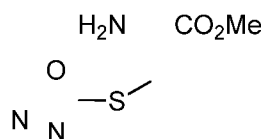
[0330] To a solution of 1-dodecanethiol (8.0 mg, 0.040 mmol) in THF (2 mL) and phosphate buffer (200 mM, pH=7.4, 2 mL) was added 2-(methylsulfonyl)-5-phenyl-1,3,4-oxadiazole (10.8 mg, 0.048 mmol) at room temperature. The reaction mixture was stirred for 30 min at room temperature and quenched with brine. The mixture was extracted with EtOAc. The organic layer was dried over MgSO₄, filtered and evaporated. Purification by silica gel column chromatography (hexane to hexane/EtOAc = 10/1) gave the desired product, 2-(methylsulfonyl)-5-phenyl-1,3,4-oxadiazole (12.5 mg, 90%) as a white solid. ¹H NMR (400 MHz, CDCl₃) δ 8.02-7.99 (m, 2H), 7.53-7.47 (m, 3H), 3.30 (t, *J* = 7.4 Hz, 2H), 1.84 (dt, *J* = 15.0, 7.4 Hz, 2H), 1.47 (dt, *J* = 15.0, 7.4 Hz, 2H), 1.37 – 1.20 (m, 16H), 0.88 (t, *J* = 7.0 Hz, 3H); ¹³C NMR (126 MHz, CDCl₃) δ 165.59, 164.58, 131.51, 128.97, 126.59, 123.72, 32.63, 31.88, 29.59, 29.52, 29.42, 29.30, 29.25, 29.00, 28.58, 22.65, 14.08; HRMS calcd for C₂₀H₃₀N₂OS (M+H)⁺ 347.2151, found 347.2153.

[0331] (*R*)-2-acetamido-*N*-benzyl-3-((5-phenyl-1,3,4-oxadiazol-2-yl)thio)propanamide

[0332] To a solution of (*R*)-2-acetamido-*N*-benzyl-3-mercaptopropanamide (25.2 mg, 0.10 mmol) in THF (5 mL) and phosphate buffer (200 mM, pH=7.4, 5 mL) was added 2-(methylsulfonyl)-5-phenyl-1,3,4-oxadiazole (26.9 mg, 0.12 mmol) at room temperature. The reaction mixture was stirred for 30 min at room temperature and quenched with brine. The mixture was extracted with EtOAc. The organic layer was dried over MgSO₄, filtered and evaporated. Purification by silica gel column chromatography (hexane to EtOAc followed by EtOAc/MeOH = 25/1) gave (*R*)-2-acetamido-*N*-benzyl-3-((5-phenyl-1,3,4-oxadiazol-2-yl)thio)propanamide (37.7 mg, 95%) as a white solid. ¹H NMR (500 MHz, CDCl₃) δ 7.96 (d, *J* = 7.1 Hz, 2H), 7.56-7.46 (m, 3H), 7.44 (t, *J* = 5.6 Hz, 1H), 7.36 (d, *J* = 7.3 Hz, 1H), 7.33-7.20 (m, 5H), 4.98 (td, *J* = 7.3, 4.7 Hz, 1H), 4.43 (d, *J* = 5.8 Hz, 2H), 3.73-3.64 (m, 2H), 1.99

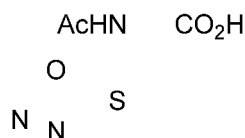
(s, 3H); ^{13}C NMR (126 MHz, CDCl_3) δ 171.13, 169.44, 166.15, 165.05, 137.60, 131.90, 129.08, 128.65, 127.57, 127.46, 126.68, 123.19, 53.48, 43.64, 34.72, 23.06; HRMS calcd for $\text{C}_{20}\text{H}_{20}\text{N}_4\text{O}_3\text{S}$ ($\text{M}+\text{H}$) $^+$ 397.1329, found 397.1318.

[0333] (*R*)-methyl 2-amino-3-((5-phenyl-1,3,4-oxadiazol-2-yl)thio)propanoate



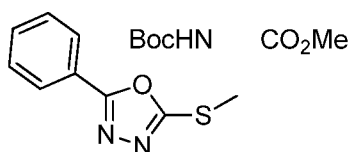
[0334] ^1H NMR (600 MHz, CDCl_3) δ 7.78 (d, $J = 7.4$ Hz, 2H), 7.49 (t, $J = 7.4$ Hz, 1H), 7.42 (t, $J = 7.4$ Hz, 2H), 4.68 (s, 1H), 3.76 (s, 3H), 3.61-3.54 (m, 2H); ^{13}C NMR (151 MHz, CDCl_3) δ 171.02, 165.23, 164.61, 133.27, 131.59, 128.66, 127.01, 60.49, 52.93, 32.92; HRMS calculated for $\text{C}_{12}\text{H}_{13}\text{N}_3\text{O}_3\text{S}$ ($\text{M}+\text{H}$) $^+$ 280.0750, found 280.0749.

[0335] (*R*)-2-acetamido-3-((5-phenyl-1,3,4-oxadiazol-2-yl)thio)propanoic acid



[0336] ^1H NMR (400 MHz, DMSO-d_6) δ 13.12 (br s, 1H), 8.46 (d, $J = 8.0$ Hz, 1H), 7.98 (dd, $J = 7.9, 1.5$ Hz, 2H), 7.66-7.58 (m, 3H), 4.67 (td, $J = 8.5, 4.8$ Hz, 1H), 3.78 (dd, $J = 13.8, 4.8$ Hz, 1H), 3.51 (dd, $J = 13.8, 8.5$ Hz, 1H), 1.82 (s, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 172.17, 170.33, 165.99, 164.18, 132.88, 130.28, 127.26, 123.91, 52.34, 34.43, 23.15; HRMS calcd for $\text{C}_{13}\text{H}_{13}\text{N}_3\text{O}_4\text{S}$ ($\text{M}+\text{H}$) $^+$ 308.0699, found 308.0704.

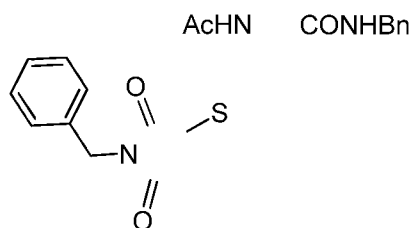
[0337] (*R*)-methyl 2-((tert-butoxycarbonyl)amino)-3-((5-phenyl-1,3,4-oxadiazol-2-yl)thio)propanoate



[0338] ^1H NMR (500 MHz, CDCl_3) δ 8.00 (d, $J = 7.7$ Hz, 1H), 7.55-7.48 (m, 3H), 5.62 (d, $J = 6.6$ Hz, 1H), 4.79-4.72 (m, 1H), 3.84 (dd, $J = 14.0, 4.7$ Hz, 1H), 3.77 (s, 3H), 3.75-3.71 (m, 1H), 1.42 (s, 9H); ^{13}C NMR (126 MHz, CDCl_3) δ 170.35, 165.95, 163.41, 155.01, 131.70,

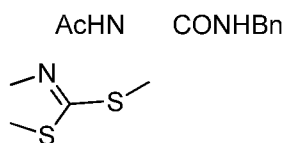
128.98, 126.62, 123.37, 80.38, 53.16, 52.81, 34.85, 28.14; HRMS calcd for $C_{17}H_{21}N_3O_5S$ (M+H)⁺ 380.1275, found 380.1281.

[0339] (2*R*)-2-acetamido-*N*-benzyl-3-((1-benzyl-2,5-dioxopyrrolidin-3-yl)thio)propanamide



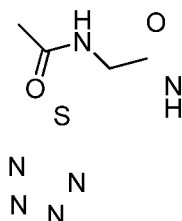
[0340] To a solution of (*R*)-2-acetamido-*N*-benzyl-3-mercaptopropanamide (25.2 mg, 0.10 mmol) in THF (5 mL) and phosphate buffer (200 mM, pH=7.4, 5 mL) was added *N*-benzyl maleimide (22.5 mg, 0.12 mmol) at room temperature. The reaction mixture was stirred for 30 min at room temperature and quenched with brine. The mixture was extracted with EtOAc. The organic layer was dried over $MgSO_4$, filtered and evaporated. Purification by silica gel column chromatography (hexane to EtOAc followed by EtOAc/MeOH = 25/1) gave (2*R*)-2-acetamido-*N*-benzyl-3-((1-benzyl-2,5-dioxopyrrolidin-3-yl)thio)propanamide (40.6 mg, 92%) as a colorless oil. ¹H NMR (500 MHz, $CDCl_3$) δ 7.45 (t, J = 5.3 Hz, 0.5H), 7.35-7.25 (m, 10.5H), 7.07 (d, J = 7.4 Hz, 0.5H), 6.79 (d, J = 7.6 Hz, 0.5H), 4.80 (q, J = 7.0 Hz, 0.5H), 4.70-4.54 (m, 2.5H), 4.52-4.36 (m, 2H), 4.05 (dd, J = 9.4, 4.5 Hz, 0.5H), 3.70 (dd, J = 9.2, 4.4 Hz, 0.5H), 3.46 (dd, J = 14.4, 4.6 Hz, 0.5H), 3.194 (dd, J = 18.8, 9.4 Hz, 0.5H), 3.09-3.03 (m, 1.5H), 2.89 (dd, J = 14.4, 6.6 Hz, 0.5H), 2.57 – 2.50 (m, 1H), 2.01 (s, 1.5H), 1.97 (s, 1.5H); ¹³C NMR (126 MHz, $CDCl_3$) δ 177.81, 177.54, 173.94, 173.80, 170.49, 170.32, 169.77, 169.74, 137.58, 137.53, 135.11, 135.04, 128.71, 128.66, 128.62, 128.59, 128.12, 128.08, 127.71, 127.54, 127.47, 52.68, 52.42, 43.66, 42.82, 40.28, 40.01, 36.24, 35.73, 35.52, 33.89, 23.07, 22.94; HRMS calcd for $C_{23}H_{25}N_3O_4S$ (M+H)⁺ 440.1638, found 440.1624.

[0341] (*R*)-2-acetamido-3-(benzo[*d*]thiazol-2-ylthio)-*N*-benzylpropanamide



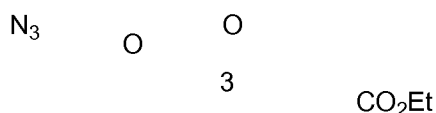
[0342] This compound was prepared according to a reported literature procedure.

[0343] (*R*)-2-Acetamido-*N*-benzyl-3-((1-phenyl-1H-tetrazol-5-yl)thio)propanamide



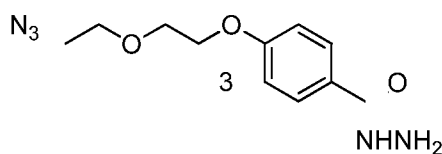
[0344] The above conjugation compound was prepared from 5-(methylsulfonyl)-1-phenyl-1H-tetrazole in a manner similar to that described for conjugation example 2 with a yield of >99% as a white solid. ¹H NMR (600 MHz, CDCl₃) δ 7.57 (m, 5H), 7.45 (d, *J* = 6.8 Hz, 1H), 7.39 (t, *J* = 5.8 Hz, 1H), 7.31 (m, 2H), 7.27 (m, 3H), 4.45 (d, *J* = 5.8 Hz, 2H), 3.77 (dd, *J* = 4.6, 3.7 Hz, 1H), 3.69 (dd, *J* = 7.7, 4.7 Hz, 1H), 2.03 (s, 3H).

[0345] Ethyl 4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)benzoate



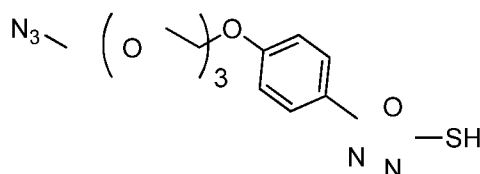
[0346] The mixture of ethyl 4-hydroxybenzoate (1.22 g, 7.35 mmol), 1-azido-2-(2-(2-(2-iodoethoxy)ethoxy)ethoxy)ethane (2.42 g, 7.35 mmol) and K₂CO₃ (2.03 g, 14.7 mmol) in DMF (10 mL) was stirred at 65 °C for 16 h. Then, water and EtOAc were added. The organic layer was washed with brine, dried over MgSO₄, filtered and evaporated. Purification by silica gel column chromatography (hexane to hexane/EtOAc = 1/1) gave ethyl 4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)benzoate (2.05 g, 76%) as a colorless oil. ¹H NMR (600 MHz, CDCl₃) δ 7.99 (d, *J* = 8.7 Hz, 2H), 6.93 (d, *J* = 8.7 Hz, 2H), 4.34 (qd, *J* = 7.1, 1.3 Hz, 2H), 4.19 – 4.18 (m, 2H), 3.89-3.87 (m, 2H), 3.74-3.66 (m, 10H), 3.38-3.37 (m, 2H), 1.38 (t, *J* = 7.1 Hz, 3H); ¹³C NMR (151 MHz, CDCl₃) δ 166.27, 162.39, 131.41, 122.97, 114.04, 70.80, 70.63, 70.62, 70.59, 69.96, 69.46, 69.46, 67.47, 60.56, 50.59, 14.31; HRMS calcd for C₁₇H₂₅N₃O₆ (M+H)⁺ 368.1816, found 368.1828.

[0347] 4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)benzohydrazide



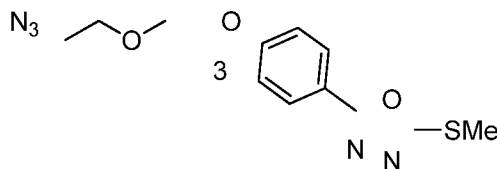
[0348] The mixture of ethyl 4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)benzoate (2.03 g, 5.53 mmol) and hydrazine monohydrate (402 μ L, 8.29 mmol) in EtOH (6 mL) was stirred at 110 °C for 18 h. The reaction mixture was evaporated *in vacuo*. The residue was purified by silica gel column chromatography (hexane to EtOAc followed by EtOAc/MeOH = 5/1) to afford 4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)benzohydrazide (248 mg, 13%) as a colorless oil. ^1H NMR (600 MHz, CDCl_3) δ 7.80 (br s, 1H), 7.72 (d, J = 8.7 Hz, 2H), 6.93 (d, J = 8.7 Hz, 2H), 4.17-4.15 (m, 2H), 3.88-3.86 (m, 2H), 3.74-3.65 (m, 10H), 3.38-3.36 (m, 2H); ^{13}C NMR (151 MHz, CDCl_3) δ 168.11, 161.53, 128.58, 124.94, 114.38, 70.75, 70.58, 70.57, 70.54, 69.92, 69.44, 67.44, 50.56; HRMS calcd for $\text{C}_{15}\text{H}_{23}\text{N}_5\text{O}_5$ ($\text{M}+\text{H}$) $^+$ 354.1772, found 354.1780.

[0349] 5-(4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)phenyl)-1,3,4-oxadiazole-2-thiol



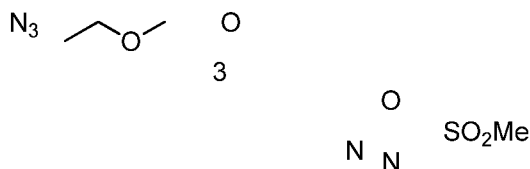
[0350] The mixture of 4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)benzohydrazide (295 mg, 0.835 mmol), carbon disulfide (332 μ L, 5.51 mmol) and KOH (47.0 mg, 0.835 mmol) in EtOH (2 mL) was stirred at 85 °C for 18 h. Then, 1N HCl and EtOAc were added. The organic layer was washed with brine, dried over MgSO_4 , filtered and evaporated. Purification by silica gel column chromatography (hexane to EtOAc) gave 5-(4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)phenyl)-1,3,4-oxadiazole-2-thiol (255 mg, 77%) as a white solid. ^1H NMR (500 MHz, CDCl_3) δ 7.62 (d, J = 8.8 Hz, 2H), 6.88 (d, J = 8.8 Hz, 2H), 4.19 (dd, J = 5.6, 3.0 Hz, 2H), 3.94-3.92 (m, 2H), 3.84 (s, 4H), 3.77 (dd, J = 5.6, 3.0 Hz, 2H), 3.771 (dd, J = 5.6, 3.0 Hz, 2H), 3.67 (t, J = 5.0 Hz, 2H), 3.38 (t, J = 5.0 Hz, 2H); ^{13}C NMR (126 MHz, CDCl_3) δ 177.41, 161.54, 160.77, 127.87, 114.68, 114.65, 70.43, 70.36, 70.35, 69.78, 69.40, 67.39, 50.46; HRMS calcd for $\text{C}_{16}\text{H}_{21}\text{N}_5\text{O}_5\text{S}$ ($\text{M}+\text{H}$) $^+$ 396.1336, found 396.1337.

[0351] 2-(4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)phenyl)-5-(methylthio)-1,3,4-oxadiazole



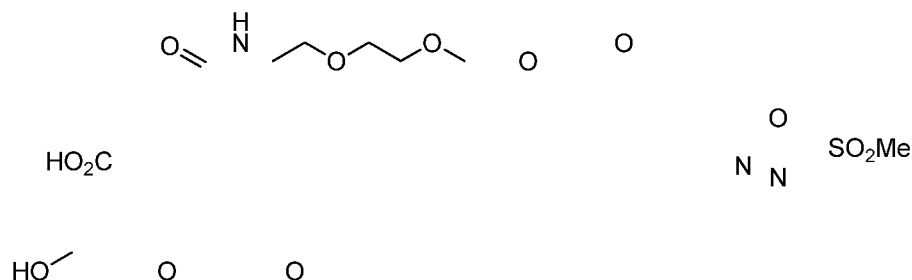
[0352] To a solution of 5-(4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)phenyl)-1,3,4-oxadiazole-2-thiol (233 mg, 0.589 mmol) in THF (5 mL) was added MeI (92.0 mg, 0.648 mmol) and Et₃N (100 μ L, 0.707 mmol) at roomtemperature and stirred for 5 h. Then, water and EtOAc were added. The organic layer was washed with 1N HCl and brine, dried over MgSO₄, concentrated *in vacuo*. Purification by silica gel column chromatography (hexane to hexane/EtOAc = 1/2) gave 2-(4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)phenyl)-5-(methylthio)-1,3,4-oxadiazole (178 mg, 74%) as a colorless oil. ¹H NMR (500 MHz, CDCl₃) δ 7.91 (d, *J* = 8.7 Hz, 2H), 7.00 (d, *J* = 8.7 Hz, 2H), 4.20-4.18 (m, 2H), 3.89-3.87 (m, 2H), 3.75-3.66 (m, 10H), 3.37 (t, *J* = 5.0 Hz, 2H), 2.76 (s, 3H); ¹³C NMR (126 MHz, CDCl₃) δ 165.47, 164.02, 161.25, 128.12, 116.08, 114.85, 70.65, 70.48, 70.46, 70.44, 69.81, 69.31, 67.44, 50.46, 14.45; HRMS calcd for C₁₇H₂₃N₅O₅S (M+H)⁺ 410.1493, found 410.1494.

[0353] 2-(4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)phenyl)-5-(methylsulfonyl)-1,3,4-oxadiazole

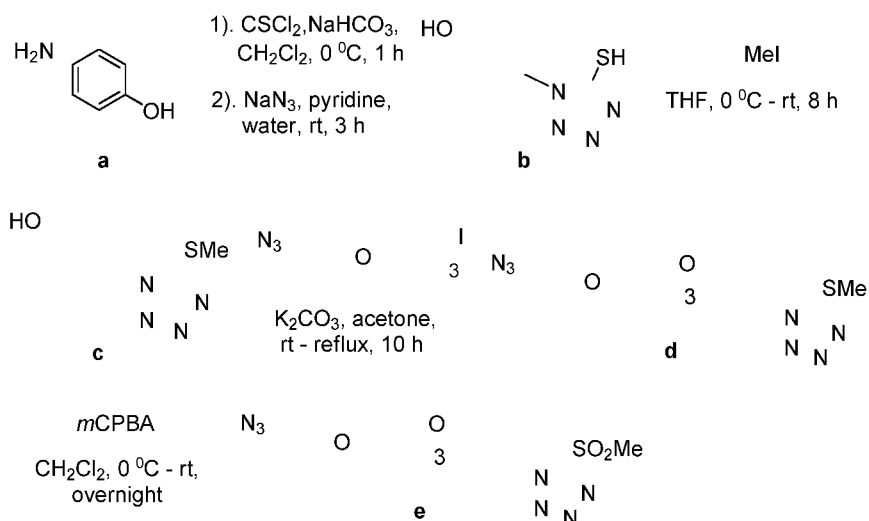


[0354] To a solution of 2-(4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)phenyl)-5-(methylthio)-1,3,4-oxadiazole (190 mg, 0.464 mmol) in CH₂Cl₂ (10 mL) was added *m*CPBA (70wt%, 457 mg, 1.86 mmol) at 0 °C and stirred for 4 h at room temperature. Insoluble material was removed by filtration and the filtrate was washed with saturated aqueous solution of NaHCO₃, dried over MgSO₄, filtered and evaporated. Purification by silica gel column chromatography (hexane to EtOAc) gave 2-(4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)phenyl)-5-(methylsulfonyl)-1,3,4-oxadiazole (76.0 mg, 37%) as a white solid. ¹H NMR (400 MHz, CDCl₃) δ 8.04 (d, *J* = 8.9 Hz, 2H), 7.06 (d, *J* = 8.9 Hz, 2H), 4.24-4.23 (m, 2H), 3.92-3.90 (m, 2H), 3.76-3.66 (m, 10H), 3.52 (s, 3H), 3.39 (t, *J* = 5.0 Hz, 2H); ¹³C NMR (126 MHz, CDCl₃) δ 166.46, 162.68, 161.47, 129.54, 115.28,

114.26, 70.54, 70.52, 70.49, 69.86, 69.30, 67.67, 50.52, 42.86; HRMS calcd for $C_{17}H_{23}N_5O_7S$ ($M+H$)⁺ 442.1391, found 442.1396.

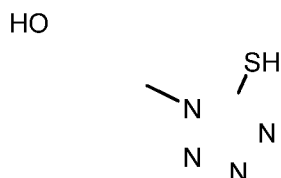


[0355] To a solution of 2-(4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)phenyl)-5-(methylsulfonyl)-1,3,4-oxadiazole (30.0 mg, 0.0680 mmol) in THF (1 mL) and water (0.1 mL) was added PPh_3 (53.0 mg, 0.204 mmol) at room temperature and stirred for 2 h at 37 °C. Then, trifluoroacetic acid (15.6 μ L, 0.204 mmol) and water were added. The aqueous phase was washed with EtOAc and lyophilized to give the corresponding amine (13.9 mg). The amine was dissolved in DMSO (500 μ L). To a DMSO solution of the amine (370 μ L) was added 5-(and 6-)carboxyfluorescein succinimidyl ester (6.4 mg, 0.0135 mmol) and Et_3N (3.3 μ L, 0.0237 mmol) at room temperature. After 2 h at room temperature, the reaction mixture was neutralized with aqueous TFA solution and lyophilized. The residue was purified by preparative TLC (EtOAc/MeOH = 20/1) to afford the title compound (5.3 mg, 2 steps 14%) as an orange amorphous. HRMS calcd for $C_{38}H_{35}N_3O_{13}S$ ($M+H$)⁺ 774.1963, found 774.1963; 92% purity by LCMS.



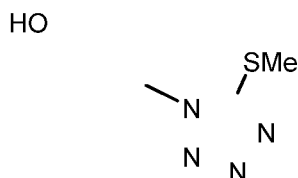
Scheme 9

[0356] (b) 4-(5-mercapto-1H-tetrazol-1-yl)phenol:



[0357] (b) A saturated solution of aqueous sodium bicarbonate (50 mL) was added to a solution of 4-aminophenol (10.0 mmol, 1.09 g) in dichloromethane (50 mL) at 0 °C. The mixture was stirred for 10 minutes, stirring stopped, and thiophosgene (0.84 mL, 11.0 mmol) added to the dichloromethane (lower) layer in one portion via syringe. The resulting mixture was stirred (~500 rpm) for 50 minutes at 0 °C. The reaction was then transferred to a separatory funnel and the organic layer removed. The aqueous phase was extracted with dichloromethane (50 mL) and the combined organics dried over Na₂SO₄. Solvent removal *in vacuo* afforded analytically pure 4-isothiocyanatophenol (1.45g, 96% yield) as yellow oil, which was used without further purification for the subsequent reactions. A mixture of 4-isothiocyanatophenol (9.6 mmol, 1.45g), sodium azide (11.52 mmol, 0.75 g) and pyridine (28.8 mmol, 2.23 g) in water (35 mL) was stirred for a period of 3 h at room temperature. The resulting mixture was then washed with EtOAc (30 mL) and aqueous layer was acidified within concentrated HCl to pH 2. The acidified solution was extracted with EtOAc (50 mL) three times. The organic layer was washed with brine and dried over Na₂SO₄. Solvent removed under vacuum to yield 4-(5-mercapto-1H-tetrazol-1-yl)phenol (**b**, 1.26 g, 68% yield) as a white solid. ¹H NMR (400 MHz, [D⁶]DMSO): δ 11.11(s, 1 H), 9.49(s, 1 H), 7.33-7.36(dd, *J* = 4 Hz, *J* = 8 Hz, 2 H), 6.81-6.84(dd, *J* = 4 Hz, *J* = 8 Hz, 2 H); ¹³C NMR (100 MHz, [D⁶]DMSO): δ 154.9, 132.2, 121.1, 116.7, 116.4. ESI-MS: *m/z* 195.03 [M⁺].

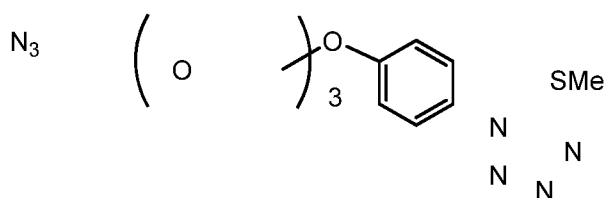
[0358] (c) 4-(5-(methylthio)-1H-tetrazol-1-yl)phenol:



[0359] (c) To a solution of 4-(5-mercapto-1H-tetrazol-1-yl)phenol (3.0 mmol, 0.58 g) in THF (30 mL) was added MeI (3.3 mmol, 0.47 g) and Et₃N (3.6 mmol, 0.51 mL) at 0 °C. Then, the mixture was stirred for 8 h at room temperature. The reaction was then added EtOAc and

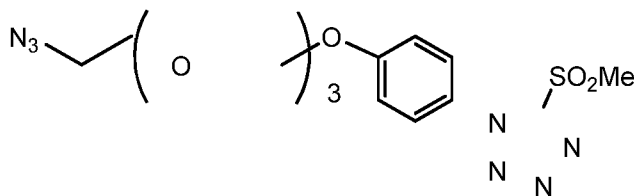
water. The organic layer was washed with 1N HCl and brine, dried over Na₂SO₄. Solvent removal *in vacuo*. Purification by silica gel column chromatography (Hexane/EtOAc = 1:1) gave 4-(5-(methylthio)-1H-tetrazol-1-yl)phenol (**c**, 0.39 g, 63% yield). ¹H NMR (400 MHz, [D⁶]DMSO): δ 10.29(s, 1 H), 7.47-7.49(dd, *J* = 4 Hz, *J* = 8 Hz, 2 H), 7.02-7.04(d, *J* = 8 Hz, 2 H), 2.81(s, 3 H); ¹³C NMR (100 MHz, [D⁶]DMSO): δ 159.7, 155.8, 126.8, 116.7, 15.5. ESI-MS: *m/z* 209.05 [M⁺].

[0360] (d) 1-(4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)phenyl)-5-(methylthio)-1H-tetrazole:



[0361] (d) To a solution of 4-(5-(methylthio)-1H-tetrazol-1-yl)phenol (0.67 mmol, 0.14 g) in acetone (15 mL) was added 1-azido-2-(2-(2-(2-iodoethoxy)ethoxy)ethoxy)ethane (0.804 mmol, 0.264 g) and K₂CO₃ (2.21 mmol, 0.31 g) at room temperature. The mixture was then refluxed for 10 h. Insoluble material was removed by filtration and the solvent was concentrated under vacuum. Purification by silica gel column chromatography (Hexane/EtOAc = 1:1) gave 1-(4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)phenyl)-5-(methylthio)-1H-tetrazole (**d**, 0.21 g, 76.6% yield). ¹H NMR (400 MHz, CDCl₃): δ 7.44-7.46(d, *J* = 8 Hz, 2 H), 7.05-7.07(d, *J* = 8 Hz, 2 H), 4.18-4.21(dd, *J* = 4 Hz, *J* = 8 Hz, 2 H), 3.88-3.91(dd, *J* = 4 Hz, *J* = 8 Hz, 2 H), 3.66-3.75(m, 10 H), 3.37-3.39(m, 2 H), 2.81(s, 3 H); ¹³C NMR (100 MHz, CDCl₃): δ 160.0, 125.4, 115.6, 70.9, 70.8, 70.7, 70.1, 69.5, 67.9, 50.7, 15.4. HRMS-ESI (*m/z*): calcd for C₁₆H₂₃N₇O₄S+H⁺: 410.1605; found: 410.1607, 0.5ppm.

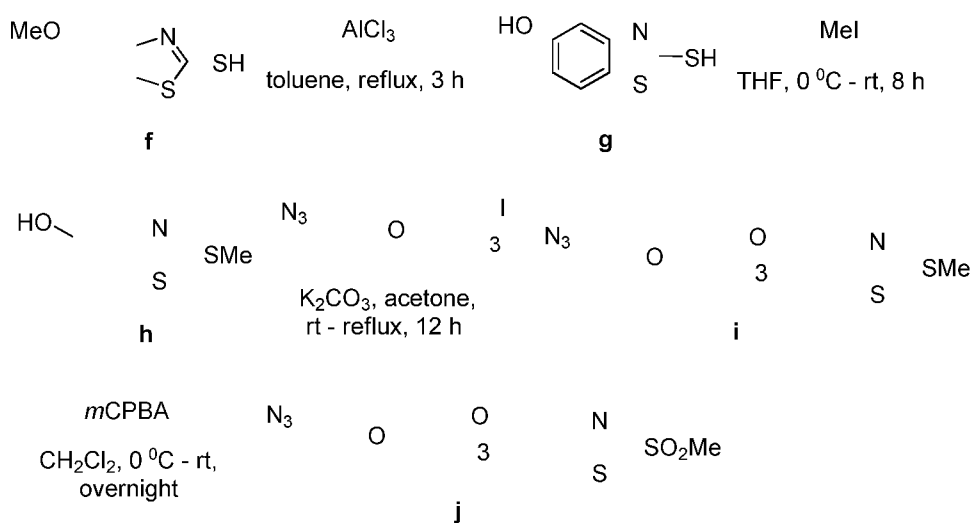
[0362] (e) 1-(4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)phenyl)-5-(methylsulfonyl)-1H-tetrazole:



[0363] (e) To a solution of 1-(4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)phenyl)-5-(methylthio)-1

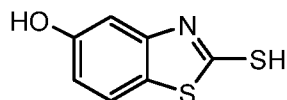
[0364] H-tetrazole (0.34 mmol, 0.14 g) in CH_2Cl_2 (15 mL) was added *m*CPBA (70wt%, 1.36 mmol, 0.34 g) at 0 °C. Then, the mixture was stirred overnight at room temperature. Insoluble material was removed by filtration and the solvent was concentrated under vacuum.

Purification by silica gel column chromatography (Hexane/EtOAc = 1:1) gave 1-(4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)phenyl)-5-(methylsulfonyl)-1H-tetrazole (**e**, 95 mg, 63% yield). $^1\text{H NMR}$ (400 MHz, CDCl_3): δ 7.55-7.57(d, J = 8 Hz, 2 H), 7.06-7.08(d, J = 8 Hz, 2 H), 4.18-4.21(dd, J = 4 Hz, J = 8 Hz, 2 H), 3.87-3.90(dd, J = 4 Hz, J = 8 Hz, 2 H), 3.65-3.74(m, 10 H), 3.60(s, 3 H), 3.36-3.38 (t, J = 4 Hz, 2 H); $^{13}\text{C NMR}$ (100 MHz, CDCl_3): δ 153.9, 126.4, 115.5, 70.71, 70.7, 70.6, 70.0, 69.5, 67.9, 50.7, 43.8. **HRMS-ESI** (m/z): calcd for $\text{C}_{16}\text{H}_{23}\text{N}_7\text{O}_6\text{S}+\text{H}^+$: 442.1503; found: 442.1502, 0.2ppm.



Scheme 10

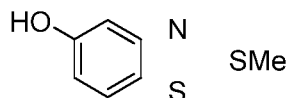
[0365] (g) 2-mercaptobenzo[d]thiazol-5-ol:



[0366] (g) 2-Mercapto-5-methoxybenzothiazole (2.0 mmol, 0.4 g) was stirred in toluene (20 mL) and AlCl_3 (1.08 g) added. The stirred mixture was heated under reflux for 3 h then allowed to cool, and 1N HCl (10 mL) added. The solid was filtered and mostly dissolved in

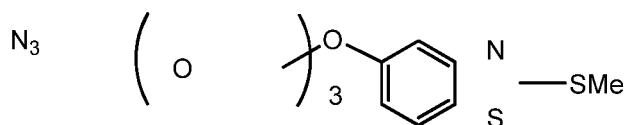
1N NaOH (5 mL). After filtration, the solution was acidified with acetic acid. The gray solid was filtered and recrystallized from methanol to give the product 2-mercaptobenzo[d]thiazol-5-ol (**g**, 0.23g, 63% yield). $^1\text{H NMR}$ (400 MHz, $[\text{D}^6]\text{DMSO}$): δ 7.41-7.43(d, $J = 8$ Hz, 1 H), 6.71-6.78(m, 2 H); $^{13}\text{C NMR}$ (100 MHz, $[\text{D}^6]\text{DMSO}$): δ 157.4, 122.4, 113.1, 100.0.

[0367] (h) 2-(methylthio)benzo[d]thiazol-5-ol:



[0368] (h) To a solution of 2-mercaptobenzo[d]thiazol-5-ol (1.26 mmol, 0.23 g) in THF (20 mL) was added MeI (1.39 mmol, 0.2 g) and Et_3N (1.51 mmol, 0.22 mL) at 0 °C. Then, the mixture was stirred for 8 h at room temperature. The reaction was then added EtOAc and water. The organic layer was washed with 1N HCl and brine, dried over Na_2SO_4 . Solvent removal *in vacuo*. Purification by silica gel column chromatography (Hexane/EtOAc = 1:1) gave 2-(methylthio)benzo[d]thiazol-5-ol (**h**, 0.2 g, 82% yield). $^1\text{H NMR}$ (400 MHz, $[\text{D}^6]\text{DMSO}$): δ 9.70(s, 1 H), 7.75-7.77(d, $J = 8$ Hz, 1 H), 7.19-7.20(d, $J = 4$ Hz, 1 H), 6.84-6.87(dd, $J = 4$ Hz, $J = 8$ Hz, 1 H), 2.77(s, 3 H); $^{13}\text{C NMR}$ (100 MHz, $[\text{D}^6]\text{DMSO}$): δ 168.9, 157.1, 154.8, 122.3, 114.3, 107.1, 15.9. ESI-MS: m/z 198.00 $[\text{M}^+]$.

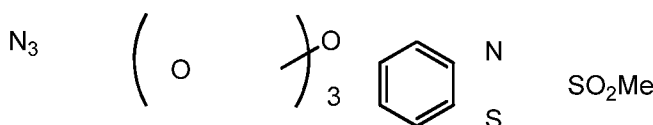
[0369] (i) 5-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)-2-(methylthio)benzo[d]thiazole:



[0370] (i) To a solution of 2-(methylthio)benzo[d]thiazol-5-ol (0.76 mmol, 0.149 g) in acetone (15 mL) was added 1-azido-2-(2-(2-(2-iodoethoxy)ethoxy)ethoxy)ethane (0.91 mmol, 0.31 g) and K_2CO_3 (2.5 mmol, 0.4 g) at room temperature. The mixture was then refluxed for 12 h. Insoluble material was removed by filtration and the solvent was concentrated under vacuum. Purification by silica gel column chromatography (Hexane/EtOAc = 1:1) gave 5-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)-2-(methylthio)benzo[d]thiazole (**i**, 0.18 g, 60% yield). $^1\text{H NMR}$ (400 MHz, CDCl_3): δ 7.57-7.59(d, $J = 8$ Hz, 1 H), 7.37-7.38(d, $J = 4$ Hz, 1 H), 6.94-6.97(dd, $J = 4$ Hz, $J = 8$ Hz, 1 H), 4.17-4.19(t, $J = 4$ Hz, 2 H), 3.87-3.90(dd, $J = 4$ Hz, $J = 8$ Hz, 2 H), 3.64-3.75(m, 10 H), 3.35-3.38(dd, $J = 4$ Hz, $J = 8$ Hz, 2 H), 2.77(s, 3 H); $^{13}\text{C NMR}$ (100 MHz, CDCl_3): δ 158.1, 154.5,

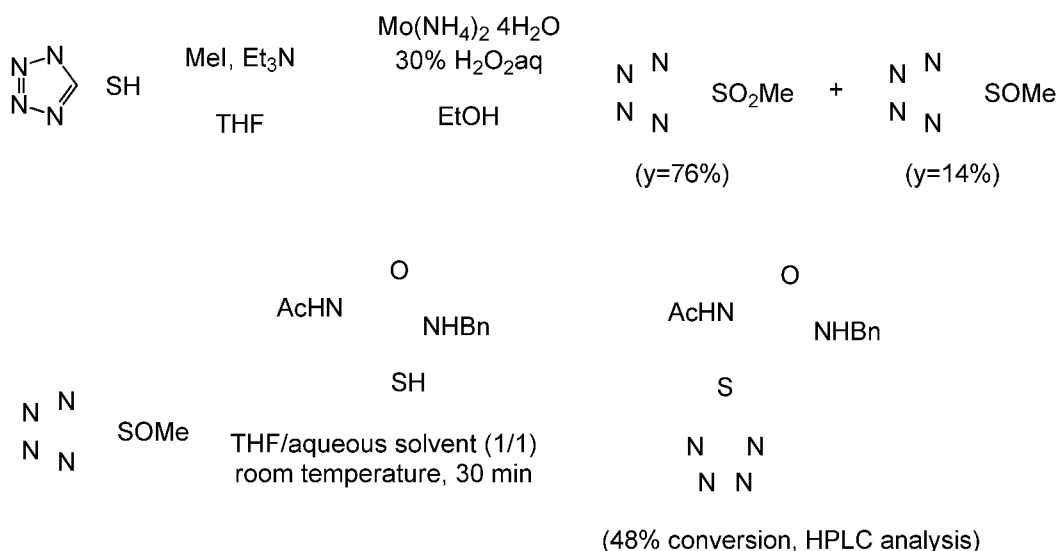
126.9, 121.1, 114.3, 105.4, 70.9, 70.7, 70.0, 69.7, 67.9, 50.7, 15.9. **HRMS-ESI** (m/z): calcd for $C_{16}H_{22}N_4O_4S_2+H^+$: 399.1155; found: 399.1159, 1.0ppm.

[0371] (j) 5-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)-2-(methylsulfonyl)benzo[d]thiazole:



[0372] (j) To a solution of 5-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)-2-(methylthio)benzo[d]thiazole (0.38 mmol, 0.15 g) in CH_2Cl_2 (15 mL) was added *m*CPBA (70wt%, 1.39 mmol, 0.36 g) at 0 °C. Then, the mixture was stirred overnight at room temperature. Insoluble material was removed by filtration and the solvent was concentrated under vacuum. Purification by silica gel column chromatography (Hexane/EtOAc = 1:1) gave 5-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)-2-(methylsulfonyl)benzo[d]thiazole (**j**, 84 mg, 51.4% yield). 1H NMR (400 MHz, $CDCl_3$): δ 7.84-7.86(d, J = 8 Hz, 1 H), 7.62-7.63(d, J = 4 Hz, 1 H), 7.26-7.29(dd, J = 4 Hz, J = 8 Hz, 1 H), 4.22-4.24(t, J = 4 Hz, 2 H), 3.92-3.94(t, J = 4 Hz, 2 H), 3.66-3.78(m, 10 H), 3.37-3.40(m, 5 H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.9, 159.1, 153.8, 128.9, 122.6, 119.8, 107.3, 70.9, 70.7, 70.6, 70.0, 69.5, 68.1, 50.7, 42.5.

[0373] Sulfoxide agents are also used for thiol-modification and subsequent conjugation reactions as shown in Scheme 11.



Scheme 11

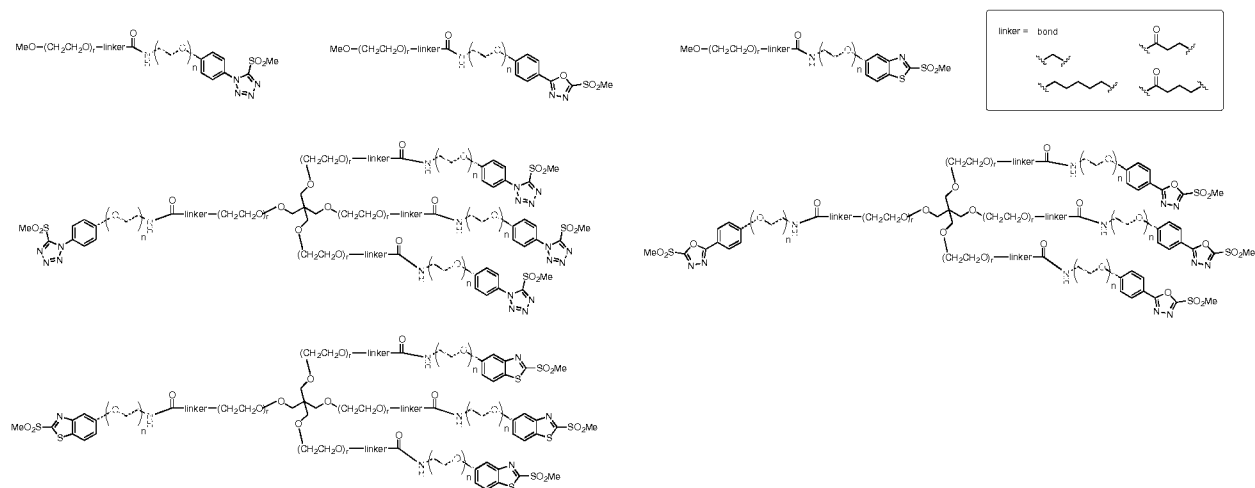
EXAMPLE 8

CONJUGATION REACTIONS

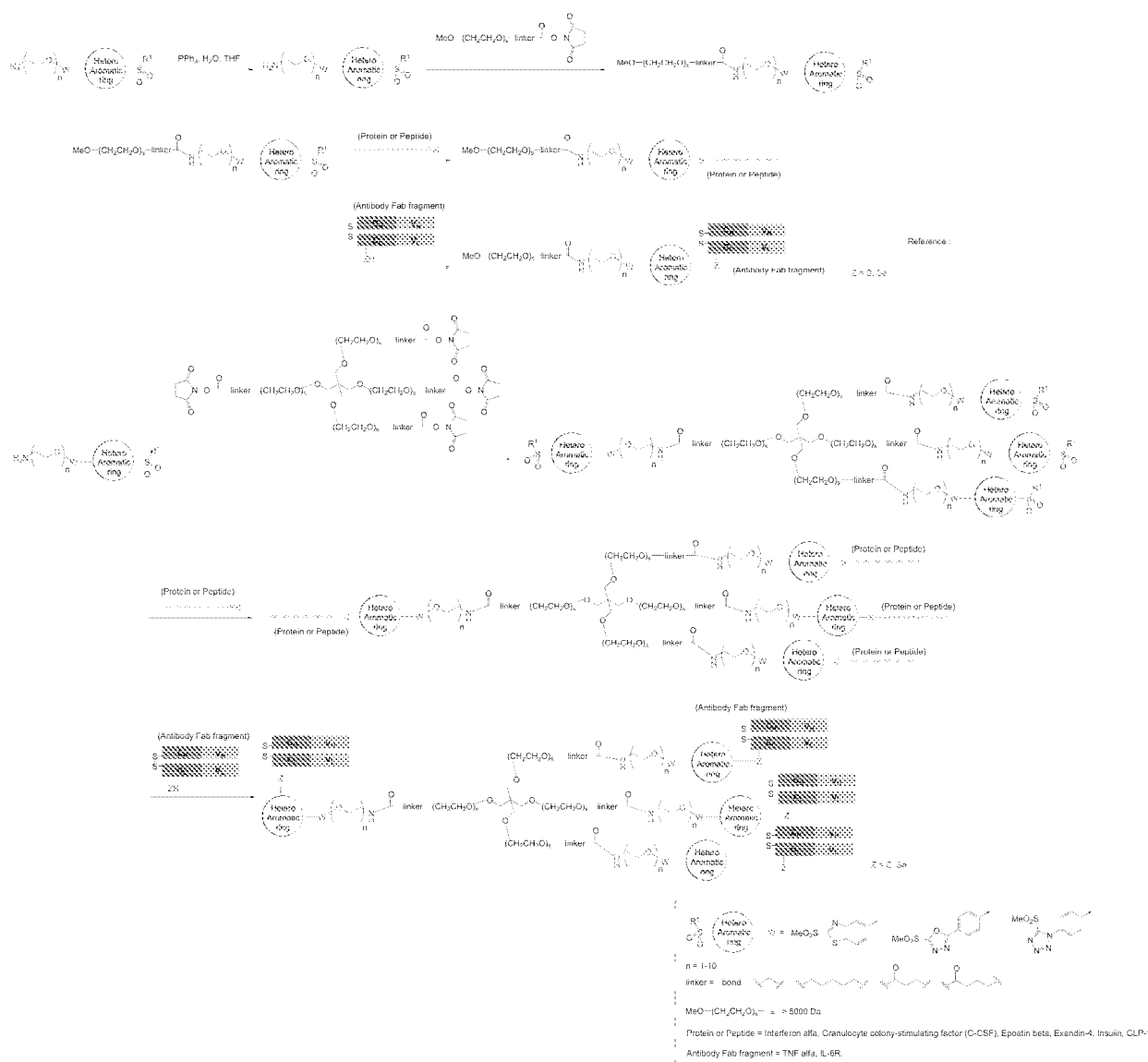
[0374] This example illustrates the applicability of the reagents and methods described herein are contemplated for uses in a fashion analogous to maleimide-thiol conjugation agents and reactions. (See Figures 25-30.) All of the below cited references are incorporated herein by reference in their entirety.

[0375] Day et. al describe the development of new glucagon and GLP-1 co-agonist peptides Aib2 C24 chimera 2 and Aib2 C24 chimera 2 lactam that eliminate obesity in rodents. To illustrate the power of the thio-click reaction in rapidly preparing long-lived peptide drugs based on Aib2 C24 chimera 2 lactam the peptide was modified using thiol-click chemistry to 1) pegylate the peptide; 2) to link the peptide to human albumin; 3) to link the peptide to humanized antibody 38C2; 4) to link the peptide to an antibody bearing a free thiol.

[0376] **Pegylation using thiol-Click:** Aib2 C24 chimera 2 lactam was synthesized as described by Day et al. and treated with sulfone-peg selected from Chart A in 7 M urea and 50 mM Tris pH 8. Reaction progress was monitored by analytical reverse-phase HPLC, and free peptide was consumed within 30 min. and the pegylated Aib2 C24 chimera 2 lactam product was purified. We anticipate that this general strategy will provide for the pegylation of a wide range of thiol-containing molecules or molecules modified to contain a free thiol such as peptides (binding peptides, GLP-1, Exendin-4), nucleic acids, antibodies, antibody fragments, affibodies, ankrin repeat proteins, cytokines, proteins, interferon-a-2b, G-CSF, FGF-21, human growth hormone, erythropoietin, small molecules, CCR5 antagonists, CXCR4 antagonists.



[0377] Chart A: Thiol-click reagents for pegylation

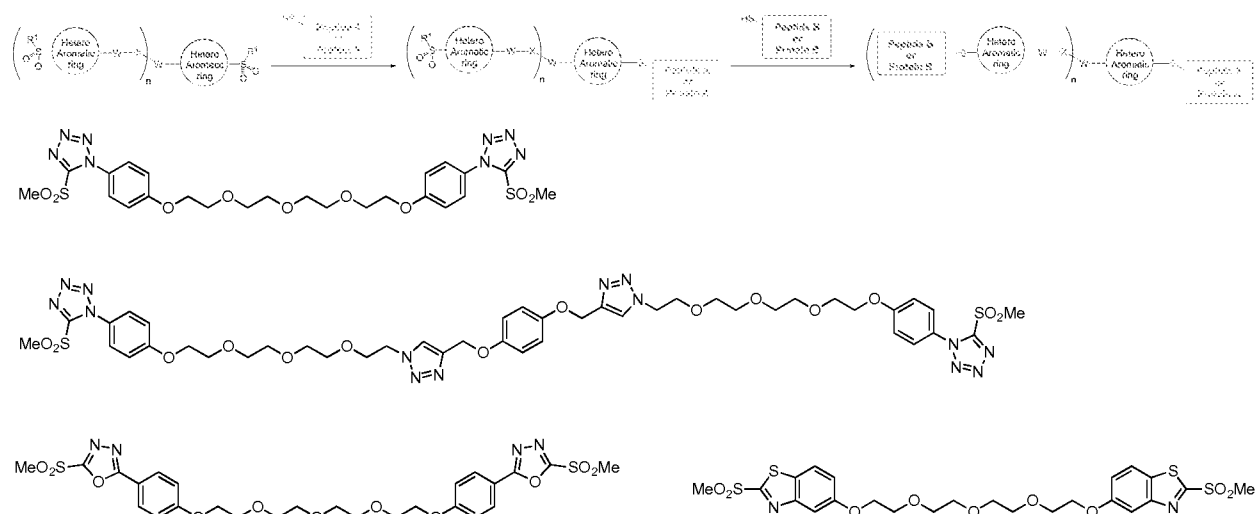


[0378] Pegylation of an Antibody Fab and Pegylation of an Antibody Fab for tetramerization.

[0379] **Albumin conjugation between albumin and a thiol containing molecule:** Aib2

C24 chimera 2 lactam was synthesized as described by Day et al. and treated with a equimolar sulfone-link-sulfone chosen from Chart B in 50 mM Tris pH 8 and purified. The resulting Aib2 C24 chimera-sulfone product was a) mixed at a ratio of peptide to purified albumin of 1.2 to 1 to form the Aib2 C24 chimera linked to albumin. Use of engineered albumins with extended half-life or albumin domains bearing a free cysteine are also anticipated; b) added to fresh human blood to form the same Aib2 C24 chimera linked to

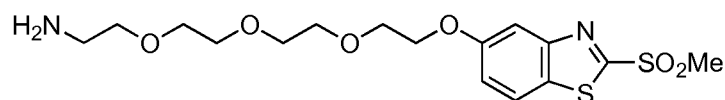
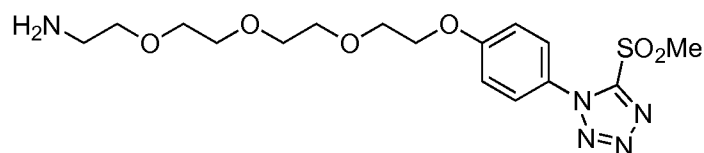
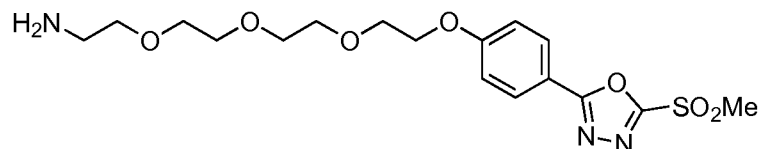
human albumin; 3) administered by injection to mice or human (intravenous injection, subcutaneous injection, or intraperitoneal injection) to form in vivo Aib2 C24 chimera linked to mouse or human albumin. When linkage is performed ex vivo, albumin conjugates may subsequently be formulated as nanoparticles for example by passage through a jet under high pressure as is done for the approved albumin nanoparticle drug Abraxane. Various nanoparticle formulations of any modified albumins described in this application are anticipated. We anticipate that this general strategy will provide for the conjugation of albumin, engineered albumins, or albumin fragments with a free thiol to a wide range of thiol-containing molecules or molecules modified to contain a free thiol such as peptides (binding peptides, GLP-1, Exendin-4), nucleic acids, antibodies, antibody fragments, scFvs, affibodies, ankrin repeat proteins, cytokines, proteins, interferon- α -2b, G-CSF, FGF-21, human growth hormone, erythropoietin, small molecules, CCR5 antagonists, CXCR4 antagonists nucleic acids, aptamers, and other bioactive molecules.



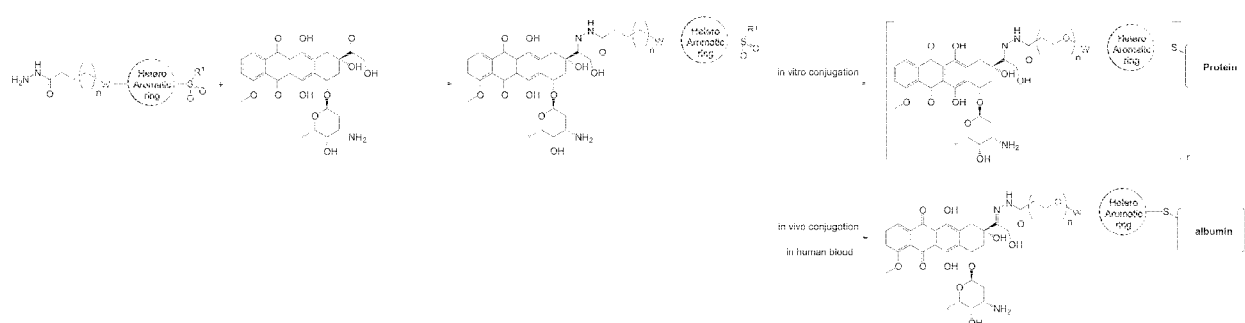
[0380] Chart B. Sulfone-linked-Sulfones for sequential linkage of thiol-bearing molecules

[0381] **Albumin conjugation between albumin and molecule synthesized to contain a thiol-reactive sulfone:** For example, Aib2 C24 chimera 2 lactam was synthesized and chemically linked at the C-terminal carboxyl group to an amino-sulfone chosen from Chart C. The resulting Aib2 C24 chimera 2 lactam-sulfone was a) mixed at a ratio of peptide to human albumin of 1.2 to 1 to form the Aib2 C24 chimera linked to human albumin; b) added to fresh human blood to form the same Aib2 C24 chimera linked to human albumin; 3) administered by injection to mice or human (intravenous injection, subcutaneous injection, or

intraperitoneal injection) to form in vivo Aib2 C24 chimera linked to human or mouse albumin. In each case HPLC verified formation of Aib2 C24 chimera linked to either human or mouse albumin. By analogy any carboxylate bearing molecule that can be coupled to an amine from Chart C may then be subsequently linked to any molecule bearing a free thiol or selenol.



[0382] Chart C. Amino sulfones for chemical synthesis and coupling to carboxylates



[0383] AA. Hydrazine derived Sulfones for in vitro or in vivo coupling of albumins to doxorubicin and derivations.

[0384] Doxorubicin linkage to albumin is known to enhance the utility of the toxin as done in the drug INNO-206. As shown in AA, hydrazine based sulfones provide for the rapid coupling of ketone or aldehyde bearing molecules to thiol- or selenol-bearing molecules to create enhanced doxorubicin-albumin conjugates with in vivo or ex vivo.

[0385] Anti-myeloma effects of the novel anthracycline derivative INNO-206.

[0386] Sanchez E, Li M, Wang C, Nichols CM, Li J, Chen H, Berenson JR.

[0387] Clin Cancer Res. 2012 Jul 15;18(14):3856-67. doi: 10.1158/1078-0432.CCR-11-3130. Epub 2012 May 22.

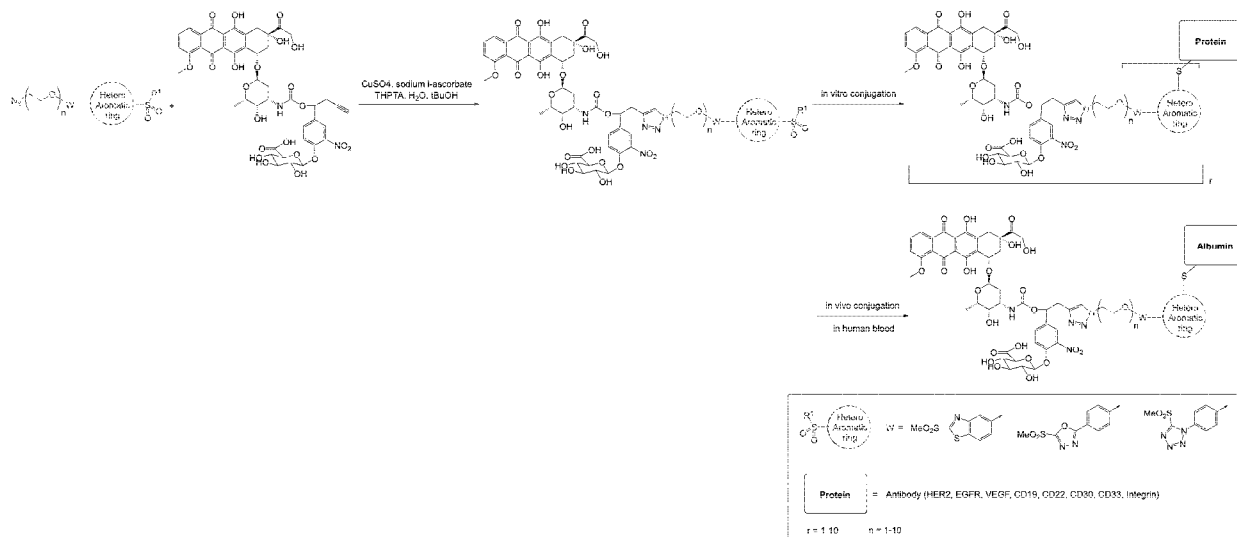
[0388] Other linkage strategies for doxorubicin and related toxins are also available. For example, thiol-click sulfones bearing azides are readily linked to drugs bearing alkynes. Subsequent labeling to a carrier such as an albumin or another targeting of PK modifying carrier bearing a free thiol then is facilitated. **BB** illustrates this approach.

[0389] See J Med Chem. 2012 May 10;55(9):4516-20. doi: 10.1021/jm300348r. Epub 2012 Apr 30, which is incorporated herein by reference.

[0390] Synthesis and antitumor efficacy of a β -glucuronidase-responsive albumin-binding prodrug of doxorubicin.

[0391] Legigan T, Clarhaut J, Renoux B, Tranoy-Opalinski I, Monvoisin A, Berjeaud JM, Guilhot F, Papot S.

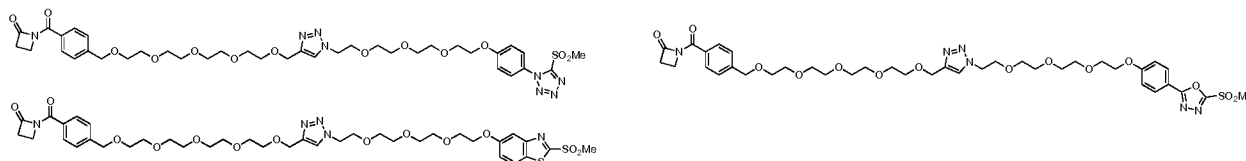
[0392] Institut de Chimie des Milieux et des Matériaux de Poitiers, IC2MP, Université de Poitiers, UMR-CNRS 7285, 4 Rue Michel Brunet, 86022 Poitiers, France.



[0393] BB. Azido Sulfones for in vitro or in vivo coupling of albumins to doxorubicin derivatives with enzymatic trigger.

[0394] Chemically programmed antibody Hu38C2 via thiol-click reaction: Aib2 C24 chimera 2 lactam was synthesized as described by Day et al. and treated with a sulfone- acyl lactam compound chosen from chart D in phosphate buffered saline at pH 7 and purified. The

resulting Aib2 C24 chimera 2 lactam-acyl lactam was then admixed at a ratio of 3 peptide to 1 antibody hu38C2 in phosphate buffer for 10 hrs. Purification of the resulting antibody conjugate and mass spectrometry analysis revealed 2 peptides labeled per antibody molecule.



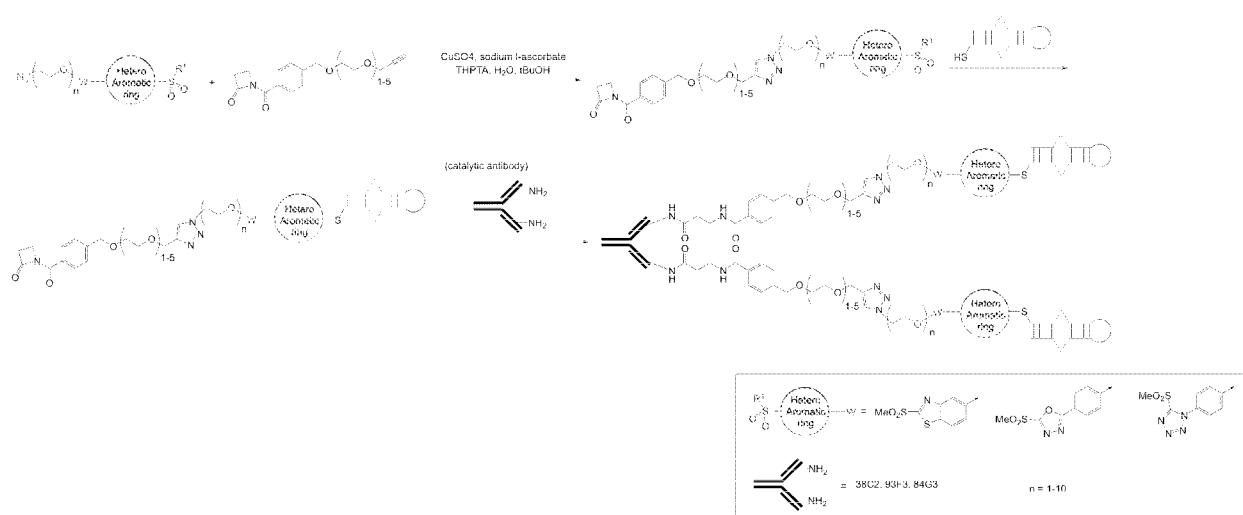
[0395] Chart D. Sulfone- acyl lactams for linkage of thiol containing molecules to aldolase antibodies.

[0396] In each of the cases illustrated the resulting products are expected to provide long-lived peptide derivatives with agonism at the glucagon and GLP-1 receptors that has potent, sustained satiation inducing and lipolytic effects.

[0397] A new glucagon and **GLP-1** co-agonist eliminates obesity in rodents.

[0398] Day JW, Ottaway N, Patterson JT, Gelfanov V, Smiley D, Gidda J, Findeisen H, Bruemmer D, Drucker DJ, Chaudhary N, Holland J, Hembree J, Abplanalp W, Grant E, Ruehl J, Wilson H, Kirchner H, Lockie SH, Hofmann S, Woods SC, Nogueiras R, Pfluger PT, Perez-Tilve D, DiMarchi R, Tschöp MH. Nat Chem Biol. 2009 Oct;5(10):749-57. doi: 10.1038/nchembio.209. Epub 2009 Jul 13, PMID:19597507 [PubMed - indexed for MEDLINE], which is incorporated herein by reference.

[0399] “Mono-PEGylated Dimeric Exendin-4 as High Receptor Binding and Long-Acting Conjugates for Type 2 Anti-Diabetes Therapeutics,” *Bioconjugate Chemistry* **2011** 22 (4), 625-632, which is incorporated herein by reference.



[0400] Thiol-Click enablement of Aptamer programming of Aldolase antibodies

[0401] Aldolase antibodies may be rapidly programmed with Aptamer determined specificities using the simple chemistry shown above. Here the thiol-click reaction is used in preference over maleimide linkages originally described by Wuellner et al. 2010. Aptamers of any given specificity may be engineered and used.

[0402] “Expanding the concept of chemically programmable antibodies to RNA aptamers: chemically programmed biotherapeutics.” Wuellner U, Gavriluk JI, Barbas CF 3rd. *Angew Chem Int Ed Engl.* 2010 Aug 9;49(34):5934-7. doi: 10.1002/anie.201001736.

[0403] Thiol-Click reactions in antibody chemistry and creation of multi-specific antibodies

[0404] Thiomab consist of antibodies or antibody fragments or forms of antibodies or domains with engineered free cysteine residues. Some preferred sites of substitution on the antibody heavy chain are Ala114 Cys (Kabat numbering) and/or on the light chain Val110Cys or Val205Cys or any terminal Cys.

[0405] **Anti-TNF anti-Ang2 bispecific antibodies.** To create this bispecific antibody, we chose Humira as the IgG and introduced the light chain Val205Cys mutation. Expression, purification and mild reduction provided Humira thiomab. Angiopoetin-2 binding peptides from Chart E were synthesized with an addition of N- or C-terminal cysteine and admixed with a sulfone-linked-sulfone from chart B. Alternatively angiopoetin-2 binding peptides from Chart E were synthesized with attachment of an amino sulfone from Chart C to the C-terminus. The resulting peptide sulfones were purified and then reacted with Humira thiomab at a ratio of 3 peptides to 1 mAb. The resulting Humira-ang2 peptide conjugates were purified and antibody was determined to have 2 peptides attached per mAb. The resulting protein bound both TNF and ang-2. Alternatively, another anti-TNF antibody like Remicade can be used as the scaffold antibody.

[0406] **Anti-Her2- anti-Ang2 bispecific antibodies.** To create this bispecific antibody, we chose Herceptin as the IgG and introduced the light chain Val205Cys mutation. Expression, purification and mild reduction provided Herceptin thiomab. Angiopoetin-2 binding peptides from Chart E were synthesized with an addition of N- or C-terminal cysteine and admixed with a sulfone-linked-sulfone from chart B. Alternatively angiopoetin-2 binding peptides from Chart E were synthesized with attachment of an amino sulfone from Chart C to the C-terminus. The resulting peptide sulfones were purified and then reacted with Herceptin

thiomab at a ratio of 3 peptides to 1 mAb. The resulting Herceptin-ang2 peptide conjugates were purified and antibody was determined to have 2 peptides attached per mAb. The resulting protein bound both HER2 and ang-2.

[0407] Anti-Her2- anti-EGFR bispecific antibodies. To create this bispecific antibody, we chose Herceptin as the IgG and introduced the light chain Val205Cys mutation. Expression, purification and mild reduction provided Herceptin thiomab. EGFR binding affibody EGFR *e1* from Chart F was expressed in *E. coli* with an addition of N- or C-terminal cysteine and admixed with a sulfone-linked-sulfone from chart B. Alternatively affibody EGFR *e1* from Chart E was synthesized with attachment of an amino sulfone from Chart C to the C-terminus. The resulting affibody sulfones were purified and then reacted with Herceptin thiomab at a ratio of 3 peptides to 1 mAb. The resulting Herceptin-affibody conjugates were purified and antibody was determined to have 2 affibodies attached per mAb. The resulting protein bound both HER2 and EGFR.

[0408] Other Anti-Her2 bispecific antibodies. Anti-Her2-anti-Her3, anti-Her2-anti-IGFR1, and anti-Her2-anti-avb3 bispecifics were created using the methods described for Anti-Her2-anti-EGFR bispecific antibodies wherein the ang2 peptides are replaced with the appropriate peptide or domain selected from Chart F.

[0409] 1. GGGSGGAQTNFMPMDQDEALLYEEFILQQGLE

[0410] 2. GGGSMGAQTNFMPMDNDELLLYEQFILQQGLE

[0411] 3. GGGSGGAGGGGSPHEECYSYPNPPHCYTMS

[0412] 4. LWDDCYFFPNPPHCYNP

[0413] 5. DEHQTNFLPLDQDEALLYEEFI LQQGLE

[0414] Chart E. Angiopoeitin-2 binding peptides for thiol-click conjugation. For use, peptides are synthesized with addition of an N- or C-terminal cysteine. Other peptides that compete with these peptides are readily prepared by phage display. Chemical alteration to enhance in vivo stability is also anticipated.

[0415] IGF1R *i1* Affibody Li, 2010 VDNKFNKEGFYAAIEI LALPNLNRKQSTAFI
SSLEDDPSQSANLLAEAKKLNDAPK

[0416] EGFR *e1* Affibody Gostring, 2010 VDNKFNKEMWAAWEEIR
NLPNLNGWQMTAFI ASLVDDPSQSANLLAEAKKLNDAPK

[0417] ErbB3 *c1* Affibody Kronqvist, 2011 VDNKFNKER YSAYYEI
WQLPNLNVRQKAIFI GSLQDDPSQSANLLAEAKKLNDAQAPK

[0418] $\alpha\beta 3$ *n1* Knottin Kimura, 2009 GCPQGRGDWAPT
SCKQDSDCRAGCVCGPNGFCG

[0419] Chart F. Other binding peptides of domains for thiol-click conjugation. For use, peptides are synthesized or domains expressed with addition of an N- or C-terminal cysteine.

[0420] **Anti-VEGF- anti-Ang2 bispecific antibodies.** To create this bispecific antibody, we chose Avastin as the IgG and introduced the light chain Val205Cys mutation. Expression, purification and mild reduction provided Avastin thiomab. Angiopoetin-2 binding peptides from Chart E were synthesized with an addition of N- or C-terminal cysteine and admixed with a sulfone-linked-sulfone from chart B. Alternatively angiopoetin-2 binding peptides from Chart E were synthesized with attachment of an amino sulfone from Chart C to the C-terminus. The resulting peptide sulfones were purified and then reacted with Avastin thiomab at a ratio of 3 peptides to 1 mAb. The resulting Avastin-ang2 peptide conjugates were purified and antibody was determined to have 2 peptides attached per mAb. The resulting protein bound both vegf and ang-2. With these examples, it should be clear that additional specificity(ies) can be added to any antibody by engineering a free cysteine on to it and labeling with a defined peptide, small molecule, or aptamer.

[0421] **Trispecific, Tetraspecific, Pentaspecific, or hexaspecific Antibodies.** The zybodies antibody variants described by LaFleur et al. provide for the expression of bi-, tri-, tetra-, and penta- specific antibodies. Introduction of thiomab mutations into zybodies then allows for the site selective introduction of peptides and domains as described above or addition of cytotoxic reagents. Addition of more free cysteines allows the valency of the peptide conjugated by the thiol-click reaction to increase from 2 to 4 and beyond. In some cases heterodimeric Fc regions might be used to allow odd numbers of labeled peptides to be added 1, 3, 5, etc. Further this approach is not limited to intact IgG molecules but can be applied to antibody fragments, scFv's, and immunoglobulin domains. Of course any other protein or domain can be engineered to display a free thiol that can then be labeled by the thiol-click reaction.

[0422] Peptides from charts E and F are from Kanakaraj 2012 and LaFleur 2013

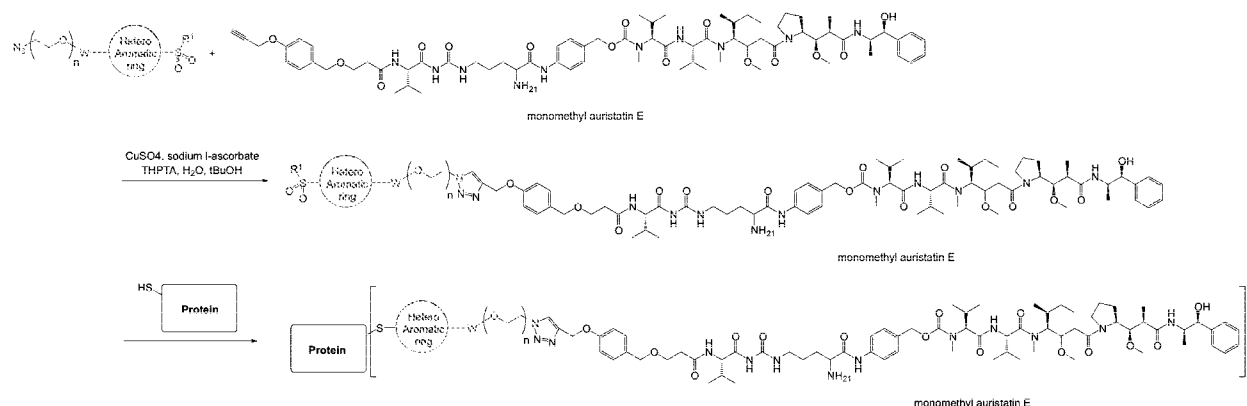
[0423] Simultaneous targeting of TNF and Ang2 with a novel bispecific antibody enhances efficacy in an in vivo model of arthritis.

[0424] Kanakaraj P, Puffer BA, Yao XT, Kankanala S, Boyd E, Shah RR, Wang G, Patel D, Krishnamurthy R, Kaithamana S, Smith RG, LaFleur DW, Barbas CF 3rd, Hilbert DM, Kiener PA, Roschke VV. *MAbs*. 2012 Sep-Oct;4(5):600-13. doi: 10.4161/mabs.21227. Epub 2012 Aug 6. PMID: 22864384

[0425] LaFleur D, Abramyan D, Kanakaraj P, Smith R, Shah R, Wang G, Yao X, Kankanala S, Boyd E, Zaritskaya L, Nam V, Puffer B, Buasen P, Kaithamana S, Burnette A, Krishnamurthy R, Patel D, Roschke V, Kiener P, Hilbert D, Barbas C. Monoclonal antibody therapeutics with up to five specificities: Functional enhancement through fusion of target-specific peptides. *mAbs* 2013; 5:208 - 218; <http://dx.doi.org>.

[0426] Thiomab reference and references therein:

[0427] *Nat Biotechnol*. 2012 Jan 22;30(2):184-9. doi: 10.1038/nbt.2108. "Conjugation site modulates the in vivo stability and therapeutic activity of antibody-drug conjugates." Shen BQ, et al.



[0428] Antibody and Albumin Toxin Drug Conjugates:

[0429] Any of the engineered Thiomabs, or Zybodies, or albumin or other free thiol- or selenol-containing molecules can be rapidly attached to cytotoxic drugs. One example shown above depicts the coupling of monomethyl auristatin E to a free thiol- displaying protein. Preferred proteins target cancer cells. IgGs, fragments thereof, engineered forms containing immunoglobulin domain, small molecule, peptide, aptamer, ankrin repeat domains, affibodies, or other molecules developed to bind a target can be used to deliver toxins such as monomethyl auristatin E by connecting the toxin and the targeting molecule as shown. Other

preferred proteins such as albumins can concentrate at the site of a tumor. As noted for doxorubicin, albumin conjugates comprising auristatin toxins should be useful anti-tumor agents also constructed as shown above. A wide variety of toxins can be adapted for linkage using this strategy, see for example Alley 2010 and Flygare 2013.

[0430] “Antibody-drug conjugates for the treatment of cancer.” Flygare JA, Pillow TH, Aristoff P. *Chem Biol Drug Des.* 2013 Jan;81(1):113-21. doi: 10.1111/cbdd.12085.

[0431] “Antibody-drug conjugates: targeted drug delivery for cancer.” Alley SC, Okeley NM, Senter PD. *Curr Opin Chem Biol.* 2010 Aug;14(4):529-37. doi: 10.1016/j.cbpa.2010.06.170. Epub 2010 Jul 17. **Review.**

[0432] Thiol-Click linked Fab-scFvs:

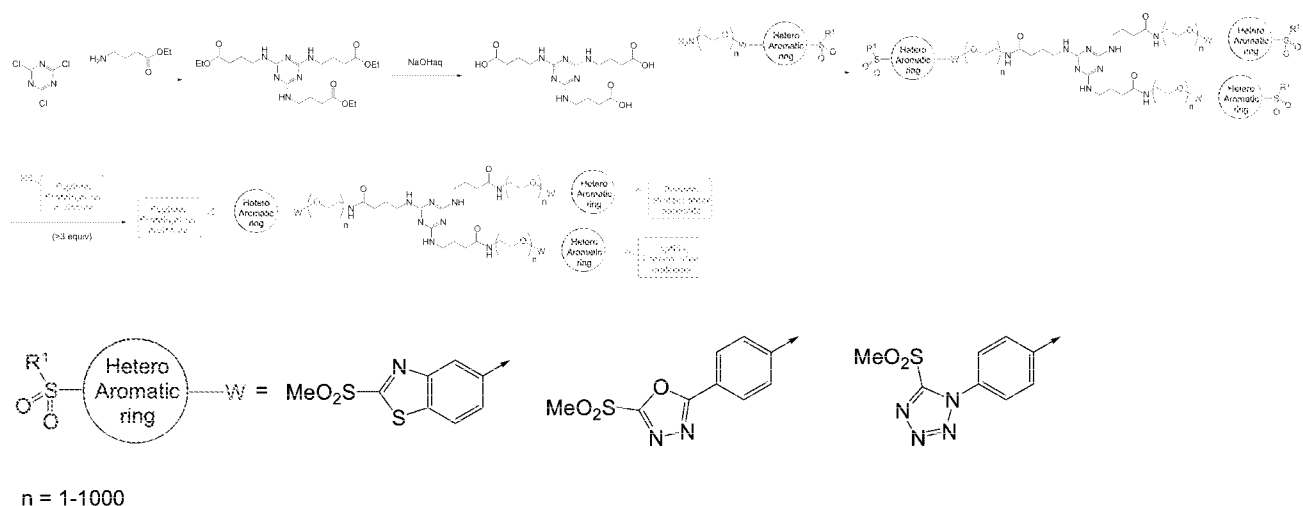
[0433] T-cell targeting with Anti-CD3-Anti-Her2 thiol clicked Fab-scFvs. anti-human CD3 Fab v9 with a selenocysteine (Sec) at the C-terminus of the heavy chain fragment followed by a hexa-histidine tag (Cui et al. 2012) or the same protein modified to have a free cysteine at the C-terminus of the heavy chain was expressed, purified, and mildly reduced as described by (Cui et al. 2012). These proteins were then reacted with a Sulfone-linked-Sulfones from Chart B by treatment with a slight molar excess of sulfone-link-sulfone in 50 mM Tris pH 8 and purified. The resulting anti-human CD3 Fab v9 -sulfone products linked via cysteine or selenocysteine. A HER2 binding scFv was engineered based on (Zhu et al. 1996) with a C-terminal cysteine (HER2 scFvCys), expressed and purified. anti-human CD3 Fab v9 – sulfones were then mixed with HER2scFv-Cys50 mM Tris pH 8. The resulting Fab-scFv bispecific antibodies were then purified. This product is useful for T-cell directed killing. Replacement of the HER2 scFv with other scFvs or targeting molecules allows for T-cell targeting of other cells.

[0434] It is anticipated that this general strategy will provide for the conjugation a wide range of thiol-containing molecules or molecules modified to contain a free thiol such as peptides (binding peptides, GLP-1, Exendin-4), nucleic acids, antibodies, antibody fragments, scFvs, immunoglobulin domains, affibodies, ankrin repeat proteins, cytokines, proteins, interferon- α -2b, G-CSF, FGF-21, human growth hormone, erythropoietin, small molecules, CCR5 antagonists, CXCR4 antagonists, anti-viral agents, on to an appropriately engineered Fab such as v9 or any other protein modified to display a free thiol.

[0435] Thiol-Click linked scFc-scFvs: In order to cross-link any 2 scFvs, each is expressed with terminal or otherwise accessible cysteine or selenocysteine. Treatment of said first scFv with a Sulfone-linked-Sulfones from Chart B provides for a scFv-sulfone. Addition of the second scFv under buffered conditions then provides for the linkage of the two proteins.

[0436] Chemically programmed bispecific antibodies that recruit and activate T cells.” Cui H, Thomas JD, Burke TR Jr, **Rader C.** J Biol Chem. 2012 Aug 17;287(34):28206-14. doi: 10.1074/jbc.M112.384594. Epub 2012 Jul 3.

[0437] “High level secretion of a humanized bispecific diabody from Escherichia coli.” Zhu Z, Zapata G, Shalaby R, Snedecor B, Chen H, Carter P. Biotechnology (N Y). 1996 Feb;14(2):192-6.

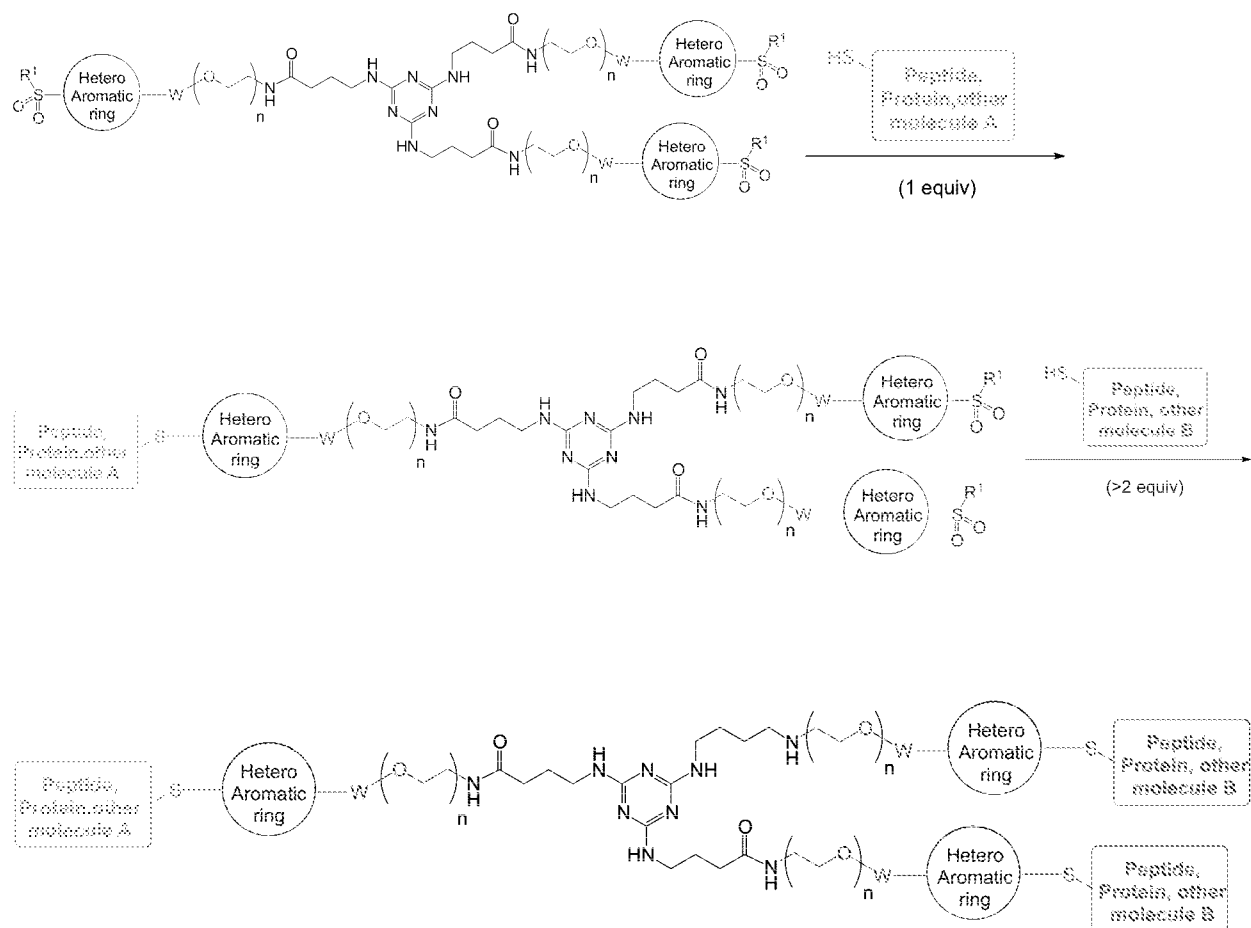


[0438] Chart G. Sulfones for Trifunctional Linkage of thiol- or selenol-containing molecules

[0439] Trifunctional Linkage of molecules.

[0440] Trifunctional linkages of thiol- or selenol containing molecules are rapidly prepared by simple mixing of the molecule in various buffer/mixed solvent media with a trimeric sulfone shown at **Chart G**. The linkage is then formed displaying the molecule trivalently. The melamine core molecule used for trifunctional branching can be replaced with a wide range of other three branched molecules. Other dendritic forms are also anticipated. We anticipate that this general strategy will provide for the trimerization of a wide range of thiol-containing molecules or molecules modified to contain a free thiol such as peptides (binding peptides, GLP-1, Exendin-4), nucleic acids, antibodies, antibody fragments, scFvs, affibodies, ankrin repeat proteins, cytokines, proteins, interferon-a-2b, G-CSF, FGF-21,

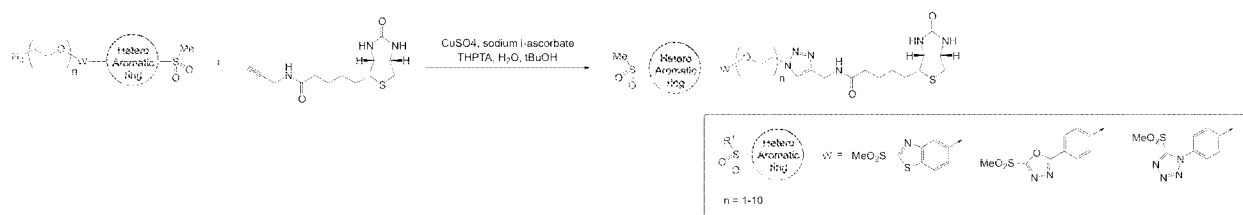
human growth hormone, erythropoietin, small molecules, CCR5 antagonists, CXCR4 antagonists, nucleic acids, aptamers, and other bioactive molecules. Trifunctional linkage molecules are also useful for immobilization on surfaces.



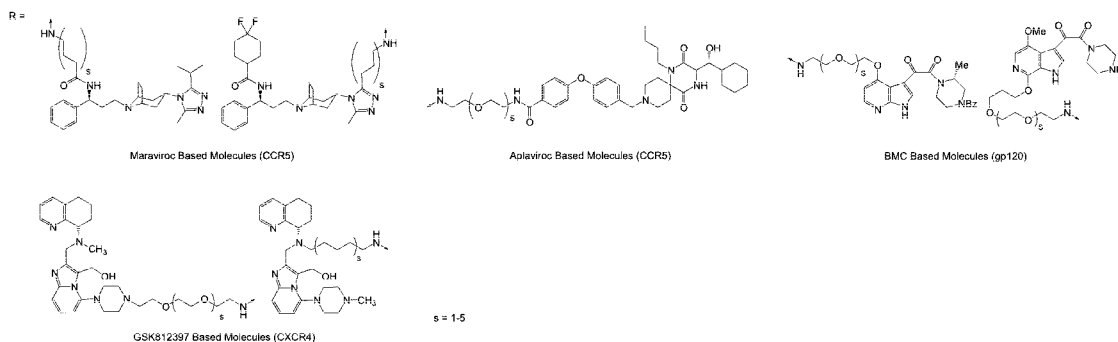
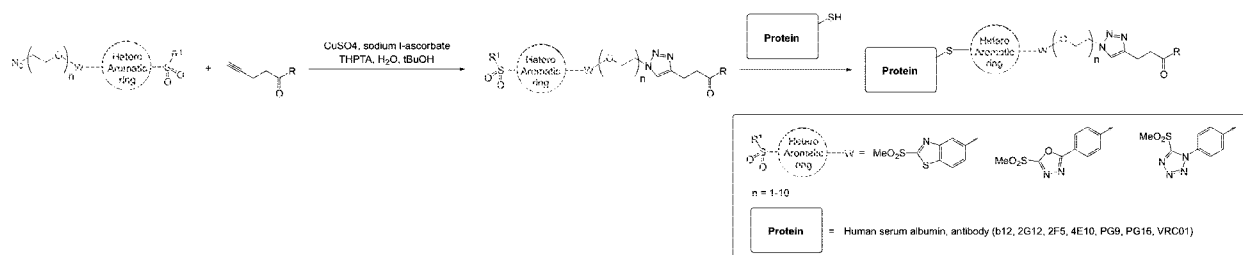
[0441] Chart H. Linkage of one thiol- or selenol-containing molecule to two of a different thiol- or selenol containing molecules.

[0442] As shown in Chart H, a molecule A may be rapidly linked to 2 molecules B wherein each contain a free thiol- or selenol group. Examples would include T-cell targeting wherein the first molecule is a CD3 targeting scFv and the second molecule has affinity to a cancer cell to be targeted. Bivalent binding of the second molecule to the target cell surface is expected to improve overall T-cell targeting for therapeutic applications. We anticipate that this general strategy will provide for the linkage of a wide range of thiol-containing molecules A and B or molecules A and B modified to contain a free thiol such as peptides (binding peptides, GLP-1, Exendin-4), nucleic acids, antibodies, antibody fragments, scFvs,

affibodies, ankrin repeat proteins, cytokines, proteins, interferon- α -2b, G-CSF, FGF-21, human growth hormone, erythropoietin, small molecules, CCR5 antagonists, CXCR4 antagonists, nucleic acids, aptamers, and other bioactive molecules. Trifunctional sulfone molecules are also useful for immobilization on surfaces.



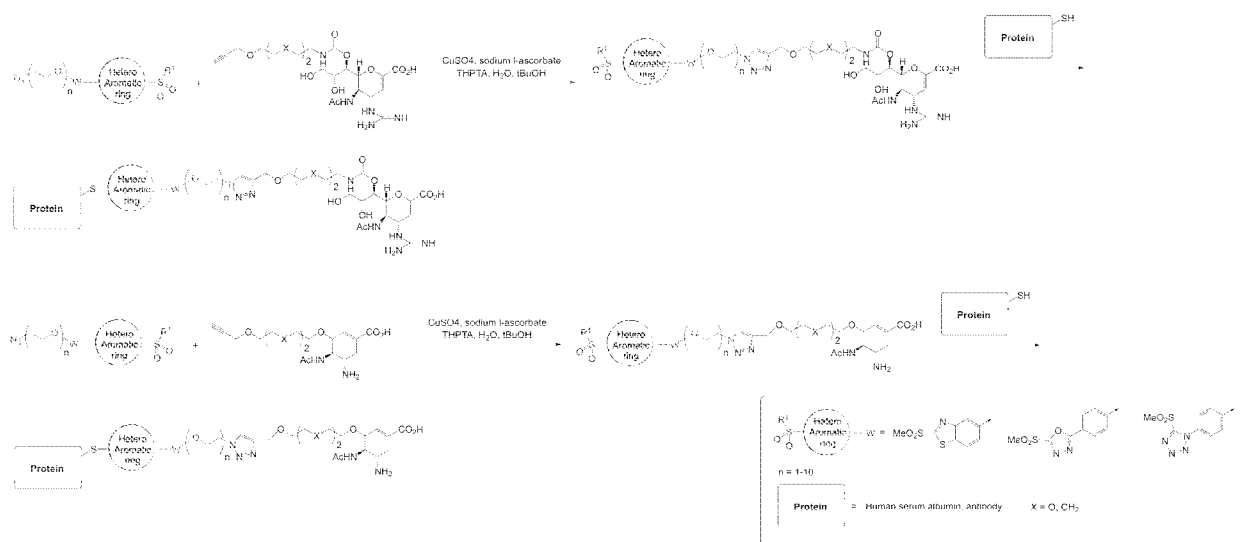
[0443] CC. Biotinylation enabled with thiol-click sulfones. For fluorophore attachment biotin is exchanged for a fluorophore.



[0444] DD. Enhancing the potential of anti-HIV entry inhibitors. Long-lived inhibitors are created by linkage to albumins ex vivo or albumin in vivo. Coupling to other proteins like other anti-HIV antibodies is anticipated.

[0445] “Potent inhibition of HIV-1 entry with a chemically programmed antibody aided by an efficient organocatalytic synthesis.” Gavriluk J, Uehara H, Otsubo N, Hessel A, Burton DR, Barbas CF 3rd. *Chembiochem.* 2010 Oct 18;11(15):2113-8. doi: 10.1002/cbic.201000432.

[0446] “Antibody Conjugation Approach Enhances Breadth and Potency of Neutralization of anti-HIV-1 Antibodies and CD4-IgG.” Gavriluk J, Ban H, Uehara H, Sirk S, Saye-Francisco K, Cuevas A, Zablowsky E, Oza A, Seaman MS, Burton DR, Barbas CF 3rd. J Virol. 2013 Feb 20.



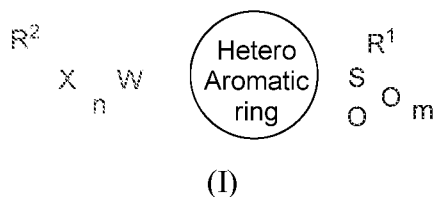
[00100] EE. Enhancing the potential of influenza inhibitors. Long-lived inhibitors are created by linkage to albumins ex vivo or albumin in vivo. Coupling to other proteins like other proteins like antibodies or other molecules is anticipated.

[00101] See “A chemically programmed antibody is a long-lasting and potent inhibitor of influenza neuraminidase.” Hayakawa M, Toda N, Carrillo N, Thornburg NJ, Crowe JE Jr, Barbas CF 3rd. Chembiochem. 2012 Oct 15;13(15):2191-5. doi: 10.1002/cbic.201200439. Epub 2012 Sep 10.

[00102] Illustrative examples of the invention are shown in Exhibit A, which is hereby incorporated by reference. Although the invention has been described with reference to the above example, it will be understood that modifications and variations are encompassed within the spirit and scope of the invention. Accordingly, the invention is limited only by the following claims.

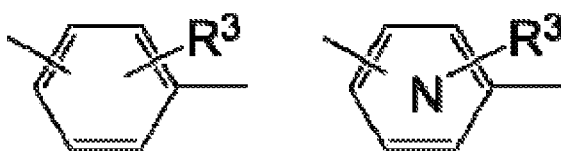
What is claimed is:

1. A compound of structural Formula I:



or salt thereof, wherein:

W is selected from bond, hydrogen, and



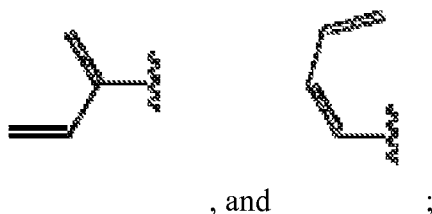
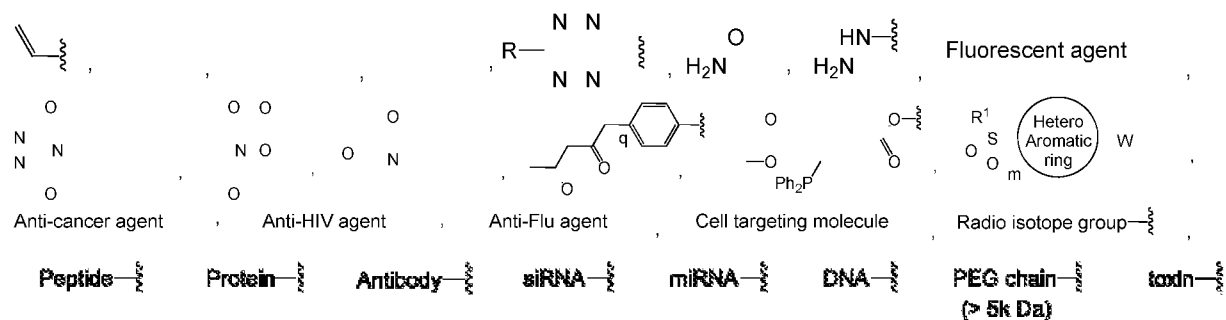
X is a linear or branched connecting chain of atoms comprising any of C, H, O, N, P, S, Si, F, Cl, Br, or I, or a salt thereof;

m is 0 or 1;

n is an integer from 0 to 5;

R¹ is selected from substituted or unsubstituted alkyl or substituted and unsubstituted aryl;

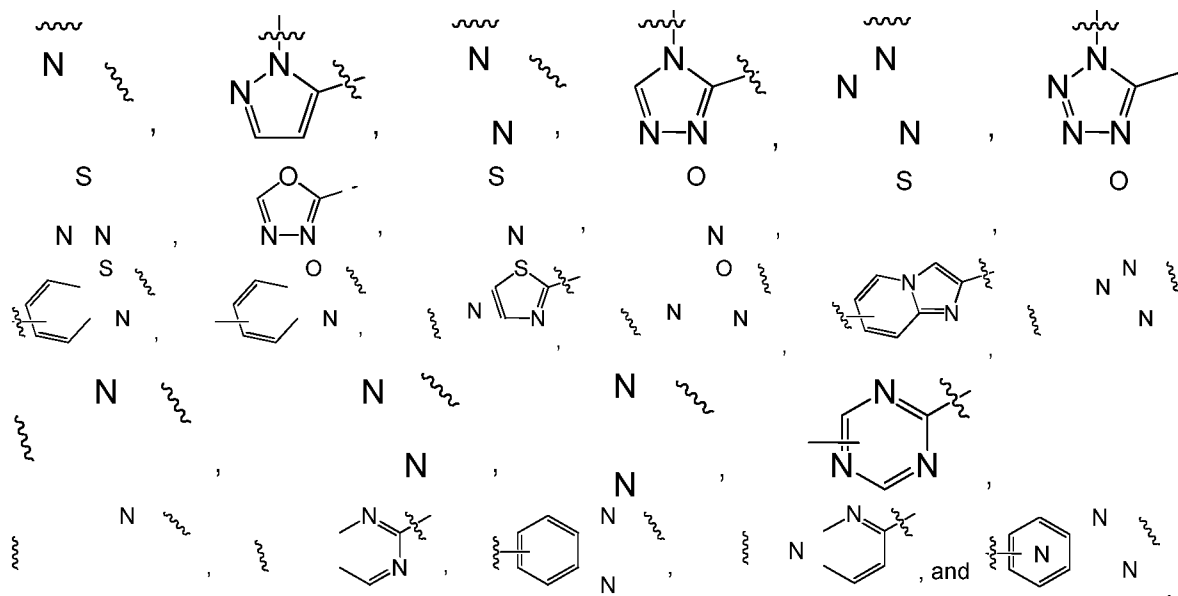
R² is selected from substituted or unsubstituted alkyl, substituted or unsubstituted aryl, $-(OCH_2CH_2)_p-$, $-CO_2H$, $-NH_2$, OH, $-N_3$,



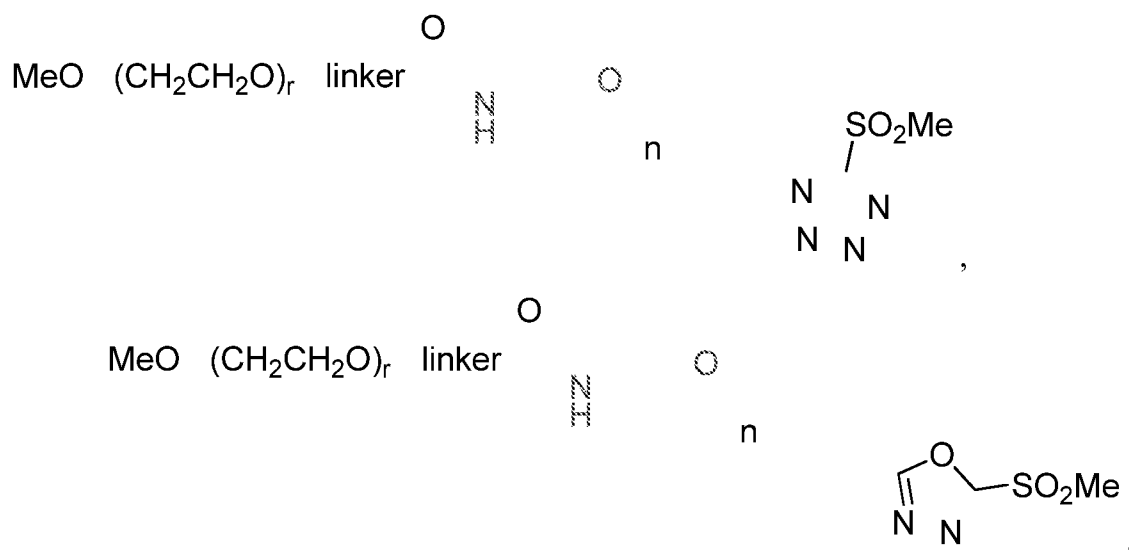
p is an integer from 1 to 1000;

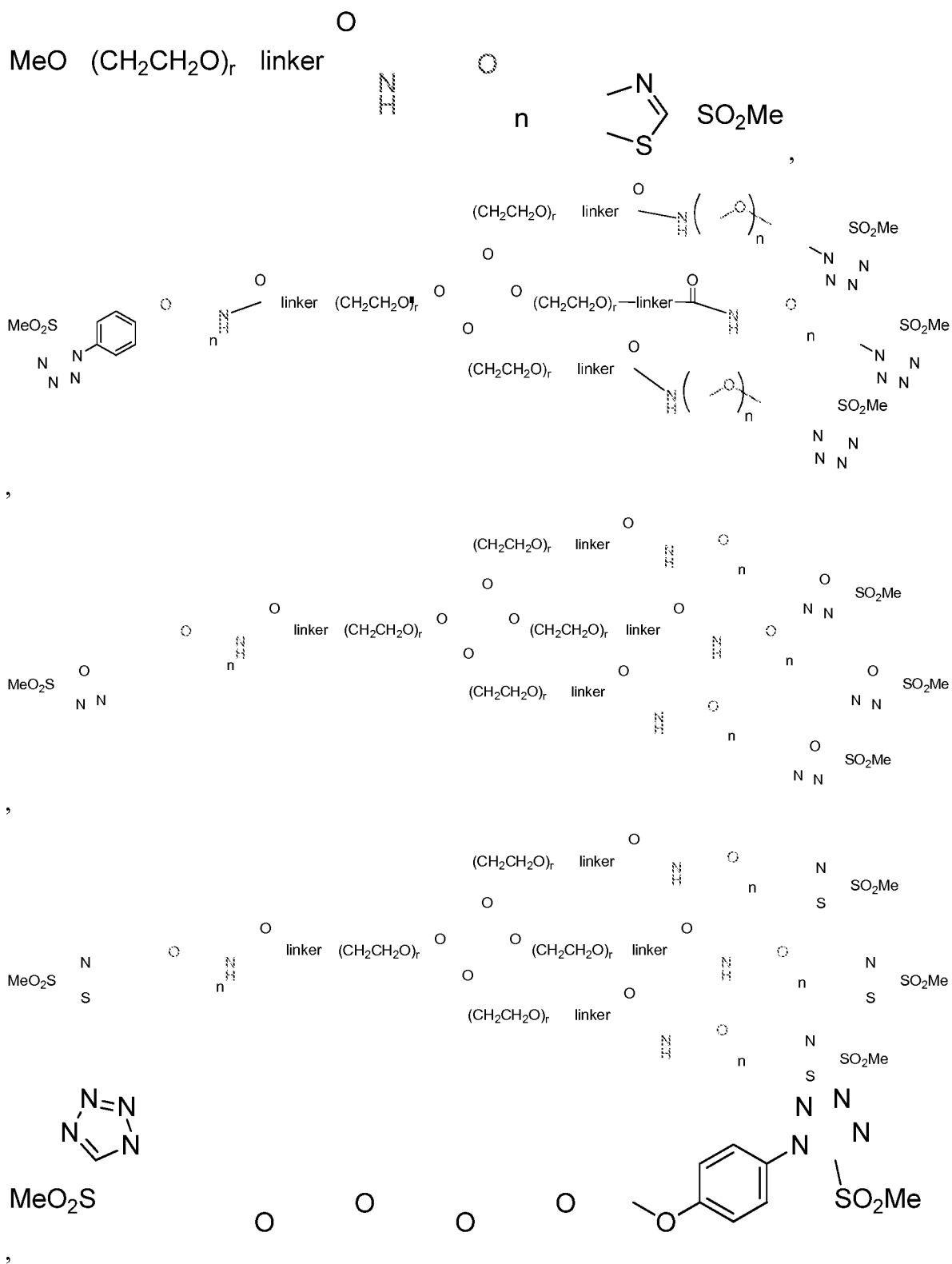
R³ is selected from hydrogen, halogen, -CO₂H, -NH₂, OH, -N₃, and -alkyne; and

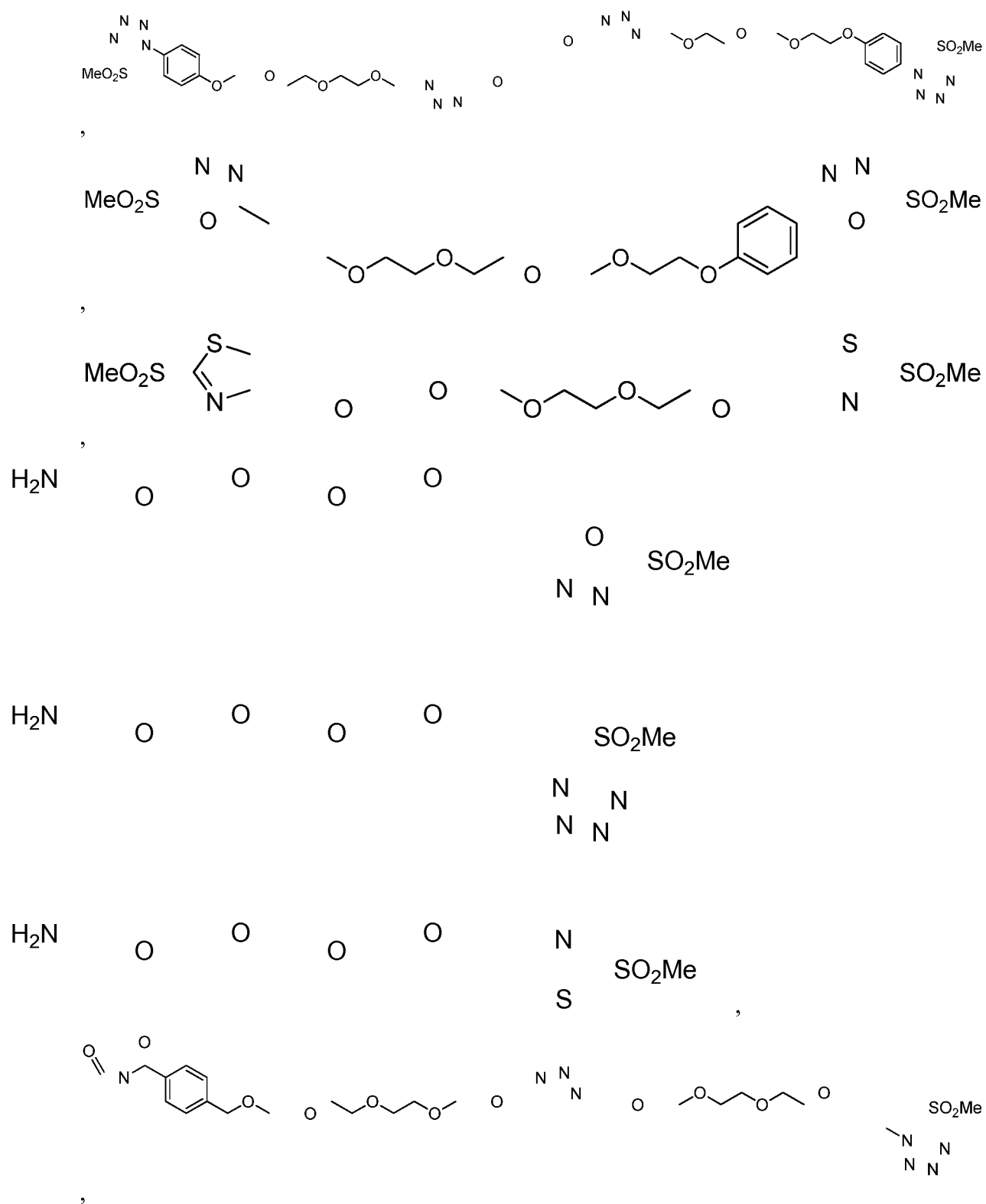
Heteroaromatic ring is selected from

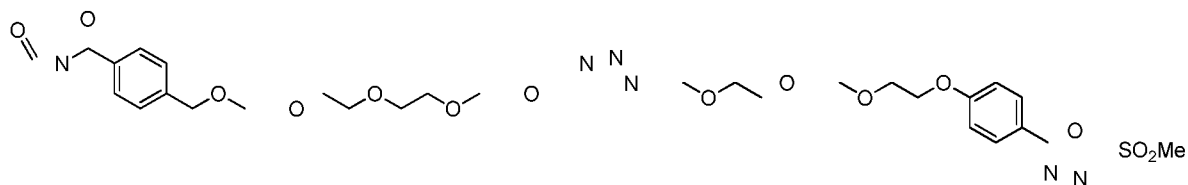


2. A compound formed by reaction of a compound of Formula I with a thiol- or selenol-containing compound, wherein the reaction is performed a) outside the body by admixture of a thiol- or selenol- containing compound or b) within a living organism, wherein the thiol- or selenol- containing compound is found in the organism.
3. Polyethylene glycol linked sulfones or sulfoxides selected from:

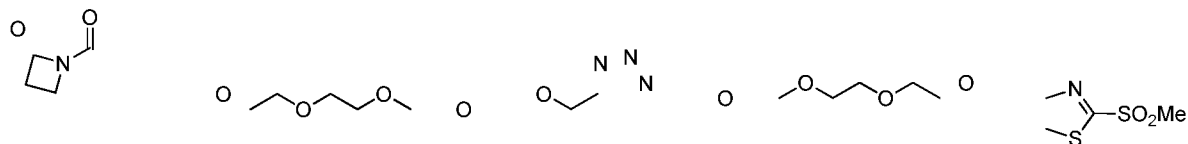




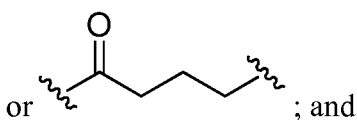




,
and

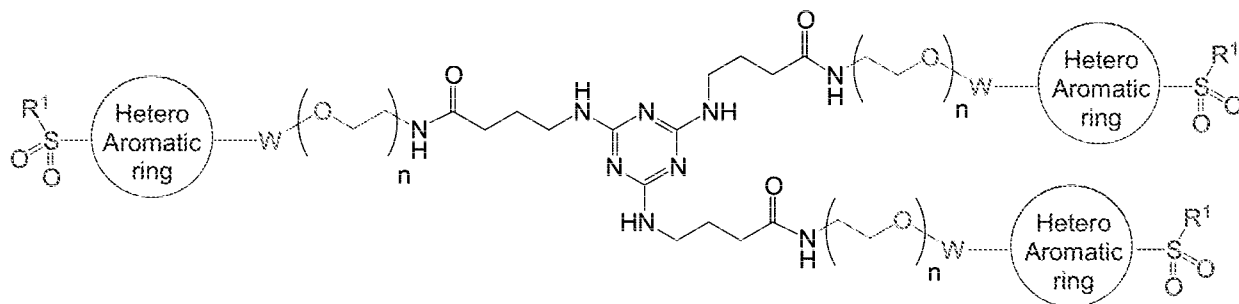


wherein linker is bond,



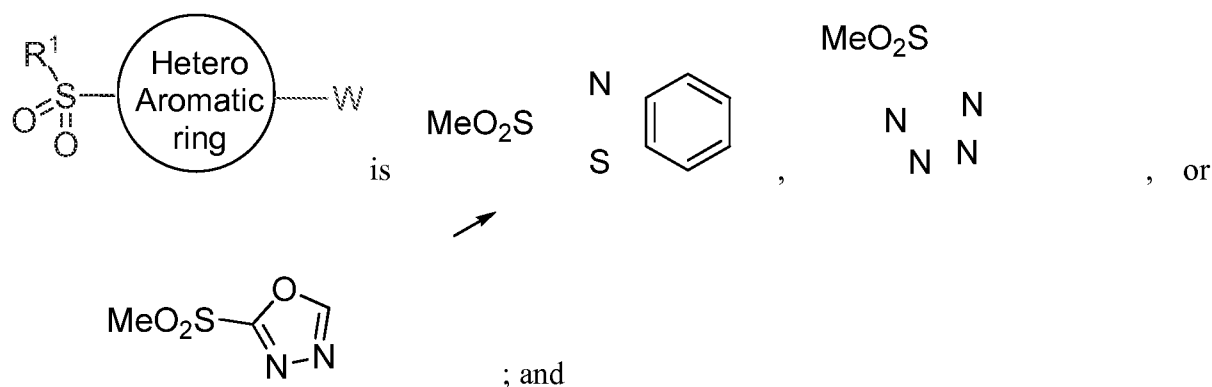
r and n are each independently an integer from 0 to 1000.

4. A compound formed by reaction of a compound of claim 3 with a thiol- or selenol-containing compound.
5. A method for conjugating two thiol containing compounds, two selenol containing compounds, or a selenol containing compound and a thiol containing compound, comprising:
 - (i) reacting a first compound containing a thiol or selenol group with a compound of Formula I of claim 1; and
 - (ii) adding the product of the reaction of (i) to a medium containing a second compound containing a thiol or selenol group.
6. The method of claim 5, wherein the second step is performed outside the body.
7. The method of claim 5, wherein the second step is performed inside the body and the second molecule is a protein.
8. The method of claim 5, wherein the second step is performed inside the body and the second molecule is albumin.
9. A compound of structural Formula II:



(II)

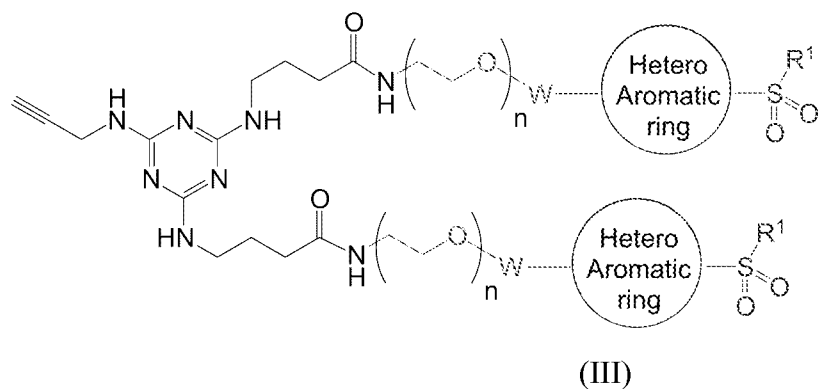
or salt thereof, wherein:



n is a integer from 1 to 1000.

10. A method for conjugating two thiol containing compounds, two selenol containing compounds, or a selenol containing compound and a thiol containing compound, comprising:
 - (i) reacting a first compound containing a thiol or selenol group with a compound of Formula II of claim 9; and
 - (ii) adding the product of the reaction of (i) to a medium containing a second compound containing a thiol or selenol group.
11. The method of claim 10, wherein the second step is performed outside the body.
12. The method of claim 10, wherein the second step is performed inside the body and the second molecule is a protein.
13. The method of claim 10, wherein the second step is performed inside the body and the second molecule is albumin.
14. The method of claim 5, wherein the first and/or second thiol or selenol-containing compounds are each independently selected from the group consisting of a) antibodies, aldolase antibodies, zybodyes, or antibody fragments, antibody Fc,

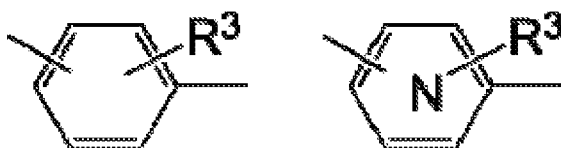
- antibodies engineered for increased half-life or effector function, scFvs, domain antibodies, diabodies, and immunoglobulin domains or variants therein engineered to possess a free thiol(s) (cysteine) or free selenol(s) (selenocysteine) residue; b) albumin or albumin fragments comprising a free thiol (cysteine) or free selenol (selenocysteine) or engineered variants of albumins or muteins with extended half-lives; c) an affibody or an engineered ankrin repeat protein; d) a nucleic acid; e) a peptide; f) an organic molecule of mw at least 200 Daltons; g) a protein; h) a polyethylene glycol chain; i) a toxin; j) FGF21 and known muteins; k) GLP-1; l) doxorubicin; m) aptamer; n) Aib2 C24 chimera lactam; o) biotin; p) a fluorescent molecule; q) aurastatin and derivatives; and r) maytansinoid and derivatives.
15. The method of claim 10, wherein the first and/or second thiol or selenol-containing compounds are each independently selected from the group consisting of a) antibodies, aldolase antibodies, zybodies, or antibody fragments, antibody Fc, antibodies engineered for increased half-life or effector function, scFvs, domain antibodies, diabodies, and immunoglobulin domains or variants therein engineered to possess a free thiol(s) (cysteine) or free selenol(s) (selenocysteine) residue; b) albumin or albumin fragments comprising a free thiol (cysteine) or free selenol (selenocysteine) or engineered variants of albumins or muteins with extended half-lives; c) an affibody or an engineered ankrin repeat protein; d) a nucleic acid; e) a peptide; f) an organic molecule of mw at least 200 Daltons; g) a protein; h) a polyethylene glycol chain; i) a toxin; j) FGF21 and known muteins; k) GLP-1; l) doxorubicin; m) aptamer; n) Aib2 C24 chimera lactam; o) biotin; p) a fluorescent molecule; q) aurastatin and derivatives; and r) maytansinoid and derivatives.
16. An antibody or antibody fragment or immunoglobulin domain drug conjugate prepared by the method of claims 5 or 10.
17. A bi-, tri-, tetra-, penta-, or hexa- specific antibody prepared by the method of claims 5 or 10.
18. A modified albumin, albumin mutein, or albumin fragment linked to one or more molecules prepared by the method of claims 5 or 10.
19. An intermediate of structural Formula III:



or salt thereof, wherein:

n is an integer from 1 to 1000;

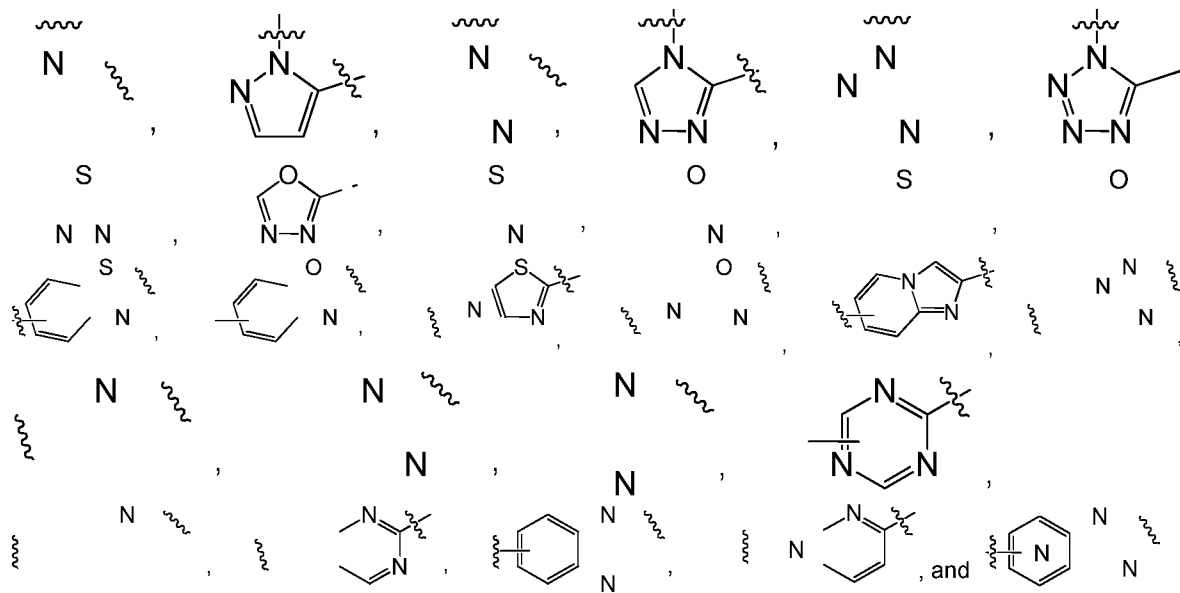
W is selected from bond, hydrogen, and



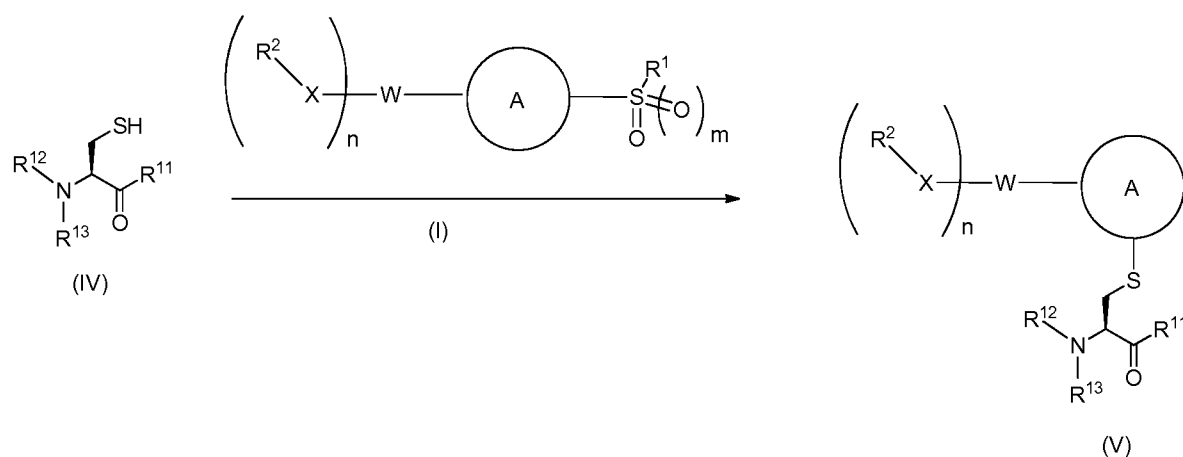
;

R¹ is selected from substituted or unsubstituted alkyl or substituted and unsubstituted aryl; and

Heteroaromatic ring is selected from

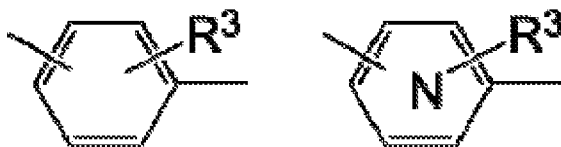


20. A method of chemoselectively modifying a moiety containing the amino acid cysteine, comprising reacting a compound of Formula I with a compound of Formula IV to produce a compound of Formula V, thereby modifying the moiety containing the amino acid cysteine:



wherein:

W is selected from bond, hydrogen, and



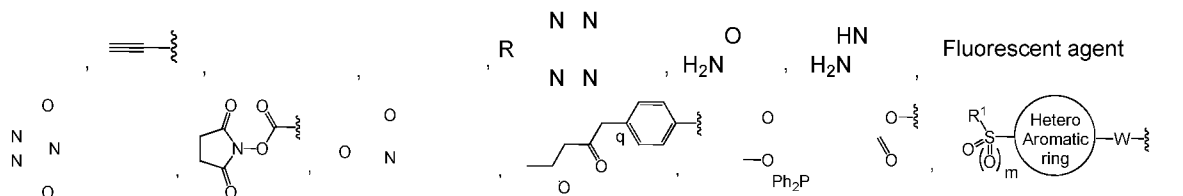
X is a linear or branched connecting chain of atoms comprising any of C, H, O, N, P, S, Si, F, Cl, Br, or I, or a salt thereof;

m is 0 or 1;

n is an integer from 0 to 5;

R¹ is selected from substituted or unsubstituted alkyl or substituted and unsubstituted aryl;

R² is selected from unsubstituted alkyl, substituted or unsubstituted aryl, (-OCH₂CH₂)_p, -CO₂H, -NH₂, OH, -N₃,



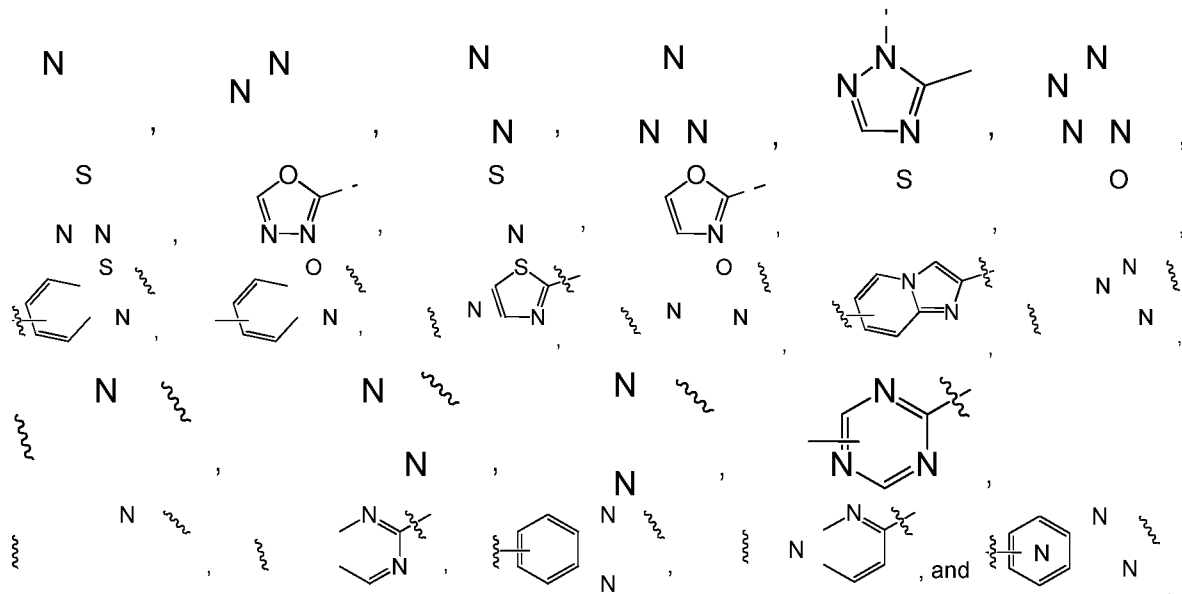
Anti-cancer agent , Anti-HIV agent , Anti-Flu agent , Cell targeting molecule , Radio isotope group

Peptide , Protein , Antibody , siRNA , miRNA , DNA— $\begin{matrix} \text{---} \\ \text{---} \\ \text{---} \end{matrix}$,and PEG chain (> 5k Da) ;

p is an integer from 1 to 1000;

R³ is selected from hydrogen, halogen, -CO₂H, -NH₂, OH, -N₃, and -alkyne; and

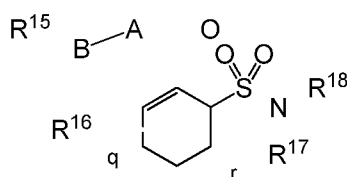
A is selected from



R¹², R¹³, and R¹⁴ are each independently hydrogen, hydroxyl, amino, substituted or unsubstituted alkyl, substituted or unsubstituted thioalkyl, perfluoroalkyl, substituted or unsubstituted alkoxy, substituted or unsubstituted aryl, substituted or unsubstituted aryloxy, substituted or unsubstituted heteroaryl, substituted or unsubstituted heteroaryloxy, substituted or unsubstituted alkylaryl, substituted or unsubstituted alkylheteroaryl, or R¹², R¹³, and R¹⁴ are in a cysteine residue of a peptide or a protein or a thiol group on an organic molecule.

21. The method of claim 20, wherein reaction occurs in an aqueous media at a pH between 2 and 10.
22. The method of claim 21, wherein the aqueous media is a phosphate buffer at about a pH of 7.4.

23. The method of claim 20, wherein the reaction occurs in a mixed organic/aqueous media.
24. A compound of Formula IV:



(IV)

or salt thereof, wherein:

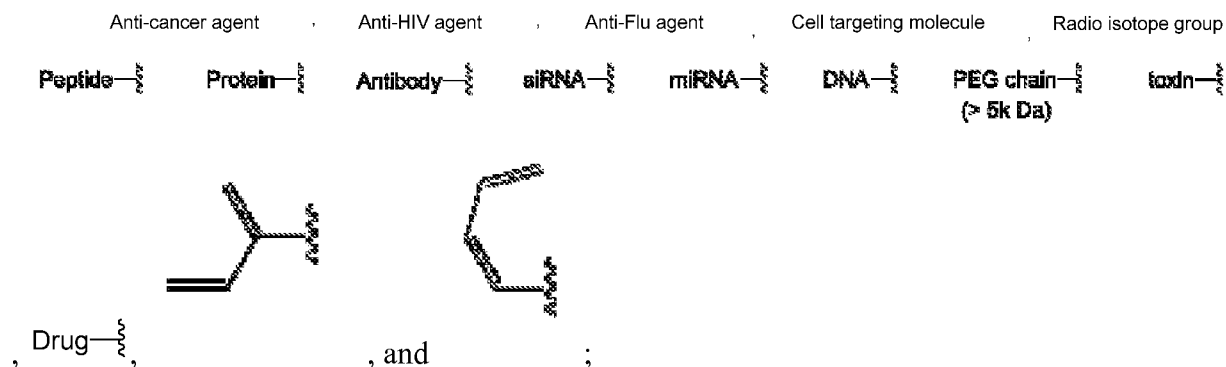
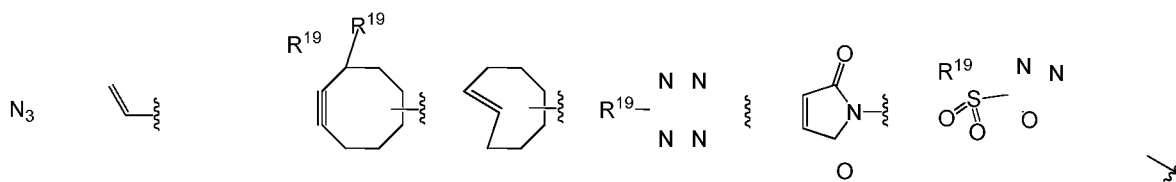
q is an integer from 0 to 5;

r is an integer from 0 to 3;

A is O or CH₂;

B is aryl, heteroaryl, or a linear or branched connecting chain of atoms comprising any of C, H, O, N, P, S, Si, F, Cl, Br, and I or a salt thereof;

R¹⁵ is selected from substituted or unsubstituted alkyl, substituted or unsubstituted aryl, (-OCH₂CH₂)_p, (-OCH₂CH₂)_p-N₃, -CO₂H, -NH₂, OH, -N₃,



p is an integer from 1 to 1000;

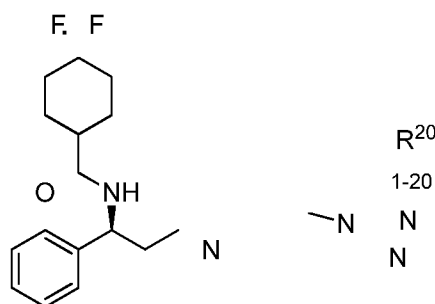
R¹⁶ is H, C₁₋₅alkyl or F;

R¹⁷ is H or C₁₋₅alkyl;

R¹⁸ is substituted or unsubstituted aryl and heteroaryl; and

R¹⁹ is H, F, substituted or unsubstituted C₁₋₅alkyl, aryl or heteroaryl.

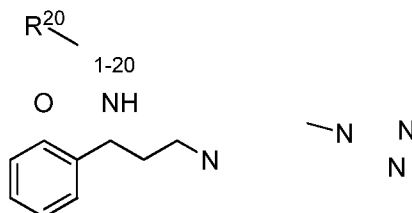
25. A compound formed by reaction of a compound of Formula IV with an amino group-containing compound, wherein the reaction is performed a) outside the body by admixture of an amino group-containing compound or b) within a living organism, wherein the amino group-containing compound is found in the organism.
26. The compound of claim 25, wherein the amino group-containing compound is a protein.
27. The compound of claim 26, wherein the protein is human serum albumin (HSA).
28. The compound of claim 25, wherein the anti-HIV agent is a CCR5 or CXCR4 antagonist.
29. The compound of claim 28, wherein the CCR5 antagonist is Maraviroc.
30. The compound of claim 28, wherein the CXCR4 antagonist is GSK812397.
31. The compound of claim 1, wherein the anti-HIV agent is a CCR5 or CXCR4 antagonist.
32. The compound of claim 31, wherein the CCR5 antagonist is Maraviroc.
33. The compound of claim 31, wherein the CXCR4 antagonist is GSK812397.
34. A compound of structural Formula V:



or salt thereof, wherein:

R²⁰ is (CH₂)₁₀N₃, (CH₂)₁₀N₃, 4,4-difluoro-cycloHx, -(OCH₂CH₂)_p-, -N₃, -(OCH₂CH₂)_p-N₃, a compound of formula I, a compound of formula IV, an antibody, or a protein; and p is an integer from 0 to 1000.

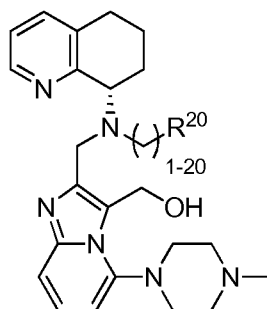
35. A compound of structural Formula VI:



or salt thereof, wherein:

R²⁰ is (CH₂)₁₀N₃, (CH₂)₁₀N₃, 4,4-difluoro-cycloHx, -(OCH₂CH₂)_p-, -N₃, -(OCH₂CH₂)_p-N₃, a compound of formula I, a compound of formula IV, an antibody, or a protein; and p is an integer from 0 to 1000.

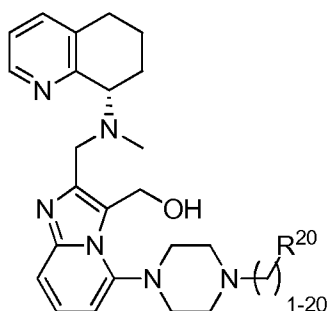
36. A compound of structural Formula VII:



or salt thereof, wherein:

R²⁰ is (CH₂)₁₀N₃, (CH₂)₁₀N₃, 4,4-difluoro-cycloHx, -(OCH₂CH₂)_p-, -N₃, -(OCH₂CH₂)_p-N₃, a compound of formula I, a compound of formula IV, an antibody, or a protein; and p is an integer from 0 to 1000.

37. A compound of structural Formula VIII:

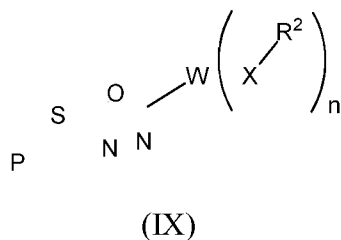


VIII

or salt thereof, wherein:

R^{20} is $(CH_2)_{10}N_3$, $(CH_2)_{10}N_3$, 4,4-difluoro-cycloHx, $-(OCH_2CH_2)_p-$, $-N_3$, $-(OCH_2CH_2)_p-N_3$, a compound of formula I, a compound of formula IV, an antibody, or a protein; and p is an integer from 0 to 1000.

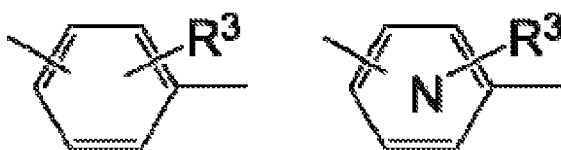
38. A compound of structural Formula IX:



or salt thereof, wherein:

P is protein, peptide, nucleic acid, or other molecule;

W is selected from bond, hydrogen, and



;

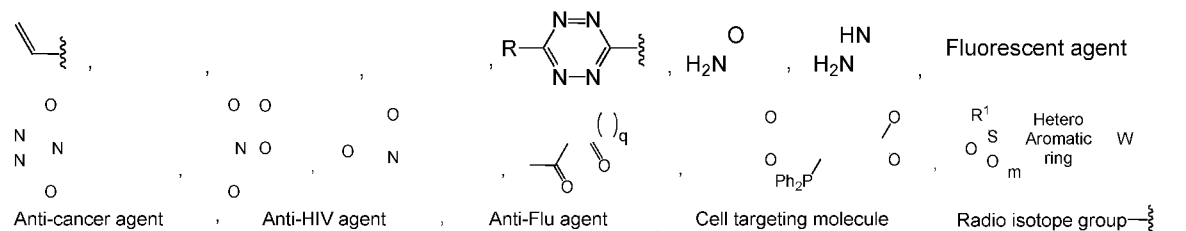
X is a linear or branched connecting chain of atoms comprising any of C, H, O, N, P, S, Si, F, Cl, Br, or I, or a salt thereof;

m is 0 or 1;

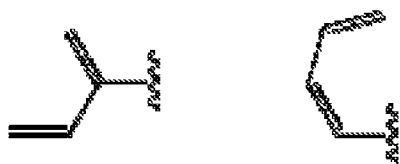
n is an integer from 0 to 5;

R^1 is selected from substituted or unsubstituted alkyl or substituted and unsubstituted aryl;

R^2 is selected from substituted or unsubstituted alkyl, substituted or unsubstituted aryl, $-(OCH_2CH_2)_p-$, $-CO_2H$, $-NH_2$, OH , $-N_3$,



Peptide— —R^1 Protein— —R^1 Antibody— —R^1 siRNA— —R^1 miRNA— —R^1 DNA— —R^1 PEG chain— —R^1
 (> 5k Da) toxin— —R^1

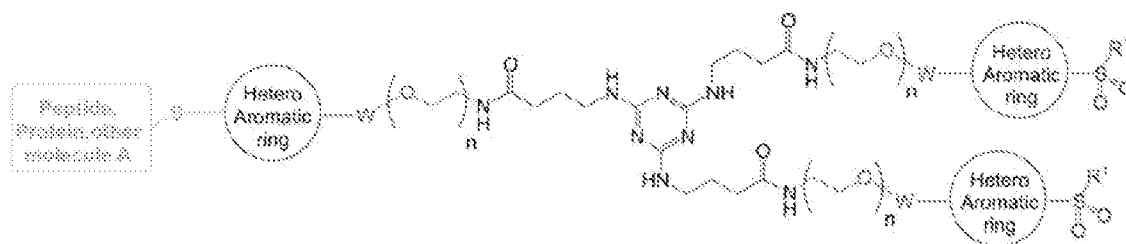


, and ;

p is an integer from 1 to 1000; and

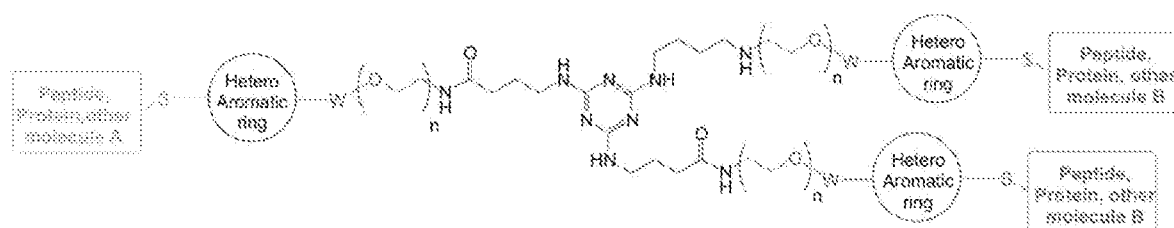
R^3 is selected from hydrogen, halogen, $-\text{CO}_2\text{H}$, $-\text{NH}_2$, OH , $-\text{N}_3$, and $-\text{alkyne}$.

39. A compound of structural formulas X or XI:



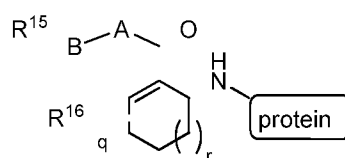
or

(X)



(XI).

40. A compound of structural formula XII:



(XII)

or salt thereof, wherein:

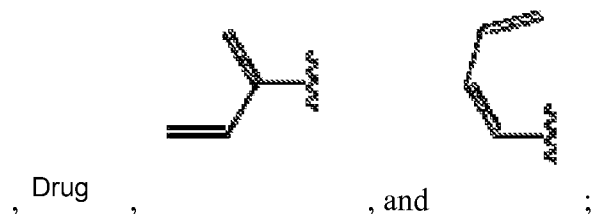
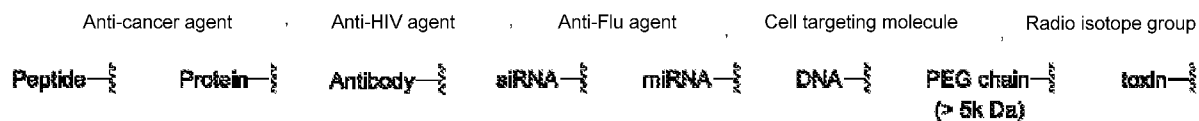
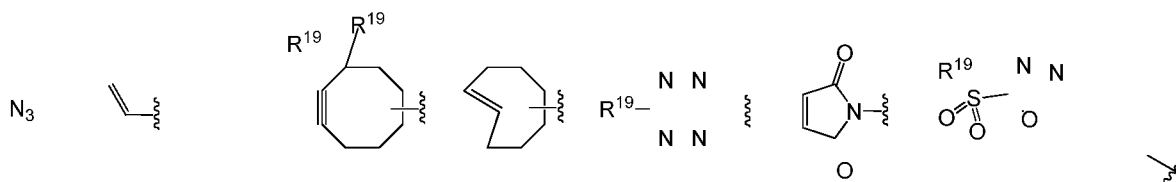
q is an integer from 0 to 5;

r is an integer from 0 to 3;

A is O or CH₂;

B is aryl, heteroaryl, or a linear or branched connecting chain of atoms comprising any of C, H, O, N, P, S, Si, F, Cl, Br, and I or a salt thereof;

R¹⁵ is selected from substituted or unsubstituted alkyl, substituted or unsubstituted aryl, (-OCH₂CH₂)_p, (-OCH₂CH₂)_p-N₃, -CO₂H, -NH₂, OH, -N₃,

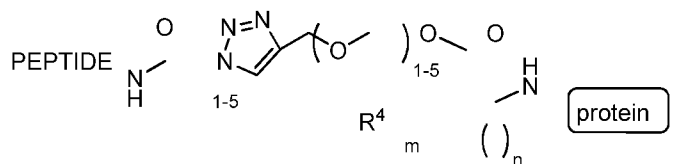


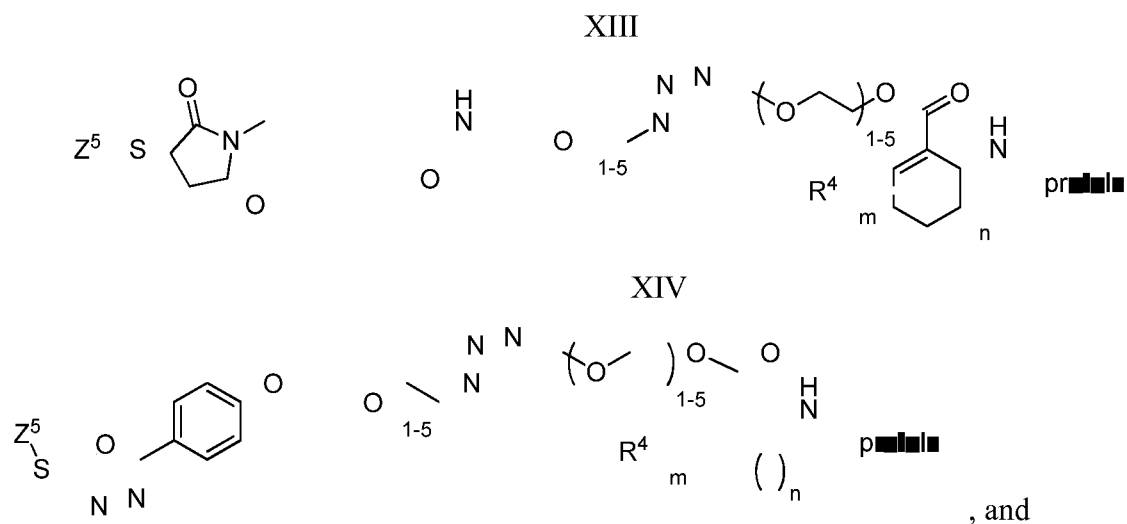
p is an integer from 1 to 1000;

R¹⁶ is H, C₁₋₅alkyl or F; and

R¹⁹ is H, F, substituted or unsubstituted C₁₋₅alkyl, aryl or heteroaryl.

41. A compound selected from the group consisting of structural formulas XIII, XIV and XV:

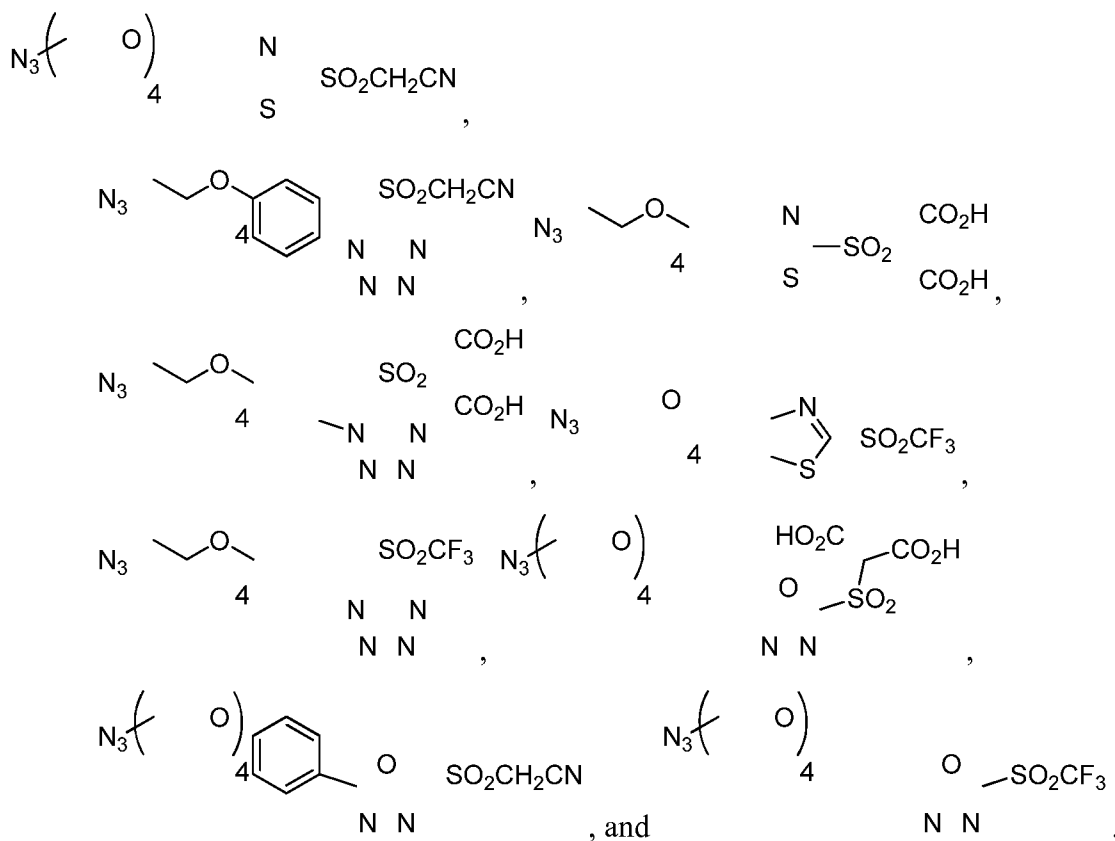




or salt thereof, wherein:

Z⁵ is protein, peptide, nucleic acid, or other molecule.

42. The compound of claim 1, wherein the compound is selected from the group consisting of



1/35

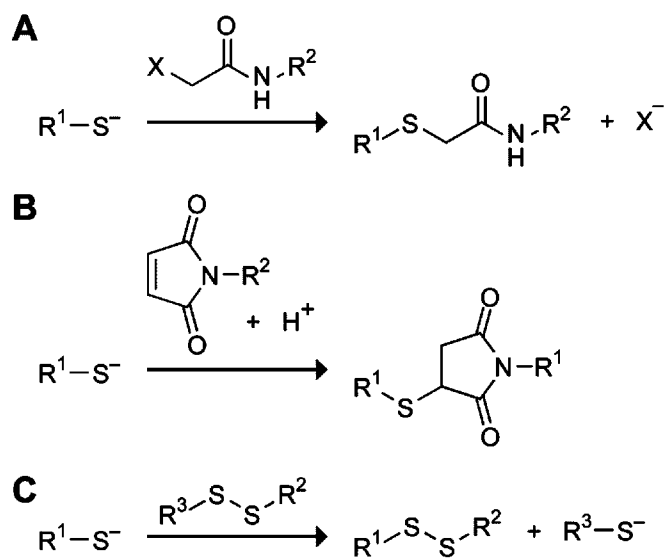


FIG. 1

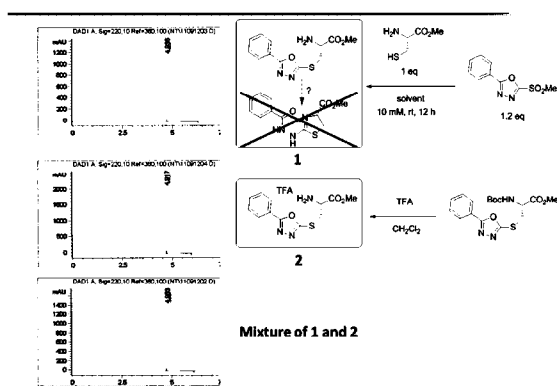


FIG. 2

2/35

Compounds (100ug/mL) were incubated in human plasma at 37°C for 72 h.

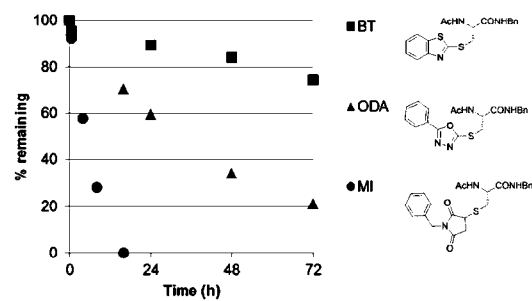


FIG. 3

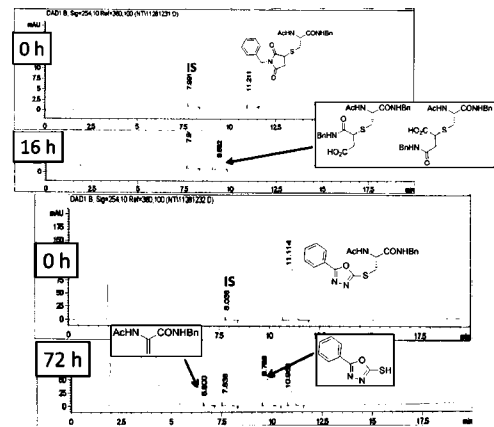


FIG. 4

3/35

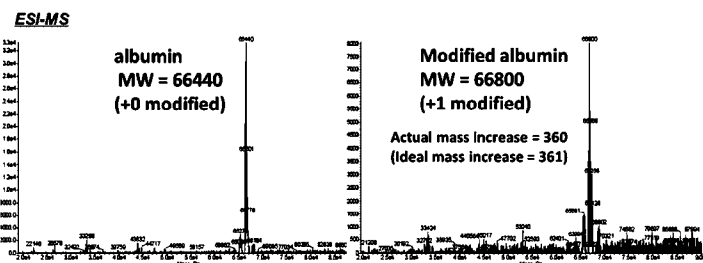


FIG. 5

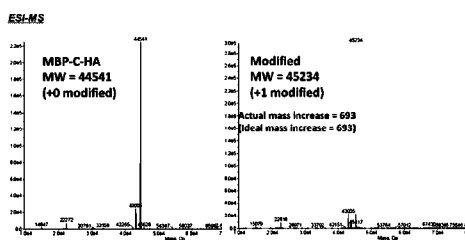


FIG. 6

Compounds (100 µg/mL) were incubated in human plasma at 37°C for 72 h

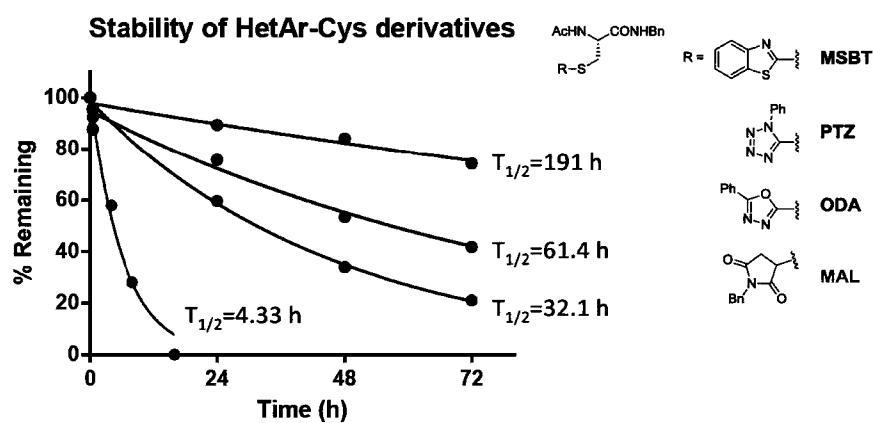


FIG. 7

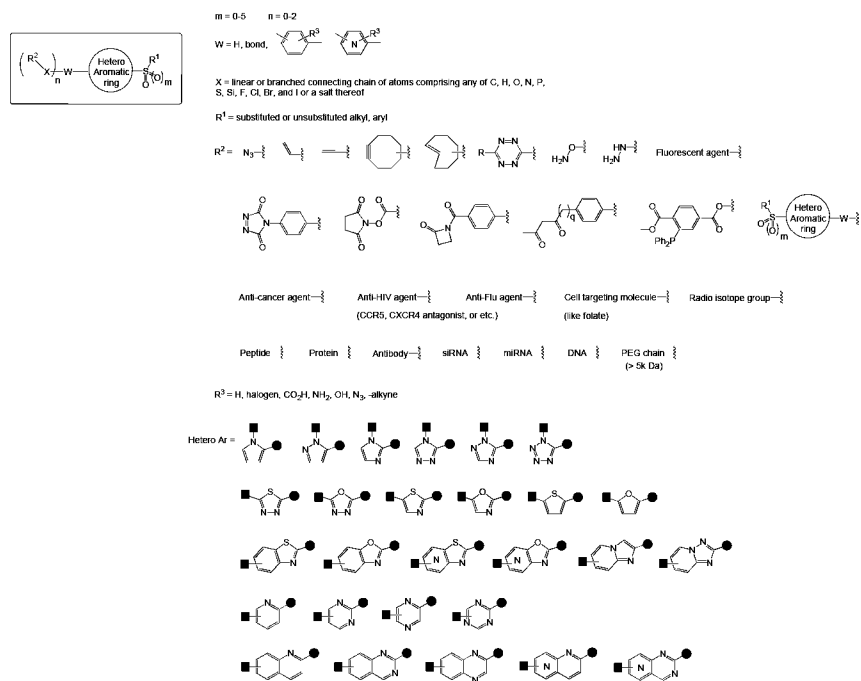


FIG. 8

5/35

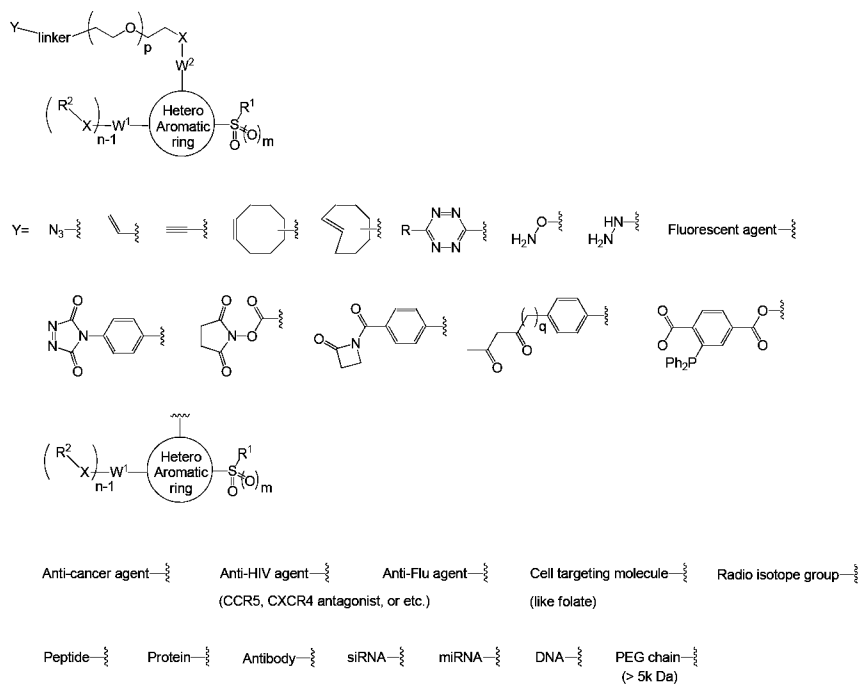


FIG. 9

6/35

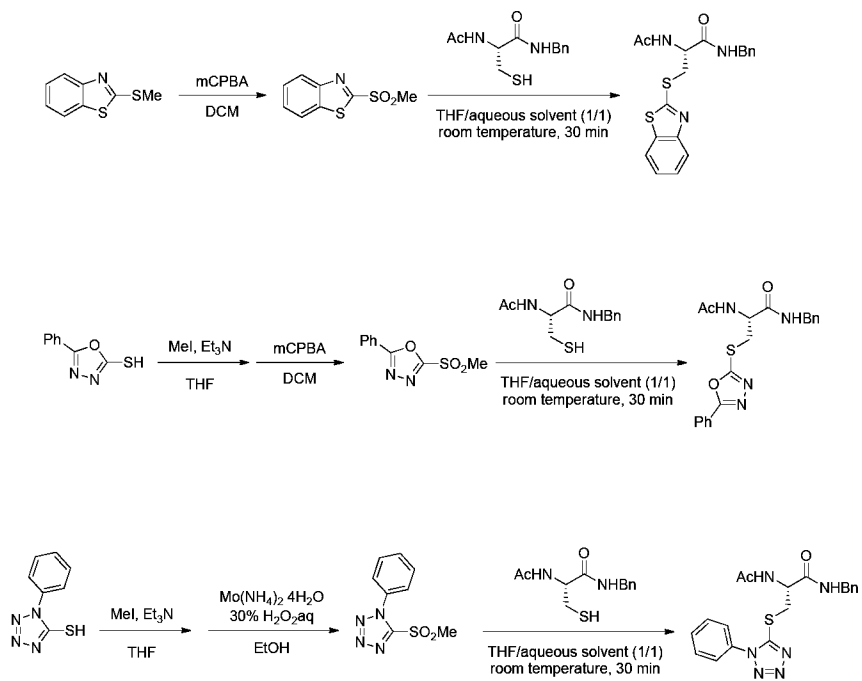
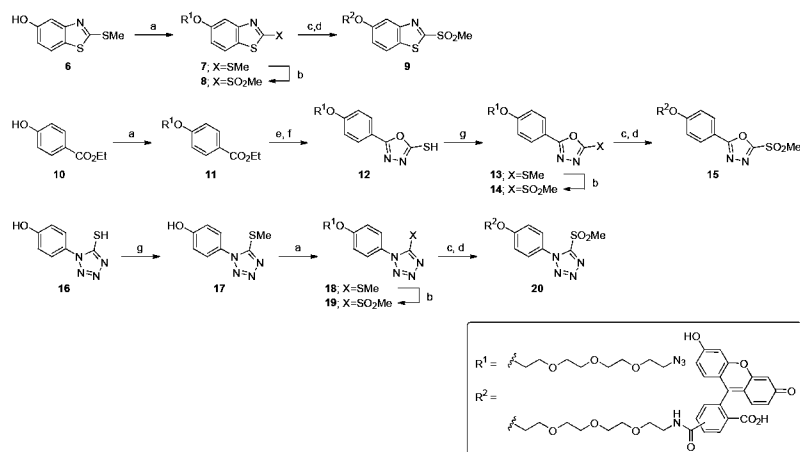


FIG. 10

7/35



a Reagents and Conditions: (a) R1-I (24), K₂CO₃, N, N-dimethylformamide; (b) mCPBA, CH₂Cl₂; (c) triphenylphosphine, H₂O, tetrahydrofuran; (d) NHS-5(6)-fluorescein, DMSO; (e) hydrazine monohydrate, C₂H₅OH; (f) carbon disulfide, KOH, C₂H₅OH; (g) CH₃I, triethylamine, tetrahydrofuran; (h) trifluoroacetic acid, CH₂Cl₂; (i) Sulfo-SMCCR, triethylamine, N, N-dimethylformamide.

FIG. 11

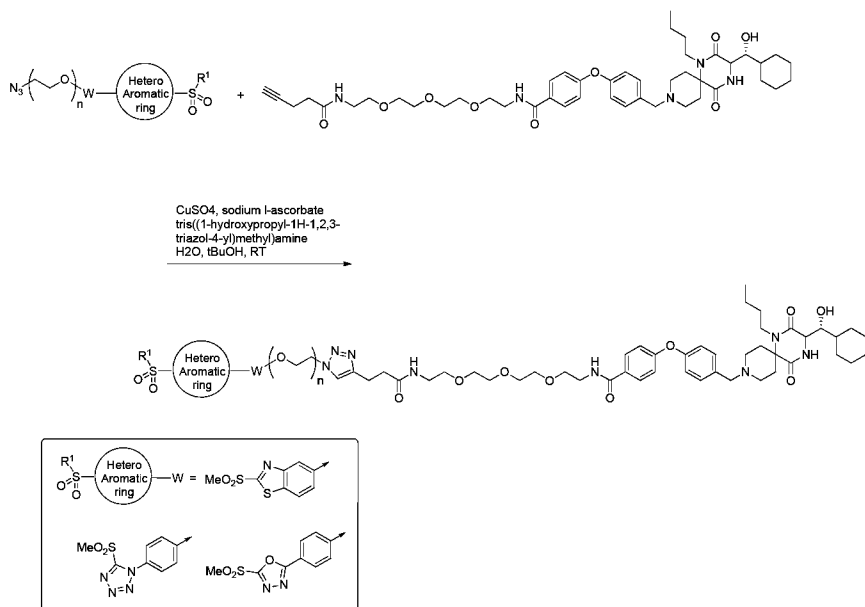


FIG. 12

8/35

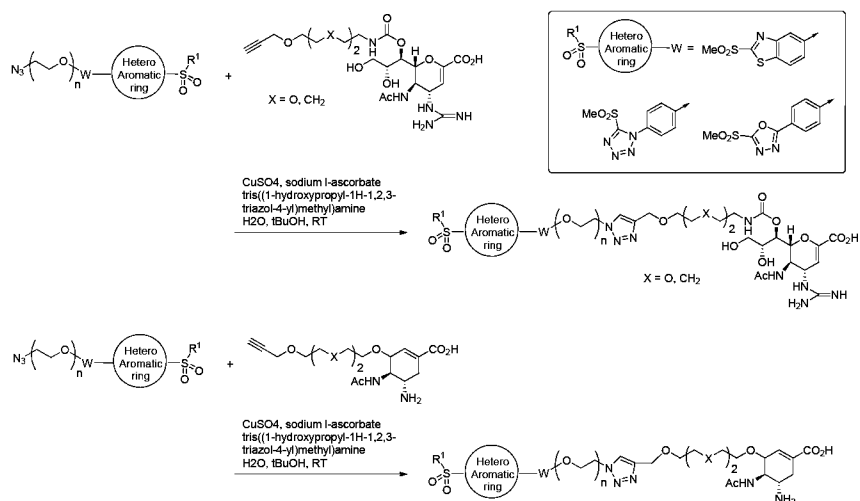


FIG. 13

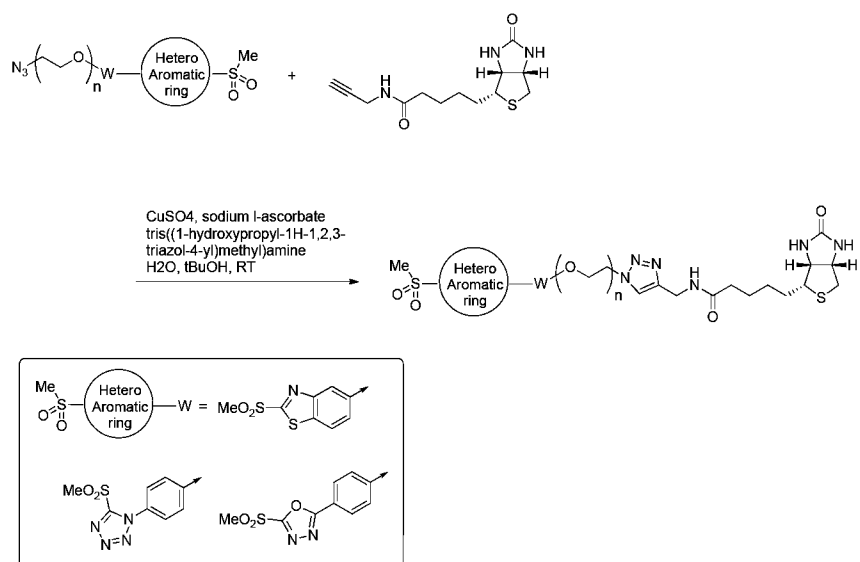


FIG. 14

9/35

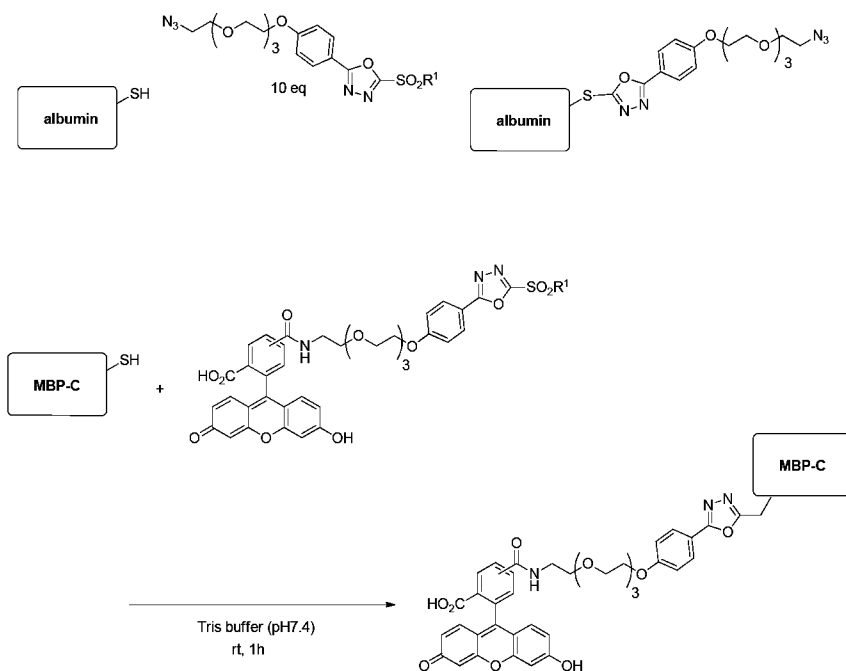
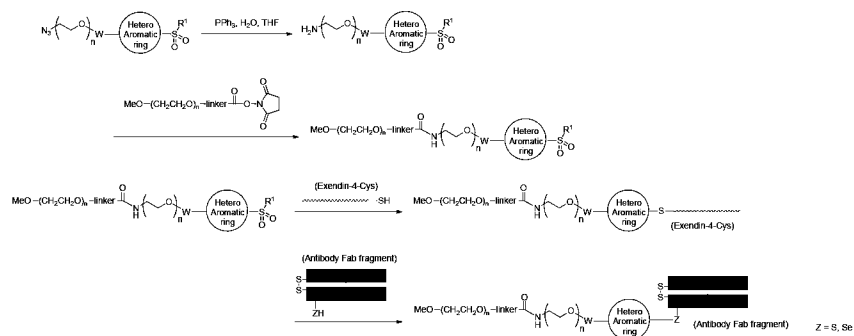


FIG. 15



Reference : THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 36, pp. 25599-25614, August 17, 2012
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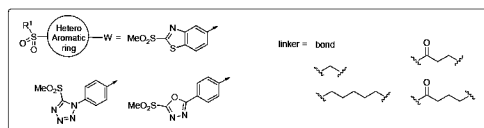


FIG. 16

10/35

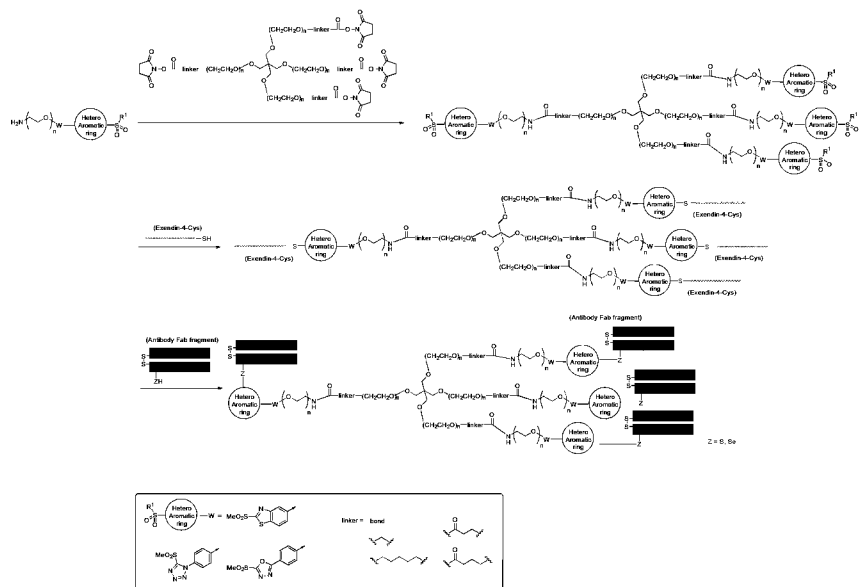


FIG. 17

11/35

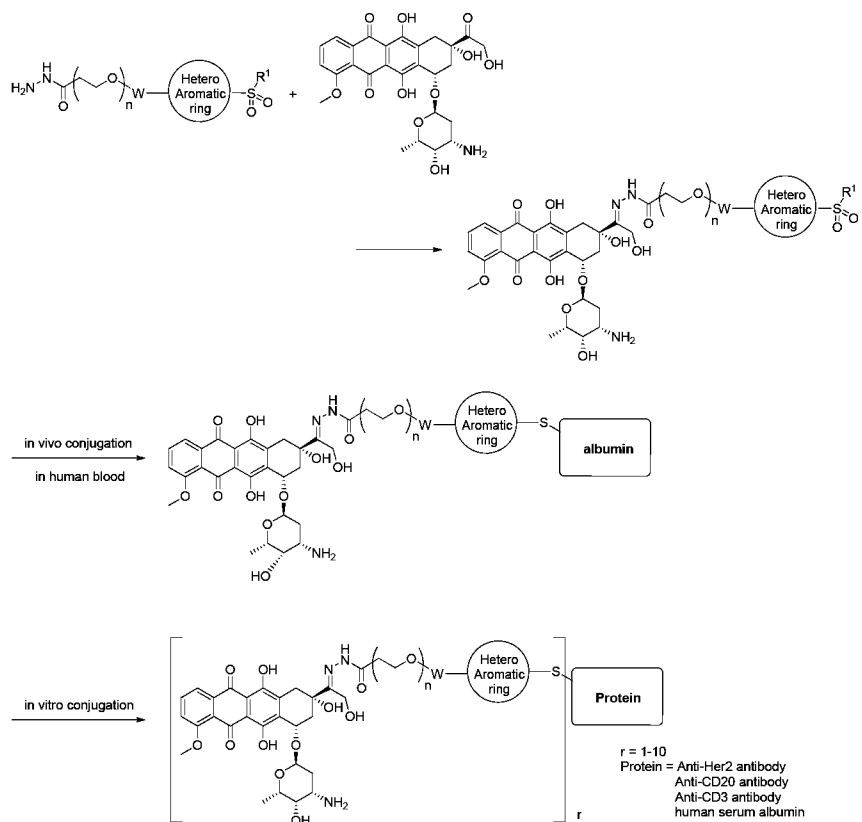


FIG. 18

12/35

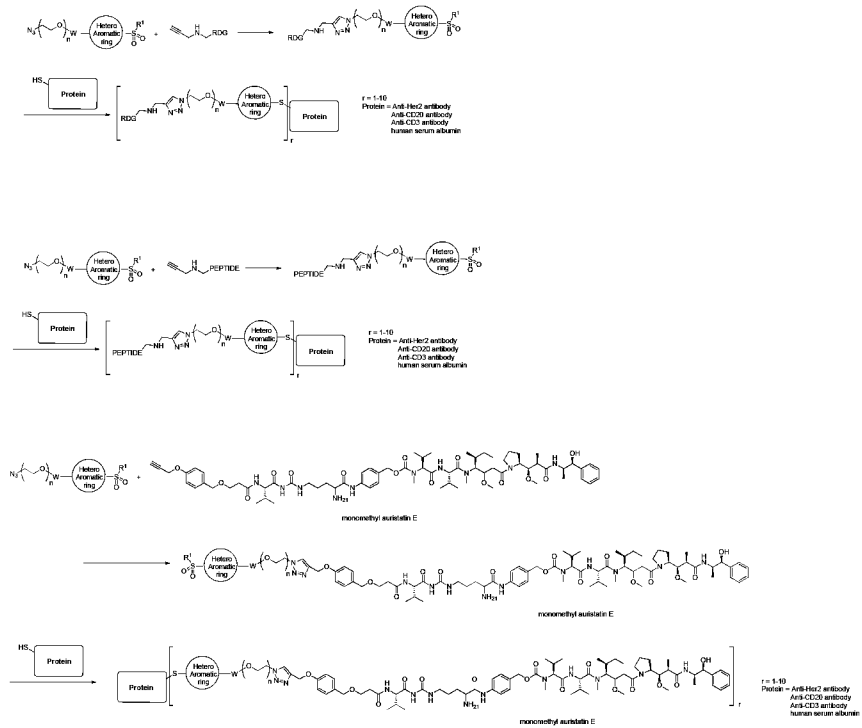


FIG. 19

13/35

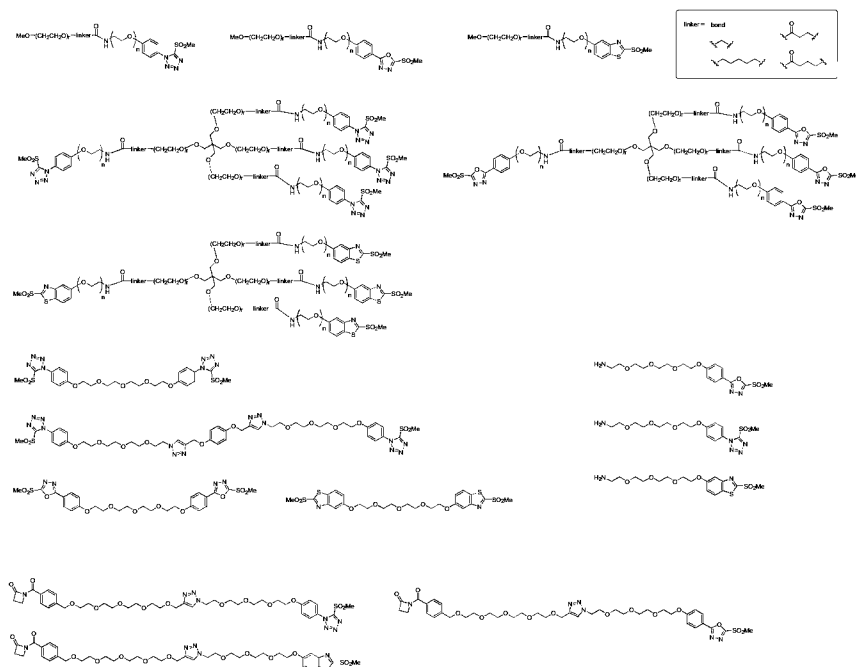


FIG. 20

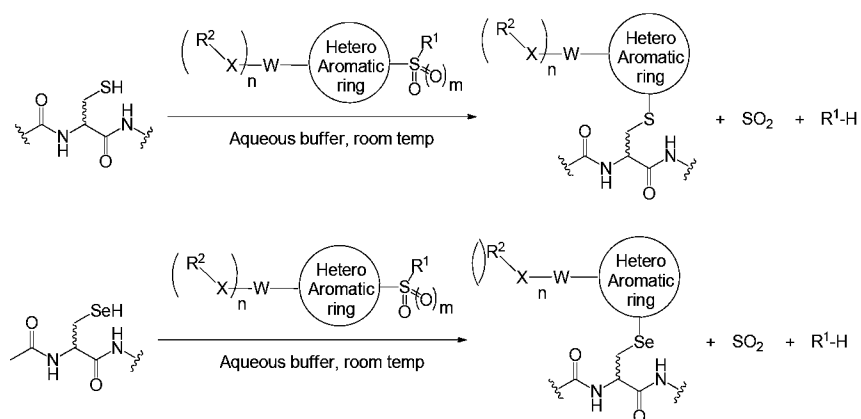


FIG. 21

14/35

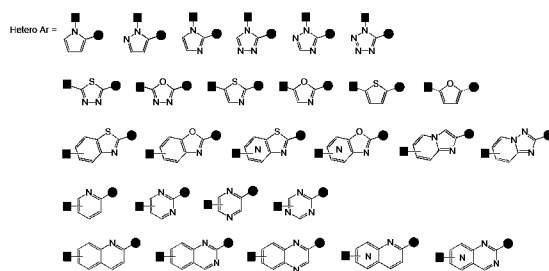
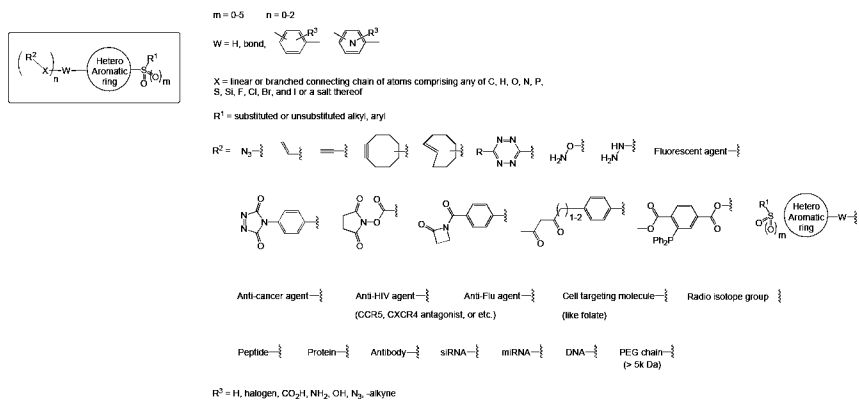


FIG. 22

15/35

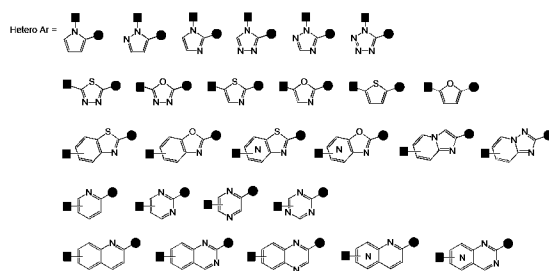
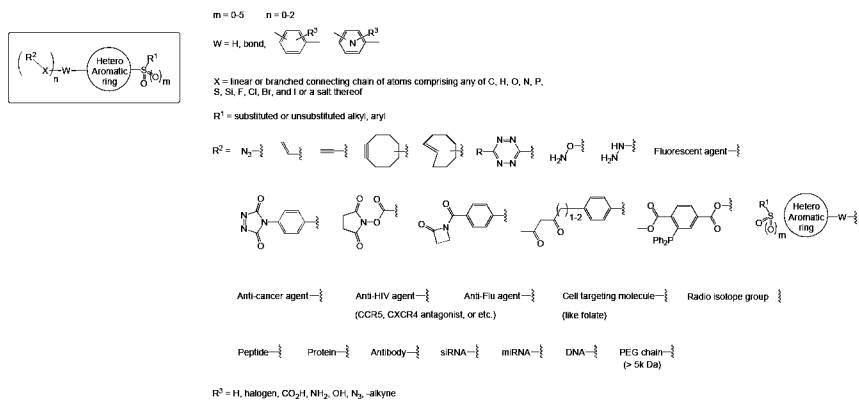


FIG. 23

[illegible]

FIG. 24

17/35

Typical application

PEGylat

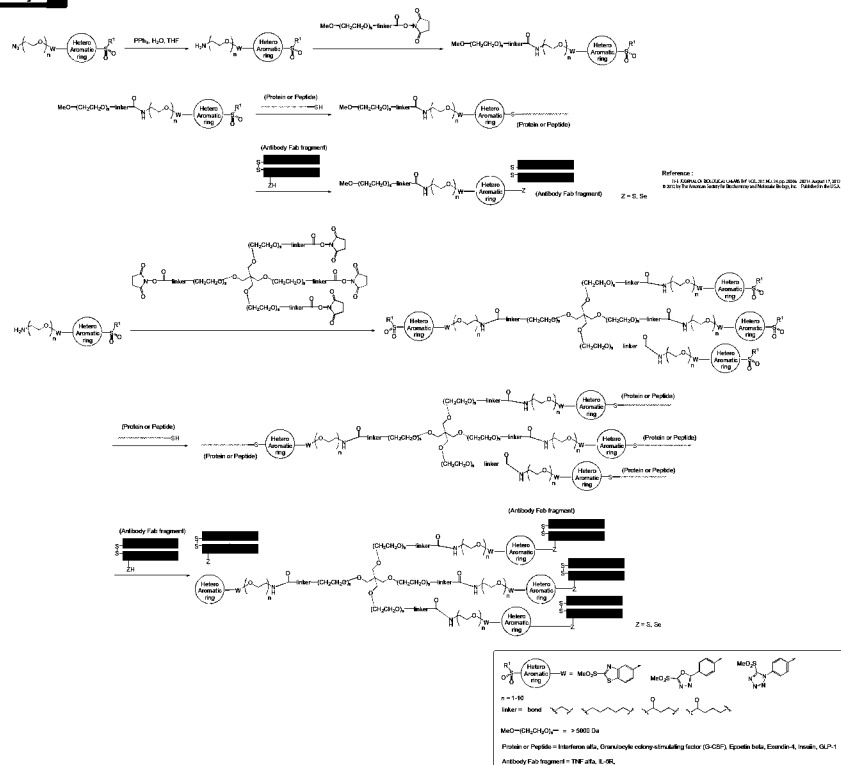


FIG. 25

Disfunctional come

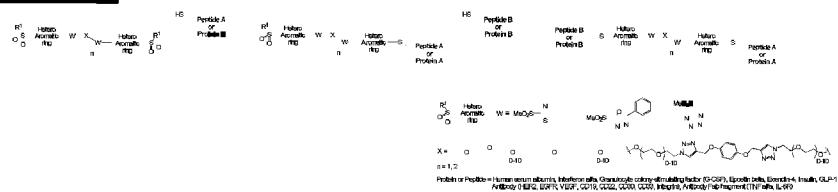


FIG. 26

18/35

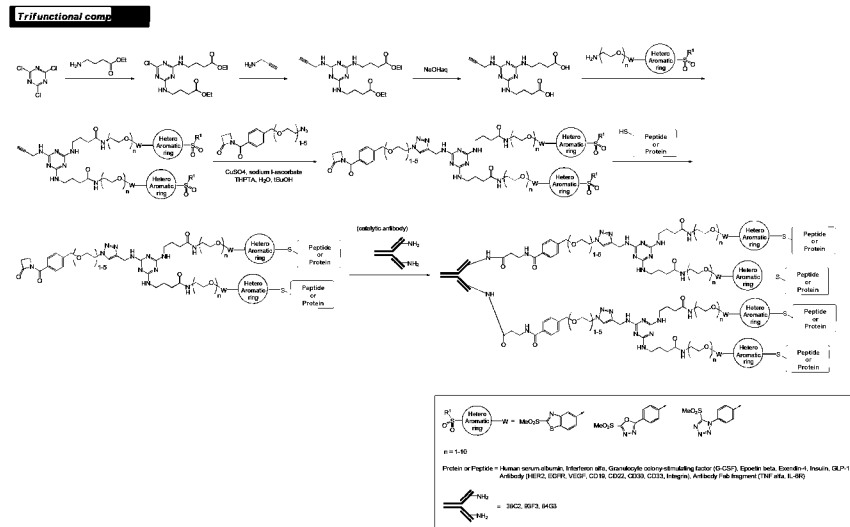


FIG. 27

19/35

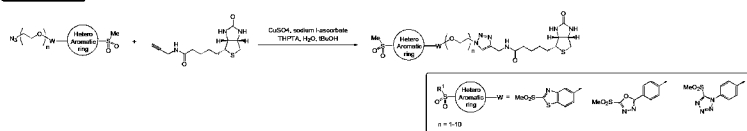
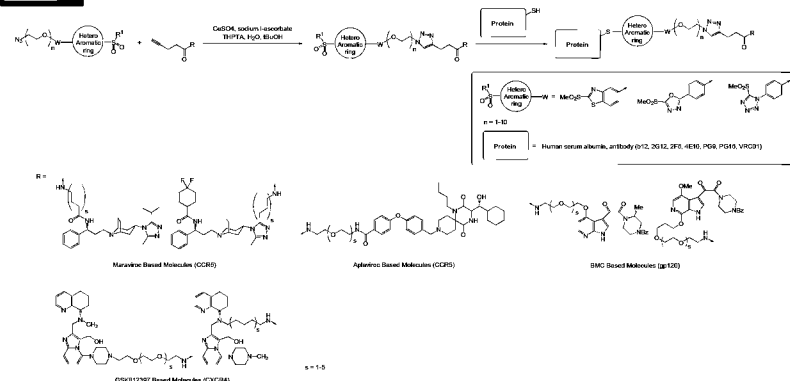
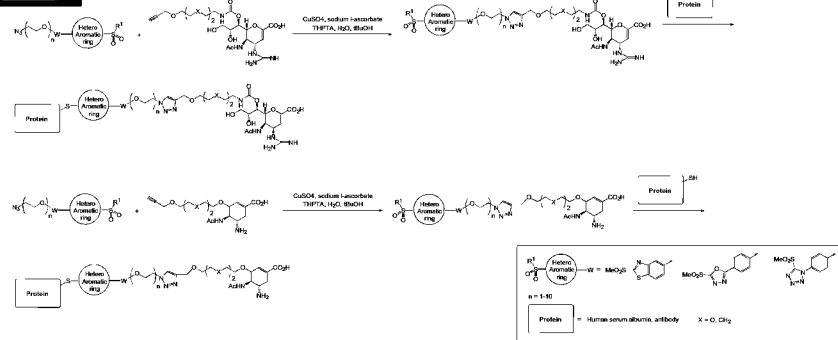
Typical application**Biotinylated****HIV ther****Flu ther**

FIG. 28

20/35

Typical application

Cancer ther

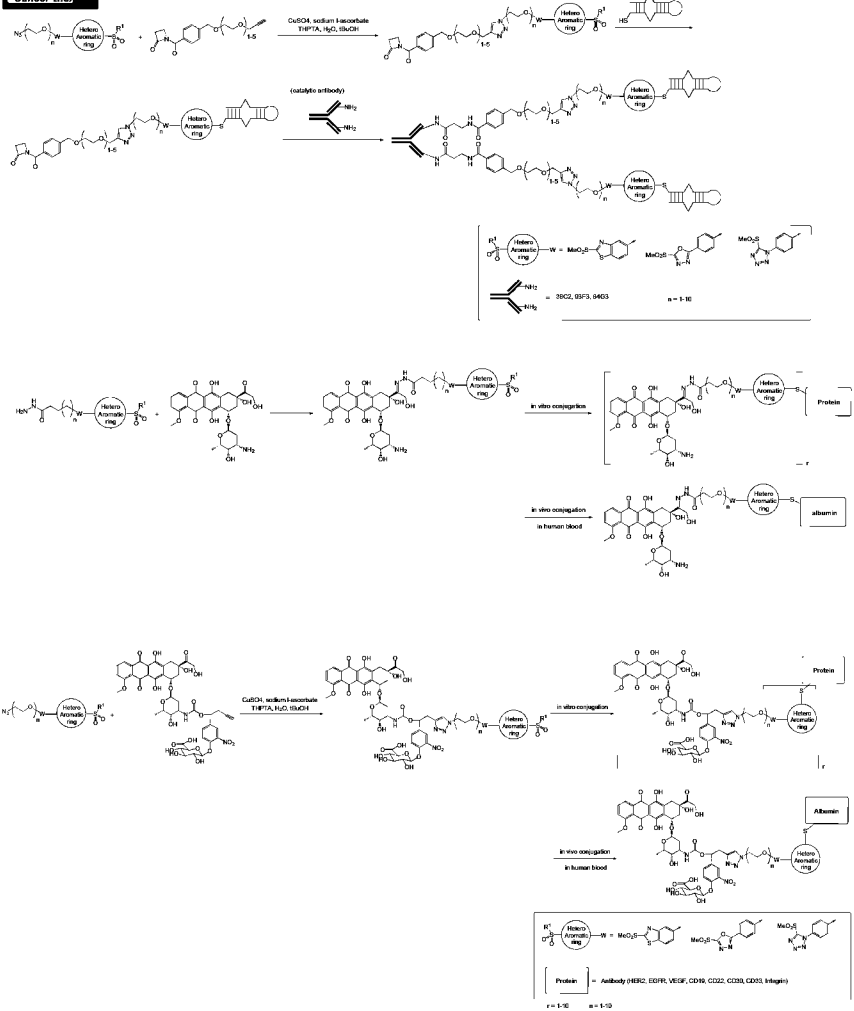


FIG. 29

21/35

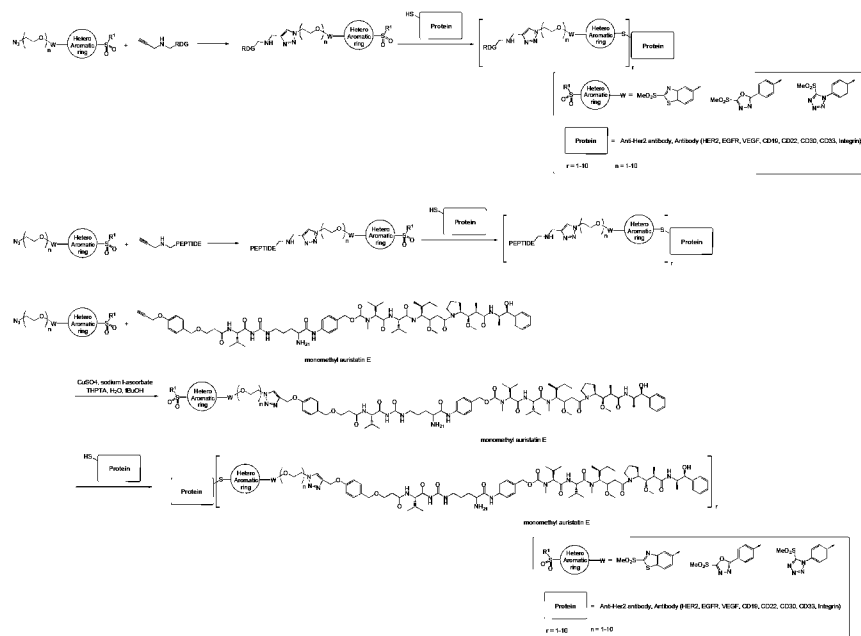
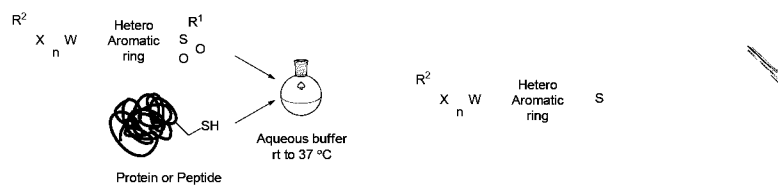


FIG. 30

22/35

in vitro conjugation



in vivo conjugation

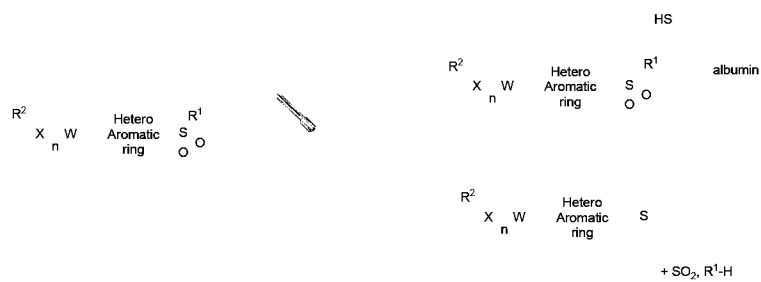


FIG. 31

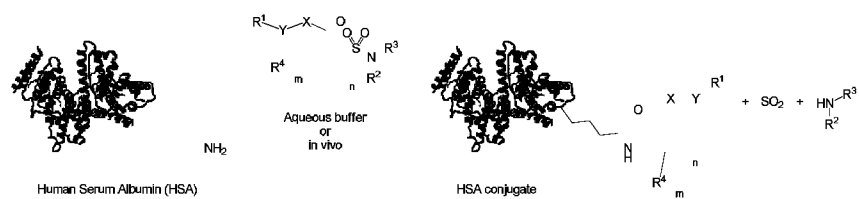
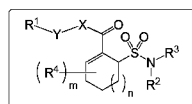


FIG. 32

23/35

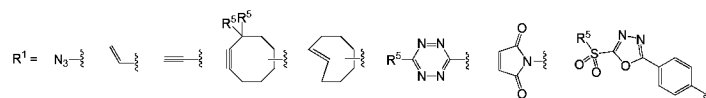


m = 0-5

n = 0-3

X = O, CH₂

Y = aryl or heteroaryl (un)included linear or branched connecting chain of atoms comprising any of C, H, O, N, P, S, Si, F, Cl, Br, and I or a salt thereof



Fluorescent agent—

Biotin—

Radio isotope group—

Anti-cancer agent—

Anti-HIV agent—

Anti-Flu agent—

Cell targeting molecule—

(CCR5, CXCR4 antagonist, or etc.)

(like folate)

Peptide—

Protein—

Antibody—

siRNA—

miRNA—

DNA—

PEG chain—

(> 5k Da)

R² = H, C₁₋₅ alkylR³ = substituted or unsubstituted aryl and heteroarylR⁴ = H, C₁₋₅ alkyl, FR⁵ = H, F, (un)substituted C₁₋₅ alkyl or phenyl or heteroaryl

Aryl = phenyl, naphthalenyl

Heteroaryl = diazolyl, triazolyl, tetrazolyl, thiazolyl, oxazolyl, pyridyl, pyrimidyl, triazinyl

FIG. 33

24/35

<General synthetic methods>

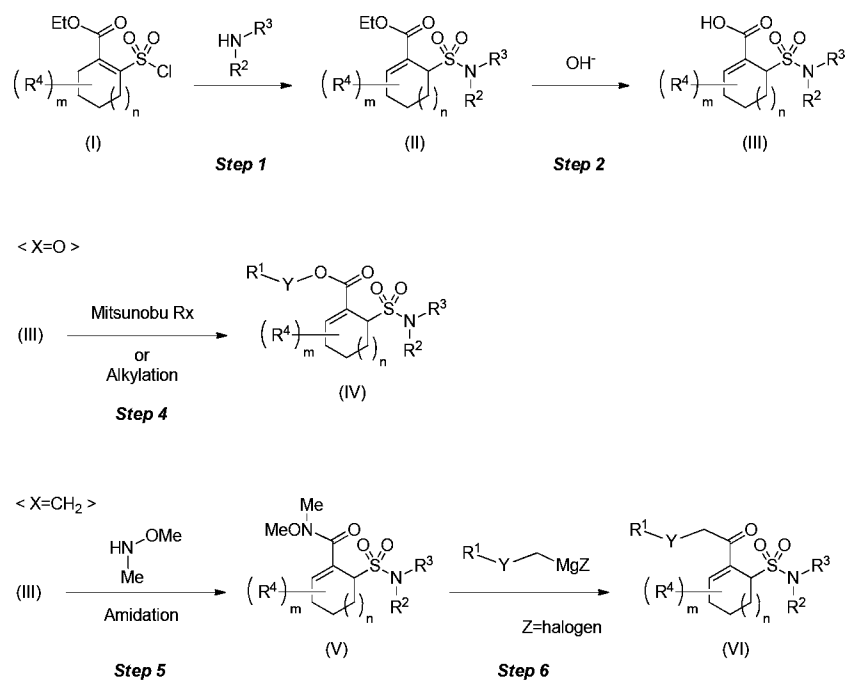


FIG. 34

25/35

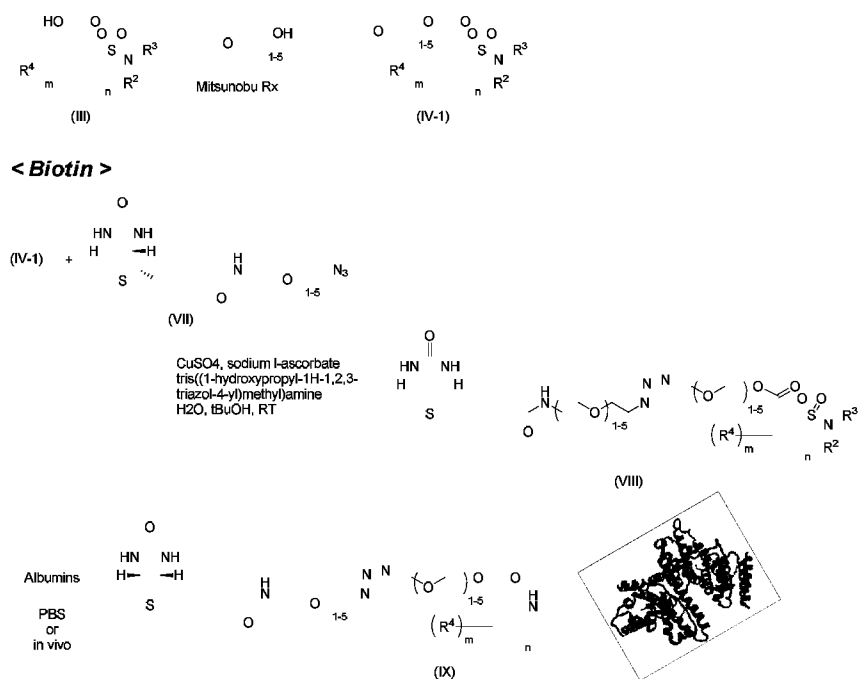


FIG. 35

< Fluorescein (Rhodamine) >

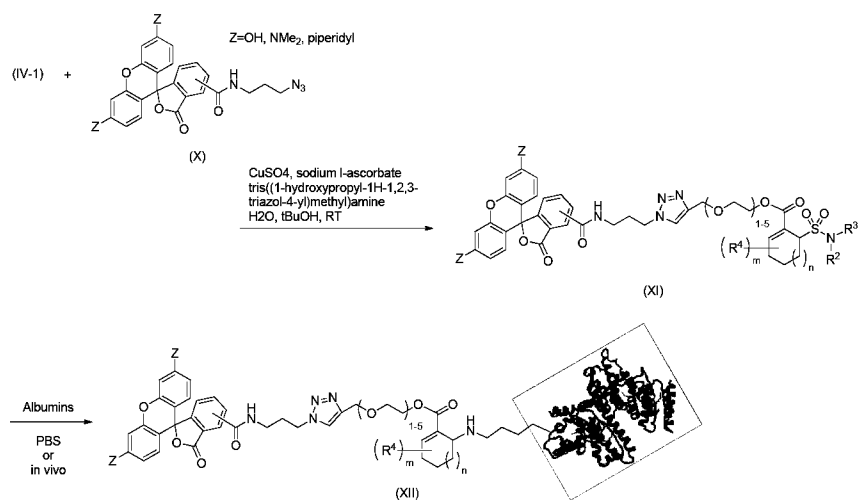


FIG. 36

< CCR5 antagonists >

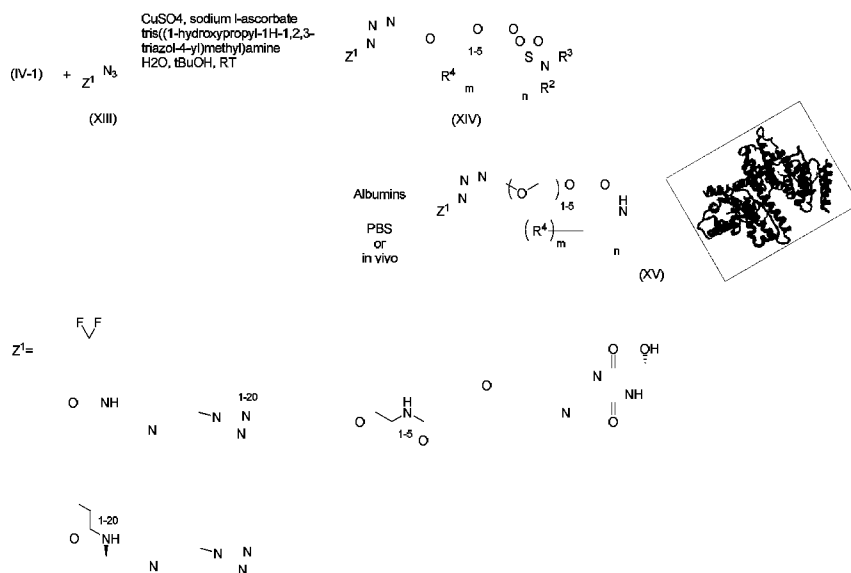


FIG. 37

27/35

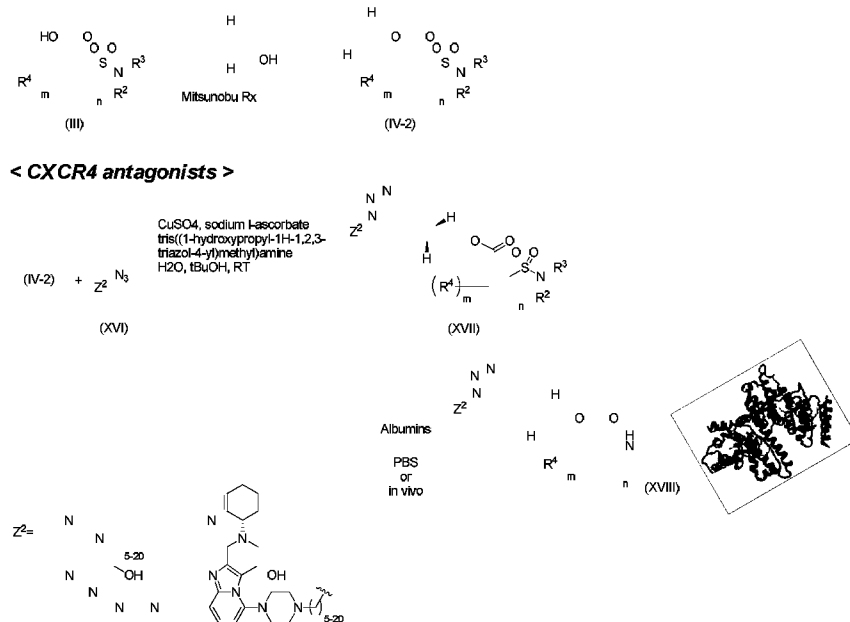


FIG. 38

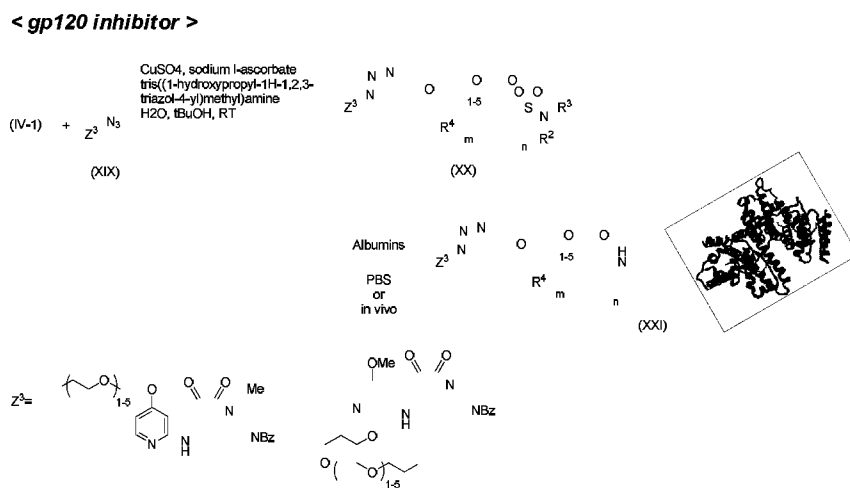
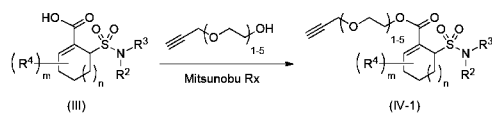


FIG. 39

28/35



< Anti-Flu (Neuraminidase inhibitors) >

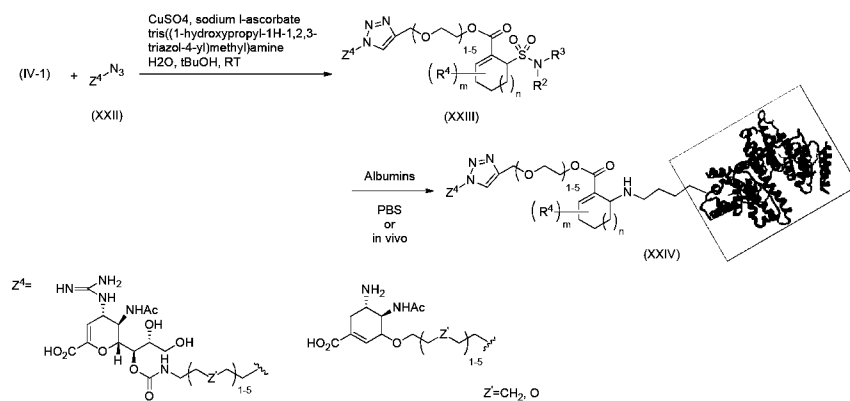


FIG. 40

< Peptides >

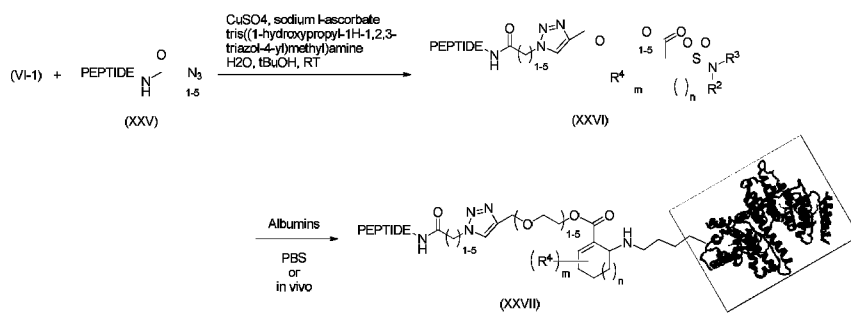


FIG. 41

< Peptides and Proteins >

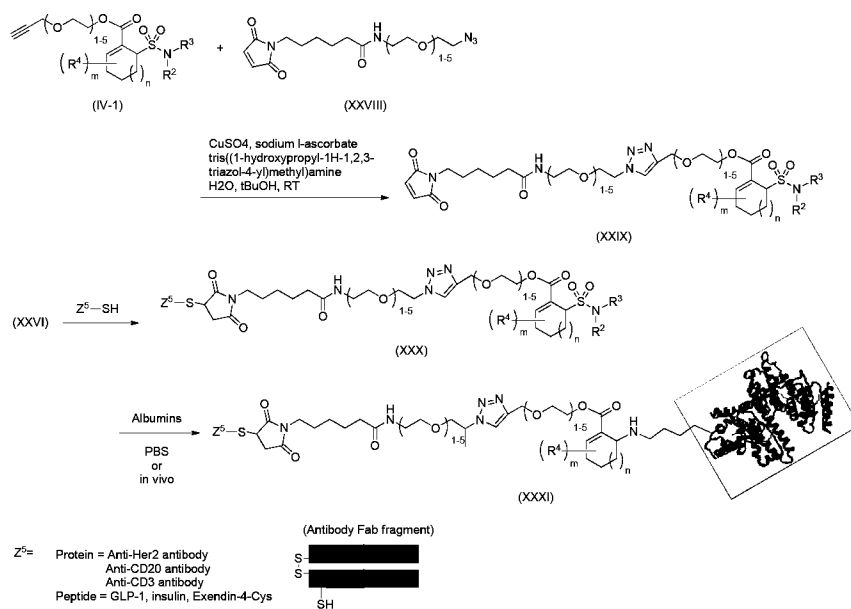


FIG. 42

30/35

< Peptides and Proteins >

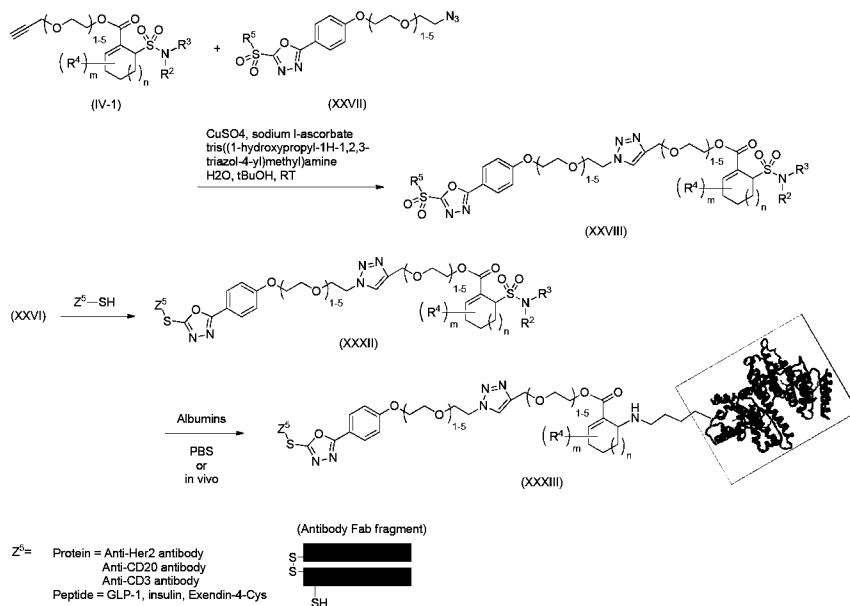


FIG. 43

31/35

< Cancer >

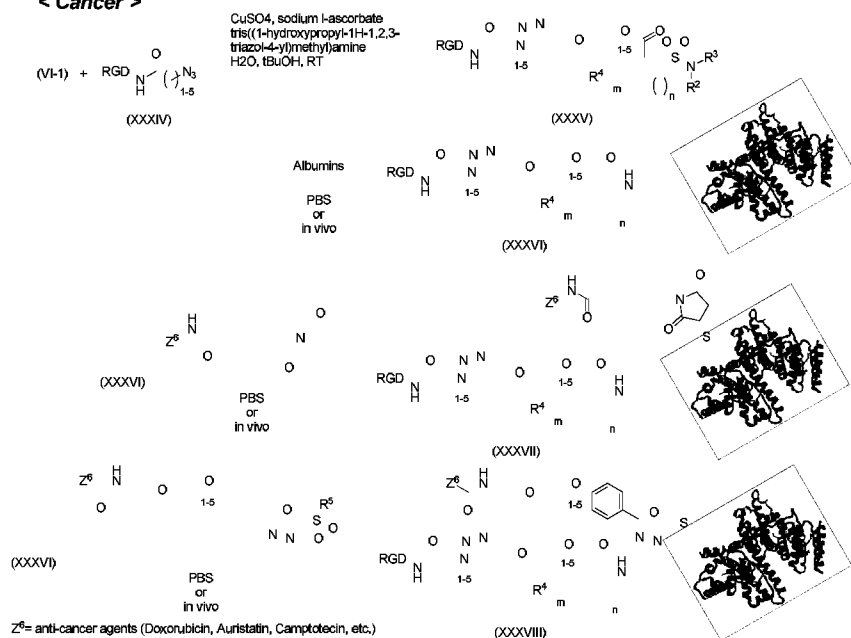


FIG. 44

32/35

< Cancer >

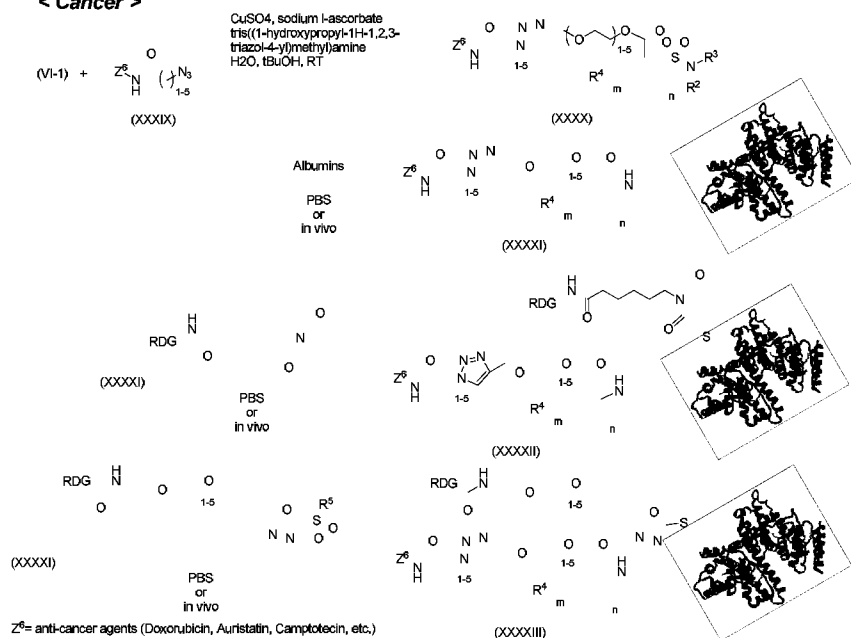


FIG. 45

< Cancer >

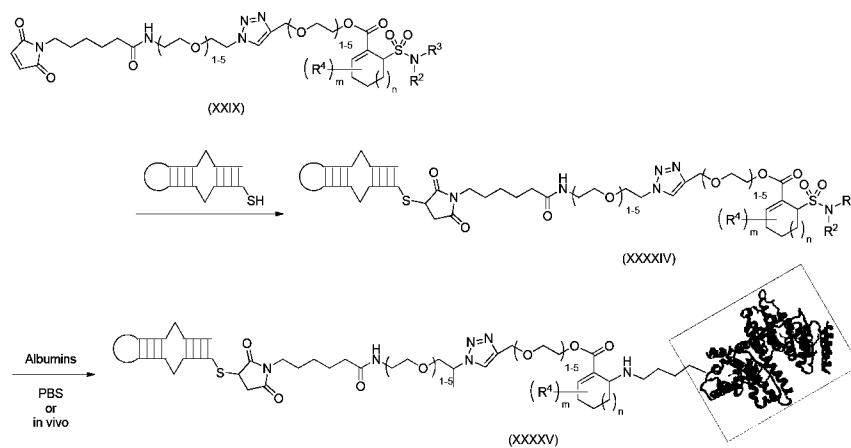


FIG. 46

33/35

< Cancer >

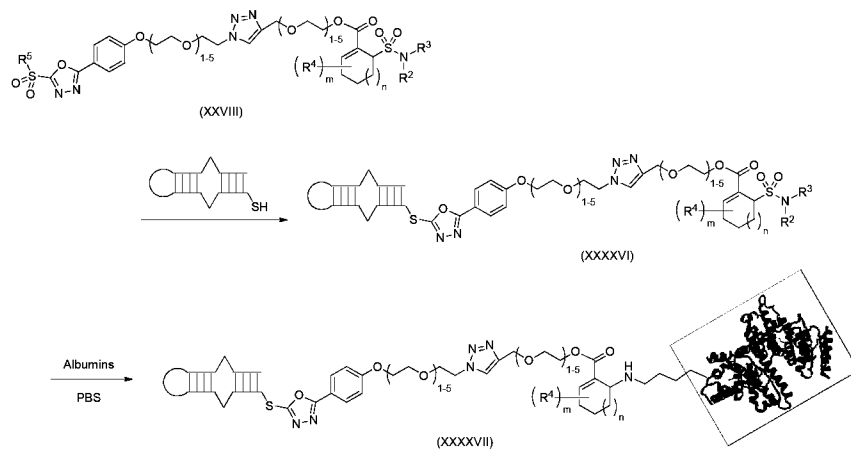


FIG. 47

< Cancer >

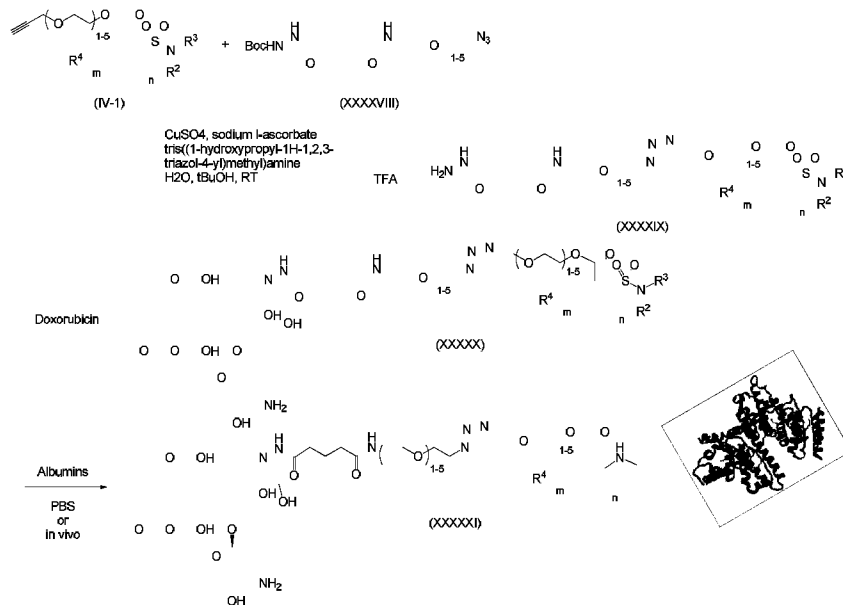


FIG. 48

34/35

< Cancer >

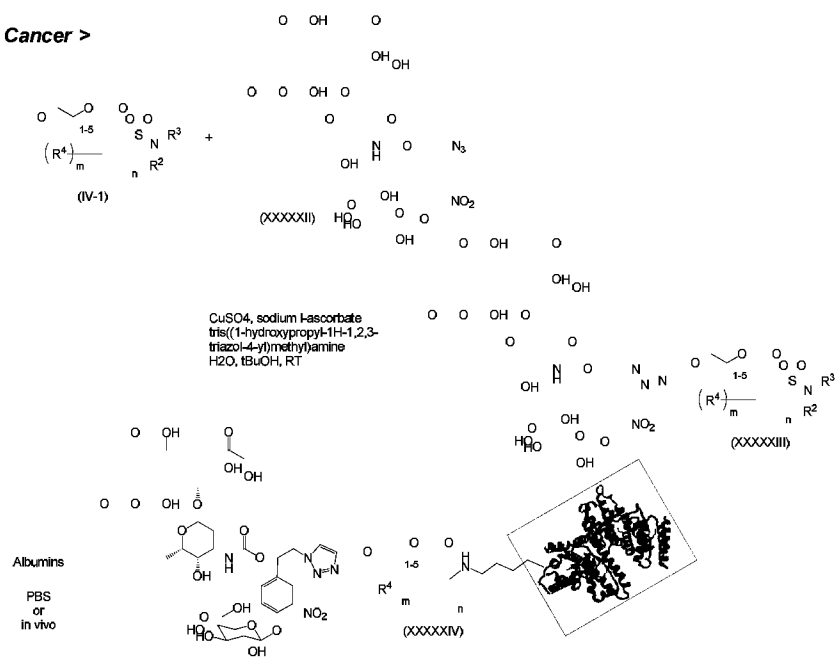


FIG. 49

< Cancer >

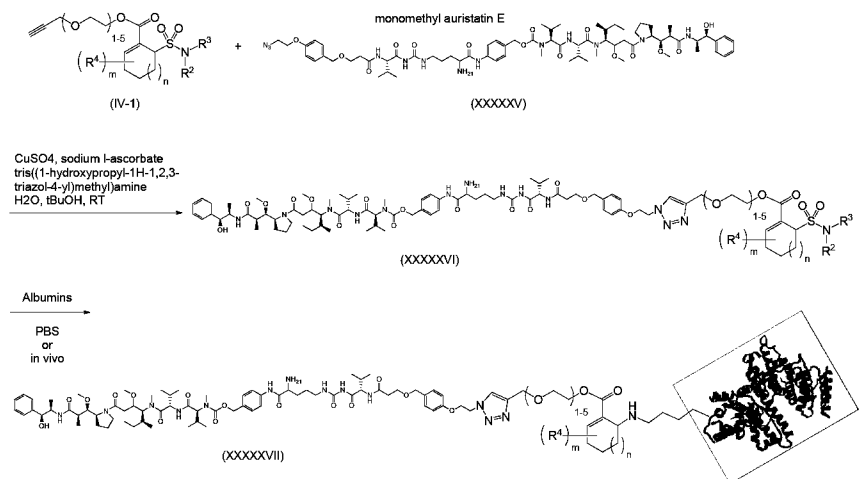


FIG. 50

35/35



FIG. 51

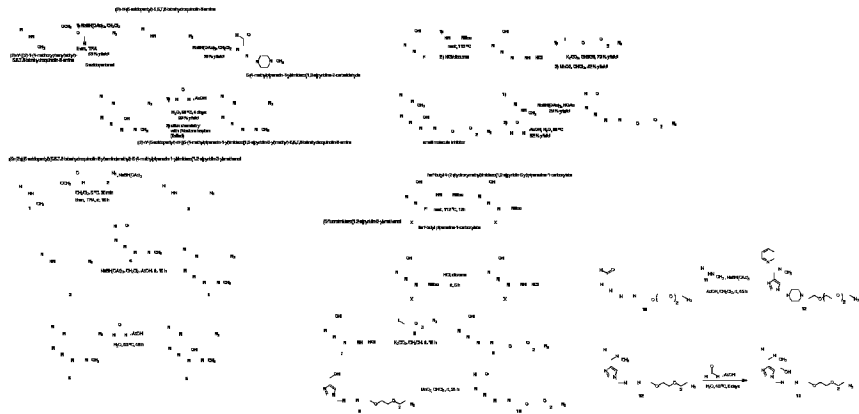


FIG. 52