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(54) Title: CONJUGATES OF BIOMOLECULE AND USE THEREOF

(57) Abstract: Provided are conjugates of biomolecule and use thereof. The provided conjugates of biomolecule contain a biomolecule and a functional moiety covalently linked to the biomolecule. The functional moiety contains a group that prevents the biomolecule from binding to its ligand or receptor, a cleavable linker arm that can be activated by proteolytic enzymes or can be acidically activated in a microenvironment of a disease, a linker arm that will automatically shed after the cleavable linker arm is cleaved, and a group that maintains or promotes the binding capacity of the biomolecule to its ligand or receptor. The conjugates of biomolecule can effectively reduce immunogenicity of the biomolecule, increase its half-life, and breakthrough the immune barrier of an individual, reach a pathologic microenvironment and be activated and released in the pathologic microenvironment, selectively promoting proliferation or killing effect of T cells and the like in the tumor, thereby preventing on target off tumor toxicity or in the inflammatory microenvironment of autoimmune disease and achieving a low autoimmunity and high efficacy.



Conjugates of Biomolecule and Use thereof

5 **Technical Field**

The present invention relates to conjugates of biomolecule and use thereof.

Technical Background

10 Conjugating agents to antibodies by linking to native cystine has been used to further advance the use of antibody. Molecules such as toxins and drugs have been conjugated to antibodies to generate antibody-drug conjugates (ADCs). Fusion of a cleaving, masking peptide sequence to an antibody to generate a probody has been used for a local activation in Tumor. But the peptides are limited in terminal of antibody, and the activation sequence is limited with peptide with low activation rate and high immunogenicity from masking peptide.

15 The common side effect of the currently commercial macromolecular drugs is immunotoxicity. Immunotoxicity includes immunosuppression, generation of immunogenicity, hypersensitivity, autoreaction and adverse immune stimulation. These side effects are mainly caused by extraneous macromolecules. After entering into the body, heterogenous macromolecules will elicit an immune response in the patient due to its immunogenicity. In
20 normal tissues, immunity also will be stimulated and autoimmunity response will be generated after a macromolecule drug, such as antibody or cytokine, binds to the antigens or receptors. At this time it is very dangerous to the patient. For example, during the treatment of non-small cell lung cancer with the current PD-1 antibodies, Keytruda and Opdivo, serious pneumonia may occur, which may even lead to death of patients. Similar effects were also reported in the
25 clinical use of CTLA4 antibodies (yervoy), 41BB, IL-2, IL-10 etc. In combination therapy of Opdivo and Yervoy to non-small cell lung cancer, 55% patients showed the high grade3-4 AEs and 36% patients had to stop drug treatment due to drug toxicity. Therefore, intelligent conjugates of biomolecule with new functions are required in this field to decrease the overall toxicity and immunotoxicity by blocking drug activity in blood and normal tissue and to
30 enhance the active drug in pathologic microenvironment, to adjust DMPK and half-life in serum, to decrease immunogenicity of Fab of antibody, to adjust the binding affinity of active biomolecule, and to enhance the efficacy.

Summary of Invention

35 The present disclosure provides conjugates of biomolecule having the following structure:



wherein,

- 5 R5 represents a biomolecule with one or more cysteine residues introduced by mutation;
 cys represents the cysteine residue(s) contained in R5;
 S represents sulfur atom(s) of the cysteine residue(s);
 R1 is a group that prevents R5 from binding to its antigen, ligand or receptor;

10 R2 is absent, or R2 is a cleavable linker arm capable of being activated by one or more
 proteolytic enzymes or a chemical bond capable of being acidically activated in a pathologic
 microenvironment;

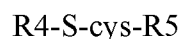
R3 is absent, or R3 is a linker arm capable of automatically shedding after R2 is cleaved or
 a chemical bond capable of being acidically activated in a pathologic microenvironment; with
 the proviso that when R2 is absent, R3 is the chemical bond capable of being acidically
 15 activated in a pathologic microenvironment; and

R4 is a group covalently linked to R5 via the sulfur atom(s) of the cysteine residue(s)
 contained in R5 that recovers, maintains or promotes the binding capacity of R5 to its antigen,
 ligand or receptor after the moiety R1-R2-R3 is cleaved.

20 In the above formula, when R1-R2 is cleaved from R3-R4-S-cys-R5 by a proteolytic
 enzyme or under an acid condition in the pathologic microenvironment, R3-R4-S-cys-R5 is
 released. And the binding capacity of R4-S-cys-R5 to the antigen, ligand or receptor of R5 will
 be recovered, maintained or improved after R3 automatically sheds and R4-S-cys-R5 is
 released.

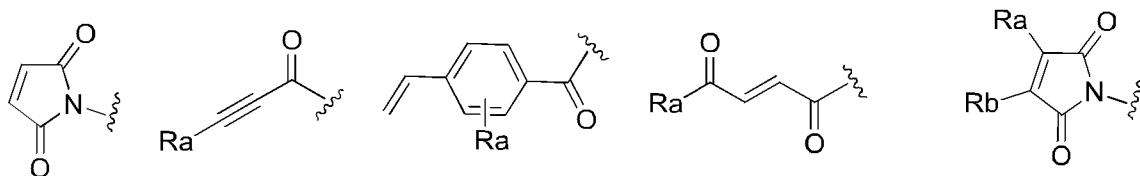
25 In one or more embodiments of the present disclosure, R1, R2, R3, R4 and R5 of the
 conjugates of biomolecule are described as in other parts of the present disclosure.

The present disclosure also provides a compound having the following structure:



wherein,

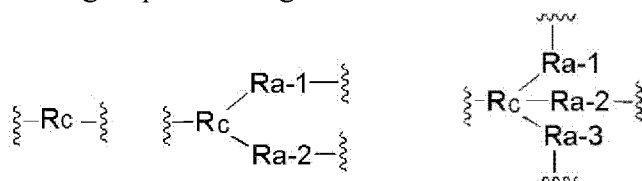
30 R4 is represented by $R_{4-a}-R_{4-b}-R_{4-c}$ -, wherein R4 is represented by $R_{4-a}-R_{4-b}-R_{4-c}$ -, wherein
 R_{4-a} is selected from the group consisting of:



R_{4-a1} , R_{4-a2} , R_{4-a3} , R_{4-a4} , and R_{4-a5} ;

wherein Ra and Rb are each independently selected from the group consisting of H and C₁₋₆ alkyl or C₁₋₆ alkoxy;

R_{4-b} is selected from the group consisting of:



R_{4-b1} , R_{4-b2} , and R_{4-b3} ;

wherein in formula R_{4-b1}, R_c is absent, or is selected from the group consisting of C₁₋₁₂ alkyl, C₁₋₁₂alkoxy-C₁₋₁₂alkyl, C₁₋₁₂alkyl-C₃₋₈cycloalkyl, (C₁₋₄alkyl-O)_p-C₁₋₁₂alkyl,

C₁₋₁₂alkylcarbonylamino-(C₁₋₄alkyl-O)_p-C₁₋₁₂alkyl, phenyl-C₁₋₁₂alkyl, C₃₋₈cycloalkyl,

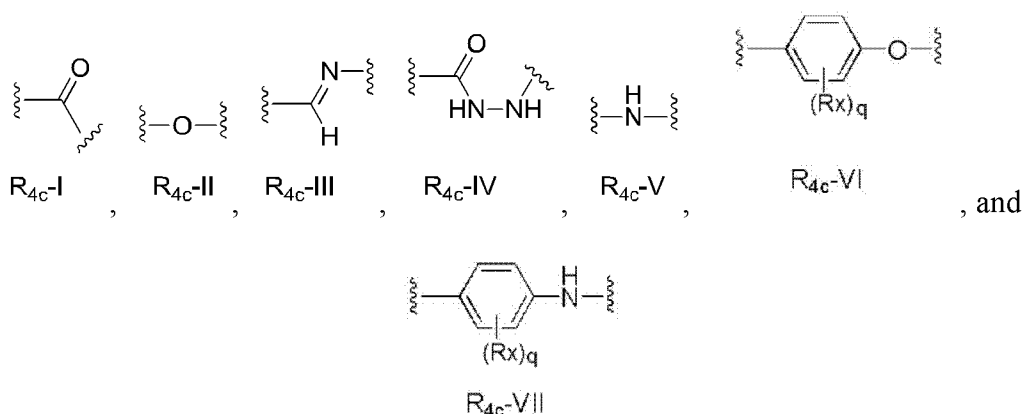
C₁₋₁₂alkyl-C₃₋₈cycloalkyl-C₁₋₁₂alkyl, and C₁₋₁₂alkyl-phenyl-C₁₋₁₂alkyl; in formula

R_{4-b2}, R_c is a C₁₋₁₂alkylamino with Ra-1 and Ra-2 substituted at N atom of the amino group, and in formula

R_{4-b3}, R_c is a C₁₋₁₂alkyl with the last C atom at the end of the alkyl being substituted by Ra-1, Ra-2 and Ra-3, wherein Ra-1, Ra-2 and Ra-3 are each independently selected from the group consisting of C₁₋₁₂alkyl, C₁₋₁₂alkyl-OH, and C₁₋₁₂alkyl-NR''R''', wherein R'' and R''' are each independently selected from the group consisting of H and C₁₋₁₂alkyl; wherein in formulae

R_{4-b2} and R_{4-b3}, R_{4-b} links to R_{4-c} via at least one of the Ra-1, Ra-2 and Ra-3;

R_{4-c} is selected from the group consisting of:



wherein Rx is selected from the group consisting of H, halo and C₁₋₄alkyl; p is an integer in a range of 1 to 10, such as an integer in a range of 1 to 5; and q is an integer in a range of 1 to 4,

such as an integer in a range of 1 to 2; with the proviso that R4-c is absent when R4-a is selected from the group consisting of formulae R4-a2, R4-a3 and R4-a4; wherein R3 links to R4 via the R_{4-c} of R4, and the wave line shown in each formula of R4-a indicates the position at which R4-a links to R4-b.

5 Also provided is a compound represented by R1-R2-R3-R4, wherein R1, R2, R3 and R4 are defined as in any embodiments of the present disclosure.

The present disclosure provides use of the conjugates of biomolecule or the R4-S-cys-R5 compound as described herein in the manufacture of an anti-tumor drug.

10 The present disclosure provides a pharmaceutical composition comprising the conjugates of biomolecule as described herein.

The present disclosure provides a method for treating or preventing tumor, comprising providing to a subject in need thereof a therapeutically effective amount of the conjugates of biomolecule or the R4-S-cys-R5 compound as described herein.

15 The present disclosure also provides compounds, including compounds S1-S64 and compounds described in other parts of the present disclosure, and antibodies and cytokines containing mutation as described herein.

Brief Description of Drawings

- Fig. 1: Alignment of the amino acid sequences of the light chains of antibodies.
- 20 Fig. 2: Co-IP screening for point mutation in the light chain of anti-PD1 antibody.
- Fig. 3: Structure of antibody.
- Fig. 4: The sequences of loop 4 in the variable region are conservative.
- Fig. 5: Variable regions of two antibodies may come from the variable region framework of antibodies of same germline.
- 25 Fig. 6: Alignment of amino acid sequences of heavy chains of several antibodies.
- Fig. 7: Alignment of amino acid sequences of light chains of several antibodies.
- Fig. 8: Conjugation of PD1 Ab-C28.
- Fig. 9: Binding of the PD1 Ab-C28 conjugate to PD1 before and after activation.
- Fig. 10: Schematic drawing of conjugate of biomolecule which contains an activatable and
- 30 binding arm and is activated in a pathologic microenvironment.
- Fig. 11: Secretion of IFN-gamma.
- Fig. 12: Survival curve.
- Fig. 13: Death curve.
- Fig. 14: Proliferation of T cells.
- 35 Fig. 15: Conjugate of anti-PD1 antibody inhibits growth of tumor on mice suffering from

MC38 colon cancer.

Fig. 16: Conjugation of IL2-C41 mutant.

Fig. 17: Binding of the IL2-C41 conjugate to IL2 receptor alpha before and after activation.

5 Fig. 18: Effect of IL2 mutant conjugation (IL2-Thr41Cys, i.e., IL2-C41) on proliferation of T cells.

Fig. 19: Effect of IL2 conjugate on CD4/CD8 proliferation.

Fig. 20: Conjugate of IL2 inhibits growth of B16F10 tumor.

Fig. 21: Conjugate of IL2 in combination with anti-PD-1 antibody inhibits growth of

10 MC38 tumor.

Fig. 22: The SDS-PAGE results of mutant IL2, IL2 TMEAkine and the recovery active IL2 after enzyme cleavage *in vitro*.

Fig. 23: ELISA results indicating the binding activity of IL2 TMEAkine to IL2R α or R β before and after enzyme cleavage *in vitro*.

15 Fig. 24: The amount of IL2-T41C-S47 detected by western blot and the corresponding results.

Fig. 25: The pharmacokinetic study *in vivo* indicating that IL2 TMEAkine has a long half-life and a high exposure in plasma when compared with IL2.

20 Fig. 26: The measurement of pulmonary (wet weight) and the sections stained with hematoxylin and eosin indicating that IL2 TMEAkine induces less toxicity to lung than wild-type IL2.

Fig. 27: The measurement of pulmonary indicating that IL2-T41C/S87C-S47 and IL2-R38D/E61R/S87C-S47 induce almost no toxicity to lung.

Fig. 28: Tumor volumes after treatment.

25 Fig. 29: High exposure of active IL2 in tumor, which is consistent with high efficacy of anti-tumor effect, and low exposure of active IL2 in lung and heart, which is consistent with low toxicity of pulmonary edema.

Fig. 30: Reduced SDS-PAGE gel results showing that all five position are conjugated with S13 linker in a high efficiency.

30 Fig. 31: The 8 selected antibodies sequences.

Fig. 32: By site-specially conjugating different R4 groups to the selected mutant site of antibody, the R4 group could be selectively changed for screening.

Fig. 33: Conjugating to different sites shows different degree of binding activity recovery.

35 Fig. 34: The TMEAbodies with significant decreased binding activity to human CTLA-4 protein also show dramatic decrease of receptor blocking activity.

Fig. 35: TMEAbody with decreased binding activity against CTLA-4 shows impairment of functional efficacy of T cell activation, and protease mediated activation can restore the activity of TMEAbody.

Fig. 36: Ipilimumab TMEAbodies significantly down regulate Treg population in the tumor, with the similar efficacy as WT Ipilimumab.

Fig. 37: TMEAbody shows high stability in mouse serum, without significant degradation after 96h at 37°C.

Fig. 38: CTLA-4 TMEAbodies show increased half-life and exposure by conjugation with S47 functional group comparing with WT-Ipilimumab and CTLA-4 probody.

Fig. 39: Ipilimumab TMEAbodies control the tumor size with comparable efficacy as WT Ipilimumab, whereas control human IgG fails to show any efficacy.

Fig. 40: TMEAbody causes very low immune response in the animals, with comparable or lower antibody titer than WT Ipilimumab.

Fig. 41: Protection of an immune system by a conjugate of anti-CTLA4 TMEAbody in combination therapy conjugate could reduce autoimmunity as compared to the primary antibody.

Fig. 42: Niv-se001 shows increased activity after R4-7 conjugation or protease cleavage of 40kD conjugated TMEAbody, which is 432% of WT Nivolumab.

Fig. 43: After conjugation with R4-7, or after protease cleavage of Niv-se005 conjugated with 40kD functional group, the binding activity of Niv-se005 is restored with comparable level than WT Nivolumab (125% of WT).

Fig. 44: Pembrolizumab and Nivolumab TMEAbodies control the tumor size with comparable efficacy as WT Pembrolizumab or Nivolumab antibodies, whereas control human IgG failed to show any efficacy.

Fig. 45: Anti-CTLA4 or anti-PD1 TMEAbody in combination therapy could reduce autoimmunity as compared to the primary antibody.

Fig. 46: Tumor volumes after treatment.

Embodiments

It should be understood that, within the scope of the present disclosure, each of the technical features mentioned above and each of those mentioned hereinafter, such as in the Examples, can be combined with each other to constitute preferred technical solutions. Additionally, the present disclosure is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that, unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of

ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

It must be noted that as used herein and in the appended claims, the singular forms "a," "an," "and," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an antibody" includes a plurality of antibodies and reference to "the antibody" includes reference to one or more antibodies and equivalents thereof known to those skilled in the art, and so forth. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely", "only" and the like in connection with the recitation of claim elements, or use of a "negative" limitation.

One of the purposes of the present disclosure is to provide modified biomolecules, which are only activated in a pathologic microenvironment, such as in a tumor microenvironment or an inflammatory environment, to release a biomolecule (R5) in the pathologic microenvironment which has the same or even improved binding capacity to its ligand, thereby improving the targeting and efficacy of the biomolecule, overcoming drug resistance and reducing toxicity.

In the present disclosure, the modified biomolecule is a conjugate, which comprises a biomolecule and a functional moiety covalently linked to the biomolecule. Biomolecules suitable for use in the present disclosure may be biomolecules having a biological function or activity of interest, including but is not limited to various functional proteins. Biological function or activity of interest includes but is not limited to functions or activities in enzymology and immunology. Therefore, biomolecules suitable for use in the present disclosure include but are not limited to antibodies or functional fragments thereof, enzymes, fusion proteins (such as protein-antibody fusions), antibody drug conjugates, cytokines, and any other genetically engineered biological molecules.

As used herein, the term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies, polyclonal antibodies, dimers, multimers, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments, so long as they exhibit the desired biological activity (Miller et al (2003) Jour. of Immunology 170:4854-4861). In the context and in the Figures, an antibody is abbreviated as "Ab".

The basic antibody structural unit is known to comprise a tetramer composed of two identical pairs of polypeptide chains, each pair having one light and one heavy chain. The N

terminal portion of each chain includes a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The C terminal portion of each chain defines a constant region primarily responsible for effector function. The variable region of heavy chain (VH) and the variable region of light chain (VL) each contain 3 complementarity determining region (CDR), including HCDR1, HCDR2, HCDR3 and LCDR1, LCDR2 and LCDR3. These six CDRs form antigen-binding site of an antibody. The remaining amino acids of the variable region are relatively conservative and are termed as framework region (FR). VH and VL each contain 4 framework regions, called as FR1, FR2, FR3 and FR4, respectively.

Antibodies may be murine, human, humanized, chimeric, or derived from other species.

The immunoglobulin disclosed herein can be of any type (e.g., IgG, IgE, IgM, IgD, and IgA), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. The immunoglobulins can be derived from any species. In one aspect, however, the immunoglobulin is of human, murine, or rabbit origin.

Antibody fragment comprise a portion of a full length antibody, generally the antigen binding or variable region thereof. Preferably, fragment of an antibody is a functional fragment, i.e., retaining the antigen-binding capability of the intact antibody. Examples of antibody fragments or functional fragments include Fab, Fab', F(ab')₂, and Fv fragments; diabodies; linear antibodies; and single chain antibody molecules (scFv); etc.

Fusion protein used herein may contain an antigen binding domain of an antibody and optionally a cytokine. The antigen binding domain of an antibody contained in fusion protein may be an antigen binding domain to an antigen selected from the group consisting of HER2, CD19, EGFR, CD22, CD3, TROP2, Glycoprotein NMB, Guanylyl cyclase C, CEA, CD79b, PSMA, ENPP3, Mesothelin, CD138, NaPi2b, CD56, CD74, FOLR1, DLL3, CEACAM5, CD142, SLAMF7, CD25, SLTRK6, CD37, CD70, AGS-22, C4.4A, FGFR2, Ly6E, MUC16, BCMA, pCadherin, Ephrin-A, LAMP1, MUC1, CD19, PDL1, HER2, NY-ESO-1, BCMA, WT1, MUC1, CD20, CD23, ROR1, CD123, CD33, CD44v6, CD174, CD30, CD133, cMet, EGFR, FAP, EphA2, GD2, GPC3, IL-13Ra2, LewisY, Mesothelin, SS1, CEA, CD171, EGFR, EGFRvIII, VEGFR2, NY-ESO-1, MUC-1 and MAGE-A3, or may be an antigen binding domain of any antibodies as described herein. In some embodiments, the fusion protein is a bispecific antibody, which contain an antigen binding domain to an antigen selected from the group consisting of HER2, CD19, EGFR, CD22, CD3, TROP2, Glycoprotein NMB, Guanylyl cyclase C, CEA, CD79b, PSMA, ENPP3, Mesothelin, CD138, NaPi2b, CD56, CD74, FOLR1, DLL3, CEACAM5, CD142, SLAMF7, CD25, SLTRK6, CD37, CD70, AGS-22, C4.4A, FGFR2, Ly6E, MUC16, BCMA, pCadherin, Ephrin-A, LAMP1, MUC1, CD19, PDL1, HER2, NY-ESO-1, BCMA, WT1, MUC1, CD20, CD23, ROR1, CD123, CD33, CD44v6, CD174,

CD30, CD133, cMet, EGFR, FAP, EphA2, GD2, GPC3, IL-13Ra2, LewisY, Mesothelin, SS1, CEA, CD171, EGFR, EGFRvIII, VEGFR2, NY-ESO-1, MUC-1 and MAGE-A3, or which contain an antigen binding domain from any antibodies as described herein. Preferably, the bispecific antibody is a single chain bispecific antibody, which contains two scFv from the same or different antibodies.

In some embodiments, the fusion protein is an antibody-cytokine fusion protein, which contains an antibody or functional fragment thereof and a cytokine selected from the group consisting of IL-2, IL-7, IL-10, IL-11, IL-12, IL-15, IL-21, IFN- α , IFN- β , IFN- γ , G-CSF, GM-CSF, TNF- α , TRAP, and TRAIL. Example of such fusion protein is a fusion protein of anti-PD-1 antibody or anti-CD3 antibody or antigen binding domain thereof with IL2.

Antibody used herein may be any antibodies known in the art and functional fragments thereof. For example, antibody used herein may be an antibody or functional fragment thereof selected from the group consisting of anti-Her2 antibody, anti-EGFR antibody, anti-VEGFR antibody, anti-CD20 antibody, anti-CD33 antibody, anti-PD-L1 antibody, anti-PD-1 antibody, anti-CTLA-4 antibody, anti-TNF α antibody, anti-CD28 antibody, anti-4-1BB antibody, anti-OX40 antibody, anti-GITR antibody, anti-CD27 antibody, anti-CD40 antibody, or anti-ICOS antibody, anti-CD25 antibody, anti-CD30 antibody, anti-CD3 antibody, anti-CD22 antibody, anti-CCR4 antibody, anti-CD38 antibody, anti-CD52 antibody, anti-Complement C5 antibody, anti-F protein of RSV, anti-GD2 antibody, anti-GITR antibody, anti-Glycoprotein receptor lib/IIla antibody, anti-ICOS antibody, anti-IL2R antibody, anti-LAG3 antibody, anti-Integrin α 4 antibody, anti-IgE antibody, anti-PDGFR α antibody, anti-RANKL antibody, anti-SLAMF7 antibody, anti-LTIGIT antibody, anti-TIM-3 antibody, anti-VEGFR2 antibody, anti-VISTA antibody.

In preferred embodiments, antibody used herein may be an antibody or functional fragment thereof selected from the group consisting of Utomilumab, Urelumab, ADG106, PoteligeoTM (Mogamulizumab), PoteligeoTM (Mogamulizumab), BexxarTM (tositumomab), ZevalinTM (ibritumomab tiuxetan), RituxanTM (rituximab), ArzerraTM (Ofatumumab), GazyvaTM (Obinutuzumab), BesponsaTM (Inotuzumab ozogamicin), ZenapaxTM (daclizumab), Varlilumab, Theralizumab, AdcetrisTM (Brentuximab vedotin), MyelotargTM (gemtuzumab), DarzalexTM (Daratumumab), CDX-1140, SEA-CD40, RO7009789, JNJ-64457107, APX-005M, Chi Lob 7/4, CampathTM (alemtuzumab), RaptivaTM (efalizumab), SolirisTM (eculizumab), YervoyTM (ipilimumab), tremelimumab, ErbituxTM (cetuximab), VectibixTM (panitumumab), PortrazzaTM (Necitumumab), TheraCIMTM (Nimotuzumab), SynagisTM (palivizumab), UnituxinTM (Dinutuximab), TRX-518, MK-4166, MK-1248, GWN-323, INCAGN0186, BMS-986156, AMG-228, ReoProTM (abiximab), HerceptinTM (trastuzumab), PerjetaTM (Pertuzumab), KadcylaTM (Ado-trastuzumab emtansine), GSK-3359609, JTX-2011, SimulectTM

(basiliximab), Tysabri™ (natalizumab), BMS-986016, REGN3767, LAG525, Xolair™ (omalizumab), Tavolimab, PF-04518600, BMS-986178, MOXR-0916, GSK-3174998, INCAGN01949, IBI-101, Keytruda™ (Pembrolizumab), Opdivo™ (Nivolumab), Lartruvo™ (Olaratumab), Tencentriq™ (Atezolizumab), BMS-936559, Bavencio™ (Avelumab), Imfinzi™ (Duralumab), Prolia™ (Denosumab), Empliciti™ (Elotuzumab), MTIG7192A, TSR-022, MBG-453, Remicade™ (infliximab), Humira™ (adalimumab), Avastin™ (bevacizumab), Lucentis™ (ranibizumab), Cyramza™ (Ramucirumab), and JNJ-61610588.

In the present disclosure, cytokine may have a meaning and structure commonly used in the art. It generally refers to a kind of small proteins having wide biological activities, which are synthesized and secreted by stimulating immunocytes, such as monocytes, macrophages, T cells, B cells, NK cells, etc., or nonimmune cells, such as endothelial cells, epidermal cells, fibroblasts, etc. Cytokine may regulate cell growth, differentiation and effect, and immune response through binding to a corresponding receptor. Suitable cytokines include interleukins, interferons, tumor necrosis factor superfamily, colony stimulating factors, chemotactic factors and growth factors, etc.

Exemplary cytokines include but are not limited to IL-2, IL-7, IL-10, IL-11, IL-12, IL-15, IL-21, IFN- α , IFN- β , IFN- γ , G-CSF, GM-CSF, TNF- α , TRAP, and TRAIL.

In the present disclosure, one or more (such as 5 or less or 3 or less) amino acids in suitable position(s) of the amino acid sequence of the biomolecule are mutated to cysteine and the biomolecule is covalently linked to the functional moiety of the present disclosure (R1-R2-R3-R4) via the thiol group of the cysteine. For example, one or two amino acids of interesting in the biomolecule of interesting are mutated to cysteine for conjugation to the functional moiety. For an antibody, the mutation position may be present in a complementarity determining region or a non-complementarity determining region of a variable region.

Preferably, the mutation is a mutation by substitution. More preferably, the mutation occurs in a variable region of the light chain of antibody. Generally, a mutant can be prepared and then its binding activity to a corresponding antigen is tested. If a mutant retains 70% or more, preferably 80% or more, more preferably 90% or more of binding activity as compared to the wild-type antibody, it is believed that the amino acid residue at the mutation position may be mutated to cysteine to covalently link to a functional moiety. Alternatively, in certain embodiments, if a conjugate produced by linking a mutant to R4 retains 80% or more, preferably 90% or more, more preferably 95% or more of binding activity, it is believed that the amino acid residue at the mutation position may be mutated to cysteine to covalently link to a functional moiety.

In general, one or more of G, A, S, T, L, I, F, E, K, D and Y, etc., more preferably one or

more of G, A, S, T, L, I, K and Y, more preferably one or more of G, A, T, L and S, in a CDR of a variable region of a light chain of an antibody may be mutated to cysteine. In some embodiments, one or more of G, A, S, T, L, I, F, E, K, D and Y, etc., more preferably one or more of G, A, S, T, L, I, K and Y, more preferably one or more of G, A, T, L, Y and S, in a CDR of a variable region of a heavy chain of an antibody may be mutated to cysteine. If mutation occurs in a non-CDR of a light chain of an antibody, one or more of G, A, S, T, L, I, F, E, K, D and Y, etc., preferably G, A, S, T, K, I, Y and L, more preferably one or more of G, A, T, Y and S, in the non-CDR in the variable region of a light chain or a heavy chain may be mutated to cysteine. In some embodiments, one or more of S, T, L, I, F, E, K, D, N, Q, R and Y residues, etc., in non-complementarity determining region (such as in FR1, FR2 or FR3) of a variable region of a light chain or a heavy chain may be mutated to cysteine. In some embodiments, substitution mutation may be introduced into one or more the following conservative sites: Gln3, Ser7, Ser26, Glu46, Thr68 and Asp72 in non-complementarity determining region (FR1, FR2 or FR3) of VH, and Thr5, Tyr49, Arg61, Ser63, Ser65, Ser67, Thr72, Thr74, Ser76 and Asp82 in non-complementarity determining region (FR1, FR2 or FR3) of VL.

For example, in some embodiments of the present disclosure, the mutation position in a heavy chain of anti-PD-1 antibody (such as Pembrolizumab) may be selected from the group consisting of: Ser7, Gly8, Gly15, Ala16, Ser17, Ala24, Ser25, Gly26, Tyr27, Thr28, Thr30, Asn31, Tyr32, Tyr33, Tyr35, Ala40, Gly42, Gly44, Leu45, Gly49, Gly50, Ile51, Asn52, Ser54, Asn55, Gly56, Gly57, Thr58, Asn59, Lys63, Lys65, Thr69, Leu70, Thr71, Thr72, Asp73, Ser74, Ser75, Thr76, Thr77, Thr78, Ala79, Leu83, Ser85, Leu86, Thr91, Ala92, Arg99, Asp100, Tyr101, Arg102, Asp104, Gly106, Gly111, Gly113, Thr114, 115Thr, 117Thr, Ser119, Ser120, Ala121, Ser122, Thr123, Lys124, Gly125 and Ser127; and the mutation position in a light chain may be selected from the group consisting of: Ile2, Thr5, Ser7, Ala9, Thr10, Leu11, Ser12, Leu13, Ser14, Gly16, Ala19, Thr20, Ala25, Ser26, Lys27, Gly28, Ser30, Thr31, Ser32, Gly33, Tyr34, Ser35, Tyr36, Leu37, Gly45, Ala47, Leu50, Leu51, Ile52, Tyr53, Leu54, Ala55, Ser56, Tyr57, Leu58, Ser60, Gly61, Ala64, Ser67, Gly68, Ser69, Gly70, Ser71, Gly72, Thr73, Ala76, Thr78, Ser80, Ser81, Ser95, Arg96, Asp97, Leu98, Leu100, Thr101, Phe102, Gly104, Ile110, Lys111 and K130.

Alternatively, the mutation position in a heavy chain of anti-PD-1 antibody (such as Nivolumab) may be selected from the group consisting of: Gln3, Ser7, Gly8, Gly9, Gly10, Gly15, Ser17, Lys23, Ala24, Ser25, Gly26, Ile27, Asn31, Thr28, Ser30, Ser32, Gly33, Ala40, Gly42, Gly44, Leu45, Ala49, Ile51, Tyr53, Asp54, Gly55, Ser56, Lys57, Tyr59, Tyr60, Ala61, Asp62, Ser63, Lys65, Gly66, Thr69, Ile70, Ser71, Arg72, Asp73, Asn74, Ser75, Lys76, Asn77,

Thr78, Leu79, Leu81, Ser85, Leu86, Ala88, Thr91, Ala92, Thr98, Asn99, Asp100, Asp101, Tyr102, Gly104, Gly106, Thr107, Leu108, Thr110, Ser112, Ser113, Ala114, Ser115, Thr116, Lys117, Gly118 and Ser120; and the mutation position in a light chain may be selected from the group consisting of: Ile2, Leu4, Thr5, Ser7, Ala9, Thr10, Leu11, Ser12, Leu13, Ser14, Gly16, Ala19, Thr20, Leu21, Ala25, Ser26, Ser28, Ser30, Ser31, Tyr32, Leu33, Ala34, Tyr36, Gly41, Ala43, Leu46, Leu47, Ile48, Tyr49, Asp50, Ala51, Ser52, Asn53, Arg54, Ala55, Thr56, Gly57, Ile58, Ala60, Arg61, Ser63, Gly64, Ser65, Gly66, Ser67, Gly68, Thr69, Thr72, Leu73, Thr74, Ile75, Ser76, Ser77, Leu78, Ala84, Ser91, Ser92, Asn93, Arg96, Thr97, Phe98, Gly99, Gly101, Thr102, Ile106, Lys107, Thr109, Ala111, Ala112, Ser114, Ile117 and Ser121.

The mutation position in a heavy chain of anti-CTLA-4 antibody may be selected from the group consisting of: Ser7, Gly8, Gly9, Gly10, Gly15, Ser17, Leu18, Leu20, Ala24, Ser25, Gly26, Phe27, Thr28, Phe29, Ser30, Ser31, Tyr32, Thr33, Ala40, Gly42, Lys43, Gly44, Leu45, Glu46, Thr49, Phe50, Ile51, Ser52, Tyr53, Asp54, Gly55, Lys58, Tyr59, Tyr60, Ala61, Asp62, Ser63, Lys65, Gly66, Thr69, Ile70, Ser71, Ser75, Lys76, Thr78, Leu79, Leu81, Ser85, Leu86, Ala88, Gly89, Asp90, Thr91, Ala92, Tyr94, Tyr95, Ala97, Phe98, Thr99, Gly100, Leu102, Gly103, Asp106, Tyr107, Gly109, Gly111, Thr112, Leu113, Thr115, Ser117, Ser118, Ala119, Ser120, Thr121 and Lys122; and the mutation position in a light chain may be selected from the group consisting of: Ile2, Leu4, Thr5, Ser7, Gly9, Thr10, Leu11, Ser12, Leu13, Ser14, Gly16, Ala19, Thr20, Leu21, Ala25, Ser26, Ser28, Gly30, Ser31, Ser32, Tyr33, Leu34, Ala35, Tyr37, Lys40, Gly42, Ala44, Leu47, Leu48, Ile49, Tyr50, Gly51, Ala52, Phe53, Ser54, Ala56, Thr57, Gly58, Ile59, Ser64, Gly65, Ser66, Gly67, Ser68, Gly69, Thr70, Asp71, Thr73, Leu74, Thr75, Ile76, Ser77, Leu79, Ala85, Tyr92, Gly93, Ser94, Ser95, Thr98, Phe99, Gly100, Gly102, Thr103, Lys104, Ile107, Lys108, Thr110, Ala112, Ala113, Ser115, Ser128, Gly129 and Thr130.

The mutation position in a heavy chain of the anti-CTLA-4 antibody Ipilimumab may be selected from the group consisting of: Gln3, Arg19, Leu20, Ser25, Gly26, Phe27, Thr28, Phe29, Ser30, Ser31, Tyr32, Thr33, Met34, His35, Gly44, Phe50, Ile51, Ser52, Tyr53, Asp54, Gly55, Asn56, Asn57, Lys58, Tyr59, Tyr60, Thr69, Ser71, Arg72, Asp73, Asn74, Ser75, Lys76, Asn77, Thr99, Gly100, Trp101, Leu102, Gly103 and Pro104; the mutation position in a light chain is selected from the group consisting of: Gln6, Arg24, Ala25, Ser26, Gln27, Ser28, Val29, Gly30, Ser31, Ser32, Tyr33, Ile49, Tyr50, Gly51, Ala52, Phe53, Ser54, Arg55, Ala56, Phe53, Ser54, Arg55, Ala56, Thr57, Gly58, Ile59, Pro60, Asp61, Arg62, Ser68, Gly69, Thr70, Gln90, Gln91, Tyr92, Gly93, Ser94, Ser95, Pro96 and Trp 97

The mutation position in a heavy chain of anti-TNF α antibody may be selected from the group consisting of: Ser7, Gly8, Gly9, Gly10, Leu11, Gly15, Ser17, Leu18, Leu20, Ala24,

Ser25, Gly26, Thr28, Asp30, Asp31, Tyr32, Ala33, Ala40, Gly42, Gly44, Leu45, Ser49, Ala50, Ile51, Thr52, Asn54, Ser55, Gly56, Ile58, Asp59, Tyr60, Ala61, Asp62, Ser63, Glu65, Gly66, Phe68, Thr69, Ile70, Ser71, Asp73, Asn74, Ala75, Lys76, Ser78, Leu79, Tyr80, Leu81, Ser85, Leu86, Ala88, Thr91, Ala92, Lys98, Ser100, Tyr101, Leu102, Ser103, Thr104, Ala105, Ser106, Ser107, Leu108, Asp109, Tyr110, Gly112, Gly114, Thr115, Leu116, Thr118, Ser120, Ser121, Ala122, Ser123 and Thr124; and the mutation position in a light chain may be selected from the group consisting of: Asp1, Thr5, Ser7, Ser9, Ser10, Leu11, Ser12, Ala13, Ser14, Gly16, Thr20, Ile21, Ala25, Ser26, Gln27, Gly28, Ile29, Arg30, Asn31, Tyr32, Leu33, Ala34, Tyr36, Lys39, Gly41, Lys42, Ala43, Leu48, Leu47, Ile48, Tyr49, Ala50, Ala51, Ser52, Thr53, Leu54, Gln55, Ser56, Gly57, Ser60, Ser63, Gly64, Ser65, Gly66, Ser67, Gly68, Thr69, Asp70, Thr72, Leu73, Thr74, Ile75, Ser76, Ser77, Leu78, Ala84, Thr85, Tyr91, Asn92, Arg93, Ala94, Tyr96, Thr97, Phe98, Gly99, Gly101, Thr102, Ile106, Lys107, Thr109 and Ala111.

The mutation position in a heavy chain of anti-CD28 antibody may be selected from the group consisting of: Ser7, Gly8, Gly15, Ala16, Ser17, Ser21, Ala24, Ser25, Gly26, Tyr27, Thr28, Thr30, Ser31, Tyr32, Ala40, Gly42, Gly44, Gly49, Tyr52, Gly54, Thr58, Ala68, Thr69, Thr71, Thr74, Ser75, Ser77, Thr78, Ala79, Ser84, Leu86, Ser88, Thr91, Ala92, Thr97, Ser99, Tyr101, Gly102, Leu103, Gly113, Thr114, Thr115, Thr117, Ser119, Ser120, Ala121, Ser122 and Thr123; and the mutation position in a light chain may be selected from the group consisting of: Thr5, Ser7, Ser9, Ser10, Ser11, Ser12, Ala13, Ser14, Gly16, Thr20, Thr22, Ala25, Ser26, Ser27, Ile29, Tyr30, Ala43, Leu46, Leu47, Tyr49, Lys50, Ala51, Ser52, Leu54, Thr56, Gly57, Ser60, Ser63, Gly64, Ser65, Gly66, Ser67, Gly68, Thr69, Asp70, Thr72, Thr74, Ser76, Ser77, Ala84, Thr85, Gly91, Thr93, Tyr94, Tyr96, Thr97, Phe98, Gly99, Gly100, Gly101, Thr102, Thr109 and Ala111.

The mutation position in a heavy chain of the anti-4-1BB antibody may be selected from the group consisting of: Thr31, Tyr32, Ser35, Lys50, Tyr52, Asp55, Ser56, Tyr57, Thr58, Asn59, Tyr60, Ser61, Gln65, Gly66, Gly99, Tyr100, Gly101, Asp104 and Tyr105; the mutation position in a light chain may be selected from the group consisting of: Ser23, Gly24, Asp25, Asn26, Gly28, Asp29, Gln30, Tyr31, Gln49, Asp50, Lys51, Asn52, Arg53, Ser55, Gly56, Thr89, Tyr90, Thr91, Gly92, Gly94 and Ser95.

The mutation position in a heavy chain of the anti-Her2 antibody (such as Trastuzumab) may be selected from the group consisting of: Arg19, Lys30, Asp31, Tyr33, Arg50, Tyr62, Asn55, Tyr57, Arg59, Tyr60, Asp62, Lys65, Asp102 and Tyr105; the mutation position in a light chain is selected from the group consisting of: Asp1, Gln3, Gln27, Asp28, Asn30, Tyr49, Tyr55, Arg66, Asp70 and Tyr92.

The mutation position in a heavy chain of the anti-PD-L1 antibody (such as Atezolizumab)

is selected from the group consisting of: Gln3, Asp31, Tyr54, Tyr59, Tyr60, Asp62, Lys65, Asp73, Lys76, Asn77 and Arg99; the mutation position in a light chain is selected from the group consisting of: Arg24, Gln27, Asp28, Tyr49, Tyr55, Asp70, Gln89, Gln90, Tyr91 and Tyr93.

5 In some preferred embodiments, the mutation positions of various antibodies are preferably selected from the positions listed in Tables 5 to 19 which retain 70% or more, preferably 80% or more, more preferably 90% or more of binding activity after mutation or retain 80% or more, preferably 90% or more of binding activity after conjugating to R4. These mutation positions include those in CDR and non-CDR. For example, preferred mutation
10 positions of a light chain of anti-PD-1 antibody 1 may include Ala25, Ser26, Gly28, Ser30, Thr31, Ser32, Gly33, Ser35, Tyr36, Leu37, Leu54, Ala55, Ser56, Tyr57, Ser60, Gly61, Ser95, Thr101 and Gly104; more preferably, the mutation positions include Ser26, Gly28, Ser30, Ser32, Gly33, Ser35, Ala55, Ser56, Ser60, Gly61 and Ser95. Preferred mutation positions of a light chain of anti-PD-1 antibody 2 may include Ala25, Ser26, Ser28, Ser30, Ser31, Ala34,
15 Ala51, Ser52, Ser54, Ala55, Thr56, Gly57, Ile58, Ala60, Ser91, Ser92, Thr97 and Gly99; more preferably, the mutation positions include Ser26, Ser28, Ser30, Ser31, Ala34, Ser52, Ser54, Thr56, Gly57, Ser91, Ser92, Thr97 and Gly99. Preferred mutation positions of a light chain of anti-CTLA-4 antibody may include Ala25, Ser26, Ser28, Gly30, Ser31, Ser32, Leu34, Ala35, Gly51, Ala52, Ser54, Ala56, Thr57, Gly58, Gly93, Ser94, Ser95, Thr98 and Gly100; more
20 preferably, the mutation positions include Ala25, Ser26, Ser28, Gly30, Ser31, Ser32, Gly51, Ser54, Thr57, Gly58, Gly93, Ser94, Ser95 and Gly100. Preferred mutation positions of a light chain of anti-CD28 antibody may include Ser26, Ile29, Tyr30, Lys50, Ala51, Ser52, Gly91, Thr93, Tyr94, Tyr96, Thr97 and Gly99; more preferably, the mutation positions include Tyr30, Ala51, Tyr96, Thr97 and Gly99. Preferred mutation positions of a light chain of anti-TNF α
25 antibody may include Ala25, Ser26, Gly28, Ile29, Tyr32, Leu33, Ala34, Ala50, Ala51, Ser52, Thr53, Leu54, Ser56, Gly57, Tyr91, Ala94 and Thr97; more preferably, the mutation positions include Ser26, Gly28, Ala51, Ser56, Gly57, Tyr91 and Ala94.

Preferred mutation positions of a heavy chain of anti-PD-1 antibody 1 may include Thr30, Tyr32, Tyr35, Gly50, Ile51, Ser54, Gly56, Gly57, Thr58, Lys63, Tyr101 and Gly106; more
30 preferably, the mutation positions include Thr30, Gly50, Ser54, Gly56, Gly57, Thr58, Lys63 and Gly106. Preferred mutation positions of a heavy chain of anti-PD-1 antibody 2 may include Ser30, Ser32, Gly33, Ile51, Tyr53, Asp54, Gly55, Ser56, Lys57, Ala61, Ser63, Lys65, Gly66, Asp101, Gly104, Gly106, Thr107 and Leu108; more preferably, the mutation positions include Ser30, Ser32, Gly33, Tyr53, Gly55, Ser56, Ser63, Lys65, Gly66, Gly104, Gly106 and Thr107.
35 Preferred mutation positions of a heavy chain of anti-CTLA-4 antibody may include Ser30,

Ser31, Tyr32, Thr33, Ile51, Asp54, Gly55, Lys58, Tyr59, Tyr60, Ala61, Asp62, Ser63, Lys65, Gly66, Gly100, Leu102, Gly103, Asp106 and Tyr107; more preferably, the mutation positions include Ser30, Ser31, Thr33, Gly55, Tyr60, Ala61, Ser63, Lys65, Gly66, Gly100, and Gly103. Preferred mutation positions of a heavy chain of anti-CD28 antibody may include Ser25, Gly26, Tyr27, Thr28, Thr30, Ser31, Tyr32, Tyr33, Tyr52, Gly54, Thr58, Ser99, Tyr101, Gly102 and Leu103; more preferably, the mutation positions include Gly26, Tyr27, Thr28, Thr30, Ser31, Tyr52, Gly54 and Leu103. Preferred mutation positions of a heavy chain of anti-TNF α antibody may include Tyr32, Ala33, Ile51, Thr52, Ser55, Gly56, Ile58, Tyr60, Ala61, Ser63, Gly66, Ser100, Tyr101, Leu102, Ser103, Thr104, Ala105, Ser106, Ser107, Leu108 and Tyr110; more preferably, the mutation positions include Ala33, Ile51, Ala61, Ser63, Gly66, Ser100, Thr104 and Ser106.

Similarly, when the mutation occurs in a non-CDR, i.e., in a framework region of a variable region of a light chain, mutation positions of various antibodies may be preferably selected from those listed in Tables 7 and 8 which retain 80% or more, preferably 90% or more of binding activity after mutation. For example, for the light chain of the anti-PD-1 antibody 1 as disclosed herein, the preferred mutation positions in the framework region include Thr5, Ser7, Ala9, Thr10, Leu11, Ser12, Leu13, Ser14, Gly16, Ala19, Thr20, Gly45, Ala47, Leu50, Leu51, Ile52, Ala64, Ser67, Gly68, Ser69, Gly70, Ser71, Gly72, Thr73, Ala76, Thr78, Ser80, Ser81, Ile110 and Lys111; more preferably, the mutation positions include Thr5, Ser7, Ala9, Leu11, Leu13, Ser14, Ala47, Leu50, Ala64, Gly68, Ser69, Ser71, Gly72, Thr73, Ser80 and Ile110. For the heavy chain of anti-PD-1 antibody 2 as disclosed herein, the preferred mutation positions of the framework region include Ser7, Gly8, Gly9, Gly10, Gly15, Ser17, Ala24, Ser25, Gly26, Thr28, Ser30, Ala40, Gly42, Gly44, Thr68, Ser71, Ser75, Thr78, Thr85, Ala88, Thr91, Ala92, Thr98, Thr110, Ser112, Ser113, Ala114, Ser115, Thr116, Lys117, Gly118 and Ser120; more preferably, the mutation positions include Ser7, Gly8, Gly9, Ala24, Ser25, Gly26, Gly44, Thr68, Ser75, Thr78, Thr85, Thr110, Ser112, Ser113, Ser115, Thr116, Lys117, Gly118 and Ser120.

With respect to other functional proteins, such as cytokines, if its mutant retains 70% or more, preferably 80% or more, more preferably 90% or more of binding activity (e.g., binding capacity to its nature ligand) of a wild-type protein, it is believed that the amino acid residue at the position may be mutated to cysteine to link to a functional moiety. For example, the mutation position of IL2 may be selected from the group consisting of Lys32, Lys35, Thr37, Met39, Thr41, Lys43, Lys48, Lys49, Lys64, Leu72, Ala73, Ser75, Lys76, Leu94, Thr101, Thr102, Tyr107, Ala108, Thr111 and Ala112, so as to block an alpha receptor; or is selected from the group consisting of Leu12, His16, Leu19, Met23, Gly27, Ser75, Arg81, Leu85, Ser87,

Leu94, Gly98, Ser99, Thr101 and Thr133, so as to block a beta receptor; or is selected from the group consisting of Leu36, Ala50, Thr51, Thr123, Ser126, Ser127 and Ser130, so as to block a gamma receptor. In some embodiments, the mutation position of the IL2 may be selected from the group consisting of: Lys32, Lys35, Thr37, Thr41, Lys43, Lys48, Lys49, Ala50, Leu72, Ala73, Ser75, Lys76, Leu94, Thr101, Thr102, Ala108, Thr111 and Ala112, so as to block alpha receptor of IL2; or may be selected from the group consisting of Leu19, Gly27, Ser75, Leu80, Ser87, Leu94, Gly98, Ser99, Thr101 and Thr133, so as to block beta receptor of IL2; or is selected from the group consisting of Leu36, Ala50, Thr51, Thr123, Ser126, Ser127 and Ser130, so as to block gamma receptor of IL2.

One or more of Ala, Gly, Ser, Thr, Leu, Lys, Tyr, Phe, Asp, Glu and Ile of cytokines, such as IL2, IL10 and IL15, may be mutated to cysteine. Preferably, any of Thr, Leu, Ala, Gly and Ser in the amino acid sequence of cytokines is mutated to cysteine.

In some embodiments, the mutation position in a Human IL10 may be selected from the group consisting of: Thr6, Ser8, Ser11, Thr13, Gly17, Arg24, Ser31, Arg32, Lys34, Thr35, Lys40, Leu46, Lys49, Ser51, Lys57, Gly58, Ser66, Tyr72, Lys88, His90, Ser93, Lys99, Thr100, Arg104, Lys117, Ser118, Lys119, Lys125, Lys130, Lys134, Gly135, Tyr137, Tyr149, Thr155, Lys157 and Arg159.

For the fusion protein of the present disclosure, one or more of cysteines may be introduced into any protein of the fusion protein. For example, for a bispecific antibody, the mutation(s) may be introduced into the CDR or non-CDR of either or both of the scFv, or into the either or both of the antigen binding domain. For a fusion protein of an antibody and a cytokine, either or both of the antibody and cytokine may contain the mutation(s).

Exemplified sequences may be referred to SEQ ID NO: 13-83.

Mutation, transfection, expression and purification of a biomolecule may be performed by the methods known in the art. For example, a nucleic acid of a biomolecule having a mutation position may be directly synthesized, then nucleic acid molecules of different fragments obtained by enzyme digestion may be ligated into an expression vector and the expression vector is transformed to bacteria or eukaryotic host cells. The biomolecule containing the cysteine mutation may be obtained through recombination in the host cells.

Bacteria or eukaryotic host cells suitable for use in the present disclosure may be host cells commonly used in the art, including but not limited to bacteria, yeast and mammal cells. Useful eukaryotic host cells include CHO cells, HEK293T cells, or *Pichia pastoris*.

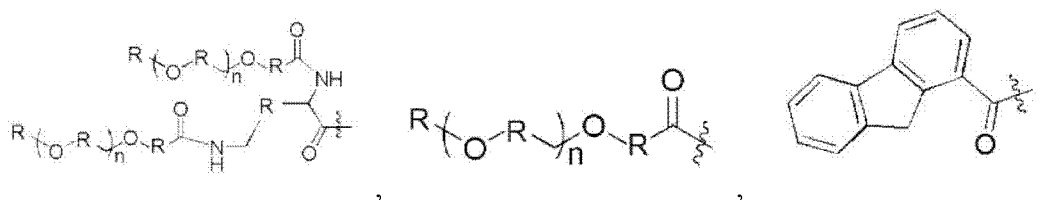
Expression vectors suitable for use in the present disclosure may be virus-based expression vectors known in the art, including but not limited to baculovirus, simian virus (SV40), retrovirus or vaccinia based viral vectors. Expression vectors containing suitable regulatory

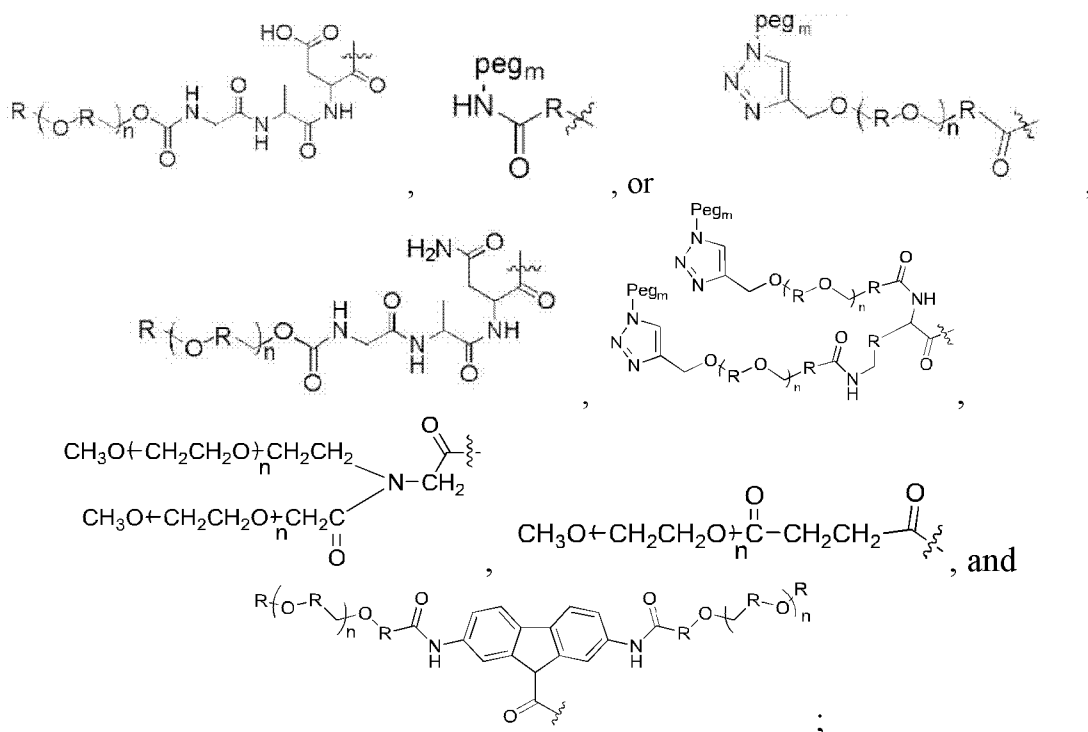
elements and selecting markers may be used to prepare mammal cell lines which stably express a mutant. For example, GS Eukaryotic Expression System (Lonza), DHFR Eukaryotic Expression System (Invitrogen) and *Pichia pastoris* Expression System (Invitrogen) may be used in the expression and preparation.

Biomolecules of the present disclosure may be purified by the isolation methods known in the art. These methods include but are not limited to DEAE ionic exchange, gel filtration and hydroxyapatite chromatography. For example, protein G column may be used to isolate biomolecules in the supernatant of cell cultures or in extracts of cytoplasm. In some embodiments, the biomolecules may be subjected to “engineering modification” to make them to contain an amino acid sequence that allows the biomolecules to be captured to an affinity matrix. For example, a tag may be used to facilitate purification of a polypeptide. The tag includes but is not limited to c-myc, hemagionium, poly-His (e.g., 6His) or Flag™ (Kodak). Such kind of tags may be inserted to any position within a polypeptide, including a carboxyl terminus or an amino terminus. Biomolecules of the present disclosure may also be purified by immunoaffinity chromatography.

The functional moiety suitable for use in the present disclosure may be represented by formula R1-R2-R3-R4. In the functional moiety, R1, R2, R3 and R4 are linked together via any suitable linkage manner, including but not limited to amide, ester, carbamate, urea or hydrazone bond. In the present disclosure, amide bond may be represented by “-CO-NH-”, ester bond may be represented by “-C(O)-O-”, carbamate bond may be represented by “-NH-C(O)-O-”, urea bond may be represented by “-NH-CO-NH-”, and hydrazone bond may be represented by “-CH=N-NH-”.

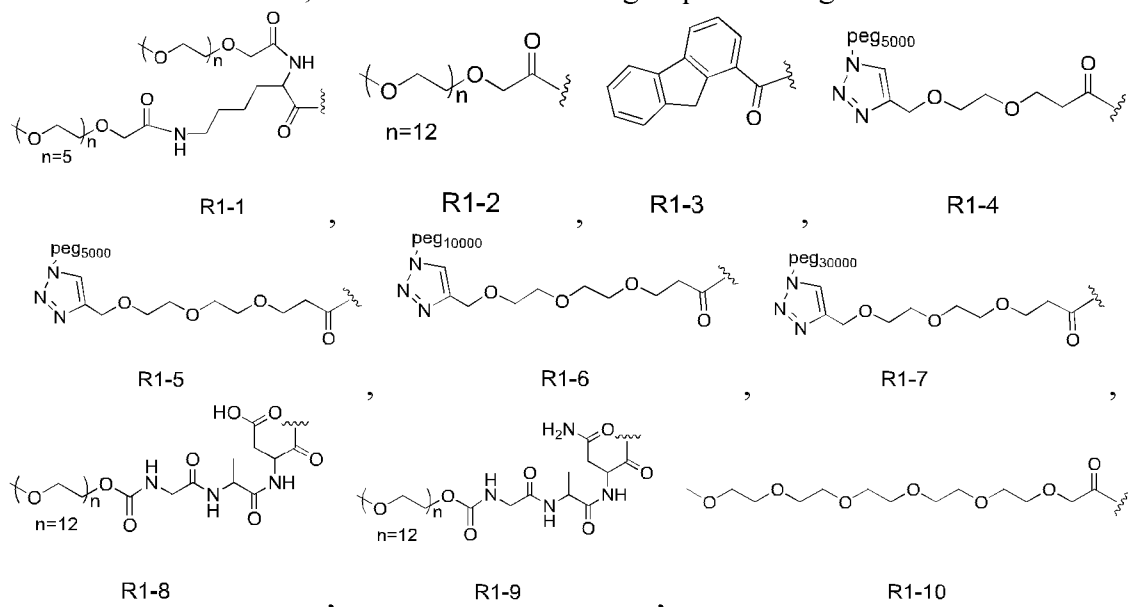
R1 is a protective group for the biomolecule R5. It may be selected from any group that can prevent the biomolecule from binding to its ligand or receptor, so as to prevent it from being interfered by other molecules, for example, those that prevent it from binding to its ligand or receptor, before it reaches a pathologic microenvironment, such as tumor or inflammatory microenvironment. Suitable R1 may be selected from the group consisting of polyethylene glycol-C₁₋₅ alkylcarbonyl, naphthylcarbonyl, quinolylcarbonyl, fluorenylcarbonyl, adamantylcarbonyl,

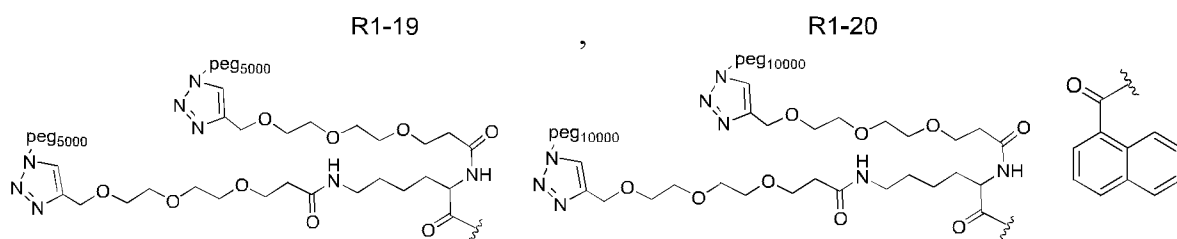
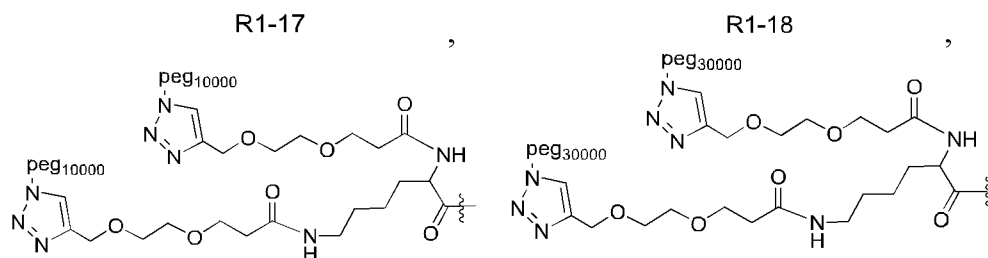
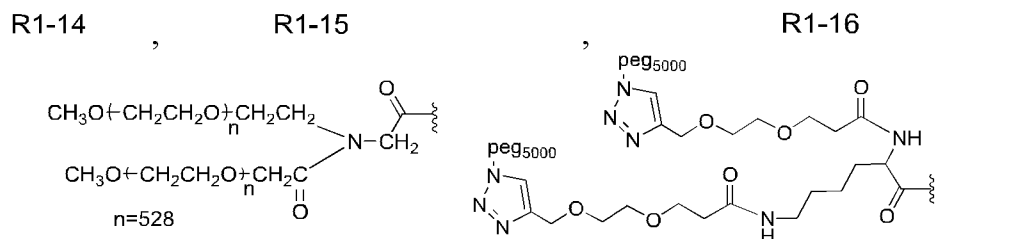
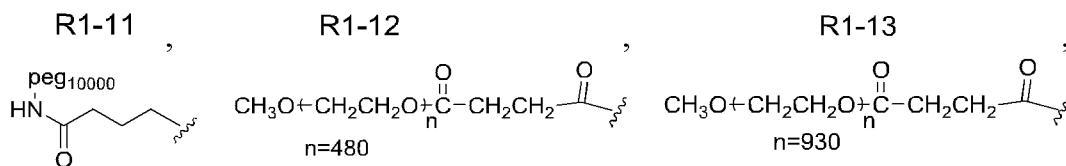
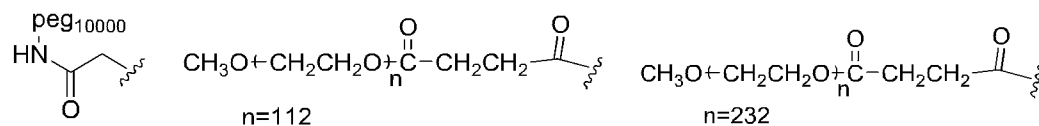




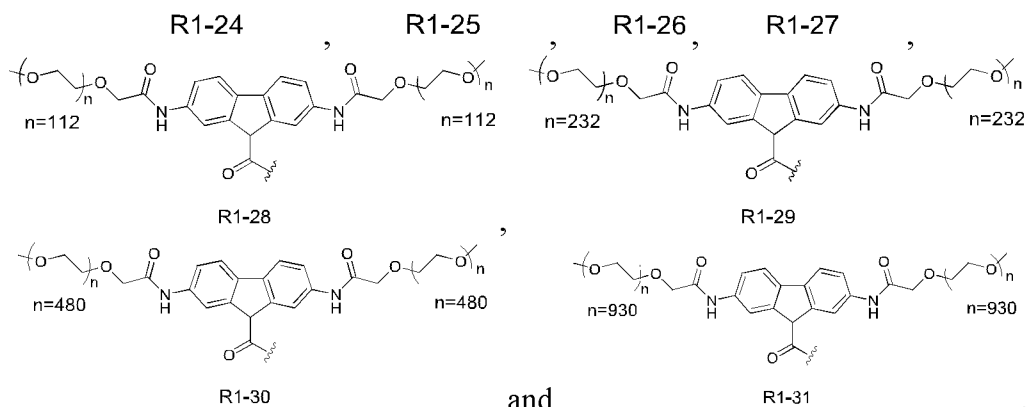
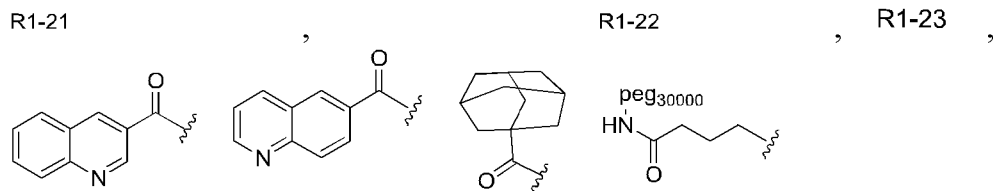
- 5 wherein each R is independently a C₁₋₄alkyl; each n is independently an integer in a range of 1 to 30000, such as an integer in a range of 1 to 15000, 1 to 5000, 1 to 2000, 1 to 300, 1 to 150, 1 to 50, 1 to 20 or 3 to 12; polyethylene glycol or peg_m represents a polyethylene glycol having a molecular weight in a range of 44 to 132000, such as that in a range of 1000 to 50000 or 10000 to 30000; m represents the molecular weight of the polyethylene glycol; and the wave line
- 10 indicates the position of R1 linking to R2.

In some embodiments, R1 is selected from the group consisting of:





5



Generally, if a mutation position is present in the functional domain of a biomolecule, for

example, in a CDR of an antibody, there is no specific limitation on the molecular weight of R1 and R1 may have a relatively low molecular weight. If a mutation position is present outside the functional domain of a biomolecule, for example, in a non-CDR of an antibody, R1 is preferably selected to make the molecular weight of R1-R2-R3-R4 to be higher than 200, preferably higher than 500, more preferably to be higher than 1000, so as to make the molecular weight of the conjugate of biomolecule to be 5000 or more, preferably 8000 or more, more preferably 10000 or more, thereby better preventing the biomolecule from binding to its ligand or receptor before arriving at a pathologic microenvironment.

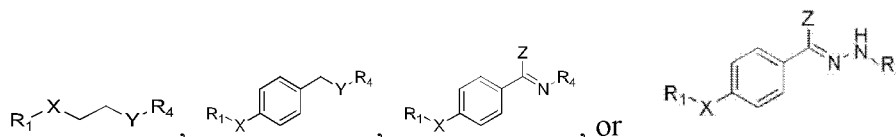
In the present disclosure, R2 is a cleavable linker arm. It may be a peptide capable of being activated by a proteolytic enzyme, protease or peptidase or a chemical bond capable of being acidically activated in a pathologic microenvironment. In the present disclosure, the proteolytic enzyme, protease or peptidase may be various proteolytic enzymes, proteases or peptidases present in a pathologic microenvironment. For example, protease may be cysteine protease, , aspartate protease, glutamic acid protease, threonine protease, gelatinase, metallopro-teinase, or asparagine peptide lyase. In some embodiments, R2 may be cleaved by at least one of a Legumain, Cathepsin B, Cathepsin C, Cathepsin D, Cathepsin E, Cathepsin K, Cathepsin L, kallikrein, hK1, hK10, hK15, plasmin, collagenase, Type IV collagenase, astromelysin, Factor Xa, chymotrypsin-like protease, trypsin-like protease, elastase-like protease, subtilisin-like protease, actinidain, bromelain, calpain, caspase, caspase-3, Mirl-CP, papain, HIV-1 protease, HSV protease, CMV protease, chymosm, pepsin, matriptase, legumain, plasmepsin, nepenthesin, metalloexopeptidase, metalloendopeptidase, matrix metalloprotease (MMP), MMP1, MMP2, MMP3, MMP8, MMP9, MMP10, MMP11, MMP12, MMP13, MMP14, ADAM10, ADAM12, urokinase plasminogen activator (uPA), nenterokinase, prostate-specific antigen (PSA, hK3), interleukin-13 converting enzyme, thrombin, FAP (FAP-a), meprin, granzyme, dipeptidyl peptidase, and dipeptidyl peptidase IV (DPPIV/CD26). In preferred embodiments, the present disclosure specifically relates to Legumain, which is largely expressed and secreted by tumor cells in a tumor microenvironment. Tumor-associated macrophage (M2 type) is also different from monocyte and inflammatory macrophage (M1 type) by the expression of Legumain. In the present disclosure, the peptide is a substrate of the proteolytic enzyme. It can be recognized and cleaved by the proteolytic enzyme.

R2 of the present disclosure may be represented by -R2a-, -R2b-, -R2a-N-, -R2a-D-, -R2a-AAN-, -R2a-AAD-, or -R2a-R2b-; wherein R2a is a peptide capable of being cleaved at amide bond by one or more proteolytic enzymes; R2b is a kind of peptide with its nitrogen in side chain forming a carbamate with R3 and the carbamate can be cleaved by one or more proteolytic enzymes; A is alanine; N is asparagine with its nitrogen in side chain forming a

carbamate with R3 and the carbamate can be cleaved by Legumain; D is aspartic acid with its nitrogen in side chain forming a carbamate with R3 and the carbamate can be cleaved by Granzyme B. R2a and R2b can be linked by forming an amide bond. After legumain, granzyme B cleaving the bonds (such as carbamate) between R2 and R3, R3 can quickly undergo auto-releasing. Then the R4 moiety may be retained. The other enzymes may cleave at amide bond of R2, which may cause some amino acid residues remaining in the linker and thus auto-releasing of R3 will not occur. Examples of such R2 include but are limited to LTPRLGPAAN (SEQ ID NO:84), GPAAN (SEQ ID NO:85) and LSGRSDN (SEQ ID NO:86).

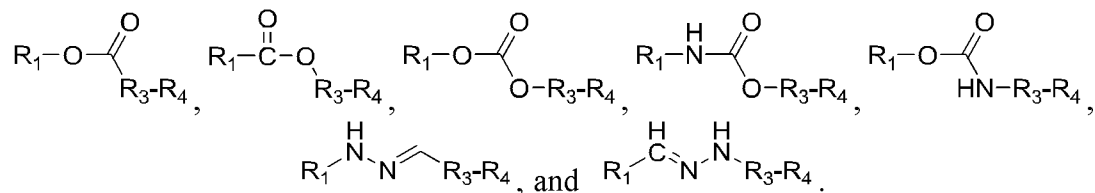
In some embodiments, suitable peptide capable of being activated by a proteolytic enzyme may be a tripeptide. Any substrate peptide capable of being recognized and cleaved (activated) by a proteolytic enzyme in a pathologic microenvironment known in the art may be used as R2 as disclosed herein. Such peptides may have structures disclosed in WO 2016/026458, the entity of which is incorporated in the present disclosure by reference. In some embodiments, in the tripeptide structure suitable for use in the present disclosure, the amino acid residue linked to R1 may be selected from the group consisting of Ala, Thr, Val and Ile, the middle amino acid residue may be selected from the group consisting of Ala, Thr, Val and Asn, and the amino acid residue linked to R3 may be selected from the group consisting of Asn and Asp. Generally, R2 links to R1 via an amino group of its amino acid residue in a linkage manner of amide, ester, carbamate, urea or hydrazone bond, and links to R3 via a carboxyl group of its amino acid residue in a linkage manner of amide, ester, carbamate, urea or hydrazone bond. In some preferred embodiments of the present disclosure, the tripeptide is selected from the group consisting of Ala-Ala-Asn and Ala-Ala-Asp. Ala-Ala-Asn may be recognized and cleaved by Legumain, and Ala-Ala-Asp may be recognized and cleaved by granzyme.

In some embodiments, R2 may be a chemical bond capable of being acidically activated in a pathologic microenvironment. Such bond includes but is not limited to amide bond, ester bond, carbamate bond, urea bond or hydrazone bond. When R2 is a chemical bond, the functional moiety as disclosed herein may be represented by formula R1-R2-R3-R4, wherein R1 links to R3 by the chemical bond capable of being acidically activated in a pathologic microenvironment. For example, in some embodiments, the structure of R1-R2-R3-R4 may be represented as follows:

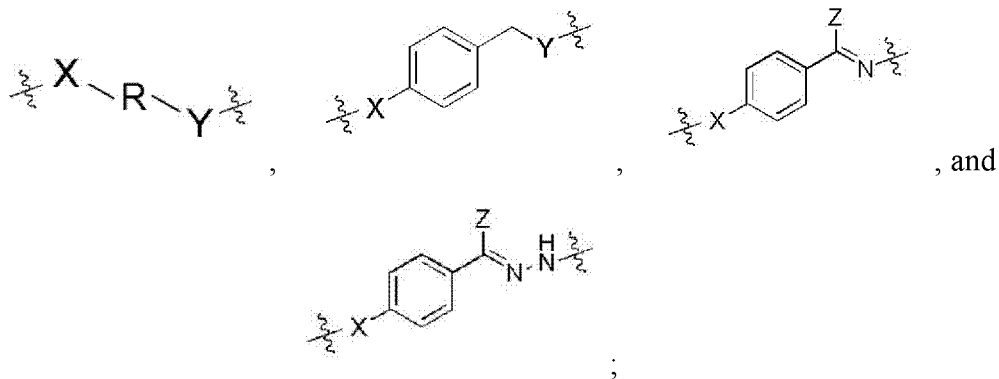


wherein X and Y are each independently NR' or O, Z is H or C₁₋₁₀alkyl, preferably C₁₋₄alkyl, and R' is H or C₁₋₄alkyl; R3 links to R1 and R4, respectively, via X and Y in a linkage manner of amide, ester, carbamate, urea or hydrazone bond.

In some embodiments, the structure of R1-R2-R3-R4 may be represented as follows:



In the present disclosure, R3 may be an automatically cleavable linker arm capable of automatically sheeding after cleavage of R2 to release R4-S-cys-R5. For example, such linker arm includes but is not limited to:

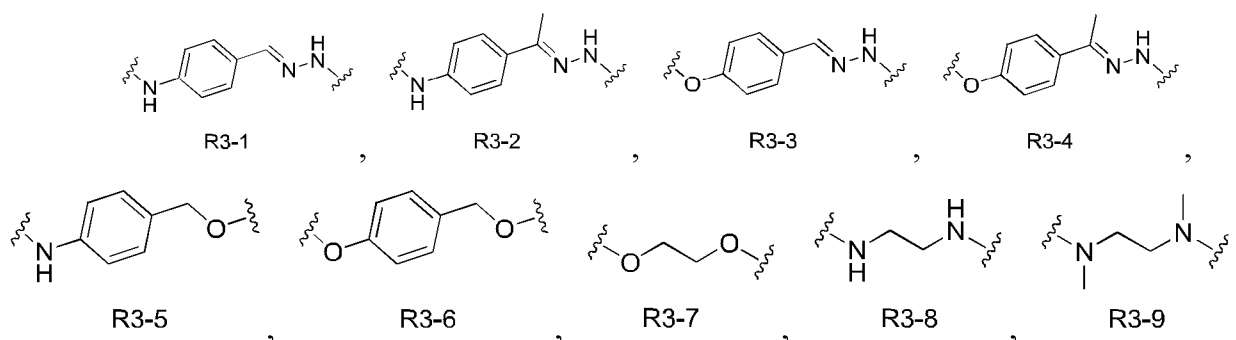


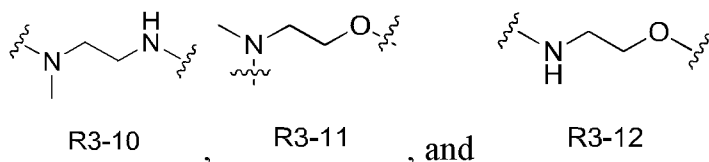
wherein X and Y are each independently NR' or O, Z is H or C₁₋₁₀alkyl, preferably C₁₋₄alkyl; R is C₁₋₄alkyl; and R' is H or C₁₋₄alkyl; wherein R4 links to R3 via Y or N in above formula in a manner of, such as amide bond, ester bond, carbamate bond, urea bond and hydrazone bond. In some embodiments, R3 is selected from the group consisting of -NH-phenyl-CH₂O-, -NH-phenyl-CH=N-, -NH-phenyl-C(CH₃)=N-, -O-phenyl-CH=N- and -O-phenyl-C(CH₃)=N-.

In some embodiments, R3 is a chemical bond capable of being acidically activated in a pathologic microenvironment. The chemical bond may be selected from the group consisting of amide bond, ester bond, carbamate bond, urea bond and hydrazone bond.

When R3 is a chemical bond capable of being acidically activated, R2 may be absent, such that the R1-R3-R4-S-cys-R5 can merely be acidically activated. On the other hand, when R2 is absent, R3 must be a chemical bond capable of being acidically activated.

In some embodiments, R3 is represented by any of the following structures:

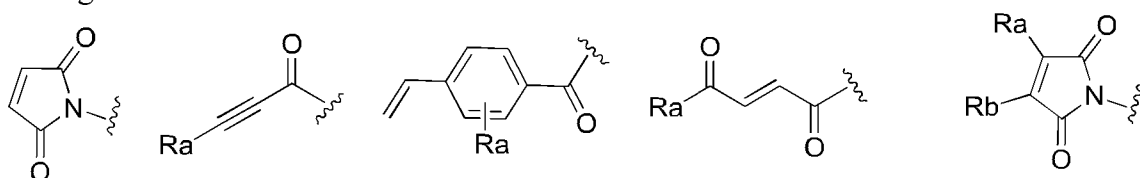




In the above formulae R3-1 to R3-12, R2 or R1 if R2 is absent may link to either end of the formulae as long as they form amide bond, ester bond, carbamate bond, urea bond and hydrazone bond.

- 5 In the present disclosure, R4 is a binding group capable of recovering, maintaining, reducing or promoting the binding capacity of a biomolecule to its antigen, ligand or receptor after cleavage of R2 and R3. In some embodiments, the resultant R4-s-Cys-R5 exhibits >60% affinity of native R5 to its antigen, ligand or receptor after cleavage of R2 and R3.

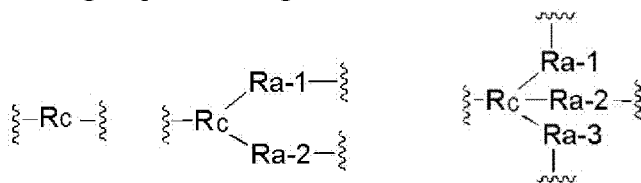
10 Suitable R4 may be represented by -R_{4-a}-R_{4-b}-R_{4-c}-, wherein R_{4-a} is selected from the group consisting of:



R_{4-a1} , R_{4-a2} , R_{4-a3} , R_{4-a4} , and R_{4-a5} ;

wherein Ra and Rb are each independently selected from the group consisting of H and C₁₋₆ alkyl or C₁₋₆ alkoxy;

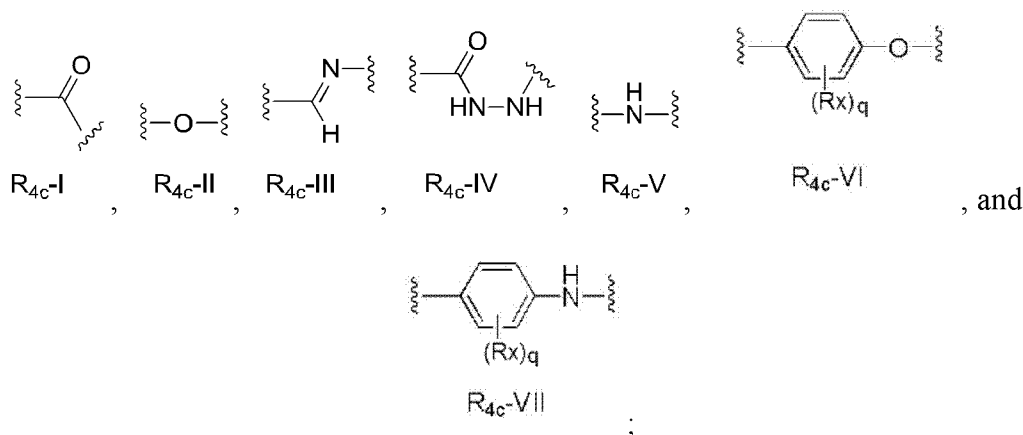
R_{4-b} is selected from the group consisting of:



R_{4-b1} , R_{4-b2} , and R_{4-b3} ;

- 15 wherein in formula R4-b1, Rc is absent, or is selected from the group consisting of C₁₋₁₂ alkyl, C₁₋₁₂alkoxy-C₁₋₁₂alkyl, C₁₋₁₂alkyl-C₃₋₈cycloalkyl, (C₁₋₄alkyl-O)_p-C₁₋₁₂alkyl, C₁₋₁₂alkylcarbonylamino-(C₁₋₄alkyl-O)_p-C₁₋₁₂alkyl, phenyl-C₁₋₁₂alkyl, C₃₋₈cycloalkyl, C₁₋₁₂alkyl-C₃₋₈cycloalkyl-C₁₋₁₂alkyl, and C₁₋₁₂alkyl-phenyl-C₁₋₁₂alkyl; in formula R4-b2, Rc is a C₁₋₁₂alkylamino with Ra-1 and Ra-2 substituted at N atom of the amino group, and in formula R4-b3, Rc is a C₁₋₁₂alkyl with the last C atom at the end of the alkyl being substituted by Ra-1, Ra-2 and Ra-3, wherein Ra-1, Ra-2 and Ra-3 are each independently selected from the group consisting of C₁₋₁₂alkyl, C₁₋₁₂alkyl-OH, and C₁₋₁₂alkyl-NR''R''', wherein R'' and R''' are each independently selected from the group consisting of H and C₁₋₁₂alkyl; wherein in formulae R4-b2 and R4-b3, R4-b links to R4-c via at least one of the Ra-1, Ra-2 and Ra-3;
- 20
- 25

R_{4-c} is selected from the group consisting of:

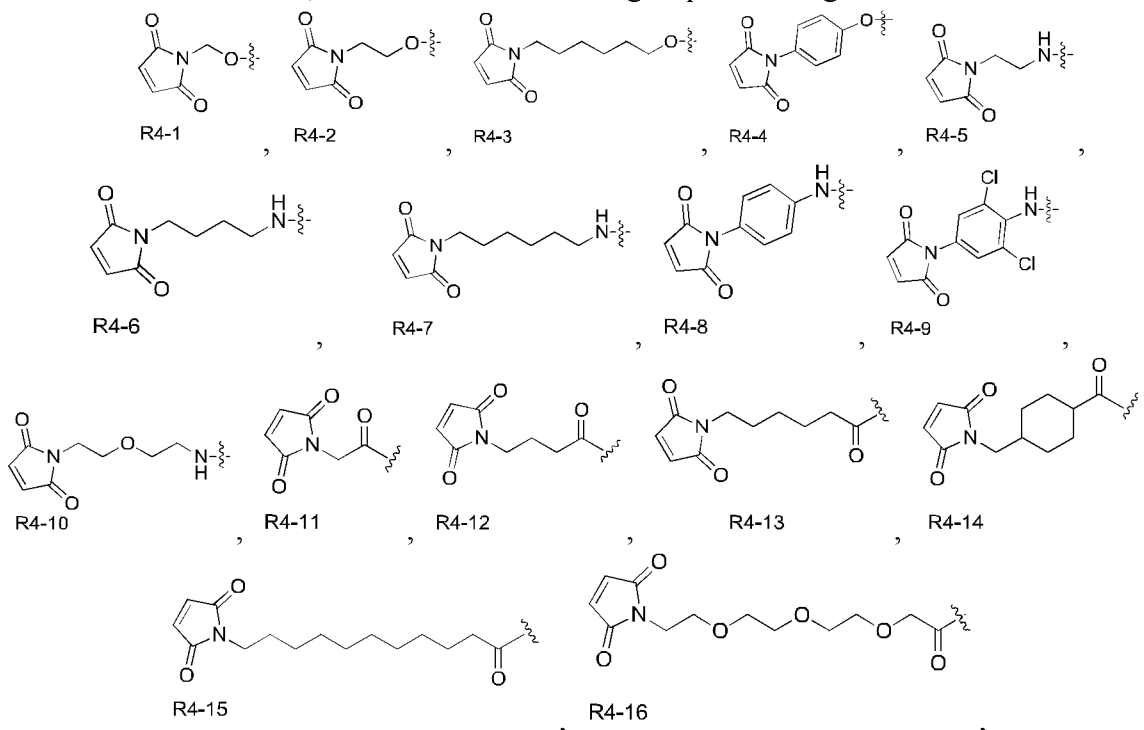


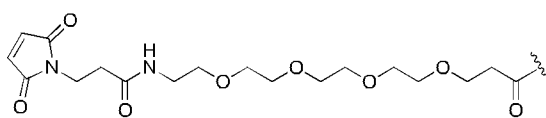
- 5 wherein Rx is selected from the group consisting of H, halo and C₁₋₄alkyl; p is an integer in a range of 1 to 10, such as an integer in a range of 1 to 5; and q is an integer in a range of 1 to 4, such as an integer in a range of 1 to 2; with the proviso that R_{4-c} is absent when R_{4-a} is selected from the group consisting of formulae R_{4-a2}, R_{4-a3} and R_{4-a4};

- 10 wherein R₃ links to R₄ via the R_{4-c} of R₄, and the wave line shown in each formula of R_{4-a} indicates the position at which R_{4-a} links to R_{4-b}. Preferably, in formulae R_{4c}-III, R_{4c}-IV, R_{4c}-VI and R_{4c}-VII, R₃ links to the carbon atom of these groups.

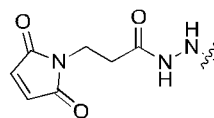
Generally, R₄ links to the S atom of the cysteine of R₅ via maleimide (R_{4-a1}), acetylene (R_{4-a2}), vinyl (R_{4-a3}), mono-substituted butenedioic acid (R_{4-a4}), or di-substituted maleimide (R_{4-a4}).

- 15 In some embodiments, R₄ is selected from the group consisting of:

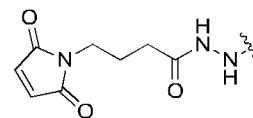




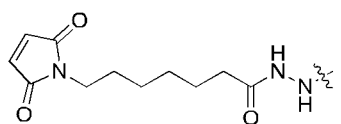
R4-17



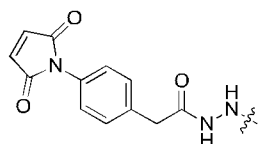
R4-18



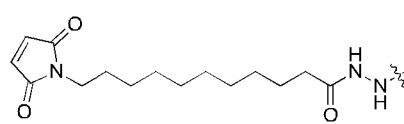
R4-19



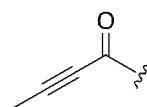
R4-20



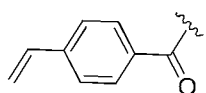
R4-21



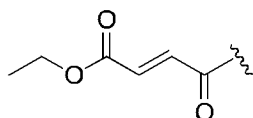
R4-22



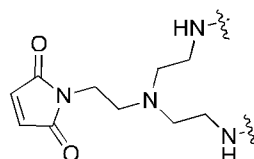
R4-23



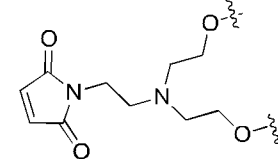
R4-24



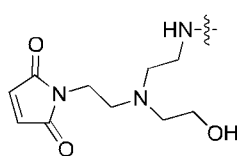
R4-25



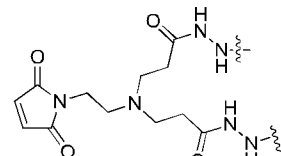
R4-26



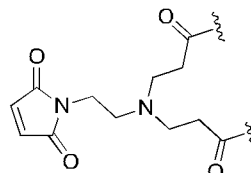
R4-27



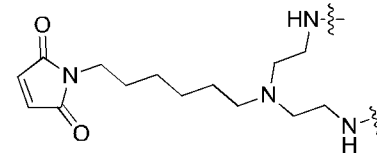
R4-28



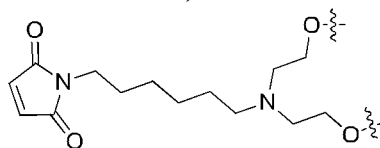
R4-29



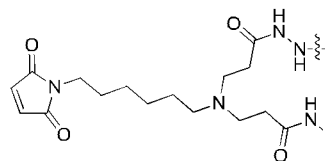
R4-30



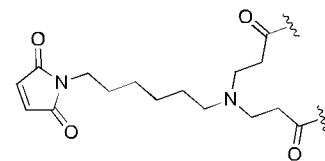
R4-31



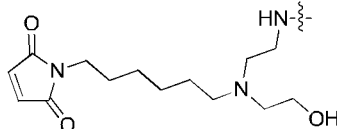
R4-32



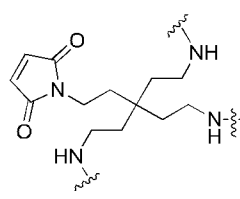
R4-33



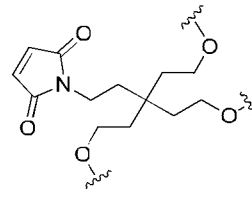
R4-34



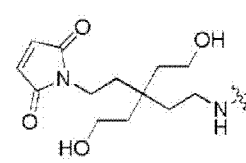
R4-35



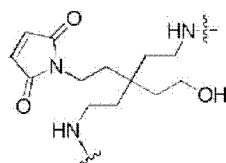
R4-36



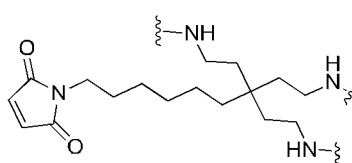
R4-37



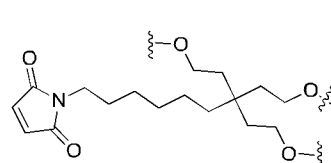
R4-38a



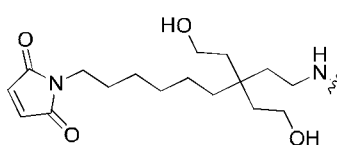
R4-38b



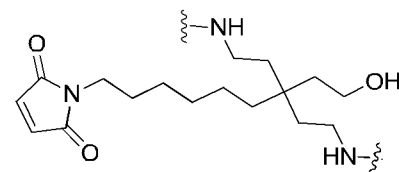
R4-39



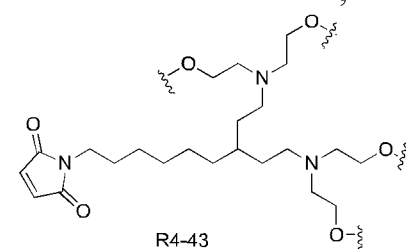
R4-40



R4-41

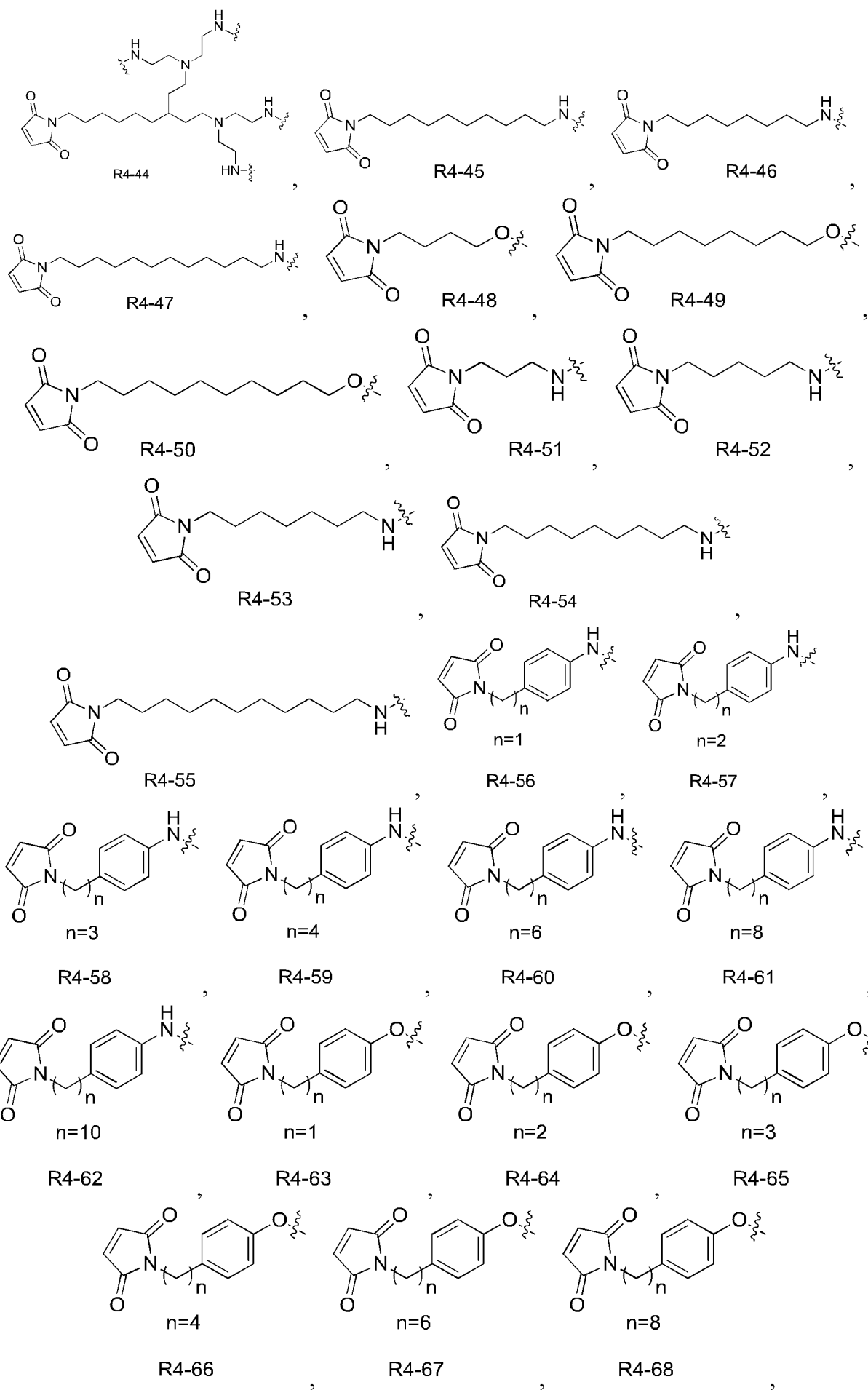


R4-42

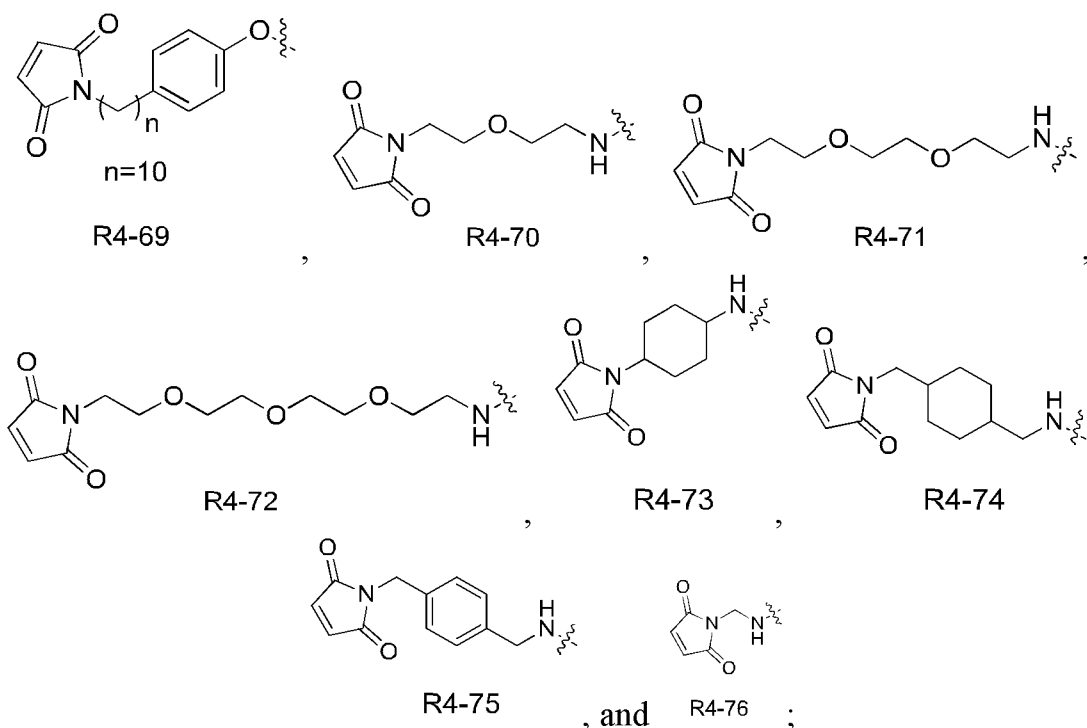


R4-43

5

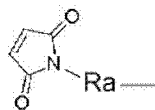


5



wherein the wave line indicates the position of R4 linking to R3.

5 In some embodiments, R4 is represented by:



wherein:

Ra is selected from the group consisting of C₁₋₁₂ alkyl, C₁₋₁₂ alkoxy, C₁₋₁₂alkylcarbonyl, phenoxy or phenyl amino optionally substituted by one or two halogens, C₁₋₁₂alkylamino, C₁₋₁₂alkoxy-C₁₋₁₂alkylamino, C₁₋₁₂alkylcarbonyloxy, C₁₋₁₂alkyl-C₃₋₈cycloalkylcarbonyloxy, (C₁₋₄alkyl-O)_p-C₁₋₁₂alkylcarbonyloxy, C₁₋₁₂alkylcarbonylamino-(C₁₋₄alkyl-O)_p-C₁₋₁₂alkylcarbonyloxy, C₁₋₁₂alkylcarbonylamino and phenyl-C₁₋₁₂alkylcarbonylamino;

p is an integer in a range of 1 to 10, such as an integer in a range of 1 to 5;

15 wherein R3 links to R4 via the Ra of R4, and to the thiol group of the cysteine of R5 via the maleimide group of R4.

In the conjugate of the present disclosure, R4 covalently links to R5 via the S of cysteine contained in R5. R1-R2 is cleaved from R3-R4-S-cys-R5 by a proteolytic enzyme or under an acidic condition of a pathologic microenvironment, and R3-R4-S-cys-R5 is released. Then R3 automatically sheds and R4-S-cys-R5 is released. The R4-S-cys-R5 can recover or promote the binding capacity of R5 to its ligand or receptor.

It should be understood that in the present disclosure, the wave line(s) used in each of the

indicated formulae indicate the linking position of the group containing the wave line(s) to other groups, and all position numbers of the amino acid mentioned for amino acid residue of an antibody is based on Kabat numbering.

R5, as described above, represents a biomolecule with one or more amino acid residues mutated to cysteine. R5 in fact is a moiety of the biomolecule without the hydrogen atom of the thiol group of the introduced cysteine. Absence of the hydrogen atom of the thiol group allows R5 being regarded as a group to link to R4 of the present disclosure.

Conjugates of the present disclosure may be prepared by a method comprising reducing the mutant biomolecule by DTT, TCEP or other reducing agent; oxidizing by Cu_2SO_4 , dehydroascorbic acid or other oxidizing agent; and then conjugating the oxidized biomolecule (R5) to R1-R2-R3-R4 in a liquid phase or solid phase condition. The final product may be collected in a liquid phase.

Therefore, in addition to the conjugate, the present disclosure also comprises the functional moiety, i.e., R1-R2-R3-R4; R2-R3-R4; R3-R4-S-cys-R5; R4-S-cys-R5; and the mutated biomolecule; wherein R1, R2, R3, R3, R5 and their linkage manner and the mutated biomolecule are defined as in any part or any embodiments of the present disclosure. In some embodiments, the functional moieties are shown by S1-S64. In the present disclosure, the -S-cys- indicates that R4 covalently links to R5 via the thiol group of cysteine introduced by mutation in R5. The R3-R4-S-cys-R5 is a conjugate produced by cleavage of R1-R2 by a proteolytic enzyme or under an acidic condition of a pathologic microenvironment. Generally, after separation of R3 from R2, the group of R3 previously linked to R2 forms a hydroxyl (-OH) or an amino group (-NH₂). R4-S-cys-R5 is a conjugate formed after automatic shedding of R3. Generally, after automatic shedding of R3, the group of R4 previously linked to R3 forms a hydroxyl (-OH) or an amino group (-NH₂).

The conjugate, the functional moiety, R2-R3-R4, R3-R4-S-cys-R5 and R4-S-cys-R5 as described herein may be synthesized by the methods known in the art. For example, they may be prepared according to the method described in Example 1 of the present application.

The present disclosure also includes a pharmaceutical composition which comprises the conjugate as described herein. The pharmaceutical composition may further comprise a pharmaceutically acceptable carrier. The carrier may be any pharmaceutically acceptable carrier or excipient, which may be varied according to the dosage form and administration mode. The pharmaceutically acceptable carrier is generally safe and non-toxic, and may comprise any known substance used in formulating a pharmaceutical composition in the pharmaceutical industry, including filler, diluent, coagulant, adhesive, lubricant, glidant, stabilizer, colorant, wetting agent, and disintegrant, etc. Suitable pharmaceutically acceptable carrier include sugars,

such as lactose or sucrose, mannitol or sorbitol; cellulose formulation and/or calcium phosphate, such as tricalcium phosphate or calcium hydrogen phosphate; amylum, including corn starch, wheat starch, rice starch, potato starch, gelatin, tragacanth, methyl cellulose, hydroxylpropylmethyl cellulose, sodium carboxyl methyl cellulose and/or polyvinylpyrrolidone; silica, talc, stearic acid or salt thereof, such as magnesium stearate or calcium stearate; and/or polyethylene glycol; and the like. When selecting a pharmaceutically acceptable carrier, the main consideration is the administration mode of the pharmaceutical composition. This is well known in the art.

The pharmaceutical composition may comprise a conjugate in a therapeutically or in a prophylactically effective amount. The “effective amount” indicates that the amount of an ingredient is sufficient to produce a desired reaction. The specific effective amount will depend on various factors, such as the specific disease to be treated, the physical condition of the patient, such as body weight, age and sex, the duration time of treatment, the therapy co-administered (if any), and the specific formulation used. Generally, the “effective amount” as described herein is a conventional amount of the biomolecule. However, in some embodiments, the therapeutically or prophylactically effective amount of the conjugate contained in the present pharmaceutical composition may be lower than the conventional amount of the biomolecule but may produce better treatment or prevention effect, because the biomolecule is protected by a protective group from binding to its ligand or receptor before arriving at a pathologic microenvironment.

The pharmaceutical composition of the present disclosure may be formulated into various suitable dosage forms, including but not limited to tablet, capsule, injection, etc., and it can be administered via any suitable route to achieve the expected purpose. For example, it can be administered parenterally, subcutaneously, intravenously, muscularly, intraperitoneally, transdermally, orally, intrathecally, intracranially, nasally or externally. The dose of a drug may depend on age, health status and body weight of a patient, treatment carried out in parallel, and frequency of treatment, etc. The pharmaceutical composition of the present disclosure may be administered to any subject in need thereof, such as a mammal, especially a human being.

In a tumor patient, tumor cells or antigen-presenting cells (APC) bearing a tumor antigen partially or fully inhibit immunological killing of the tumor by a host via binding to T cells. However, the conjugate of the present disclosure is activated and released by a proteolytic enzyme, especially Legumain or granzyme, or under an acidic condition, in a pathologic microenvironment. For example, the conjugate of the present disclosure in which the biomolecule is IL2, anti-CD28 antibody or anti-PD-1 antibody and the like can selectively stimulate proliferation of T cell or enhance its function to secrete anti-tumor cytokines.

Therefore, the conjugate of the present disclosure can effectively break through the immune barrier of an individual, arrive at a pathologic microenvironment and then be activated and released in the pathologic microenvironment. As a result, it can selectively promote proliferation or killing effect of T cells, etc., in a tumor or inflammatory microenvironment, thereby realizing low autoimmunity and high efficacy.

Therefore, each of the conjugates, R4-S-cys-R5 or mutated biomolecules disclosed in the present disclosure may be used for treating tumor or inflammation, or can be used as an active ingredient for preparing a medicament for treating tumor or inflammation. The tumor or inflammation described herein can be any tumor or inflammation which is known to be treated by the biomolecule as described herein, including but not limited to a cancer in bladder, brain, breast, cervix, colon-rectum, esophagus, kidney, liver, lung, nasopharynx, pancreas, prostate, skin, stomach, uterus, ovary, testiculus and blood, etc. Specifically, the cancer includes bladder cancer, brain cancer, breast cancer, cervical cancer, colorectal cancer, esophageal cancer, renal cancer, liver cancer, lung cancer, nasopharyngeal cancer, pancreatic cancer, prostate cancer, skin cancer, gastric cancer, uterus cancer, ovarian cancer, testicular cancer and blood cancer.

Further included is a method for treating or preventing tumor or inflammation, comprising administering a subject in need thereof a therapeutically or prophylactically effective amount of a conjugate as described herein or a pharmaceutical composition thereof. The method may be used in combination with any known radiotherapy or immunotherapy.

It should be understood that the term “comprising” and “including” or similar expressions used in the present disclosure also means “consisting of” or the like. The sum of all weight percentages or volume percentages should be equal to 100%. Unless otherwise specified, various reagents and products used in the Examples are commercial products. Unless otherwise specified, the methods mentioned in the Examples were implemented according to the conventional technique. The following examples are not intended to limit the scope of the present disclosure.

Information of sequence is summarized below:

SEQ ID NO:	Description	SEQ ID NO:	Description
1	heavy chain of anti-PD-1 antibody	45	Interleukin-2 with mutation
2	light chain of anti-PD-1 antibody	46	Interleukin-2 with mutation
3	heavy chain of anti-PD-1 antibody	47	Interleukin-2 with mutation
4	light chain anti-PD-1 antibody	48	Interleukin-2 with mutation
5	heavy chain of anti-CTLA-4 antibody	49	Interleukin-2 with mutation
6	light chain of anti-CTLA-4 antibody	50	Interleukin-2 with mutation
7	heavy chain of anti-TNF α antibody	51	Interleukin-2 with mutation
8	light chain of anti-TNF α antibody	52	Interleukin-2 with mutation

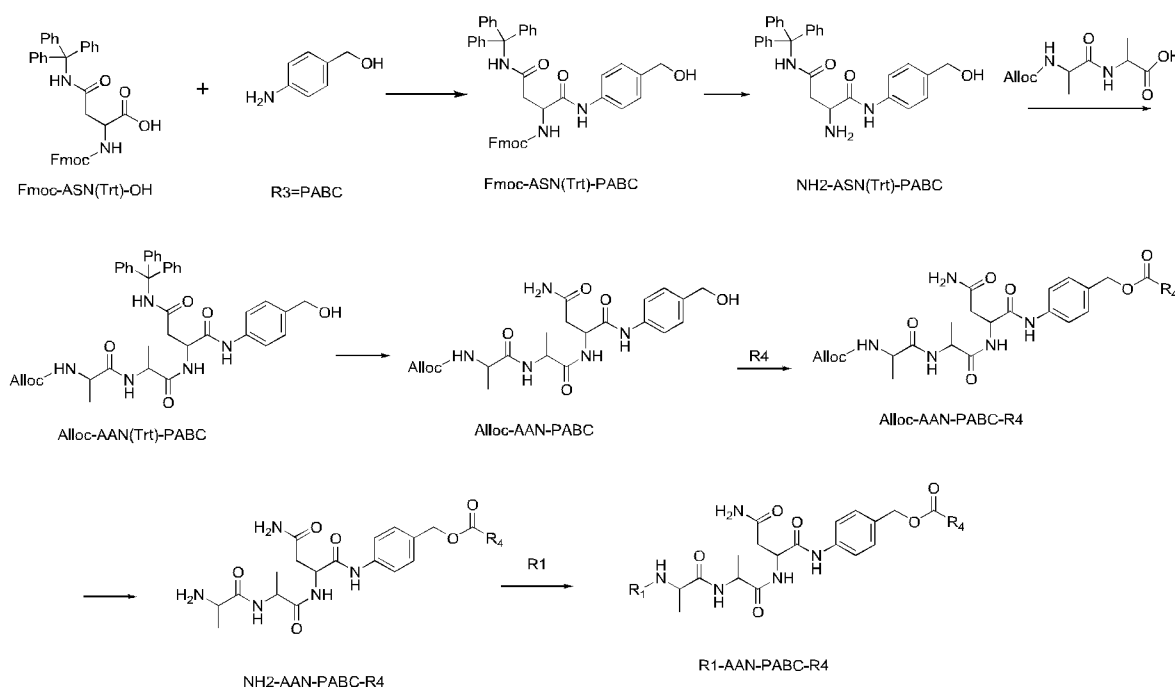
9	heavy chain of anti-CD28 antibody	53	Interleukin-2 with mutation
10	light chain of anti-CD28 antibody	54	Interleukin-10 with mutation
11	amino acid sequence of wild type IL2	55	Interleukin-10 with mutation
12	amino acid sequence of wild type IL2	56	Interleukin-10 with mutation
13	Ipilimumab heavy chain	57	Interleukin-10 with mutation
14	Ipilimumab light chain	58	Interleukin-10 with mutation
15	Pembrolizumab heavy chain	59	Interleukin-10 with mutation
16	Pembrolizumab light chain	60	Anti-human VEGF antibody heavy chain
17	Nivolumab heavy chain	61	Anti-human VEGF antibody light chain
18	Nivolumab light chain	62	Anti-human CD20 antibody heavy chain
19	Anti-human PD-1 antibody (WO 2017/124050 A1) heavy chain	63	Anti-human CD20 antibody light chain
20	Anti-human PD-1 antibody (WO 2017/124050 A1) light chain	64	VH of Atezolizumab
21	Anti-mouse PD-1 antibody J43v2 heavy chain	65	VL of Atezolizumab
22	Anti-mouse PD-1 antibody J43v2 light chain	66	VH of Cetuximab with mutation
23	Anti-mouse CTLA-4 9D9 antibody heavy chain	67	VL of Cetuximab with mutation
24	Anti-mouse CTLA-4 9D9 antibody light chain	68	VH of Ramucirumab with mutation
25	Anti-human 4-1BB antibody heavy chain	69	VL of Ramucirumab with mutation
26	Anti-human 4-1BB antibody light chain	70	Fusion protein of scFv of anti-Her2 antibody with CD3, with mutation
27	Trastuzumab heavy chain	71	VH of anti Her2 antibody with mutation
28	Trastuzumab light chain	72	VL of anti Her2 antibody with mutation
29	Adalimumab heavy chain	73	Fusion protein of scFvs of anti-Her2 antibody with anti-CD3 antibody
30	Adalimumab light chain	74	Fusion protein of scFvs of anti-Her2 antibody with anti-CD3 antibody
31	Atezolizumab heavy chain	75	anti CD3 antibody
32	Atezolizumab light chain	76	VH of Cetuximab
33	Anti-human CD28 antibody heavy chain	77	VL of Cetuximab
34	Anti-human CD28 antibody light chain	78	VH of Ramucirumab
35	Ipi-se008 light chain	79	VL of Ramucirumab
36	Ipi-se010 light chain	80	VH of anti Her2 antibody
37	Ipi-se009 light chain	81	VL of Her2 antibody

38	Pem-se010 light chain	82	Fusion protein of scFvs of anti-Her2 antibody with anti-CD3 antibody, with mutation
39	Pem-se009 light chain	83	Fusion protein of scFvs of anti-Her2 antibody with anti-CD3 antibody, with mutation
40	Pem-se007 light chain	84	Sequence of R2
41	Niv-se001 heavy chain variable domain	85	Sequence of R2
42	Niv-se005 light chain	86	Sequence of R2
43	Niv-se007 light chain	87	forward primer
44	Interleukin-2 with mutation	88	reverse primer

Example 1: Synthesis of Chemical Structure of Activatable and Binding Arms

When R2 has an amino acid sequence of Ala-Ala-Asn and R3 is PABC (R3-5), the synthetic scheme is shown below:

5

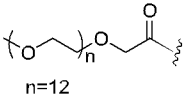
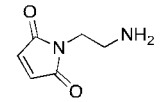
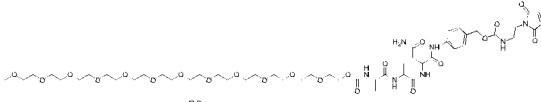
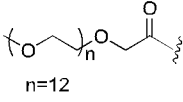
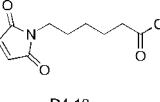
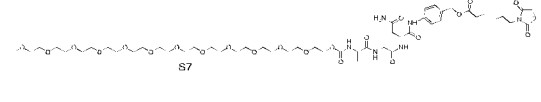
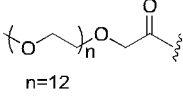
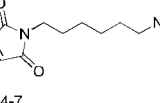
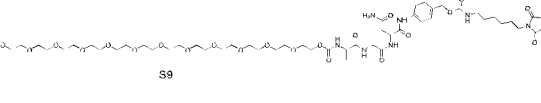
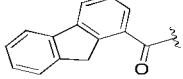
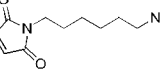
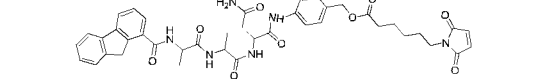
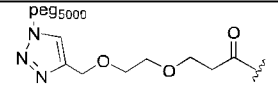
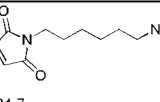
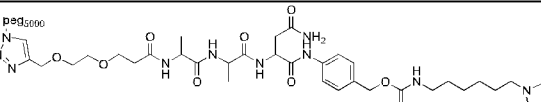
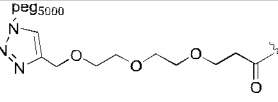
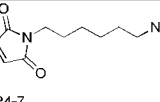
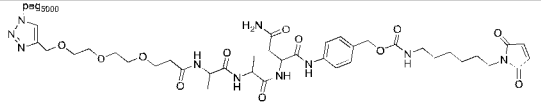
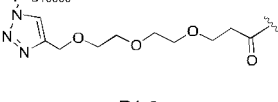
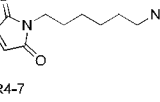
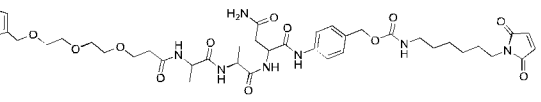
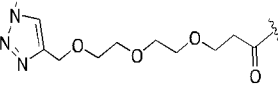
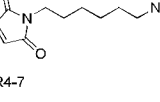
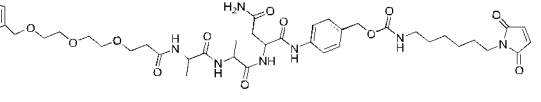
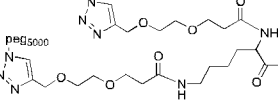
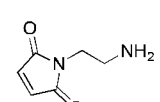
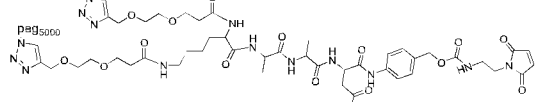
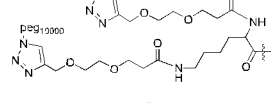
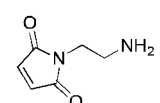
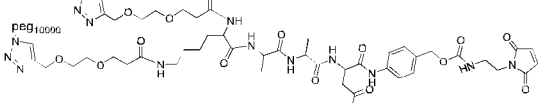
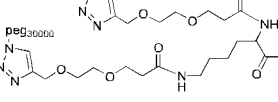
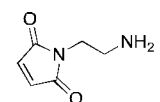
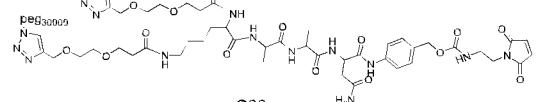


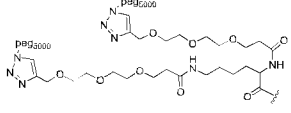
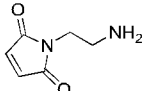
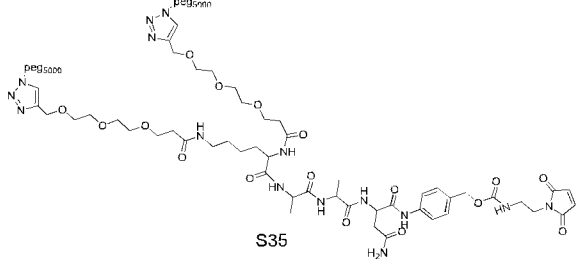
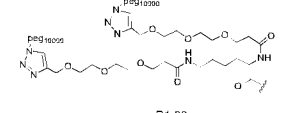
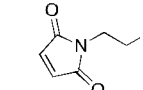
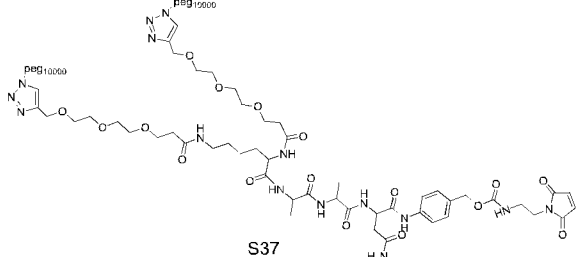
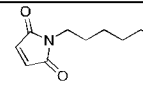
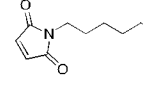
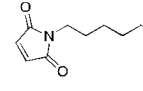
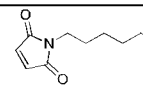
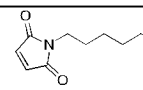
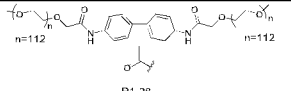
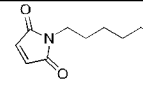
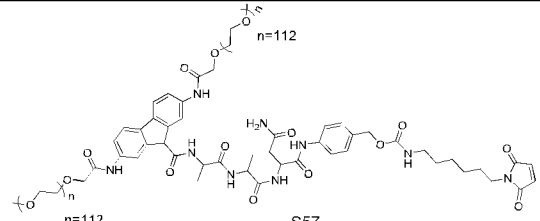
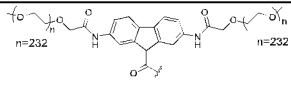
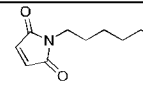
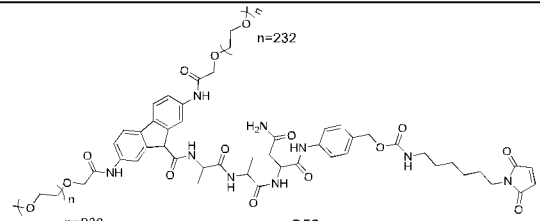
When R1 and R4 are different substituents, the following compounds shown in Table 1 were obtained.

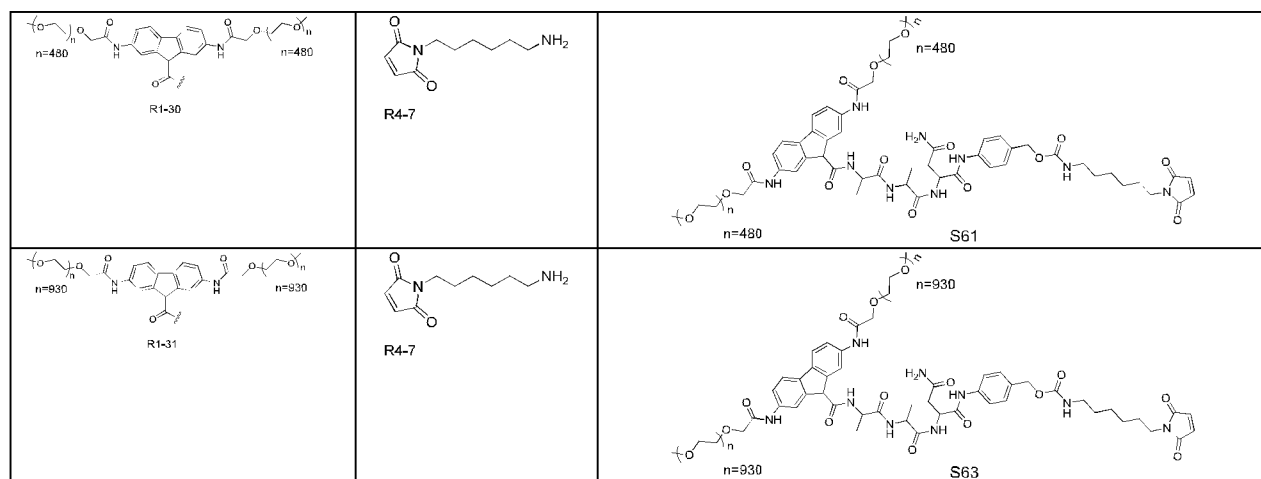
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Table 1

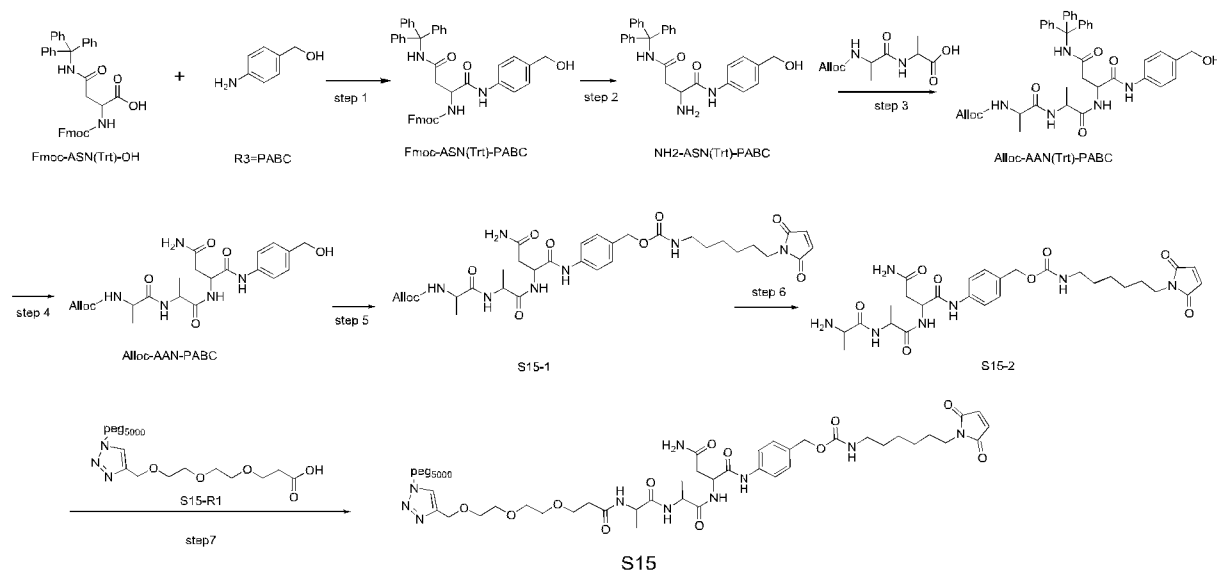
R1	R4	Compound
R1-1	R4-5	S1

 n=12 R1-2	 R4-5	 S3
 n=12 R1-2	 R4-13	 S7
 n=12 R1-2	 R4-7	 S9
 R1-3	 R4-7	 S11
 peg₅₀₀₀ R1-4	 R4-7	 peg₅₀₀₀ S13
 peg₅₀₀₀ R1-5	 R4-7	 peg₅₀₀₀ S15
 peg₁₀₀₀₀ R1-6	 R4-7	 peg₁₀₀₀₀ S17
 peg₃₀₀₀₀ R1-7	 R4-7	 peg₃₀₀₀₀ S25
 peg₅₀₀₀ peg₅₀₀₀ R1-18	 R4-5	 peg₅₀₀₀ peg₅₀₀₀ S29
 peg₁₀₀₀₀ peg₁₀₀₀₀ R1-19	 R4-5	 peg₁₀₀₀₀ peg₁₀₀₀₀ S31
 peg₃₀₀₀₀ peg₃₀₀₀₀ R1-20	 R4-5	 peg₃₀₀₀₀ peg₃₀₀₀₀ S33

 R1-21	 R4-5	 S35
 R1-22	 R4-5	 S37
$\text{CH}_3\text{O}+(\text{CH}_2\text{CH}_2\text{O})_n+\text{C}(=\text{O})\text{CH}_2\text{CH}_2\text{C}(=\text{O})\text{OCH}_3$ n=112 R1-12	 R4-7	$\text{CH}_3\text{O}+(\text{CH}_2\text{CH}_2\text{O})_n+\text{C}(=\text{O})\text{CH}_2\text{CH}_2\text{C}(=\text{O})\text{OCH}_3$ n=112 S39
$\text{CH}_3\text{O}+(\text{CH}_2\text{CH}_2\text{O})_n+\text{C}(=\text{O})\text{CH}_2\text{CH}_2\text{C}(=\text{O})\text{OCH}_3$ n=232 R1-13	 R4-7	$\text{CH}_3\text{O}+(\text{CH}_2\text{CH}_2\text{O})_n+\text{C}(=\text{O})\text{CH}_2\text{CH}_2\text{C}(=\text{O})\text{OCH}_3$ n=232 S41
$\text{CH}_3\text{O}+(\text{CH}_2\text{CH}_2\text{O})_n+\text{C}(=\text{O})\text{CH}_2\text{CH}_2\text{C}(=\text{O})\text{OCH}_3$ n=480 R1-15	 R4-7	$\text{CH}_3\text{O}+(\text{CH}_2\text{CH}_2\text{O})_n+\text{C}(=\text{O})\text{CH}_2\text{CH}_2\text{C}(=\text{O})\text{OCH}_3$ n=480 S43
$\text{CH}_3\text{O}+(\text{CH}_2\text{CH}_2\text{O})_n+\text{C}(=\text{O})\text{CH}_2\text{CH}_2\text{C}(=\text{O})\text{OCH}_3$ n=930 R1-16	 R4-7	$\text{CH}_3\text{O}+(\text{CH}_2\text{CH}_2\text{O})_n+\text{C}(=\text{O})\text{CH}_2\text{CH}_2\text{C}(=\text{O})\text{OCH}_3$ n=930 S45
$\text{CH}_3\text{O}+(\text{CH}_2\text{CH}_2\text{O})_n+\text{CH}_2\text{CH}_2\text{N}(\text{CH}_2)_2\text{C}(=\text{O})\text{OCH}_3$ n=528 R1-17	 R4-7	$\text{CH}_3\text{O}+(\text{CH}_2\text{CH}_2\text{O})_n+\text{CH}_2\text{CH}_2\text{N}(\text{CH}_2)_2\text{C}(=\text{O})\text{OCH}_3$ n=528 S47
 R1-28	 R4-7	 n=112 S57
 R1-29	 R4-7	 n=232 S59



As exemplified by S15, the specific synthesis process was shown below:



5

1) Fmoc-Asn(Trt)-OH (20 g, 0.03 mol),

2-(7-azabenzotriazole)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU) (15 g, 0.04 mol), and DMF (200mL) were added to a three-necked flask and stirred for 30min.

p-Aminobenzyl alcohol (4.1 g, 0.03 mol) and N,N-diisopropyl ethylamine (8.7 g, 0.06 mol)

10 were added at 0°C, respectively and then stirred at room temperature for 3 hours. Most of DMF were removed by rotary evaporation. The residue was dissolved in acetic acetate (200mL), washed with saturated ammonia chloride solution and saturated sodium chloride solution subsequently, dried over anhydrous sodium sulfate followed by filtration. The solvents were removed by evaporation. The crude product was beaten to obtain a white solid

15 Fmoc-Asn(Trt)-PABC (21.3g; Yield: 90%).

2) Fmoc-Asn(Trt)-PABC (16.0g, 22mmol) was dissolved in N,N-dimethyl formamide (80mL). Piperidine (30mL) were added and then stirred at room temperature for 2 hours. The

solvents were removed by evaporation under reduced pressure. The residue was drier under high vacuum in a vacuum oven to remove a small amount of piperidine to produce 9.8g pale yellow solid NH₂-Asn(Trt)-PABC which could be used in the next step without purification.

3) Alloc-Ala-Ala-OH (5.0g, 20.4mmol), benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) (11.6g, 30.6mmol) and DMF (50mL) were added into a three-necked flask and stirred for 30 min in an ice bath. NH₂-Asn(Trt)-PABC (9.8g, 20.4mmol) and N,N-diisopropyl ethylamine (7.89 g, 61.2 mmol) were added respectively at 0°C and then stirred at room temperature overnight. The solvents were removed by evaporation under reduced pressure. The residue was dissolved in acetic acetate (200mL), washed with saturated ammonia chloride solution and saturated sodium chloride solution subsequently, dried over anhydrous sodium sulfate followed by filtration. The solvents were removed by evaporation. The resulted crude product was subjected to recrystallization to obtain a white solid Alloc-AAN(Trt)-PABC (13.0g; Yield: 90%).

4) Alloc-AAN(Trt)-PABC (10.0 g, 14.2 mmol) was dissolved in dichloromethane (100 mL). Trifluoroacetic acid (20 mL) were added and then stirred at room temperature for 4 hours. After washing with water and fraction, the organic phase was dried over anhydrous sodium sulfate. The solvents were removed by evaporation under reduced pressure and the residual trifluoroacetic acid was removed by evaporation under high vacuum. The crude product was isolated by column chromatography to obtain Alloc-AAN-PABC(5.9g; Yield: 89%).

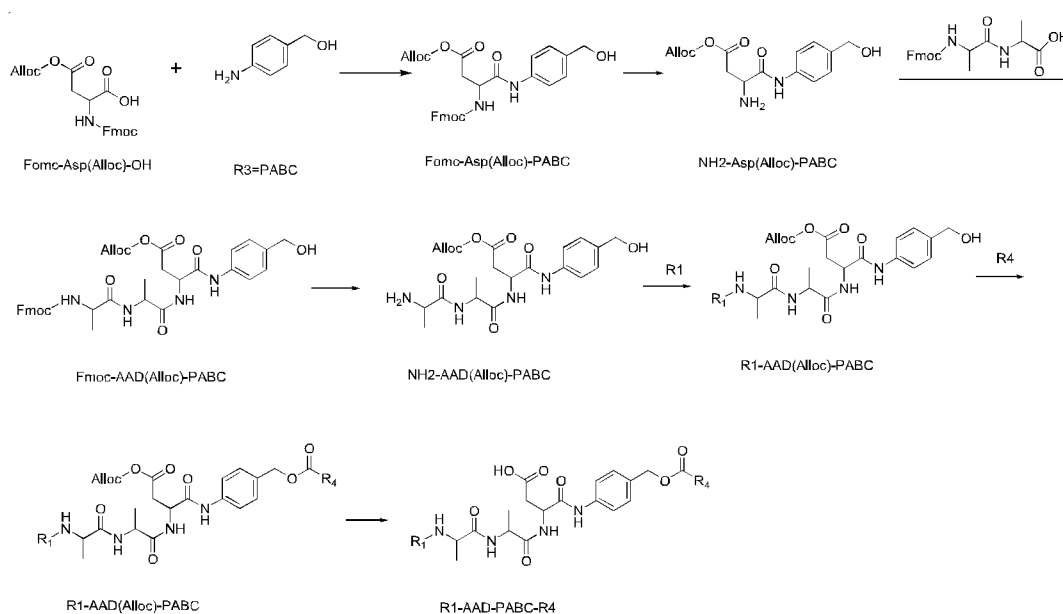
5) Alloc-AAN-PABC (467mg, 1.01 mmol) dissolved in dichloromethane (10mL) were added to a three-necked flask. 4-Nitrophenyl chloroformate (406 mg, 2.02 mmol) in dichloromethane and pyridine (160 mg, 2.03 mmol) in dichloromethane were dropped into the flask, respectively, in an ice bath and under nitrogen gas protection and then stirred at room temperature overnight. 1-(6-Aminohexyl)-1H-pyrrolo-2,5-dione (235mg, 1.2mmol) were added in batches into the above solution and was allowed to react at room temperature for 4 hours. The reaction solution was dried by rotary evaporation. The resulted crude product was purified by silica gel column chromatography to obtain a white solid S15-1 (540mg; Yield: 80%).

6) DMF (10ml), S15-1 (208mg, 0.31mmol), acetic acid (274mg, 4.65mmol), triphenylphosphine palladium (72mg, 0.062mmol) and tributyltin hydride (1.17g, 4.03mmol) were added successively into an one-neck flask. After replacing the air in the flask with nitrogen gas, the mixture was stirred at room temperature until S15-1 is reacted completely. After the reaction completed, DMF was removed by evaporation under reduced pressure. The crude product was isolated and purified by silica gel column chromatography to obtain S15-2 (white solid, 116mg, Yield: 62%).

7) S15-R1 (940mg, 0.18 mmol), benzotriazole-N,N,N',N'-tetramethyluronium

hexafluorophosphate (HBTU) (95mg, 0.25mmol) and DMF (10mL) were added to a three-necked flask, and then stirred in an ice bath for 30 min. Then compound S15-2 (110mg, 0.18 mmol) and N,N-diisopropyl ethylamine (70mg, 0.54mmol) were added respectively at 0°C and then stirred at room temperature overnight. The solvents were removed by evaporation under reduced pressure. The crude product was isolated and purified by silica gel column chromatography to obtain a white solid (418mg; Yield: 40%), which was Compound S15.

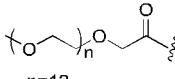
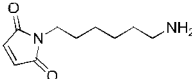
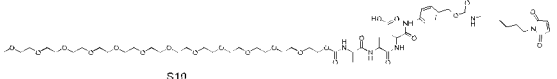
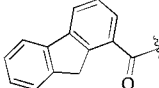
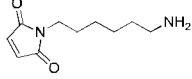
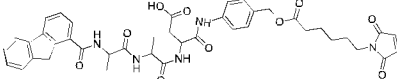
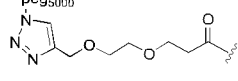
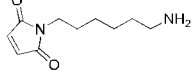
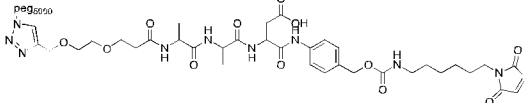
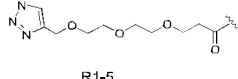
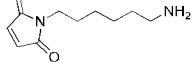
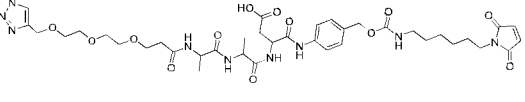
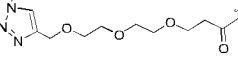
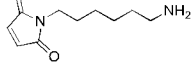
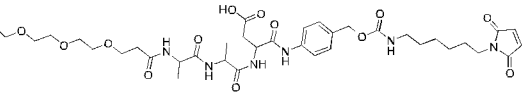
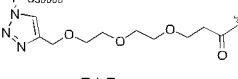
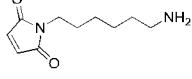
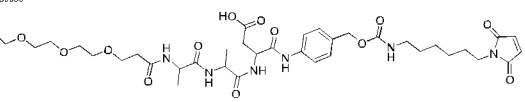
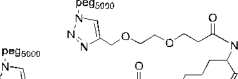
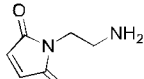
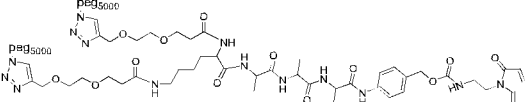
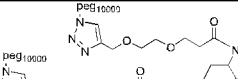
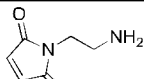
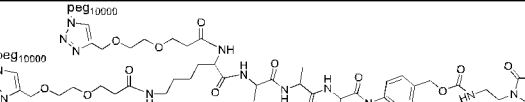
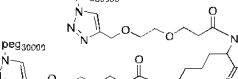
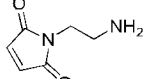
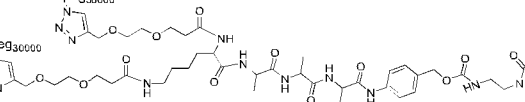
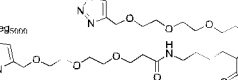
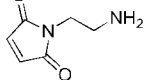
When R2 has an amino acid sequence of Ala-Ala-Asp and R3 is PABC, the synthetic scheme is shown below:

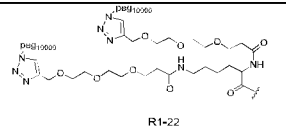
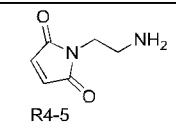
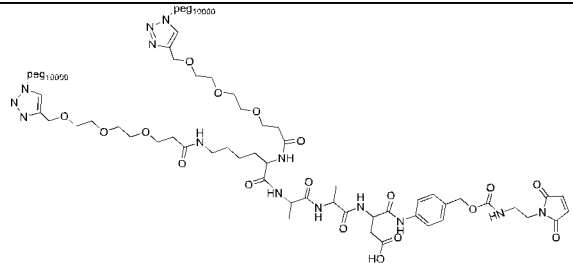
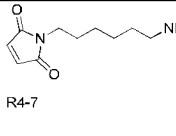
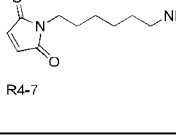
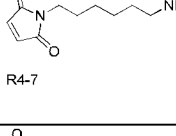
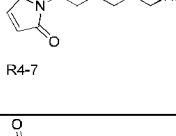
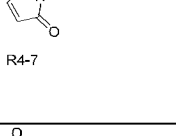
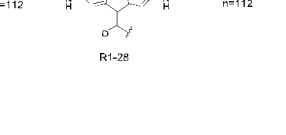
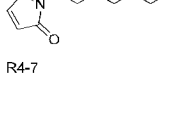
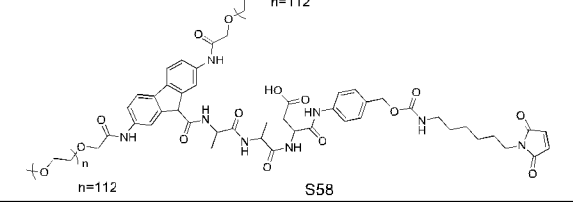
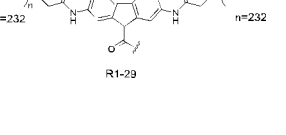
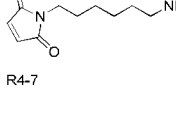
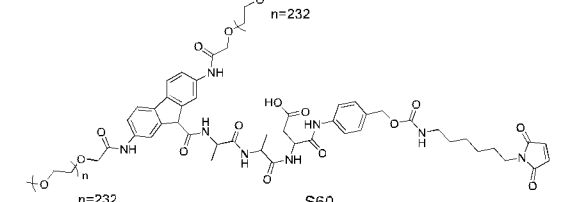
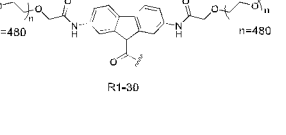
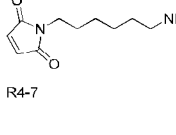
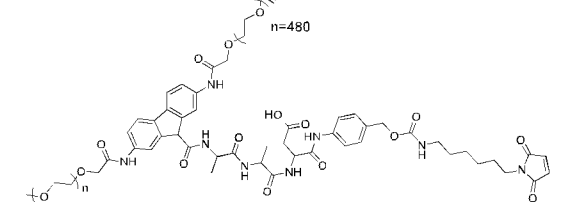


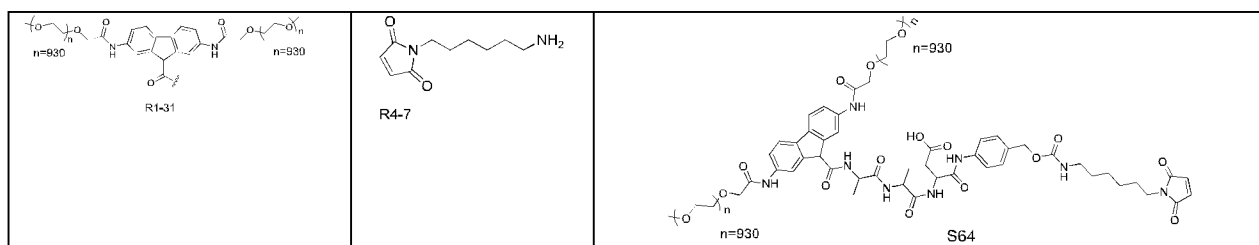
When R1 and R4 are different substituents, the following compounds in Table 2 were obtained.

Table 2

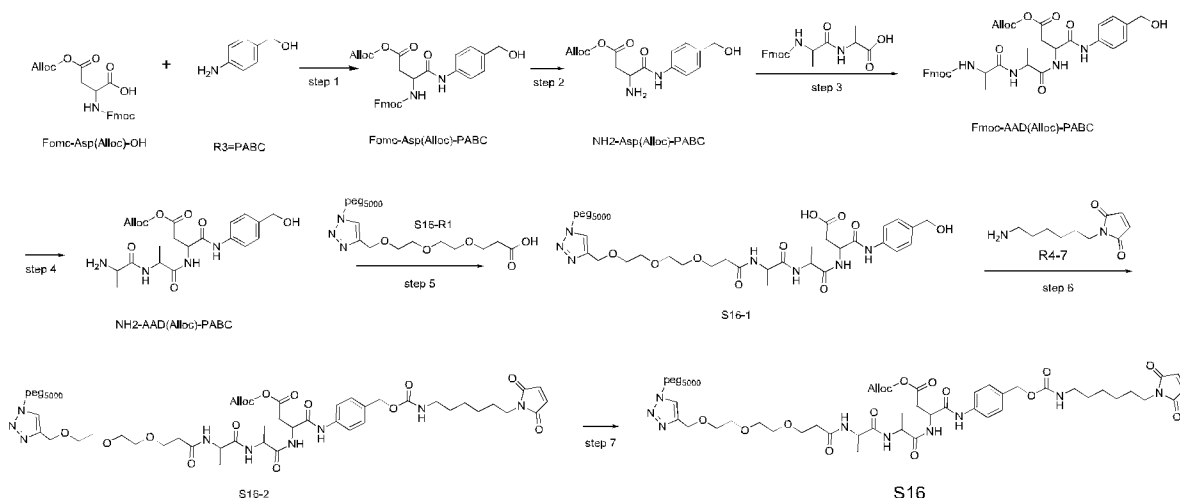
R1	R4	Compound
R1-1	R4-5	S2
R1-2	R4-5	S4
R1-2	R4-13	S8

 n=12 R1-2	 R4-7	 S10
 R1-3	 R4-7	 S12
 R1-4	 R4-7	 S14
 R1-5	 R4-7	 S16
 R1-6	 R4-7	 S18
 R1-7	 R4-7	 S26
 R1-18	 R4-5	 S30
 R1-19	 R4-5	 S32
 R1-20	 R4-5	 S34
 R1-21	 R4-5	 S36

 R1-22	 R4-5	 S38
$\text{CH}_3\text{O} + \text{CH}_2\text{CH}_2\text{O} + \text{C}(=\text{O}) - \text{CH}_2\text{CH}_2 - \text{C}(=\text{O})$ $n=112$ R1-12	 R4-7	$\text{CH}_3\text{O} + \text{CH}_2\text{CH}_2\text{O} + \text{C}(=\text{O}) - \text{CH}_2\text{CH}_2 - \text{C}(=\text{O})$ $n=112$ S40
$\text{CH}_3\text{O} + \text{CH}_2\text{CH}_2\text{O} + \text{C}(=\text{O}) - \text{CH}_2\text{CH}_2 - \text{C}(=\text{O})$ $n=232$ R1-13	 R4-7	$\text{CH}_3\text{O} + \text{CH}_2\text{CH}_2\text{O} + \text{C}(=\text{O}) - \text{CH}_2\text{CH}_2 - \text{C}(=\text{O})$ $n=232$ S42
$\text{CH}_3\text{O} + \text{CH}_2\text{CH}_2\text{O} + \text{C}(=\text{O}) - \text{CH}_2\text{CH}_2 - \text{C}(=\text{O})$ $n=480$ R1-15	 R4-7	$\text{CH}_3\text{O} + \text{CH}_2\text{CH}_2\text{O} + \text{C}(=\text{O}) - \text{CH}_2\text{CH}_2 - \text{C}(=\text{O})$ $n=480$ S44
$\text{CH}_3\text{O} + \text{CH}_2\text{CH}_2\text{O} + \text{C}(=\text{O}) - \text{CH}_2\text{CH}_2 - \text{C}(=\text{O})$ $n=930$ R1-16	 R4-7	$\text{CH}_3\text{O} + \text{CH}_2\text{CH}_2\text{O} + \text{C}(=\text{O}) - \text{CH}_2\text{CH}_2 - \text{C}(=\text{O})$ $n=930$ S46
$\text{CH}_3\text{O} + \text{CH}_2\text{CH}_2\text{O} + \text{CH}_2\text{CH}_2 - \text{N}(\text{CH}_2)_2 - \text{C}(=\text{O})$ $\text{CH}_3\text{O} + \text{CH}_2\text{CH}_2\text{O} + \text{CH}_2\text{C}(=\text{O}) - \text{N}(\text{CH}_2)_2 - \text{CH}_2 - \text{C}(=\text{O})$ $n=528$ R1-17	 R4-7	$\text{CH}_3\text{O} + \text{CH}_2\text{CH}_2\text{O} + \text{CH}_2\text{CH}_2 - \text{N}(\text{CH}_2)_2 - \text{C}(=\text{O})$ $\text{CH}_3\text{O} + \text{CH}_2\text{CH}_2\text{O} + \text{CH}_2\text{C}(=\text{O}) - \text{N}(\text{CH}_2)_2 - \text{CH}_2 - \text{C}(=\text{O})$ $n=528$ S48
 R1-28	 R4-7	 S58
 R1-29	 R4-7	 S60
 R1-30	 R4-7	 S62



As exemplified by S16, the specific synthesis process was shown below:



5

1) Fmoc-Asp(Alloc)-OH (13.2 g, 0.03 mol),

2-(7-azabenzotriazole)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU) (15 g, 0.04 mol), and DMF (200mL) were added to a three-necked flask and stirred for 30min.

p-Aminobenzyl alcohol (4.1 g, 0.03 mol) and N,N-diisopropyl ethylamine (8.7 g, 0.06 mol)

10 were added at 0°C, respectively and then stirred at room temperature for 3 hours. DMF was removed by rotary evaporation. The residue was purified by column chromatography to obtain a white solid Fmoc-Asp(Alloc)-PABC (14.7g; Yield: 89%).

2) Fmoc-Asp(Alloc)-PABC (14.0 g, 25mmol) was dissolved in N,N-dimethyl formamide (80mL). Piperidine (30mL) were added and then stirred at room temperature for 2 hours. The solvents were removed by evaporation under reduced pressure. The residue was drier under high vacuum in a vacuum oven to produce 8.0g pale yellow solid NH₂-Asp(Alloc)-PABC which can be used in the next step without purification.

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3) Fmoc-Ala-Ala-OH (7.8 g, 20.4 mmol), benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) (11.6 g, 30.6 mmol) and DMF (50mL) were added into a three-necked flask and stirred for 30 min in an ice bath. NH₂-Asp(Alloc)-PABC (6.6g, 20.4mmol) and N,N-diisopropyl ethylamine (7.89 g, 61.2 mmol) were added respectively at 0°C and then stirred at room temperature overnight. The solvents were removed by evaporation under reduced pressure. The residue was purified by silica gel column chromatography to

20

obtain a white solid Fmoc-AAD(Alloc)-PABC (12.6g; Yield: 90%).

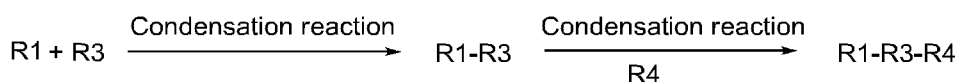
4) Fmoc-AAD(Alloc)-PABC (12 g, 17.5mmol) was dissolved in dichloromethane (80 mL). Piperidine (30 mL) were added and then stirred at room temperature for 2 hours. The solvents were removed by evaporation under reduced pressure. The residue was drier under high vacuum in a vacuum oven to obtain 7.0 g pale yellow solid, which was directly used in the next step.

5) Intermediate S16-R1 (522mg, 0.1mmol), 2-(7-azabenzotriazole)-N,N,N',N'-tetramethyluronium hexafluorophosphate (46mg, 0.12mmol), and DMF(20mL) were added into a three-necked flask and stirred for 30 min. NH₂-AAD(Alloc)-PABC (46mg, 0.1mmol) and N,N-diisopropyl ethylamine (38.7mg, 0.3mmol) were added, respectively, at 0°C and then stirred at room temperature for 3 hours. DMF was removed by rotary evaporation. The residue was purified by column chromatography to obtain S16-1 (white solid, 251mg, Yield: 45%).

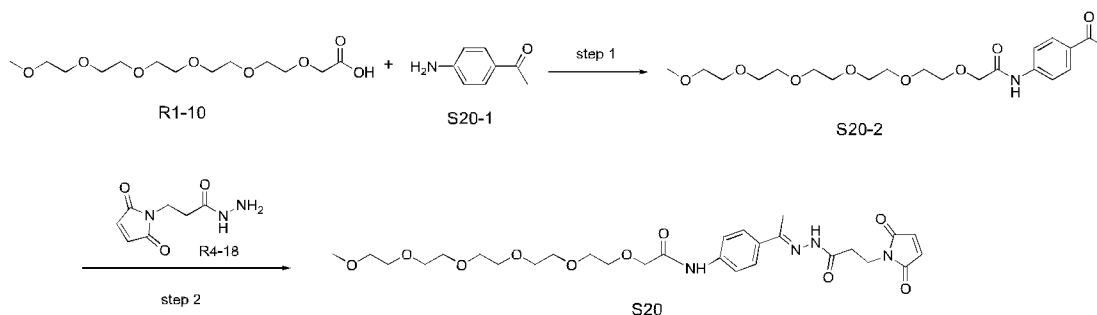
6) S16-1 (240mg, 0.046 mmol) dissolved in dichloromethane (10mL) were added into a three-necked flask. 4-Nitrophenyl chloroformate (18 mg, 0.093 mmol) in dichloromethane and pyridine (7.3 mg, 0.093mmol) in dichloromethane were dropped into the flask, respectively, in an ice bath and under nitrogen gas protection and then stirred at room temperature overnight. R4-7 (11mg,0.055mmol) were added into the above solution and allowed to react at room temperature for 4 hours. The reaction solution was dried by rotary evaporation. The resultant crude product was purified by silica gel column chromatography to obtain a white solid S16-2 (96mg; Yield: 38%).

7) DMF (10ml), compound S16-2(96mg, 0.016mmol), acetic acid (127mg, 2.15mmol), triphenylphosphine palladium (33mg, 0.029mmol) and tributyltin hydride (0.54g, 1.86mmol) were added successively into an one-necked flask. After replacing the air in the flask with nitrogen gas, the mixture was stirred at room temperature until compound S16-2 was reacted completely. After the reaction completed, DMF was removed by evaporation under reduced pressure. The crude product was isolated and purified by silica gel column chromatography to obtain a white solid (54mg; Yield: 62%), which was compound S16.

When an acidically activatable group was contained, the synthetic scheme was shown as follows:



As exemplified by S20, the specific synthesis process was shown below:



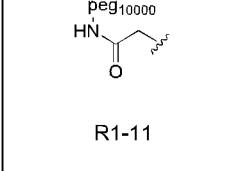
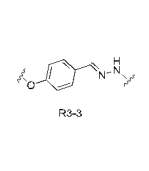
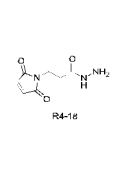
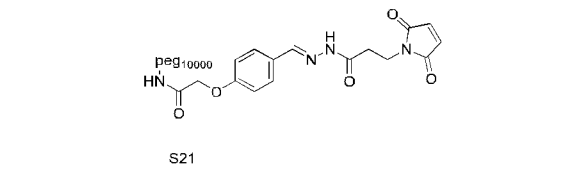
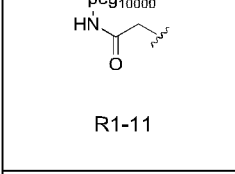
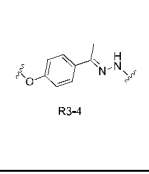
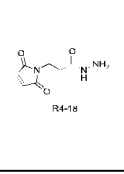
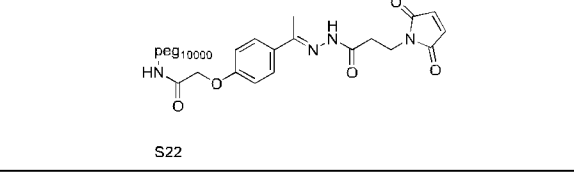
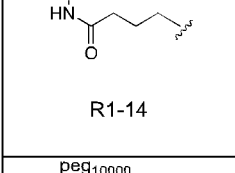
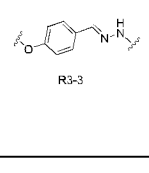
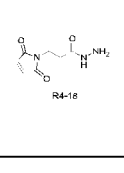
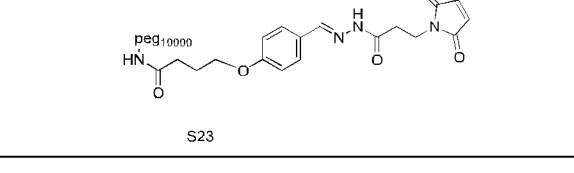
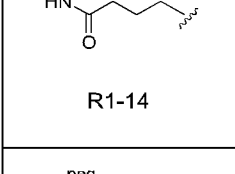
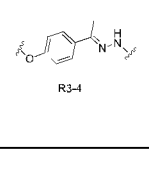
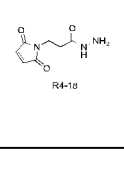
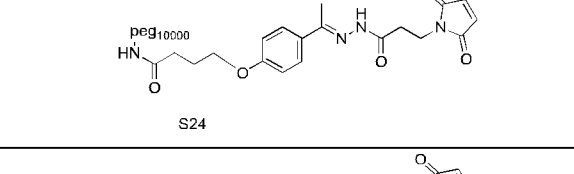
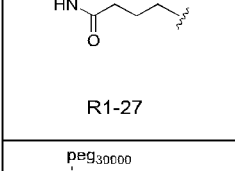
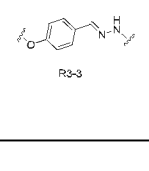
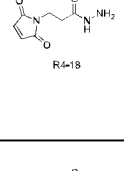
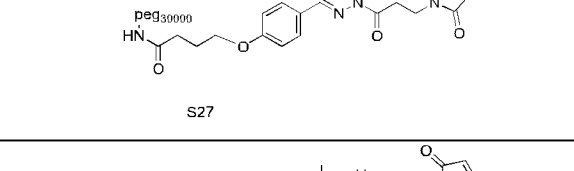
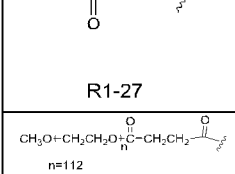
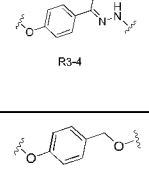
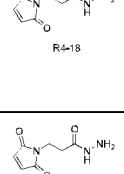
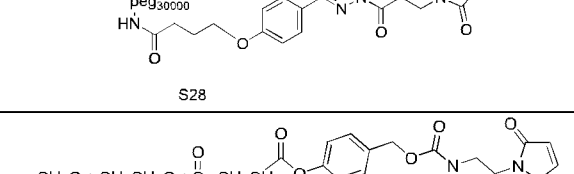
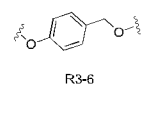
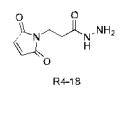
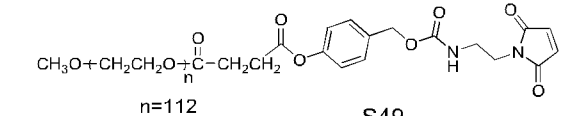
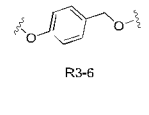
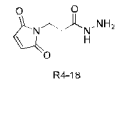
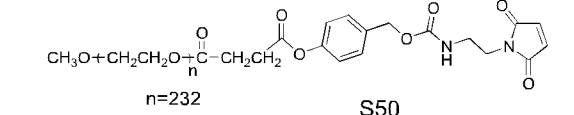
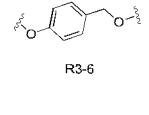
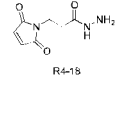
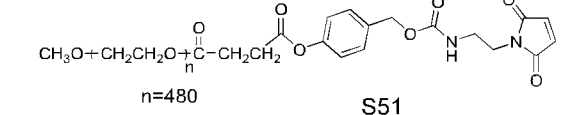
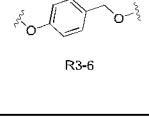
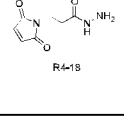
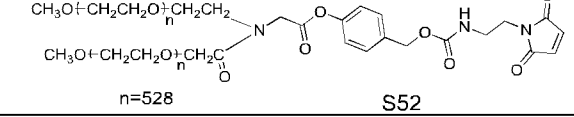
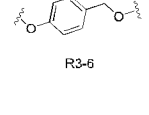
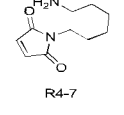
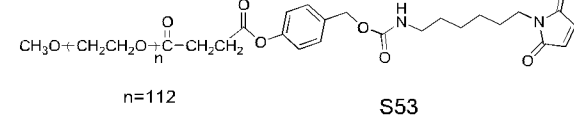
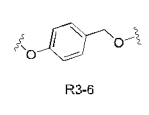
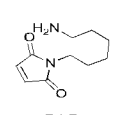
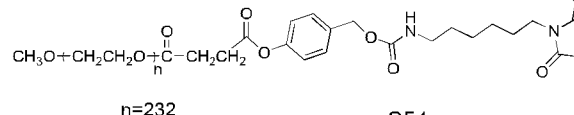
1) R1-10 (3.1 g, 0.01 mol), 2-(7-azabenzotriazole)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU) (4.56 g, 0.012 mol), and DMF (20mL) were added into a three-necked flask and stirred for 30 min. S20-1 (1.35g, 0.01 mol) and N,N-diisopropyl ethylamine (3.87 g, 0.03 mol) were added respectively at 0°C and then stirred at room temperature for 3 hours. DMF was removed by rotary evaporation. The residue was purified by silica gel column chromatography to obtain a pale yellow oily substance S20-2 (2.5g; Yield: 59%).

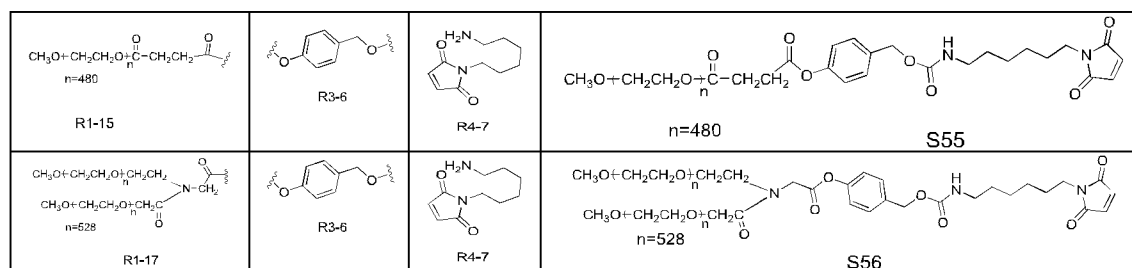
2) S20-2 (98mg, 0.23mmol) and R4-18 (42mg, 0.23mmol) were weighed and successively added into a 50ml one-necked flask. Dichloromethane (5mL) was added to dissolve S20-2 and R4-18 followed by addition of 4A molecular sieves (81mg). After replacing the air in the flask with nitrogen gas, the mixture was allowed to react at room temperature overnight. The reaction solution was dried by rotary evaporation. The crude product was purified by silica gel column chromatography to obtain a white solid (81mg; Yield: 60%), which was compound S20.

When R1, R3 and R4 were different substituents, the following compounds in Table 3 were obtained.

Table 3

R1	R3	R4	Compound

 R1-11	 R3-3	 R4-16	 S21
 R1-11	 R3-4	 R4-16	 S22
 R1-14	 R3-3	 R4-16	 S23
 R1-14	 R3-4	 R4-16	 S24
 R1-27	 R3-3	 R4-18	 S27
 R1-27	 R3-4	 R4-18	 S28
$\text{CH}_3\text{O}+\text{CH}_2\text{CH}_2\text{O}+\text{C}(=\text{O})-\text{CH}_2\text{CH}_2-\text{C}(=\text{O})-\text{F}$ n=112 R1-12	 R3-6	 R4-18	 n=112 S49
$\text{CH}_3\text{O}+\text{CH}_2\text{CH}_2\text{O}+\text{C}(=\text{O})-\text{CH}_2\text{CH}_2-\text{C}(=\text{O})-\text{F}$ n=232 R1-13	 R3-6	 R4-18	 n=232 S50
$\text{CH}_3\text{O}+\text{CH}_2\text{CH}_2\text{O}+\text{C}(=\text{O})-\text{CH}_2\text{CH}_2-\text{C}(=\text{O})-\text{F}$ n=480 R1-15	 R3-6	 R4-18	 n=480 S51
$\text{CH}_3\text{O}+\text{CH}_2\text{CH}_2\text{O}+\text{CH}_2\text{CH}_2-\text{N}(\text{CH}_3)-\text{C}(=\text{O})-\text{CH}_2\text{CH}_2-\text{C}(=\text{O})-\text{F}$ n=528 R1-17	 R3-6	 R4-18	 n=528 S52
$\text{CH}_3\text{O}+\text{CH}_2\text{CH}_2\text{O}+\text{C}(=\text{O})-\text{CH}_2\text{CH}_2-\text{C}(=\text{O})-\text{F}$ n=112 R1-12	 R3-6	 R4-7	 n=112 S53
$\text{CH}_3\text{O}+\text{CH}_2\text{CH}_2\text{O}+\text{C}(=\text{O})-\text{CH}_2\text{CH}_2-\text{C}(=\text{O})-\text{F}$ n=232 R1-13	 R3-6	 R4-7	 n=232 S54



Compounds S1-64 were verified by mass spectrum (MS) and their molecular weights were shown in Table 4, which were consistent to the calculated molecular weights based on their structures.

5

Table 4

No.	Detection by MS	Molecular Weight	Character	Yield
S1	1258	1258.37	white solid	71mg
S2	1258	1258.37	white solid	49mg
S3	1132	1132.21	white solid	236mg
S4	1133	1133.20	white solid	93mg
S5	1129	1129.21	white solid	37mg
S6	1130	1130.20	white solid	46mg
S7	1159	1159.28	white solid	158mg
S8	1160	1160.26	white solid	102mg
S9	1188	1188.32	white solid	34mg
S10	1189	1189.30	white solid	28mg
S11	765	764.82	white solid	18mg
S12	766	765.81	white solid	31mg
S13	5756	5755.81	white solid	364mg
S14	5757	5756.80	white solid	270mg
S15	5800	5799.87	white solid	418mg
S16	5801	5800.85	white solid	54mg
S17	10800	10799.87	white solid	189mg
S18	10801	10800.85	white solid	167mg
S19	579	578.61	white solid	102mg
S20	593	592.64	white solid	81mg
S21	10326	10326.32	white solid	106mg
S22	10340	10340.35	white solid	97mg
S23	10354	10354.38	white solid	139mg

S24	10368	10368.40	white solid	76mg
S25	30800	30799.87	white solid	143mg
S26	30801	30800.85	white solid	125mg
S27	30354	30354.38	white solid	136mg
S28	30368	30368.40	white solid	121mg
S29	10982	10982.37	white solid	223mg
S30	10983	10982.46	white solid	184mg
S31	20982	20982.28	white solid	274mg
S32	20983	20982.32	white solid	231mg
S33	60982	60982.19	white solid	362mg
S34	60983	60983.26	white solid	284mg
S35	11070	11070.34	white solid	164mg
S36	11071	11071.41	white solid	182mg
S37	21070	21070.18	white solid	155mg
S38	21071	21071.27	white solid	169mg
S39	5655	5655.37	white solid	156mg
S40	5656	5656.22	white solid	231mg
S41	10947	10947.42	white solid	143mg
S42	10948	10948.36	white solid	157mg
S43	21883	21883.84	white solid	241mg
S44	21884	21884.72	white solid	185mg
S45	41728	41728.86	white solid	174mg
S46	41729	41729.73	white solid	169mg
S47	47358	47358.46	white solid	248mg
S48	47359	47359.39	white solid	312mg
S49	5353	5343.61	white solid	254mg
S50	10635	10635.68	white solid	216mg
S51	21572	21572.47	white solid	198mg
S52	47047	47047.18	white solid	183mg
S53	5400	5399.76	white solid	175mg
S54	10692	10691.74	white solid	168mg
S55	21628	21628.51	white solid	156mg
S56	47103	47103.24	white solid	141mg
S57	10835	10835.22	white solid	139mg
S58	10836	10836.09	white solid	145mg

S59	21407	21407.28	white solid	182mg
S60	21408	21408.17	white solid	163mg
S61	43256	43256.02	white solid	196mg
S62	43257	43257.10	white solid	175mg
S63	81901	81901.07	white solid	154mg
S64	81902	81902.14	white solid	139mg

Example 2: Analysis on the binding activity after mutation in CDR of a variable region of an antibody and screening for R4

5 The amino acid sequence of anti-PD-1 antibody 1 was disclosed in WO200815712A1 and its DNA sequence was optimized for expression in a host and synthesized (GENEWIZ, Inc., Suzhou, China). The amino acid sequence of anti-PD-1 antibody 2 was disclosed in WO2006121168A2 and its DNA sequence was optimized for expression in a host and synthesized (GENEWIZ, Inc., Suzhou, China). The amino acid sequence of anti-CTLA-4
10 antibody was disclosed in US20150283234 and its DNA sequence was optimized and synthesized (GENEWIZ, Inc., Suzhou, China). The amino acid sequence of anti-TNF α antibody was disclosed in US009534046 and its DNA sequence was optimized for expression in a host and synthesized (GENEWIZ, Inc., Suzhou, China). The amino acid sequence of anti-CD28 antibody was disclosed in US007939638, and its DNA sequence was optimized for
15 expression in a host and synthesized (GENEWIZ, Inc., Suzhou, China). The synthesized DNAs were digested and ligated to a modified pTT5 vector (Biovector) to produce pTT5-anti-PD-1-1, pTT5-anti-PD-1-2, pTT5-anti-CTLA-4, pTT5-anti-TNF α and pTT5-anti-CD28. Cysteine mutation was introduced by using pTT5-anti-PD-1-1, pTT5-anti-CTLA-4, pTT5-anti-TNF α and pTT5-anti-CD28 as templates, designing primer to replace the codon at the mutation position
20 with that of cysteine, PCR and digesting and constructing the mutated fragment into the modified pTT5 vector.

The expression host was HEK293T cell (Life Technologies). Before transfection, HEK293T cells were cultured in a complete medium containing 10% FBS (Gibico) at 37°C and 5%CO₂. One day before transfection, the cells were inoculated onto a 15cm culture dish in an
25 appropriate density and the culture medium was changed into FBS with low IgG (Gibico). 6 hours after transfection or on day 2, the culture medium was changed into Freestyle293 (Gibico).

On the day of transfection, when the cells reached a certain confluence, lipofectamine 2000 (Life Technologies) and PEI (sigma) were used to co-transfect the plasmids expressing

the target protein to 293T cells. Antibody-expressing plasmids included heavy chain and light chain of the antibody. Culture supernatants were recovered on day 4 and day 6 after transfection, respectively. Expression and activity of the protein or antibody were detected, and the protein or antibody was purified.

The hypervariable regions of a variable region within heavy chain and light chain of an antibody (Ab) constitute an antigen (Ag) binding site of the antibody. Because the antigen binding site is complementary to a structure of an antigen epitope, the hypervariable region is also called as complementarity-determining region (CDR) of an antibody. The sequences of variable regions in light chain of anti-PD-1 antibody 1, anti-PD-1 antibody 2 and CTLA4 antibody were aligned and their CDRs was shown in Fig. 1.

In the present disclosure, the activity of each mutant having point mutation as compared to its initial antibody was detected by ELISA. Specifically, a 96-well plate was coated by an antigen of an antibody overnight and then blocked with 1% BSA blocker (ThermoFisher) for 2 hours at 37°C and washed by PBST three times. Corresponding antibody or corresponding mutant was added and allowed to bind at 37°C for 1 hour, then washed with PBST three times. HRP enzyme-conjugated anti-human IgG was added and allowed to bind at 37°C for 1 hour and then washed with PBST three times. TMB substrate (Solarbio., Inc.) was used to detect absorbance at 450nm. Effect on the binding strength of mutant was calculated by $OD_{\text{after mutation}}/OD_{\text{wild type}}$.

The binding activity of mutants of three antibodies, which had mutation at different positions as shown in Tables 5 and 6, were tested according to the above methods. In Tables 5 and 6, A represents a binding activity of 90-110%, B represents a binding activity of 70-90% and the symbol ↓ indicates a binding activity of less than 70%.

Table 5: Effect of mutation position in CDR of a variable region in a light chain of an antibody on binding activity

CDR	Position	PD-1 Antibody 1	Binding to PD-1	anti-PD-1 antibody 2	Binding to PD-1	anti-CTLA-4 antibody	Binding to CTLA-4
CDR1	25	A	B	A	B	A	A
CDR1	26	S	A	S	A	S	A
CDR1	27	K	↓				
CDR1	28	G	A	S	A	S	A
CDR1	29						
CDR1	30	S	A	S	A	G	A
CDR1	31	T	B	S	A	S	A

CDR1	32	S	A	Y	↓	S	A
CDR1	33	G	A	L	↓	Y	↓
CDR1	34	Y	↓	A	A	L	B
CDR1	35	S	A			A	B
CDR1	36	Y	↓	Y	↓		
CDR1	37	L	B			Y	↓
CDR2	51			A	B	G	A
CDR2	52			S	A	A	↓
CDR2	53	Y	↓				
CDR2	54	L	B	S	A	S	A
CDR2	55	A	B	A	B		
CDR2	56	S	A	T	A	A	B
CDR2	57			G	A	T	A
CDR2	58			I	B	G	A
CDR2	60	S	A	A	B		
CDR2	61	G	A				
CDR3	91			S	A		
CDR3	92			S	A	Y	↓
CDR3	93					G	A
CDR3	94					S	A
CDR3	95	S	A			S	A
CDR3	96		↓				
CDR3	97	D	↓	T	A		
CDR3	98	L	↓	F	↓	T	B
CDR3	99		↓	G	A	F	↓
CDR3	100	L	↓			G	A
CDR3	101	T	B	G	↓		
CDR3	102	F	↓				

Table 6: Effect of mutation position in CDR of a variable region in a heavy chain of an antibody on binding activity

CDR	Position	PD-1 Antibody 1	Binding to PD-1	anti-PD-1 antibody 2	Binding to PD-1	anti-CTLA-4 antibody	Binding to CTLA-4
CDR1	30	T	A	S	A	S	A

CDR1	31					S	A
CDR1	32	Y	↓	S	A	Y	B
CDR1	33	Y	↓	G	A	T	A
CDR1	35	Y	B				
CDR2	50	G	A				
CDR2	51	I	B	I	B	I	B
CDR2	53			Y	↓	Y	↓
CDR2	54	S	A	D	B	D	B
CDR2	55			G	A	G	A
CDR2	56	G	A	S	A	S	A
CDR2	57	G	A	K	↓		
CDR2	58	T	A			K	↓
CDR2	59			Y	↓	Y	↓
CDR2	60			Y	↓	Y	↓
CDR2	61			A	B	A	A
CDR2	62			D	↓	D	B
CDR2	63	K	B	S	A	S	A
CDR2	65	K	↓	K	↓	K	↓
CDR2	66			G	A	G	A
CDR3	100	D	↓	D	↓		
CDR3	101	Y	↓	D	B	G	A
CDR3	102			Y	↓	L	A
CDR3	104			G	A	T	A
CDR3	105					L	B
CDR3	106	G	A	G	A	D	↓
CDR3	107			T	A	Y	B

The results showed that in variable region of different antibodies, mutating G, S and a portion of T to C had less effect on the binding activity as compared to the wild type antibody, which was 90% or more of the original binding activity. Mutating A, I and a portion of T to C produced a binding activity which was 70-90% of the binding activity of the wild type antibody.

Results obtained from Co-IP screening for point mutation on light chain of anti-PD1 antibody 1 are shown in Fig. 2, wherein panel a shows the effect of mutation of a wild-type antibody on expression detected by WB, and panel b shows the binding of the mutated antibody

to PD1 detected by WB.

Analysis on the binding activity of mutant of antibody having mutation in CDR after binding to R4

5 As shown in Tables 7-16, antibodies having mutation in CDR of a light chain or a heavy chain were conjugated to R₄ in a library of small molecules for conjugation, and their binding activities were compared to wild type antibodies (binding activity of a conjugate to antibody / binding activity of wild type antibody * 100%) to obtain a conjugation manner which would provide intermolecular force and enhance the binding activity.

10

Table 7: Binding effect of mutants of anti-PD-1 antibody 1 having mutation in CDR of a light chain after conjugating to R4-1 or R4-5

CDR	Position	anti-PD-1 antibody 1	Conjugating to R4-1	Conjugating to R4-5
CDR 1	26	S	93.2%	
CDR 1	30	S	86.9%	
CDR 1	31	T	84.4%	
CDR 1	28	G	104.6%	107.6%
CDR 1	35	S	88.3%	
CDR 1	36	Y	85.4%	
CDR 1	55	A	92.1%	
CDR 2	57	Y	87.7%	
CDR 2	60	S	89.4%	
CDR 3	104	G	83.6%	

Table 8: Binding effect of mutants of anti-PD-1 antibody 1 having mutation in CDR of a heavy chain after conjugating to R4

15

CDR	Position	anti-PD-1 antibody 1	Conjugating to R4-1	Conjugating to R4-4	Conjugating to R4-7
CDR 1	30	T	91.2%		
CDR 1	32	Y		89.5%	
CDR 2	50	G	84.4%		
CDR 2	54	S	103.6%		
CDR 2	58	T	93.9%		
CDR 2	63	K			95.2%
CDR 3	101	Y		86.1%	
CDR 3	106	G	95.1%		

According to Tables 7 and 8, after mutating A, G, S, Y, T or K in the CDRs of anti-PD-1 antibody 1 to C and binding to R4, the mutants could retained a binding efficiency of >80%.

5 Table 9: Binding effect of mutants of anti-PD-1 antibody 2 having mutation in CDR of a light chain after conjugating to R4-1 or R4-5

CDR	Position	anti-PD-1 antibody 2	Conjugating to R4-1	Conjugating to R4-5
CDR 1	25	A	26.5%	87.2%
CDR 1	26	S	86.8%	
CDR 1	28	S	88.9%	
CDR 1	30	S	34.4%	
CDR 1	31	S	95.7%	
CDR 1	32	Y	25.6%	
CDR 1	33	L	1.6%	
CDR 1	34	A	1.7%	83.9%
CDR 2	51	A	95.6%	
CDR 2	52	S	34.6%	
CDR 2	55	A	99.5%	
CDR 2	56	T		89.4%
CDR 3	57	G	89.8%	
CDR 3	91	S	93.5%	
CDR 3	92	S	45.5%	
CDR 3	97	T	93.7%	

Table 10: Binding effect of mutants of anti-PD-1 antibody 2 having mutation in CDR of a heavy chain after conjugating to R4

CDR	Position	anti-PD-1 antibody 2	Conjugating to R4-1	Conjugating to R4-3	Conjugating to R4-4	Conjugating to R4-7
CDR 1	30	S	107.2%			
CDR 1	32	S	86.8%			
CDR 1	33	G	88.9%			
CDR 2	53	Y			91.6%	
CDR 2	55	G	95.7%			
CDR 2	56	S	105.6%			
CDR 2	57	K				89.7%

CDR 2	61	A	87.0%		83.9%	
CDR 2	63	S	95.6%			
CDR 2	65	K				92.1%
CDR 2	66	G	99.5%			
CDR 3	104	G	89.4%			
CDR 3	106	G	91.8%			
CDR 3	107	T	95.7%			
CDR 3	108	L		86.4%		

According to Tables 9 and 10, after mutating G, S, A, Y, K, L or T in the CDRs of anti-PD-1 antibody 2 to C and binding to R4, the mutants could retained a binding efficiency of >80%.

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Table 11: Binding effect of mutants of anti-CTLA-4 antibody having mutation in CDR of a light chain after conjugating to R4-1

CDR	Position	Anti-CTLA-4 Antibody	Conjugating to R4-1
CDR 1	25	A	95.40%
CDR 1	26	S	91.00%
CDR 1	28	S	82.00%
CDR 1	30	G	95.30%
CDR 1	31	S	80.90%
CDR 1	32	S	99.00%
CDR 1	34	L	94.90%
CDR 1	35	A	91.50%
CDR 2	51	G	97.00%
CDR 2	52	A	92%
CDR 2	54	S	97.70%
CDR 2	56	A	102.30%
CDR 2	57	T	92.50%
CDR 2	58	G	88.10%
CDR 3	93	G	99.10%
CDR 3	94	S	92.40%
CDR 3	95	S	95.40%
CDR 3	98	T	91.00%

Table 12: Binding effect of mutants of anti-CTLA-4 antibody having mutation in CDR of a heavy chain after conjugating to R4

CDR	Position	Anti-CTLA-4 Antibody	Conjugating to R4-1	Conjugating to R4-3	Conjugating to R4-4	Conjugating to R4-7
CDR 1	30	S	93.4%			
CDR 1	31	S	109.5%			
CDR 1	33	T	102.3%			
CDR 2	55	G	95.2%			
CDR 2	58	K				87.6%
CDR 2	59	Y			89.1%	
CDR 2	60	Y			91.8%	
CDR 2	65	K				96.2%
CDR 2	66	G	92.7%			
CDR 3	100	G	98.3%			
CDR 3	102	L		83.9%		
CDR 3	103	G	93.8%			
CDR 3	106	D		87.6%		

5 According to Tables 11 and 12, after mutating A, G, S, L, T, K, Y or D in the CDRs of anti-CTLA-4 antibody to C and binding to R4, the mutants could retained a binding efficiency of >80%.

Table 13: Binding effect of mutants of anti-CD28 antibody having mutation in CDR of a light chain after conjugating to R4

CDR	Position	anti-CD28 antibody	Conjugating to R4-1	Conjugating to R4-3	Conjugating to R4-4	Conjugating to R4-7
CDR 1	26	S	87.0%			
CDR 1	29	I		88.5%		
CDR 1	30	Y	19.5%		93.6%	
CDR 2	50	K	26.6%			88.9%
CDR 2	51	A	97.7%			
CDR 2	52	S	84.0%			
CDR 3	91	G	88.3%			
CDR 3	93	T	87.0%			
CDR 3	94	Y	20.1%		83.7%	

CDR3	96	Y	14.9%		91.4%	
CDR 3	97	T	95.5%			
CDR 3	99	G	96.6%			

Table 14: Binding effect of mutants of anti-CD28 antibody having mutation in CDR of a heavy chain after conjugating to R4

CDR	Position	anti-CD28 antibody	Conjugating to R4-1	Conjugating to R4-3	Conjugating to R4-4
CDR 1	25	S	87.0%		
CDR 1	26	G	89.5%		
CDR 1	27	Y			94.2%
CDR1	28	T	108.6%		
CDR1	30	T	87.3%		
CDR1	31	S	97.5%		
CDR1	32	Y			81.8%
CDR 2	52	Y			91.6%
CDR 2	54	G	97.9%		
CDR 2	58	T	89.3%		
CDR 3	99	S	87.6%		
CDR 3	101	Y			89.3%
CDR 3	102	G	80.1%		
CDR3	103	L	14.9%	94.6%	

5 According to Tables 13 and 14, by binding mutation position of G, S, A, I, L, K, Y or T in the CDRs of anti-CD28 antibody to R4, the mutants could retained a binding efficiency of >80%.

Table 15: Binding effect of mutants of anti-TNF α antibody having mutation in CDR of a light chain after conjugating to R4

CDR	Position	anti-TNF α antibody	Conjugating to R4-1	Conjugating to R4-3	Conjugating to R4-4
CDR 1	25	A	88.6%		
CDR 1	26	S	97.1%		
CDR 1	28	G	92.3%		
CDR 1	29	I		87.8%	
CDR 1	32	Y			89.1%
CDR 1	33	L		89.4%	

CDR 1	34	A	85.7%		
CDR 2	50	A	85.5%		
CDR 2	51	A	95.1%		
CDR 2	52	S	86.8%		
CDR 2	53	T	89.4%		
CDR 2	54	L		82.2%	
CDR 2	56	S	95.4%		
CDR 2	57	G	95.5%		
CDR 3	91	Y	34.5%		90.1%
CDR 3	94	A	97.5%		
CDR 3	97	T	82.5%		

Table 16: Binding effect of mutants of anti-TNF α antibody having mutation in CDR of a heavy chain after conjugating to R4

CDR	Position	anti-TNF α antibody	Conjugating to R4-1	Conjugating to R4-3	Conjugating to R4-4
CDR 1	32	Y			88.6%
CDR 1	33	A	95.1%		
CDR 2	51	I		102.6%	
CDR 2	52	T	82.8%		
CDR 2	55	S	86.9%		
CDR 2	56	G	88.7%		
CDR 2	58	I		86.7%	
CDR 2	60	Y			85.2%
CDR 2	61	A	95.7%		
CDR 2	63	S	96.8%		
CDR 2	66	G	99.4%		
CDR 3	100	S	92.2%		
CDR 3	101	Y			85.4%
CDR 3	102	L		85.5%	
CDR 3	103	S	84.5%		
CDR 3	104	T	97.3%		
CDR 3	105	A	86.5%		
CDR 3	106	S	98.3%		
CDR 3	107	S	87.4%		
CDR 3	108	L		81.4%	

CDR 3	110	Y			82.5%
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According to Tables 13 and 14, after mutating A, G, S, L, I, Y or T in the CDRs of anti-TNF α antibody to C and binding to R4, the mutants could retained a binding efficiency of >80%.

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Example 3: Analysis on the binding activity of mutants having a mutation in a sequence of high homology in the non-CDR of a variable region and screening for R1

An antibody consists of 4 peptide chains, including two identical light chains (LC) and two identical heavy chains (HC). The chains form a monomer by disulfide bond(s) and non-covalent bonds. There are two types of light chains, i.e., κ and λ , and five types of heavy chains, i.e., μ , δ , γ , ϵ and α . An antibody, as a whole, is divided into a constant region and a variable region. The variable region is located at the terminus of the two arms of the Y-shaped structure. Humanized or human antibodies have a certain generality, that is, they all contain 4 loops in heavy chain or light chain at the terminus of the two arms of the Y-shaped structure (Fig. 3). Three loops are highly variable and directly anticipate in binding to an antigen. The regions in these loops are termed CDRs, wherein CDR1, CDR2 and CDR3 are present in these three loops, respectively. Another loop is also present at the same side where antibody binds to antigen from the four-dimensional space. Some loops not only have a conservative structure but also have a conservative sequence.

Light chains from 8 commercial antibodies (Tecentriq (Atezolizumab), Yervoy (Ipilimumab), Humira (Adalimumab), Keytruda (Pembrolizumab), Opdivo (Nivolumab), Erbitux (Cetuximab), Rituxan (Rituximab) and Perjeta (Pertuzumab), respectively, shown in the first to the last antibody of Fig. 4) were aligned and the results were shown in Fig. 4. The fourth loop in the variable region of each light chain (GSGSGST) was conservative.

There were 4 loops at the same side of the CDR of the light chain of anti-PD-1 antibody, wherein 3 loops were CDRs, including CDR1, CDR2 and CDR3. The remaining loop had a structure and a sequence which was conservative compared to many drugs approved by FDA. The sequence of this loop was RFSGSGSGT, located at positions 64-72 (Fig. 4). Each of the amino acid residues in loop 4 of anti-PD-1 antibody 1 was mutated to Cys and conjugated to S9 or S13. Effect of conjugation to R1-R4 of different length on binding activity of the mutant to PD-1 was detected by ELISA. Results were shown in Table 17. It could be found that 70% or more of an activity of the mutants may be inhibited by varying the steric structure or length of a conjugating arm.

Table 17: Binding activity of conjugates of anti-PD-1 antibody 1 having a mutation in loop 4 of a variable region of a light chain

	Position	Residue	Binding Activity	Conjugating to S9	Conjugating to S13
Loop4	67	S	87.8%	27.2%	17.8%
Loop 4	68	G	93.5%	89.9%	13.2%
Loop 4	69	S	96.1%	90.6%	16.2%
Loop 4	70	G	89.3%	65.2%	17.9%
Loop 4	71	S	99.5%	89.0%	16.0%
Loop 4	72	G	98.3%	92.9%	10.9%
Loop 4	73	T	93.5%	90.2%	10.6%

Antibody was produced by recombination of genes of immunoglobulin superfamily *in vivo*.

- 5 Some framework regions of antibodies against different antigens may be derived from a gene or an amino acid sequence of a same germline antibody. For example, the heavy chain of an Her2 antibody, Herceptin (Trastuzumab) is different from that of Tecentriq (Atezolizumab), an anti-PD-1 antibody in the three CDRs. They have the same non-CDR framework sequence, which is derived from the same germline antibody (Fig. 5). Similarly to the heavy chains of
- 10 Herceptin and Tecentriq, the light chains of Opdivo (Nivolumab) and Keytruda (Pembrolizumab), which are PD-1 antibodies, are different in the 3 CDRs in the variable region. Their non-CDR framework regions are derived from a same germline antibody (Fig. 5).

- Similar situation was found in many antibodies. As exemplified by reshaping antibodies (also called as CDR-implanted antibodies) approved by FDA, to retain their specificity to an
- 15 antigen and reduce heterology, the CDRs from murine derived antibody was directly replaced by CDRs from human derived antibody. As maturation of humanization technology and genetic engineering technology, the humanized antibodies later developed were mainly the humanized antibodies or human antibodies. However, even so, the heavy chains of many commercial antibodies still exhibit very high similarity. For example, Fig. 6 shows the comparison results
- 20 for similarity of heavy chains of 7 commercial antibodies (Avastin (Bevacizumab), Herceptin (Trastuzumab), Tecentriq (Atezolizumab), Humira (Adalimumab), Yervoy (Ipilimumab), Opdivo (Nivolumab) and Perjeta (Pertuzumab), shown in the first to the last antibody in Fig. 6), which is 85% or more. They are different from each other mainly in the CDRs and their framework regions in the variable regions have similar sequences. Even more, the framework
- 25 structures of Tecentriq (Atezolizumab) and Herceptin (Trastuzumab) are identical (Fig. 5). After comparing the light chain variable regions of immune checkpoint antibodies, Opdivo, Keytruda and Yervoy (the first to the last antibody shown in Fig. 7, respectively), it could be

found that their homology is 92%. The main different is in the CDRs and the sequences in their framework regions are close to each other (Fig. 7).

For antibodies having a conservative framework region, activities of their mutants having a mutation in a framework region (non-CDR) of heavy or light chain variable region and conjugates thereof were tested. Each of the amino acid positions shown in tables 18 and 19 was mutated to cysteine and each of the resulted mutants was conjugated to S9 or S13. Effect of conjugation to a R1-R4 side chain having a different length on its binding activity to PD-1 was tested by ELISA. Results were shown in Tables 18 and 19. Fig. 8 shows conjugation of PD1 Ab-C28 (PD1-Ab-Gly28Cys) to S13, and Fig. 9 shows the binding of anti-PD1-Ab-C28 to PD1 before and after conjugating to S13.

Table 18: Binding activities of mutant of anti-PD-1 antibody 1 having a mutation in a framework region of a light chain variable region and conjugates thereof

Position	Amino acid residue	Binding activity after mutation	Conjugating to S9	Conjugating to S13
5	T	95.3%	67.4%	26.30%
7	S	98.2%	59.5%	34.20%
9	A	96.5%	78.4%	44.60%
10	T	84.3%	65.7%	37.40%
11	L	96.3%	32.50%	
12	S	85.5%	13.50%	
13	L	97.5%	80.2%	42.30%
14	S	97.8%	19.7%	
16	G	89.4%	75.3%	21.70%
19	A	86.4%	49.9%	25.00%
20	T	89.7%	69.7%	32.10%
25	A	83.3%	70.3%	37.20%
41	G	88.5%	17.4%	
43	A	99.3%	82.1%	30.80%
46	L	95.9%	80.2%	37.50%
47	L	81.1%	68.9%	22.90%
48	I	89.5%	23.8%	
51	A	92.1%	82.1%	31.2%
55	A	87.3%	72.3%	29.4%
69	T	83.4%	67.9%	20.5%
76	S	90.2%	68.7%	21.9%

77	S	83.8%	69.3%	32.1%
106	I	98.3%	76.7%	26.8%
107	K	87.1%	70.1%	35.6%

Table 19: Binding activities of mutant of anti-PD-1 antibody 2 having a mutation in a framework region of a heavy chain variable region and conjugates thereof

Position	Amino acid residue	Binding activity after mutation	Conjugating to S9	Conjugating to S13
7	S	95.3%	65.9%	26.3%
8	G	93.2%	78.3%	34.2%
9	G	97.8%	87.4%	24.6%
10	G	84.3%	69.3%	37.4%
15	G	85.6%	32.50%	21.5%
17	S	85.0%	75.3%	42.4%
24	A	99.6%	45.6%	32.3%
25	S	99.8%	48.7%	27.0%
26	G	95.4%	31.8%	11.7%
28	T	86.4%	29.9%	15.0%
30	S	89.7%	31.8%	15.3%
40	A	85.3%	50.3%	37.2%
42	G	83.4%	63.6%	29.8%
44	G	99.3%	78.3%	30.8%
69	T	95.9%	78.6%	37.5%
71	S	81.4%	56.9%	22.9%
75	S	96.3%	38.7%	14.6%
78	T	92.2%	86.3%	42.4%
85	S	98.8%	69.4%	32.3%
88	A	84.3%	46.8%	27.0%
91	T	86%	48.9%	21.7%
92	A	85.0%	64.6%	15.0%
98	T	89.6%	59.9%	32.1%
110	T	92.1%	78.4%	37.2%
112	S	95.4%	68.6%	29.8%
113	S	96.0%	70.5%	30.8%
114	A	89.7%	66.9%	37.5%
115	S	95.3%	70.4%	22.9%

116	T	93.4%	69.5%	14.6%
118	G	99.3%	72.5%	31.3%
120	S	95.9%	68.4%	36.8%

Analysis on the binding activities of mutants having a mutation in a non-CDR region of an antibody variable region showed that there was no significant effect on the binding activity when a mutation was introduced into a non-CDR and when the mutant was conjugated to a small conjugating molecule S9. However, when the mutant was conjugated to S13, the binding activity was reduced. These indicate that amino acid residue in a non-CDR could be mutated to cysteine and then the mutant could be conjugated to a R1-R2-R3-R4 having a large molecule weight or bearing a specific functional group to block the binding activity of the mutant. With this method, the activity of the mutant could be inhibited.

Anti-PD1 antibodies having two or more mutations in light chain were prepared and conjugated to S13. The mutation sites were selected from the mutation sites of the aforementioned anti-PD1 antibody having one mutation and having 95% or more binding activity to PD1 antigen. The activity of such conjugates after activation was verified and the results were shown in Table 20.

Table 20: Binding activity of conjugates of anti-PD1 antibody 1 with two or more mutations to PD1 before and after activation

R1-R2-R3-R4	R5	Binding efficiency before activation	Binding efficiency after enzymatic or acidic activation
S13	PD1 Ab-Ser7/Ser71	18.1%	109.5%
S13	PD1 Ab-Ser12/Gly72	19.6%	95.3%
S13	PD1 Ab-Ser14/Thr73	10.2%	99.8%
S13	PD1 Ab-Ser7/Ser14/Thr73	11.9%	95.1%

The above results demonstrate that conjugates of anti-PD1 antibody with two or more mutations could retain 95% or more binding activity to PD1 after activation.

Example 4: Screening for biomolecule R5, Conjugation to R1, R2-R3-R4, and activation after conjugation

During activation by a proteolytic enzyme in a pathological microenvironment, the site at which the activated linker arm binds to the biomolecule and its steric conformation has different effects on activation efficiency. Steric hindrance and structure-activity relationship determine

an effect of cleavage by activation. To investigate an effect of the biomolecular conformation of conjugates of Example 3 in a pathological environment on activation by an enzyme or an acid in the pathological environment, the following *in vitro* activation investigations were performed.

- 5 In the activation test, 10 micrograms of a proteolytic enzyme for activation were added to 1mg/ml Peg1000-R2-R3-R4-S-Cys-R5 conjugate for reaction for 1 hour at 37°C. The concentration of small compound Peg1000 produced after activation was detected by HPLC and activation or cleavage efficiency (%) in relative to a control group was calculated. Cleavage efficiency of each conjugate is shown in the following Table 21.

10

Table 21

Mutant	R1	R2	R3	R4	Cleavage efficiency
PD-1-Ab-Gly28Cys	Peg1000	-Ala-Ala-Asn-			Control, 100%
PD-1-Ab-Gly28Cys	Peg1000	-Ala-Ala-Asn-	R3-1	R4-3	99.5
PD-1-Ab-Gly28Cys	Peg1000	-Ala-Ala-Asn-	R3-2	R4-3	99.8
PD-1-Ab-Gly28Cys	Peg1000	-Ala-Ala-Asn-	R3-3	R4-3	98.6

As shown in Tables 22 and 23, the automatically shedding arm R3 substantially had no effect on the activation of Legumain due to its small molecular weight and linear structure, with

- 15 R3-2 producing the lowest effect.

Table 22: anti-PD-1 antibody 1

R2	R3	R4	R5	Cleavage efficiency
-Ala-Ala-Asn-	R3-2			Control, 100%
-Ala-Ala-Asn-	R3-2	Paclitaxel (2-OH)		42.5
-Ala-Ala-Asn-	R3-2	R4-5	PD-1-Ab1-Gly28Cys	88.4
-Ala-Ala-Asn-	R3-2	R4-6	PD-1-Ab1-Gly28Cys	93.5
-Ala-Ala-Asn-	R3-2	R4-7	PD-1-Ab1-Gly28Cys	99.8
-Ala-Ala-Asn-	R3-2	R4-8	PD-1-Ab1-Gly28Cys	63.5
-Ala-Ala-Asn-	R3-2	R4-9	PD-1-Ab1-Gly28Cys	62.4
-Ala-Ala-Asn-	R3-2	R4-1	PD-1-Ab1-Gly28Cys	83.8
-Ala-Ala-Asn-	R3-2	R4-3	PD-1-Ab1-Gly28Cys	94.5
-Ala-Ala-Asn-	R3-2	R4-1	PD-1-Ab1-Gly28Cys	86.7
-Ala-Ala-Asn-	R3-2	R4-3	PD-1-Ab1-Gly28Cys	97.5

Table 23: anti-CTLA-4 antibody

Acidic activation	R3	R4	R5	Cleavage efficiency
Amide bond linkage	R3-2			Control, 100%
Amide bond linkage	R3-2	R4-2	CTLA-4-Ser26Cys	99.8
Amide bond linkage	R3-2	R4-15	CTLA-4-Ser26Cys	99.5
Amide bond linkage	R3-2	R4-16	CTLA-4-Ser26Cys	91.7
Amide bond linkage	R3-2	R4-17	CTLA-4-Ser26Cys	94.3
Amide bond linkage	R3-2	R4-18	CTLA-4-Ser26Cys	93.4
Ester bond	R3-2			Control, 100%
Ester bond	R3-2	R4-4	CTLA-4-Ser26Cys	92.8
Ester bond	R3-2	R4-19	CTLA-4-Ser26Cys	97.4
Ester bond	R3-2	R4-20	CTLA-4-Ser26Cys	93.8
Ester bond	R3-2	R4-21	CTLA-4-Ser28Cys	95.4
Ester bond	R3-2	R4-22	CTLA-4-Ser28Cys	96.4

In Tables 22 and 23, the activation efficiency produced by molecules without conjugating with the biomolecule was used as a positive control, while the activation efficiency produced by molecules with conjugation to a hydroxyl at position 2 of a heterocyclic compound Paclitaxel was used as a negative control. The length and side chain group of R4 affected the cleavage efficiency of the whole conjugate when conjugating to a mutation site in a variable region or a non-variable region of an antibody. A longer chain structure of R4 produced enhanced cleavage efficiency. As detected, after conjugating to Ala-Ala-Asn or Ala-Ala-Asp and then binding to antibody, R4-1 to R4-25 of the present disclosure all allowed the mutants to retain >60% of the activation efficiency, and >90% of the activation efficiency in an acid-sensitive activation.

Effect of R1 selection and test for recovery of binding capacity

PBS (pH 7.2) was used to dilute ligand molecules PD1, TNF α , CTLA-4, CD28 to 1 μ g/ml, respectively. Then each of the diluted solutions was used to fix in a 96-well plate (Nunc) overnight. 1X block BSA (Thermo Fisher) was used to block the plate for 2h. Corresponding conjugate of anti-PD-1 antibody, anti-TNF α antibody, anti-CTLA-4 antibody and anti-CD28 antibody was added, respectively, in an equal concentration, and allowed to bind at 37°C for one hour. The plate was washed with PBST three times. HRP enzyme-conjugated human antibody used as secondary antibody was allowed to react at 37°C for 1 hour and then washed with PBST three times. TMB substrate (Solarbio., Inc.) recognized by HRP enzyme was used

for reaction at 37°C for 15 minutes in dark. A 1/2 volume of stopping solution was used to stop the reaction. Absorbance strength (OD450) was read. The relative binding efficiency was calculated by a percentage ratio between the binding efficiency of the conjugate and the binding efficiency of the wild type antibody before or after activation. The results were shown in Tables 24-26.

Table 24: Change of the binding activity of anti-PD-1 antibody after conjugating to R1 and after activating the conjugating arm

R1	R2	R3	R4	R5	Binding efficiency before activation	Binding efficiency after activating by enzyme or acid
	-Ala-Ala-Asn-	R3-2	R4-3	PD-1-Ab1-Gly28Cys	67.4%	143.5%
R1 from S1	-Ala-Ala-Asn-	R3-2	R4-3	PD-1-Ab1-Gly28Cys	24.4%	145.3%
R1 from S3	-Ala-Ala-Asn-	R3-2	R4-3	PD-1-Ab1-Gly28Cys	22.5%	143.4%
R1 from S11	-Ala-Ala-Asn-	R3-2	R4-3	PD-1-Ab1-Gly28Cys	15.6%	137.4%
R1 from S17	-Ala-Ala-Asn-	R3-2	R4-3	PD-1-Ab1-Gly28Cys	3.2%	156.3%

The results indicate that conjugating to R1 inhibits the activity of the anti-PD-1 antibody but does not affect the activation of the antibody. The binding efficiency of the conjugate without R1 to its antigen before activation was 67.4% of that of the wild type antibody to the antigen. However, after activation, the binding efficiency of the conjugate was improved to be 143% of that of the wild type antibody. After binding R1 to the same R2-R3-R4 and mutated antibody, it prevented the resultant conjugate from binding to its antigen. And the prevention of binding increased as the molecular weight of R1 increased. S17 could completely prevent the conjugate from binding to its antigen. However, the binding activity was recovered after cleaving by Legumain.

Table 25: Change of the binding activity of anti-TNF α antibody after conjugating to R1 and after activating the conjugating arm

R1	R2	R3	R4	R5	Binding efficiency before activation	Binding efficiency after activating by enzyme or acid
	-Ala-Ala-Asp-	R3-2	R4-2	TNF α -Ab-Ala50Cys	57.9%	125.6%
R1 from S2	-Ala-Ala-Asp-	R3-2	R4-2	TNF α -Ab-Ala50Cys	28.3%	128.9%
R1 from S4	-Ala-Ala-Asp-	R3-2	R4-2	TNF α -Ab-Ala50Cys	21.9%	127.5%

R1 from S12	-Ala-Ala-Asp-	R3-2	R4-2	TNF α -Ab-Ala50Cys	11.6%	127.4%
R1 from S18	-Ala-Ala-Asp-	R3-2	R4-2	TNF α -Ab-Ala50Cys	0.9%	125.3%

The results indicate that conjugating to R1 inhibits the activity of the anti-TNF α antibody but does not affect the activation of the antibody. The binding efficiency of the conjugate without R1 to its antigen before activation was 57.9% of that of the wild type antibody to the antigen. However, after activation, the binding efficiency of the conjugate was 125.6% of that of the wild type antibody. After binding R1 to the same R2-R3-R4 and mutated antibody, it prevented the resultant conjugate from binding to its antigen. And the prevention of binding increased as the molecular weight of R1 increased. S18 could completely prevent the conjugate from binding to its antigen. However, the binding activity was recovered after cleaving by Legumain.

Table 26: Change of the binding activities of anti-CTLA-4 antibody and anti-CD28 antibody after conjugating to the conjugate and after activating the conjugating arm

conjugating arm	R5	Binding efficiency before activation	Binding efficiency after activating by enzyme or acid
S7	CD28-Gly44Cys	22.5%	143.4%
S8	CD28-Ala40Cys	15.6%	137.4%
S9	CD28-Thr97Cys	3.2%	156.3%
S10	CTLA-4-Ser26Cys	67.4%	143.5%
S13	CTLA-4-Ser28Cys	24.4%	145.3%
S14	CTLA-4-Tyr92Cys	22.5%	143.4%
S15	CTLA-4-Leu34Cys	15.6%	137.4%
S16	CTLA-4-Gly51Cys	3.2%	156.3%

The results show that conjugating to R1 inhibits the activity of anti-CD28 antibody and anti-CTLA-4 antibody but does not affect the activation of the antibody.

From Example 4, it could be found that the activation efficiency of a conjugate and its binding capacity to antigen after recovery were influenced by the mutation position in the antibody to some extent. However, with the use of R4 expected activation efficiency by enzymatic cleavage could be produced. As for the binding between a biomolecule and its ligand, the exposed group of R4 produced after cleavage by activation could regulate the binding capacity of an antibody mutant. Different exposed groups had different effects on different mutation positions. By screening for R1 and D4, it could be found that the hindering function of

R1 led to complete lose of binding capacity of a conjugate to its antigen. However, the binding capacity was recovered or even enhanced after target cleavage by an enzyme. Such conjugate-type antibodies are only activated in a region that a target enzyme is highly expressed or secreted in a pathological microenvironment to release the antibody or protein. Thus, such microenvironment-activated antibodies are new target-activated antibodies as shown in Fig. 10.

Example 5: Promotion or inhibition of antibody on immune indexes.

1. anti-PD-1 antibody promoted T cells to secrete IFN- γ

Human whole blood (from Shang Ruijin Hospital) was diluted and uniformly mixed with PBS in an amount equal. The diluted solution was slowly transferred to another centrifugal tube containing lymphocyte separation solution along the wall of the centrifugal tube to allow the diluted solution to form a separated layer on the lymphocyte separation solution. The interface was kept clear. The tube was centrifuged at 2000rpm for 20 minutes. After centrifugation, the centrifugal tube was taken out. The solution within the tube was layered. A mononuclear cell layer was extracted to a 50ml centrifugal tube. Five-time volume of PBS was added and uniformly mixed and the mixture was centrifuged at 2000rpm for 10 minutes. The supernatant was discarded and the precipitate was resuspended with PBS. Then the resultant mixture was centrifuged at 1500rpm for 10min and the resultant human peripheral blood mononuclear cells (PBMC) were resuspend with the RPMI1640 complete medium containing 5% heat-activated human serum. The human PBMC was inoculated in a 96-well plate in a concentration of $1\sim 2\times 10^5$ cells per well. The cells were stimulated with 0.1 μ g/ml SEB for three days. The non-adherent cells (mainly mononuclear cells) were collected and uniformly inoculated to a new 96-well plate. Different concentrations of anti-PD-1 antibody without mutation or PD-1-Ab-Cys28-S-S13 conjugate were added and cultured at 37 $^{\circ}$ C, 5% CO₂ for 4 days. Supernatant was collected and concentration of cytotoxic factor IFN- γ was detected by ELISA kit. Results showed that the activated anti-PD-1 antibody exhibited a close or even a significantly improved activity of secreting IFN- γ , as shown in Fig. 11.

The results indicate that after activation of the anti-PD-1 antibody conjugate *in vitro*, not only the stimulation of anti-PD-1 antibody to T cell could be recovered, but also the remaining group produced after cleavage could introduce one or several new functional groups for the antibody. As a result, after activation, PD-1-Ab-Cys28-S-S13 enhanced the activity of such activating T cells. Such kinds of conjugated-type antibodies are merely activated in a region highly expressing or secreting a target enzyme in a pathological microenvironment to release the antibody. Thus, such microenvironment-activated antibodies are new target-activated antibodies.

2. Conjugates of anti-PD-1 antibody and anti-CD28 antibody reduce body's autoimmunity

As is well known in the art, though anti-PD-1 antibody is a drug effective for treating tumor, it was found in the current clinic research that anti-PD-1 antibody exhibited two main issues. One of the issues is that a patient would exhibit a side effect of high fever and false progress after administration of the antibody, but the mechanism is unknown. We presume these side effects may be improved if the antibody is inhibited or blocked by a conjugate and is released after arriving at a local environment of tumor so as to reduce the exposed time or dose of active drug in a non-diseased environment. For this reason, experiments were conducted with mice suffered from type I diabetes mellitus (NOD). Diabetes mellitus of this kind of mice is an autoimmune disease, wherein self-activated T lymphocyte cells destroy pancreatic islet β cells, resulting in insufficient secretion of insulin. First, female NOD of 10 weeks old (Beijing Vital River Laboratory Animal Technology Co., Ltd.) were injected with control IgG, low (1mpk), medium (5 mpk) or high (15 mpk) dose of antibody or conjugate of antibody, respectively at day 0. Indicators of diabetes mellitus, including glucose in urine and two blood sugar levels were observed every day for 12 days until no new indicator of urine glucose was observed.

Protection from autoimmunity by the PD-1-Ab-Cys28-S-S13 conjugate was showed in Fig. 12 and protection from autoimmunity by the CD28-Ab-Cys-S-S13 conjugate was showed in Fig. 13.

Results showed that protection of an immune system by a conjugate of anti-PD-1 antibody was increased by about 15 times as compared to anti-PD-1 antibody, and protection of an immune system by a conjugate of anti-CD28 antibody was increased by about 10 times as compared to anti-CD28 antibody. It can thus be seen that, when a linking moiety was used to hinder or reduce the activity of a protein or antibody, the binding of a conjugated protein or antibody to a related ligand in a normal tissue was reduced, because it was difficult to activate anti-PD-1 antibody and anti-CD28 antibody due to hydrolase and physiological environment outside a diseased microenvironment. As a result, the conjugate could reduce autoimmunity as compared to the primary antibody.

3. Conjugate of anti-CD28 antibody specifically activates CD4 or CD8 cells.

CD8⁺ T cells and CD4⁺ T cells were isolated from human peripheral blood mononuclear cells with CD8 or CD4 magnetic beads (Dynabeads) according to the specific steps provided in the specification of a kit, counted and stained according to the CFSE specification. The cells were inoculated in a 96-well plate at a concentration of $1\sim 2\times 10^5$ cells per well. A suitable amount of control anti-CD3/anti-CD28 antibody, 0.3ug/ml of anti-CD28 antibody or conjugate

of anti-CD28 antibody were added, respectively.

Results were shown in Fig. 14. According to the results, as compared to the original anti-CD28 antibody, the heavy chain conjugate CD28-Ab-Gly44Cys (CD28-Ab-Cys44-S-S13) could selectively activate CD4 T cells, and the light chain conjugate CD28-Ab-Ser52Cys (CD28-Ab-Cys52-S-S13) could selectively activated CD8 T cells. The results indicate that, when a linking moiety was used to hinder or reduce the activity of a protein or an antibody, the binding of the antibody conjugated to the linking moiety to a related antigen in a normal tissue was reduced before the antibody or protein arrives at a target tissue, because the hydrolase is expressed in a low level outside a diseased microenvironment and it is very difficult for the hydrolase to activate the linking moiety conjugated to the antibody or the protein. When the antibody conjugated to the linking moiety arrives at the diseased microenvironment, such as an inflammatory microenvironment, the conjugated linking moiety is activated by Legumain expressed on the surface of Treg, thereby stimulating proliferation of Treg and inhibiting inflammatory reaction. In a tumor region, the conjugated linking moiety is activated by Granzym-B expressed on the surface of CD8 cells, thereby stimulating proliferation of CD8 cells and inhibiting progress of tumor. The designed conjugates are regulated by hydrolase in a microenvironment and the activity of antibody is released only after the conjugated linking moiety is hydrolyzed. As a result, antibodies conjugated to a linking moiety could be greatly enriched in a microenvironment. Eventually, a whole effect, i.e., reduced side effects and improved efficacy could be achieved.

Example 6: anti-PD-1 antibody activated in a microenvironment could cure MC38 tumor

MC38 cells were subcutaneously inoculated to transgenic C57BL/6 mice (Shanghai Research Center for Model Organisms) transformed with human PD-1 fusion protein in a concentration of 2×10^6 cells per mouse. One week later, the mice implanted with MC38 tumor were randomly divided to 4 groups. Group 1 was injected with 10mg/kg anti-PD-1 antibody Keytruda, group 2 was injected with 2mg/kg anti-PD-1 antibody Keytruda, group 3 was injected with 1mg/kg PD-1-Ab-G28C-S13 conjugate, and group 4 was injected with a solvent control, twice a week for 2 weeks. The tumors in mice were recorded 3 times each week. Results were shown in Fig. 15.

Mouse transformed with human PD-1 fusion protein is a transgenic mouse. Its endogenous PDCD1 gene is replaced with human PDCD1, thereby expressing human PDCD1 protein. Such mouse could respond to human anti-PD-1 antibody and stimulate downstream immunity. The results show that 10mg/kg anti-PD-1 antibody could effectively inhibit growth of MC38 tumor,

with one mouse being cured and five mice having a significant inhibitory effect, which was 80% or more. 1mg/kg PD-1-Ab-G47C-S13 conjugate produced better inhibitory effect than 10mg/kg anti-PD-1 antibody Keytruda. The results demonstrate that the conjugate of anti-PD-1 antibody could enhance the activity of effector T cells and enrichment of the antibody in a tumor microenvironment, as proved in Example 5. Thus, the conjugate exhibits an improved efficacy than the original antibody.

Example 7a: IL2 Protein activated in a microenvironment

The amino acid sequence of IL2 is a native human IL2 protein. Its DNA sequence was optimized for expression in a host and synthesized (GENEWIZ, Inc., Suzhou, China). The synthesized DNA was digested and ligated to a modified pTT5 vector (Biovector) to produce pTT5-IL2. The mutated vector was constructed by using pTT5-IL2 as template, designing primer to replace the codon at the mutation position with that of cysteine, PCR and digesting and constructing the mutated fragment to the modified pTT5 vector. Synthesis, mutation and transfection of IL2 gene were performed according to the methods described in Example 2. Its binding activity to ligand IL2Ra or IL2Rb was analyzed by IP and ELISA in accordance with the mutation position.

To verify that activation of macromolecule in a microenvironment is not limited to antibody but is applicable to various proteins, cytokine IL2 was used as an example herein. Its mutation position, activity, linker arm, and activity and function after activation were screened. Results are shown in the following Tables 27-29.

Table 27: Activity of mutated IL2 and screening for linker arm

Amino acid	Position	Type of inhibitory ligand	Binding activity to IL2Ra after mutation	Binding activity to IL2Rb after mutation	Conjugating to S3	Conjugating to S13
Lys	32	α	86.5%	100%	26.1%	
Lys	35	α	87.1%	100%		32.4%
Thr	37	α	98.1%	100%		
Thr	41	α	99.2%	100%	18.7%	
Lys	43	α	89.9%	100%		31.3%
Lys	48	α	88.4%	100%		
Lys	49	α	79.8%	100%		39.4%
Leu	72	α	98.8%	100%		25.5%
Ala	73	α	84.3%	100%	27.9%	
Ser	75	α	86.2%	100%		

Lys	76	α	85.0%	100%		32.2%
Leu	94	α	99.6%	100%		27.2%
Thr	101	α	99.8%	100%		32.0%
Thr	102	α	95.3%	100%		29.6%
Ala	108	α	93.9%	100%		16.9%
Thr	111	α	94.9%	100%		35.3%
Ala	112	α	97.3%	100%		41.9%
Leu	19	β	100%	97.5%	39.5%	
Gly	27	β	100%	42.3%		
Ser	75	β	100%	93.3%		31.1%
Leu	80	β	100%	27.0%		
Ser	87	β	100%	31.6%		
Leu	94	β	100%	47.6%		
Gly	98	β	100%	79.5%		16.9%
Ser	99	β	100%	99.2%		18.9%
Thr	101	β	100%	21.7%		
Thr	133	β	100%	96.9%		27.8%
Thr	51	γ	100%	100%	89.70%	91.60%

It could be found from the results that, for the mutants retaining 80% or more of binding activity to its corresponding receptor as compared to the native IL2, their activity of binding receptor could be inhibited or hindered by regulating the length of a linker arm. For example, after mutating Lys32, Thr41, Ala73 or Leu19 to Cys, conjugating each of the resulted mutants to S3 may inhibit 60% of binding activity to their corresponding receptor; whereas after mutating Lys35, Lys43, Leu72, Lys76, Leu94, Thr101, Thr102, Ala108, Thr111, Ala112, Gly98, Ser99, Thr133, or the like to Cys, conjugating each of the resulted mutants to S13 may inhibit 60% or more of binding activity to their corresponding receptor.

10

Table 28: Cleavage efficiency of conjugated IL2

R2	R3	R4	R5	Cleavage efficiency
-Ala-Ala-Asp-	R3-2			Control, 100%
-Ala-Ala-Asp-	R3-2	Paclitaxel (2-OH)		45.5%
-Ala-Ala-Asp-	R3-2	R4-10	IL2-Thr37	86.5%
-Ala-Ala-Asp-	R3-2	R4-11	IL2-Thr37	92.7%
-Ala-Ala-Asp-	R3-2	R4-12	IL2-Thr37	96.7%

-Ala-Ala-Asp-	R3-2	R4-13	IL2-Thr37	73.9%
-Ala-Ala-Asp-	R3-2	R4-14	IL2-Thr37	62.4%
-Ala-Ala-Asp-	R3-2	R4-1	IL2-Thr37	73.8%
-Ala-Ala-Asn-	R3-2	R4-3	IL2-Thr37	84.7%
-Ala-Ala-Asp-	R3-2	R4-1	IL2-Thr41	86.7%
-Ala-Ala-Asp-	R3-2	R4-3	IL2-Thr41	95.5%

Results indicate that activation by Legumain is not influenced due to the small molecular linear structure of the automatically shedding arm R3.

5 Table 29: Change of binding efficiency to its receptor after conjugating IL2 to R1 an after activation of a linking arm

R1	R5	Binding efficiency before activation	Binding efficiency after enzymatic or acidic activation
S3	IL2-Thr37	28.1%	143.5%
S5	IL2-Ser87	31.6%	145.3%
S6	IL2-Thr-41	99.2%	124.8%
S13	IL2-Leu-19	97.5%	118.7%

It can thus be seen that an activation efficiency of a conjugate and its binding capacity to receptor after activation were influenced by the mutation position in a protein to some extent.

10 However, with the use of R4, activation efficiency by target enzymatic cleavage could be produced. As for the binding between a biomolecule and its ligand, the exposed group of R4 produced after cleavage by activation could regulate the binding capacity of a mutated antibody. Different exposed group had different effects on different mutation positions. By screening for R1 and D4, it could be found that the hindering function of R1 led to complete lose of binding
15 capacity of the conjugate to its antigen. However, the binding capacity was recovered or even enhanced after cleavage by a target enzyme. Such conjugated-type proteins are merely activated or released their activity in a region highly expressing or secreting a target enzyme in a pathological microenvironment. Thus, such proteins are new target-activated proteins.

20 IL2 mutants having two or more mutations were prepared and conjugated to S13. The mutation sites were selected from the mutation sites of the aforementioned IL2 mutants having one mutation and having 95% or more binding activity to its receptor. The activity of such conjugates after activation was verified and the results were shown in Table 30.

Table 30: Binding activity of conjugates of IL2 mutants with two or more mutations to its

receptor before and after activation

R1-R2-R3-R4	R5	Binding efficiency before activation	Binding efficiency after enzymatic or acidic activation
S13	IL2-Thr37/Leu19	12.1%	113.5%
S13	IL2-Ser37/Thr41	11.6%	98.3%
S13	IL2-Thr-41/Ser87	19.2%	102.8%
S13	IL2-Leu-19/Ser87	17.5%	96.7%
S13	IL2-Leu19/Ser37/Ser87	10.9%	95.9%

The above results demonstrate that conjugates of IL2 mutants with two or more mutations could retain 95% or more binding activity to corresponding receptor after activation.

5

Conjugate of IL2 protein specifically activates CD4 or CD8 cells.

CD8⁺ T cells and CD4⁺ T cells were isolated from human peripheral blood mononuclear cells with CD8 or CD4 magnetic beads (Dynabeads) according to the specific steps provided in the specification of a kit, counted and stained according to the CFSE specification. The cells were inoculated in a 96-well plate at a concentration of $1\sim 2\times 10^5$ cells per well. A suitable amount of control anti-CD3 antibody, 0.05ug/ml of IL2 protein or conjugate IL2-T41C-S16 conjugated to S16 were added, respectively. Fig. 16 shows conjugation of IL2-T41C (IL2-Thr41Cys) to S16. Fig. 17 shows the binding to IL2 receptor alpha before and after conjugation. Fig. 18 shows effect of the IL2-C41-S16 conjugation on proliferation of T cells before and after activation.

15

Native IL2 exhibited similar stimulation on CD8 and CD4. However, after conjugating to a functional moiety activated in a microenvironment, IL2-Thr37Cys and IL2-Thr41Cys could specifically activate proliferation of CD8 T cells and reduce proliferation of CD4 cells. The ratio of cell number between CD8 cells and CD4 cells increased from the original 1:1 to 410:1 and 157:1, respectively. IL2-Leu19Cys and IL2-Ser87Cys could specifically activate proliferation of CD4 T cells, with the cell ratio of CD4/CD8 being increased to 435:1 and 126:1, respectively. Results are showed in Fig. 19. The results demonstrate that CD8 or CD4 could be selectively activated by conjugating IL2 to a functional moiety to control its binding activity to each of receptors and to allow it to be activated in a specific microenvironment.

25

Conjugates of IL2 protein effectively inhibit growth of melanoma B16F10 and colon cancer MC38.

0.5×10^6 B16F10 cells per mouse were subcutaneously inoculated to C57BL/6 mice. One

week later, when the tumor's volume reached 100mm^3 , the mice implanted with melanoma were randomly divided to 4 groups, with 6 mice per group. Group 1 was injected with 2.5mg/kg of IL2-T37C-S14 protein conjugate weekly. Group 2 was injected with 0.5mg/kg of IL2-Thr37Cys protein conjugate weekly. Group 3 was injected with 3mg/kg of aldesleukin (control) twice a week. Group 4 was injected with solvent (control) twice a week. All groups were continuously administered for 3 weeks. Mice tumors were measured twice a week and mice were weighed twice a week.

MC38 cells were subcutaneously inoculated to transgenic C57BL/6 mice transformed with human PD-1 fusion protein in a concentration of 0.5×10^6 cells per mouse. One week later, the mice implanted with MC38 tumor were randomly divided to 4 groups. Group 1 was injected with 2mg/kg of IL2-T37C-S14 protein conjugate. Group 2 was injected with 0.5mg/kg of IL2-Thr37Cys conjugate and 100 μg per time of anti-PD-1 antibody twice a week. Group 3 was injected with 3mg/kg of aldesleukin (control) twice a week. Group 4 was injected with solvent (control) twice a week. All groups were continuously administered for 3 weeks. Mice tumors were measured twice a week and mice were weighed twice a week.

Results are shown in Figs. 20 and 21, which demonstrate that the IL2-Thr37Cys conjugate could not only be used in a reduced use dose, but also significantly enhance the effect of treating B16F10 tumor, as compared to the existing IL2 product, aldesleukin. And in the combination treatment group with anti-PD-1 antibody, there were MC38 mice being cured.

From the above results, it could be found that, when using a functional moiety to hinder or reduce the activity of a protein or antibody, the binding of a protein to its receptor or ligand in a normal tissue could be reduced before it arrives at a target tissue because it is very difficult to activate the functional moiety conjugated to the protein by a hydrolase and a physiological environment outside a diseased microenvironment. However, in a diseased microenvironment, IL2-Thr37Cys and IL2-Thr41Cys and the like were influenced by the hydrolase in the diseased microenvironment and activated on the surface of Granzym-B highly expressing CD8 cells, thereby binding to a receptor on the surface of CD8 cells and activating CD8 cells. As a result, the protein conjugated with a functional moiety could reduce immune toxicity while enhance targeted efficacy.

For the same reasons, IL2-Leu19Cys and IL2-Ser87Cys were activated on the surface of Legumain highly expressing Treg cells, allowing the activated IL2 to bind to a receptor on the surface of Treg cells to activate proliferation of Treg cells.

Example 7b: Tumor Microenvironment Activated IL2 cytokine (IL2 TMEAkine)

1. Expression and purification of the mutant IL2 cytokine

The mutant IL2 DNA sequence ligated to a modified pTT5 vector (Biovector) was optimized for expression in 293T cells and synthesized (GENEWIZ Inc., Suzhou, China). Transfection of the mutant IL2 DNA was performed. After incubation for 4-7 days, the supernatant containing mutant IL2 was collected.

5 In eukaryotic expression, the expression vector pPICZ α A containing the mutant IL2 genes was optimized and prepared (GENEWIZ Inc., Suzhou, China). The amino acid sequence of wild type IL2 was described is shown below:

APASSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHL
QCLEEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADETATIVE
10 FLNRWITFSQSIISTLT (SEQ ID NO:11).

The expression vector pPICZ α A was transformed in *E.coli* (DH5 α) for plasmid purification. Then pPICZ α A was transformed into GS115 by electroporation. The transformed colony was selected by obtaining the growing colonies after growing on the 100, 300, 500, 1000, 1500, 2000 ug/mL ZeocinTM containing YPD plates. After finally selecting the
15 transformant, the recombinant GS115 strain was grown in BMGY medium at 30°C, with vigorous shaking in baffled flasks to an OD600 of 2-6. The cells were then pelleted by centrifugation and suspended in BMMY to an OD600 of 1, to which was added 0.5% methanol daily in order to induce the heterologous protein expression. After a four-day induction, supernatant containing the secreted mutant IL2 protein was collected by centrifugation. The
20 total protein in the supernatant was concentrated by ultrafiltration using a 10-kDa molecular mass cutoff membrane. The concentrated protein was dialyzed with buffer A (50 mM HAc/NaAc, pH4.5) for more than 24 h, then loaded onto a cation-exchange column equilibrated with buffer A. Mutant IL2 was eluted from the column with gradient concentration of NaCl and the eluent was collected and concentrated. The condensed sample was further purified on
25 Sephacryl S-100 HR gel filtration column using 20 mM Tris-HCl, 20 mM NaCl, pH7.4, as the elution buffer.

In prokaryotic expression, the expression vector pET22b (+) containing the mutant IL2 genes was optimized and prepared (GENEWIZ Inc., Suzhou, China). The amino acid sequences of wild type IL2 were described in SEQ ID NO:12. The positive clone was selected and
30 transformed in the *E.coli* cells (BL21DE3). Standard procedure for induction of the target protein using isopropyl thiogalactoside (IPTG) was followed. The induced *E.coli* cells were centrifuged and the cell pellet was resuspended in 100 mM Tris-HCl buffer (pH 8.0) containing 5 mM EDTA and 1 mM PMSF. Cells were lysed by sonication and centrifuged to isolate IL2 protein inclusion bodies (IBs). The IB pellet was then washed with 100 mM Tris-HCl buffer
35 (pH 8.0) containing 5 mM EDTA and 2% deoxycholate, and distilled water respectively. The

IBs were solubilized in 6 M guanidine hydrochloride (GuHCl) solution (prepared in 0.1 M Tris buffer, pH 8.0) and incubated for 30 min at room temperature with gentle vortexing, followed by centrifugation. The supernatant was diluted with refolding buffer (0.1 M Tris buffer, pH 8.0 containing 10 mM reduced and 1 mM oxidized glutathione in a ratio of 10:1) so as to obtain a protein concentration and GuHCl of 0.1 mg/mL and 2 M respectively. Subsequently, the solution was kept for 16h at room temperature for slow refolding of IL2. The insoluble protein was removed by centrifugation. The supernatant was concentrated and loaded on a gel filtration column Sephacryl S-100 HR, equilibrated with 0.1 M Tris buffer containing 2 M GuHCl.

Wild type IL2 amino acid sequence produced by the transformant is shown below:

PTSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQ
CLEEELKPLEEVNLNAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADETATIVEFL
NRWITFSQSIISTLT (SEQ ID NO: 12).

2. Screening the mutants of their binding activity to IL2R α or IL2R β after mutation

Various IL2 mutants were expressed by 293T cells and secreted to the medium. Supernatant containing IL2 mutants was obtained through centrifugation as described in section 1. Then 1 μ g His-tagged IL2R α or IL2R β was added to 1 mL supernatant mentioned above and incubate for 1h at 4°C with gentle agitation. 50 μ L pre-washed Ni-NTA resin was transferred to the mixed solution of the supernatant and IL2R α or IL2R β , and incubated for 1h at 4°C with gentle agitation. The mixture was subjected to centrifugation and the supernatant was discarded. The resultant pellet was washed with 500 μ L PBS containing 25 mM imidazole for three times. The amount of IL2 mutants and IL2R α /R β was visualized by western blotting using IL2 antibody and anti His-tag monoclonal antibody.

3. Conjugating S47 to the mutant IL2

Mutant IL2 protein was generated and purified as described above in section 1. Purified mutant IL2 was incubated as the concentration of 0.3 mg/mL in 50 mM phosphate buffer (pH 7.4) containing 5 mM EDTA. TCEP solution was added to mutant IL2 in a molar ratio of 100:1 and the resultant mixture was incubated for 4h at 4°C with gentle agitation. Then the mixture was dialyzed with 50 mM phosphate buffer (pH 7.4) containing 150 mM NaCl for 2h at 4°C. Afterwards, S47 was immediately added to the mixture in a molar ratio of 20:1 and the resultant mixture was incubated for 16h at 25°C with gentle agitation. The reaction was stopped and residual S47 was removed. Before enzyme cleavage, the buffer used for the conjugate of IL2-S47 (IL2 TMEAkine, Tumor Microenvironment Activated IL2 cytokine) was changed to a buffer used for enzyme through dialysis. Then enzyme was added to the IL2 TMEAkine

solution and the mixture was incubated at 37°C for 16h. Fig.22 shows the SDS-PAGE results, with a colloidal blue stain for mutant IL2, IL2 TMEAkine and the recovery active IL2 after enzyme cleavage *in vitro*.

4. Screening IL2 TMEAkine that blocks the binding to IL2R α or IL2R β and recovers the binding activity after enzyme cleavage *in vitro*

60 ul PBS buffer containing 1 ug IL2R α -Fc/ IL2R β -Fc solution were dispensed into wells. Sealing tape was applied to the top of the plate and then the plate was incubated at 4°C overnight. After incubation, the tape was removed to aspirate each well. After washing with PBST for three times, the plate was blocked by dispensing 200 ul of PBS buffer containing 2% BSA into each well and then the plate was incubated at room temperature for 2h. The plate was washed three times and 60 ul of serial diluted samples were added to the appropriate wells. The plate was incubated at room temperature for 1.5h. After washing with PBST for three times, 60 ul of 1 ug/mL IL2 biotinylated antibody solution was dispensed to each well and the resultant mixture was incubated at room temperature for 1 hour. The plate was washed for three times and then 60 ul of streptavidin solution was dispensed to each well. Then the plate was incubated at room temperature for 30 minutes. After washing three times, 100 ul of the HRP substrate solution was dispensed into each well and the plate was incubated at 37°C for 15 minutes. After color development, 50 ul of stop solution was dispensed into each well and the absorbance of each well was immediately measured at a wavelength of 450 nm. ELISA results were shown in Fig. 23, indicating the binding activity of IL2 TMEAkine to IL2R α or R β before and after enzyme cleavage *in vitro*. Compared with binding activity of wild type IL2 to IL2R α and IL2R β , IL2 TMEAkine binds to IL2R α with much lower binding affinity but binds to IL2R β with almost the same binding affinity. After activation by enzyme, the binding affinity of IL2 TMEAkine to IL2R α equals to that of wild type IL2 to IL2R α .

5. Summary of various IL2 mutation sites

IL2 receptors may associate on the cell surface to form the following heteromers: intermediate-affinity receptor: IL2R $\beta\gamma$ to IL2 (K_d=1 nM) and low-affinity receptor: IL2R α to IL2 (K_d=10 nM). Because it is a low-affinity binding (K_d= 10nM), it is easier than CDR region of antibody to screen a position for linking to R4 group for recovering the binding affinity. Because we didn't want to increase the binding affinity between R4-S-IL2 and IL2R α , we selected the conjugated IL2 with the special R4, which can recover the native binding affinity. In some case, there are some R4 groups which can enhance the binding affinity, but we didn't select them as drug candidates. We prefer R-1, R4-7, R4-5, R4-8 and R4-12 for the large scale

synthesis in our CMC development. S47 is cleaved by Legumain. After cleaving, its R4-7 chemical group is remained. To select drug candidates, we also performed the screening expression and S47 conjugation reaction with all amino acids of IL2 in the domain that binds IL2R α and IL2R β . We acquired the possible drug candidates and results are shown in the following Tables 31-33.

Table 31: Activity of mutated IL2 and screening for linker arm

Position	Amino acid	Type of inhibitory ligand	R4	Binding recovery (>60%)	Decreased fold of binding to ligand after Conjugating with S47(>3fold)
32	K	α	R4-7	100.2%	3-fold
35	K	α	R4-7	85.4%	89-fold
37	T	α	R4-7	98.9%	51-fold
38	R	α	R4-18	81.2%	145-fold
41	T	α	R4-7	99.2%	135-fold
42	F	α	R4-8	82.3%	120-fold
43	K	α	R4-7	89.3%	110-fold
43	K	α	R4-18	109.7%	
44	F	α	R4-8	96.7%	60-fold
45	Y	α	R4-7	96.8%	125-fold
45	Y	α	R4-8	143.6%	
48	K	α	R4-7	100.2%	12-fold
49	K	α	R4-18	100.7%	
60	E	α	R4-12	97.1%	5-fold
61	E	α	R4-12	83.4%	134-fold
62	E	α	R4-12	86.6%	120-fold
63	L	α	R4-5	99.3%	13-fold
64	K	α	R4-7	97.1%	
67	E	α	R4-12	99.6%	9-fold
68	E	α	R4-12	95.4%	89-fold
71	N	α	R4-18	98.6%	5-fold
72	L	α	R4-5	90.1%	110-fold
74	Q	α	R4-19	100.0%	3-fold
75	S	α	R4-7	100.0%	4-fold
76	K	α	R4-7	100.0%	3-fold
101	T	α	R4-7	100.0%	5-fold

102	T	α	R4-7	100.0%	4-fold
102	T	α	R4-3	122.5%	
107	Y	α	R4-4	97.7%	78-fold
108	A	α	R4-5	100.0%	10-fold
111	T	α	R4-7	98.9%	20-fold
112	A	α	R4-5	100.0%	5-fold
5	S	β	R4-7	99.8%	3-fold
5	S	β	R4-2	109.5%	
8	K	β	R4-7	66.7%	6-fold
9	K	β	R4-6	97.0%	9-fold
9	K	β	R4-7	124.6%	
12	L	β	R4-5	93.4%	13-fold
13	Q	β	R4-19	85.1%	28-fold
15	E	β	R4-12	81.4%	25-fold
19	L	β	R4-5	92.3%	27-fold
20	D	β	R4-11	87.3%	31-fold
27	G	β	R4-1	100.0%	3-fold
76	K	β	R4-6	100.0%	3-fold
76	K	β	R4-7	130.4%	
80	L	β	R4-2	100.0%	5-fold
81	R	β	R4-18	95.2%	12-fold
84	D	β	R4-11	80.1%	36-fold
85	L	β	R4-7	98.4%	9-fold
85	L	β	R4-5	113.6%	
87	S	β	R4-7	99.5%	30-fold
88	N	β	R4-18	82.3%	46-fold
91	V	β	R4-5	94.5%	25-fold
92	I	β	R4-5	89.3%	19-fold
94	L	β	R4-5	99.5%	30-fold
95	E	β	R4-12	60.1%	8-fold
98	G	β	R4-1	98.6%	4-fold
99	S	β	R4-7	100.0%	3-fold
101	T	β	R4-7	100.0%	3-fold
133	T	β	R4-7	100.0%	3-fold

Table 32: Binding activity of conjugates of IL2 mutants with two or more mutation sites to its receptors before and after activation

TMEAkine	Mutation position (R α binding)	Mutation position (R β binding)	Decreased fold of binding to α ligand after Conjugating with S47	Decreased fold of binding to β ligand after Conjugating with S47	Binding recovery (>60%)
IL2-Thr37/Thr3	IL2-Thr37, IL2-Thr3		51-fold	N.D.	98.9%
IL2-Thr3/Thr41	IL2-Thr3, IL2-Thr41		135-fold	N.D.	101.8%
IL2-Thr-41/Ser87	IL2-Thr-41	IL2-Ser87	135-fold	30-fold	96.8%
IL2-Thr37/Ser87	IL2-Thr37	IL2-Ser87	51-fold	30-fold	98.4%
IL2-Thr3/Ser87	IL2-Thr3	IL2-Ser87	N.D.	30-fold	99.3%

The results demonstrate that conjugates of IL2 mutants with two or more mutation sites could retain 95% or more binding activity to corresponding receptor after activation.

After screening expression, binding activity, conjugation, cleavage, recovery and functional assay, we acquired the drug candidates with one or more stable mutations on the binding domain with R α and one conjugation on the binding domain with R β , as shown in Table 33. Particularly, stable mutation sites on the domain binding with R α were Arg38 and Glu61, in which Arg38 was mutated to Asp and Glu61 was mutated to Arg, affecting binding activity of IL2 and IL2R α . Therefore, these conjugations are releasing stable mutants on the binding domain with R α after cleaving in tumor microenvironment.

Table 33: Binding activity of candidates with one or more stable mutations and one conjugation to its receptors before and after activation

TMEAkine	Stable mutation for blocking R α binding	Mutation position (R β binding) for conjugation with S47	Decreased fold of binding to α ligand after conjugating with S47	Decreased fold of binding to β ligand after conjugating with S47	Binding recovery of β ligand (>60%)
IL2-Arg38/Ser87	Arg38Asp	Ser87	200-fold	32-fold	99.5%
IL2-Glu61/Ser87	Glu61Arg	Ser87	110-fold	30-fold	99.5%

IL2-Arg38/Glu61/Ser87	Arg38 Asp /Glu61Arg	Ser87	>200-fold	32-fold	96.4%
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Table 34: Binding activity of candidates with two stable mutants and one conjugation to its receptors before and after activation

Mutation for adjusting R α binding	Mutant of R β binding for conjugation with R4-7	Decreased fold of binding to R α	Binding recovery to R β
Arg38Asp/Glu61Arg		>200-fold	
	Lys9		124.6%
	Lys76		130.4%
Arg38Asp/Glu61Arg	Lys9,Lys76	>200-fold	165.7%

5 The results demonstrated that, with conjugation of R4 library screening and mutation, we got a new chemically modified IL2 with decreasing 200 folds of binding to R α and increasing 1.35 folds of binding to R β .

6. Stability in Human Serum

10 IL2-T41C-S47 solution and human serum were mixed in a ratio of 1:19 (v/v) and the mixture was incubated at 37°C for 0h, 8h, 24 and 48h, respectively. Then the amount of IL2-T41C-S47 was detected by western blot and the corresponding results were shown in Fig. 24. IL2-T41C-S47 was stable in human serum after 48 hours, indicating that IL2-T41C-S47 may be much more stable than IL2 in human serum *in vitro*. The stability in human serum of
15 other conjugates of IL2 mutants was shown in Table 35.

Table 35: The stability of other conjugates of IL2 mutants in human serum

R1-R2-R3-R4	R5	Stability in Human Serum
S47	IL2-Thr37	100%
S47	IL2-Thr41	99.8%
S47	IL2-Ser87	99.2%
S47	IL2-Thr41/Ser87	99.5%
S47	IL2-Ser37/Ser87	99.5%

7. Pharmacokinetics in mice

20 Mice received a single intravenous injection of IL2-T41C-S47 (0.8mg/kg), n=3 mice per sampling time. Approximately 200 uL blood was collected into K2EDTA-coated tubes. Plasma

was separated after centrifugation and frozen at -80°C until analysis. The IL2-T41C-S47 concentration was then measured using a qualified ELISA. Results were shown in Fig. 25. The pharmacokinetic study *in vivo* indicates that IL2 TMEAkine has a long half-life and a high exposure in plasma when compared with IL2.

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8. Toxicity

C57BL/6 mice received daily i.p. injection of PBS or 25 ug IL2 for 5 days or i.p. injection of equimolar IL2-T41C-S47 every 5 days for 5 doses, respectively. Mice were sacrificed and lungs were fixed in 10% formalin solution and paraffin-embedded sections were stained with hematoxylin and eosin. The results were shown in FIG. 26. The measurement of pulmonary (wet weight) and the sections stained with hematoxylin and eosin indicate that IL2 TMEAkine induces less toxicity to lung than wild-type IL2.

C57BL/6 mice received i.p. injection of PBS, 25 ug IL2-T41C/S87C-S47 and equimolar IL2-R38D/E61R/S87C-S47 every 5 days for 5 doses, respectively. Mice were sacrificed. The wet weight of lungs was measured and lungs were fixed in 10% formalin solution and paraffin-embedded sections were stained with hematoxylin and eosin. Results were shown in Fig. 27. The measurement of pulmonary indicates that IL2-T41C/S87C-S47 and IL2-R38D/E61R/S87C-S47 induce almost no toxicity to lung.

9. Study on efficacy of IL2-T41C-S47 and IL2-T41C-S47 in combination with anti-PD-1 antibody on the CT26 tumor model in BALB/C mice model

Test purpose: to investigate the anti-tumor efficacy of IL2-T41C-S47 and IL2-T41C-S47 in combination with anti-PD-1 antibody in BALB/C mice for treatment of the CT26 tumor model.

Test drug: IL2-T41C-S47, anti-PD-1 antibody and IL2 injection, diluted to corresponding concentrations by PBS when testing.

Method and results:

1. Animal: BALB/C mice of 5 weeks old, all female.

2. Production of tumor model

1) CT26 cells were purchased from American type culture collection (ATCC) and identified according the specification provided by ATCC. Cells were cultivated in RPMI 1640 culture solution containing 10% fetal bovine serum at 37°C and 5% CO₂. The cells were passaged for every three days and cells within the 9th passage were used.

2) Production of tumor model. CT26 cells were subcutaneously injected to the back of the BALB/C mice. Mice were randomly grouped after the tumor grew to about 100-200mm³ and

drug treatment began. Mice were killed after anesthesia on day 31.

3) Course of treatment

There were 5 groups with 6 animals in each group. Included were a control group treated on day 0, 5 and 11, and three single agent groups (treated by anti-PD-1 antibody on day 2, 4, 7, 9, 13 and 15, or by IL2 on day 0, 5, 11, or by IL2-T41C-S47 on day 0, 5 and 11) and one combined immunotherapy group in which IL2-T41C-S47 (given on day 0, 5 and 11) treatment was initiated prior to anti-PD-1 antibody treatment (given on day 2, 4, 7, 9, 13 and 15).

4) Grouping and test results are shown in Table 36.

Table 36: Effects of IL2-T41C-S47 and IL2-T41C-S47 in combination with anti-PD-1 antibody on the CT26 tumor model in BALB/C mice model

Group	Complete Regression
Control Group	0
Anti-PD-1 antibody	16.79%
IL2	16.79%
IL2-T41C-S47	33%
IL2-T41C-S47 in combination with Anti-PD-1 antibody	100%

Tumor volumes were monitored 2-3 times a week and were presented in the Fig. 28.

5) Results and discussion. As shown in Table 36, the regression on the CT26 tumor of BALB/C mice was greatly improved after injection of IL2-T41C-S47 in combination with anti-PD-1 antibody, indicating that IL2-T41C-S47 in combination with anti-PD-1 antibody exhibits an excellent anti-tumor efficacy on the CT26 tumor model.

10. Biodistribution of active IL2 from IL2-T41C-S47 in Tumor, Lung and Heart

BALB/C mice were implanted subcutaneously into the right flank with CT26 cells. Seven days after implantation, when tumors measured 200 mm³, animals were administered with IL2-T41C-S47 (2 mg/kg × 1). After 24h, 48h, 96h and 192h, tumor, lung and heart were harvested (n=2 per observation time), homogenized in ice-cold PBS containing protease inhibitor and 0.25% acetic acid, and centrifuged to obtain supernatant. To quantify IL2-T41C-S47 level, ELISA was performed in which PEG antibody was used as capture antibody and IL2 biotinylated antibody was used as detection antibody. To quantify IL2-T41C-S47 and active IL2 levels, ELISA was performed in which IL2 monoclonal antibody was used as capture antibody and IL2 biotinylated antibody was used as detection antibody.

The results were shown in Fig. 29, indicating high exposure of active IL2 in tumor, which is consistent with high efficacy of anti-tumor effect, and low exposure of active IL2 in lung and heart, which is consistent with low toxicity of pulmonary edema.

5 **Example 8: Section of chemical modified linker to acquire high activation efficiency**

R2-R3 is a chemical modified linker showing high activation efficiency as compared to the native peptide sequence linker. The activation of different R2-R3 linkers in which R1 was a 5 kDa PEG and R4 was R4-2 and control linker (C1-C4 shown in the the Tables) was evaluated in activation assay. The R1-R2-R3-R4 conjugates were dissolved and diluted for ten times to a
10 final concentration of 0.1 mM/ml. At 37°C, test compounds were added into 100μg acidized human breast cancer (MDA-MB435) tumor tissue homogenates (pH6.0) in a concentration of 1mg/ml. The enzyme in tumor tissue homogenates could release R1. The released R1 was detected by HPLC, thereby comparing the activation efficiency of the linkers. Results were shown in tables 37-39.

15

Table 37: Activation efficiency (%) of different linkers

R2-R3	Activation efficiency (%)
C1: AANL	68.4
C2: AANP	45.9
C3: SDNP	22.7
C4: SANP	32.6
C5: SDNH	23.9
AAN-R3-5	99.6
AAN-R3-6	97.5
AAN-R3-7	73.3
AAN-R3-8	93.3
AAN-R3-9	63.3
AAN-R3-10	55.1
AAN-R3-11	76.3
AAN-R3-12	64.5
AAN-R3-1	99.7
AAN-R3-2	99.5
AAN-R3-3	99.1
AAN-R3-4	98.9

Table 38: Activation efficiency (%) of different linkers

R2-R3	Activation efficiency (%)
C1: AADL	41.7
C2: AADP	37.8
C3: SDDP	46.8
C4: SADP	31.3
C5: SDNH	10.7
AAD-R3-5	92.3
AAD-R3-6	90.5
AAD-R3-7	84.6
AAD-R3-8	86.7
AAD-R3-9	73.4
AAD-R3-10	53.6
AAD-R3-11	49.6
AAD-R3-12	57.7
AAD-R3-1	93.7
AAD-R3-2	96.5
AAD-R3-3	90.1
AAD-R3-4	97.9

Table 39: Activation efficiency (%) of different linkers

R2-R3 (R2 is absent)	Activation Efficiency (% , pH6.0)
R3-1	43.3
R3-2	65.1
R3-3	46.3
R3-4	60.1
R3-5	8.8
R3-6	79.5

5 MDA-MB435 tumor tissue exhibits high activity of Legumain, Grazym B and MMP2 or other protease. In the assay, it was proved that AAN-R3-5, AAN-R3-6, AAD-R3-5, AAD-R3-6, AAD-R3-7 and AAD-R3-8 had relatively higher activation efficiency (>80%). When R2 is absent, R3-5 is stable at pH6.0, and R3-2, R3-4 and R3-6 are an acidically (pH6.0) activated linkers with relatively higher activation efficiency (>60%).

Example 9: Activation efficiency of different activatable linker for targeted activation in different tumor microenvironment

Six R1-R2-R3-R4 conjugates were detected in different human tumor tissue, wherein R1 was a 5kDa PEG and R4 was R4-2. The R1-R2-R3-R4 conjugates were each dissolved diluted for ten times to a concentration of 0.1 mM/ml. At 37°C, test compounds were added into 100µg different acidized human tumor tissue homogenates (pH6.0) in a concentration of 1mg/ml. The enzyme in tumor tissue homogenates could release R1. The released R1 was detected by HPLC, thereby comparing the activation efficiency of the linkers. Results were showed in Table 40.

Table 40: Activation efficiency (%) of R3-3, R3-5, R3-6, AAN-R3-5, AAN-R3-6, and AAD-R3-5 in homogenates from different tumor tissues

Tumor tissues	Cells producing tumor	Activation efficiency (%)					
		R3-2	R3-4	R3-6	AAN-R3-5	AAN-R3-6	AAD-R3-5
Human fibrosarcoma	HT-1080	63.7	64.7	78.9	87.2	84.5	89.5
Human breast cancer	MDA-MB231	66.3	53.7	89.6	97.8	84.7	92.6
Human ovarian cancer	SK-OV-3	56.6	35.7	78.4	99.4	85.8	95.8
Human colon cancer	HT-29	63.8	56.7	58.8	93.6	83.7	91.5
Human chronic leukemia	K562	64.8	53.8	58.3	88.2	84.6	84.8
Human pancreatic cancer	Panc-1	46.8	73.8	74.8	96.1	94.8	79.8
Human non-small cell lung cancer	A549	57.8	62.7	74.7	86.2	85.8	96.4
Human prostate cancer	PC-3	37.8	64.8	89.6	96.5	94.7	89.6
Human liver cancer	Hepg2	46.8	64.5	45.8	87.0	83.6	85.7
Human renal cancer	OS-RC-2	35.7	54.6	75.7	95.7	90.4	88.4
Human heart		8.6	3.7	7.4	0.1	0.1	6.8
Human Lung		6.7	6.5	4.9	0.1	0.1	1.8

Example 10: Chemical modified linker shows no steric hindrance to different biomolecules when activated by tumor tissue protease

R4-1 is the shortest chemical group in the exemplified R4 groups. Different biomolecules were conjugated to R1-R2-R3-R4, in which R1 was a 5kDa PEG, R2-R3 were shown in the following table and R4 was R4-1. The conjugates were dissolved and diluted for ten times to a concentration of 0.1 mM/ml. At 37°C, the conjugates were added into 100µg different acidized human tumor tissue homogenates (pH6.0) in a concentration of 1mg/ml or were added into a

legumain solution (0.1ug/ml) or a Granzyme B solution (0.1ug/ml), respectively. The enzyme in tumor tissue homogenates could release R1. The released R1 was detected by HPLC, thereby comparing the activation efficiency of the linkers. Results were showed in Table 41.

5 Table 41: Activation efficiency (%) of linker of different antibody

Biomolecule	Activation efficiency (%)				Legumain	Granzyme B
	AAN-R3-5	AAN-R3-6	AAD-R3-5	R3-6	AAN-R3-5	AAD-R3-5
Human IL2 (SEQ ID NO:11)	95.6	94.4	93.4	67.8	99.5	102.8
PD-1 (SEQ ID NO:19)	91.4	88.6	82.8	76.4	101.6	94.7
Nivolumab (SEQ ID NO: 18)	82.4	92.9	94.6	63.6	103.6	99.5
Pembrolizumab (SEQ ID NO:15)	87.7	76.3	73.2	67.2	100.6	97.7
Ip1(CTLA-4 Ab) (SEQ ID NO:13)	97.8	93.8	94.5	66.1	99.4	102.7
Anti-human 4-1BB (SEQ ID NO:25)	89.5	92.4	84.4	66.2	96.8	104.4
Adalimumab (SEQ ID NO:29)	100.3	101.4	79.3	76.5	99.1	93.7
Niv-se001 (SEQ ID NO:41)	88.7	87.6	76.5	77.0	103.7	95.7

The results demonstrate that the cleaving site in R2 is distant to the biomolecule (R5). Even with the shortest R4-1, cleavage of R2 is not affected by the biomolecule and the activation efficiency is not affected.

10 Example 11: Stability of chemical modified linker in human serum

The stability of the chemical modified linker R2-R3 was tested in human serum. R1-R2-R3-R4 conjugates, in which R1 was a 5kDa PEG (PEG500), R2-R3 was shown in the following Tables and R4 was R4-1, were prepared. The conjugates were dissolved and diluted for ten times to a concentration of 0.1 mM/ml. Conjugates were each added into 100μg human serum in a concentration of 1mg/ml at 37°C for 48 hr. The intact conjugate can be detected by ELISA Assay. By comparing the concentration of remain conjugates, stability could be

calculated. Results were shown in Tables 42 and 43.

Table 42: Stability (% of control) of different linkers

R2-R3	stability(% of control)
C1: AANL	97.4
C2: AANP	55.9
C3: SDNP	72.7
C4: SANP	62.6
C5: SDNH	73.9
AAN-R3-5	99.9
AAN-R3-6	99.5
AAN-R3-7	99.3
AAN-R3-8	98.3
AAN-R3-9	97.2
AAN-R3-10	98.7
AAN-R3-11	96.3
AAN-R3-12	97.5
AAN-R3-1	89.7
AAN-R3-2	89.5
AAN-R3-3	79.1
AAN-R3-4	88.9

5

Table 43: Stability (% of control) of different linkers

R2-R3 (R2 is absent)	Activation efficiency (%)
R3-1	88.8
R3-2	83.7
R3-3	94.6
R3-4	91.8
R3-5	85.8
R3-6	86.4

Example 12: Increased conjugation efficiency with increased antibody/S13 linker

For small scale conjugation , 5~10 mg IgG for different variants were buffer exchanged with ultrafiltration tubes (Merck Millipore) into 50 mM Tris-HCl, pH7.5 containing 2 mM EDTA by repeated centrifugation. Then the antibodies were mildly reduced by DTT in a

10

1:20~1:200 molar ratio at room temperature for 4~16h. Then the reduced antibodies were dialyzed into 50mM Tris-HCl, 150mM NaCl, pH7.5 and re-oxidated by Cu₂SO₄ or Dehydroascorbic acid (DHAA, Sigma) in a 1:50~1:200 molar ratio for 1~3h at room temperature. Then the re-oxidated antibodies with free sulfydryl were conjugated by S13 chemical linker in a ratio of 1:10, 1:20, 1:50 or 1:100 at room temperature for 4h. The conjugation efficiency was shown by reduced SDS-PAGE. As shown in Table 44, different conjugation efficiency was obtained.

Table 44: Increased conjugation efficiency with increased antibody (Ipilimumab, SEQ ID NO:13)/S13 linker ratio

Antibody/S13 linker molecular ratio	Conjugation efficiency
1:10	60%
1:20	72%
1:50	89%
1:100	95%

In a conjugation reaction of CTLA4-antibody (Ipilimumab, SEQ ID NO:14): S13 linker = 1:100 condition with Cys mutation in different position of CTLA4-antibody, the mutant sites were shown in Table 45. In the CTLA4-antibody:S13= 1:100 condition, all five position are conjugated with S13 linker in a high efficiency, as shown in reduced SDS-PAGE gel in Fig. 30.

Table 45: Different mutation sites for 1:100 molecular ratio conjugation

Mutation ID	Conjugation efficiency (%)
1# (FR1, L22S)	96
2# (FR2, L49Y)	99
3# (FR2, L72T)	95
4# (CDR1, L24R)	97
5# (CDR3, L13G)	99

Example 13: conjugation efficiency and affinity change of framework (non-CDR) of a variable region of human CTLA-antibody

We mutated every non-Cysteine amino acid to Cysteine in framework region (FR) of CTLA-4 antibody to make cystine mutant for experiments. Some mutation sites showed nearly 100% conjugation efficiency. The conjugation efficiency for different mutants was high in a CTLA4-antibody:S13= 1:100 condition, which was summarized in Table 46 and Table 47.

Some mutations show very low conjugation, indicating that Cys may be buried in the interior of antibody.

Table 46: Conjugation efficiency for Ipilimumab light chain framework region

Region (Kabat)	Kabat No.	Ipilimumab VL	Conjugation efficiency of S13 (%)
FR1	L1	E	35
	L2	I	58
	L3	V	99
	L4	L	29
	L5	T	96
	L6	Q	95
	L7	S	87
	L8	P	98
	L9	G	96
	L10	T	95
	L11	L	62
	L12	S	88
	L13	L	16
	L14	S	88
	L15	P	95
	L16	G	64
	L17	E	69
	L18	R	72
	L19	A	10
	L20	T	28
	L21	L	13
	L22	S	96
	L23	C	n/d
FR2	L35	W	13
	L36	Y	88
	L37	Q	16
	L38	Q	86
	L39	K	89
	L40	P	19

	L41	G	92
	L42	Q	85
	L43	A	46
	L44	P	63
	L45	R	25
	L46	L	16
	L47	L	18
	L48	I	85
	L49	Y	99
FR3	L57	G	86
	L58	I	95
	L59	P	94
	L60	D	96
	L61	R	86
	L62	F	75
	L63	S	95
	L64	G	95
	L65	S	94
	L66	G	68
	L67	S	88
	L68	G	88
	L69	T	87
	L70	D	94
	L71	F	28
	L72	T	95
	L73	L	26
	L74	T	95
	L75	I	16
	L76	S	94
	L77	R	96
	L78	L	13
	L79	E	88
	L80	P	96
	L81	E	85
	L82	D	13

	L83	F	18
	L84	A	10
	L85	V	85
	L86	Y	12
	L87	Y	19
	L88	C	n/d
FR4	L98	F	87
	L99	G	88
	L100	Q	19
	L101	G	86
	L102	T	12
	L103	K	95
	L104	V	8
	L105	E	95
	L106	I	75
	L107	K	92

Table 47: Conjugation efficiency for Ipilimumab heavy chain framework region

Region (Kabat)	Kabat No.	Ipilimumab VH	Conjugation efficiency of S13 (%)
FR1	H1	Q	68
	H2	V	95
	H3	Q	86
	H4	L	86
	H5	V	88
	H6	E	18
	H7	S	68
	H8	G	89
	H9	G	85
	H10	G	84
	H11	V	68
	H12	V	18
	H13	Q	86
	H14	P	85
	H15	G	85

	H16	R	86
	H17	S	78
	H18	L	18
	H19	R	78
	H20	L	8
	H21	S	79
	H22	C	n/d
	H23	A	89
	H24	A	12
	H25	S	86
	H26	G	89
	H27	F	88
	H28	T	n/d
	H29	F	89
	H30	S	89
FR2	H36	W	13
	H37	V	18
	H38	R	19
	H39	Q	89
	H40	A	82
	H41	P	98
	H42	G	96
	H43	K	12
	H44	G	96
	H45	L	93
	H46	E	88
	H47	W	18
	H48	V	15
	H49	T	23
FR3	H66	R	26
	H67	F	29
	H68	T	95
	H69	I	18
	H70	S	86
	H71	R	28

	H72	D	83
	H73	N	82
	H74	S	85
	H75	K	92
	H76	N	98
	H77	T	25
	H78	L	26
	H79	Y	95
	H80	L	15
	H81	Q	95
	H82	M	17
FR4	H103	W	68
	H104	G	29
	H105	Q	92
	H106	G	29
	H107	T	28
	H108	L	95
	H109	V	88
	H110	T	87
	H111	V	27
	H112	S	90
	H113	S	85

The mutant sites with high conjugation efficiency of S13 (>80%) were conjugated with R4-7 and tested for relative binding activity in an ELISA based assay according to EC50 ratio (EC50 of WT antibody: EC50 of mutant antibody-R4-7*100%). Specifically, a 96-well ELISA plate (NUNC) was coated by 1 μ g/ml His-CTLA-4 protein (Sino Biological) overnight and then blocked with 1% BSA blocker (ThermoFisher) for 2 hours at 37°C and washed by PBST three times. Corresponding antibody or corresponding mutant with R4-7 conjugation was added and allowed to bind at 37°C for 1 hour, then washed with PBST three times. HRP enzyme-conjugated anti-human IgG was added and allowed to bind at 37°C for 1 hour and then washed with PBST three times. TMB substrate (Solarbio., Inc.) was used to detect absorbance at 450nm. Data analysis was carried out with GraphPad software and EC₅₀ for each antibody or conjugate was calculated.

After comparing the binding affinity R4-7-s-cys-CTLA-4 in FR region and WT antibody

of CTLA-4, we found most of position achieved a good effect to maintain the binding affinity (Group A, >60% comparing with WT antibody) while some positions (Group B, <60% comparing with WT antibody) exhibited a lower binding affinity, as shown in Table 48 and Table 49.

5

Table 48: Binding activity of light chain framework mutants after conjugation with R4-7

	Kabat No.	Ipilimumab VL	Conjugation efficiency of S13 (%)	Binding activity after conjugation with R4-7
Region (Kabat)	L3	V	99	A
	L5	T	96	A
	L6	Q	95	A
	L7	S	87	A
	L8	P	98	B
	L9	G	96	A
	L10	T	95	A
	L12	S	88	A
	L14	S	88	A
	L15	P	95	B
	L16	G	64	A
	L22	S	96	A
	L36	Y	88	A
	L38	Q	86	A
	L39	K	89	A
	L41	G	92	A
	L42	Q	85	A
	L48	I	85	B
	L49	Y	99	A
FR3	L57	G	86	B
	L58	I	95	A
	L59	P	94	B
	L60	D	96	A
	L61	R	86	A
	L63	S	95	A
	L64	G	95	A
	L65	S	94	A

	L67	S	88	A
	L68	G	88	B
	L69	T	87	B
	L70	D	94	B
	L72	T	95	A
	L74	T	95	A
	L76	S	94	A
	L77	R	96	A
	L79	E	88	A
	L80	P	96	B
	L81	E	85	A
	L85	V	85	A
FR4	L98	F	87	A
	L99	G	88	A
	L101	G	86	A
	L103	K	95	A
	L105	E	95	A
	L107	K	92	A

Table 49: Binding activity of heavy chain framework mutants after conjugation with R4-7

Region (Kabat)	Kabat No.	Ipilimumab VH	Conjugation efficiency of S13 (%)	R4-7
	H2	V	95	A
	H3	Q	86	A
	H4	L	86	A
	H5	V	88	A
	H8	G	89	A
	H9	G	85	A
	H10	G	84	A
	H13	Q	86	A
	H14	P	85	B
	H15	G	85	A
	H16	R	86	A
	H23	A	89	A

H25	S	86	A
H26	G	89	A
H27	F	88	A
H29	F	89	A
H30	S	89	B
H39	Q	89	A
H40	A	82	A
H41	P	98	B
H42	G	96	A
H44	G	96	A
H45	L	93	A
H46	E	88	A
H68	T	95	A
H70	S	86	A
H72	D	83	B
H73	N	82	B
H74	S	85	B
H75	K	92	A
H76	N	98	A
H79	Y	95	A
H81	Q	95	A
H105	Q	92	A
H108	L	95	A
H109	V	88	A
H110	T	87	A
H112	S	90	A
H113	S	85	A

For these sites with binding activity < 60% (class B in the above tables), we conjugated them with different chemical linkers to rescue the binding activity. As shown in Table 50, for some of them, the binding activity can be restored with specific chemical modify of R4. These results indicate that the side chain of these sites might contribute to the antibody/antigen interaction and specific chemical linker can mimic the light chain structure and provide molecular interaction against antigen like the native WT amino acid.

Table 50: R4 screening to restore the activity of sites with low activity when conjugated with R4-7

Kabat No.	Original amino acid	R4-7	R4 screening						
			R4-1	R4-3	R4-4	R4-5	R4-8	R4-11	R4-27
L8	P	B	B	B	B	B	B	A	B
L15	P	B	B	B	B	B	B	B	B
L48	I	B	B	B	B	A	B	B	B
L57	G	B	A	B	B	B	B	B	B
L59	P	B	B	B	B	B	B	B	B
L68	G	B	A	B	B	B	B	B	B
L69	T	B	A	A	B	B	B	B	B
L70	D	B	B	B	B	B	B	A	B
L80	P	B	B	B	B	B	B	B	B
H14	P	B	B	B	B	B	B	B	B
H30	S	B	A	A	B	B	B	B	B
H41	P	B	B	B	B	B	B	B	B
H72	D	B	B	B	B	B	B	A	B
H73	N	B	B	B	B	B	B	A	B
H74	S	B	A	B	B	B	B	B	B

Blocking effect of selected sites when conjugated with S13 (5kD functional group) or S47 (40kD functional group) chemical linker

We selected the above sites whose activity can be restored after conjugating with R4-7 or other R4 linkers as candidate sites for tumor microenvironment activated antibody (TMEAbody) screening, because after protease cleavage, the conjugated antibody will remain the identical structure with antibody mutant-s-R4 form. Blocking effect after conjugated with S13 (5kD functional group) was first evaluated with the ELISA assay like Example 1. As shown in Table 51, after conjugation with S13 linker, some of them showed significant blocking effect of the binding activity against the antigen protein. We also categorize these sites with < 30% activity as class A and other sites as class B, as shown in the Table 51.

Table 51: Blocking efficiency of selected sites when conjugated with S13 (5kD) linker

Kabat No.	Original amino acid	Conjugation efficiency of S13 (%)	Binding Activity after S13 (5kD) blocking
L3	V	99	A

L5	T	96	B
L6	Q	95	B
L7	S	87	B
L9	G	96	B
L10	T	95	B
L12	S	88	B
L14	S	88	B
L22	S	96	A
L36	Y	88	A
L38	Q	86	B
L39	K	89	B
L41	G	92	B
L42	Q	85	B
L49	Y	99	A
L58	I	95	A
L60	D	96	A
L61	R	86	B
L63	S	95	B
L64	G	95	B
L65	S	94	A
L67	S	88	A
L69	T	87	A
L70	D	94	A
L72	T	95	B
L74	T	95	B
L76	S	94	B
L77	R	96	B
L79	E	88	B
L81	E	85	B
L85	V	85	B
L98	F	87	B
L99	G	88	B
L101	G	86	B
L103	K	95	B
L105	E	95	B

L107	K	92	B
H2	V	95	A
H3	Q	86	A
H4	L	86	A
H5	V	88	A
H8	G	89	B
H9	G	85	B
H10	G	84	B
H13	Q	86	B
H14	P	85	B
H15	G	85	B
H16	R	86	B
H23	A	89	A
H25	S	86	A
H26	G	89	A
H27	F	88	A
H29	F	89	A
H30	S	89	A
H39	Q	89	B
H40	A	82	B
H42	G	96	B
H44	G	96	B
H45	L	93	B
H46	E	88	B
H68	T	95	A
H70	S	86	A
H72	D	83	A
H73	N	82	A
H74	S	85	A
H75	K	92	A
H76	N	98	A
H79	Y	95	A
H81	Q	95	B
H105	Q	92	B
H108	L	95	B

H109	V	88	B
H110	T	87	B
H112	S	90	B
H113	S	85	B

We further investigated if blocking efficiency could be improved with higher molecular weight functional group. S47 (with 40kD functional group) and S64 (with 80kD functional group) were used for conjugation and blocking efficiency was measured with the above method in a binding ELISA assay. The results were summarized in the Table 52, which showed that the increased molecular weight can significantly improve the blocking efficiency of binding activity. All of the sites showed < 30% activity when conjugated with S64 (80kD) functional group.

Table 52: Blocking efficiency of selected sites when conjugated with S13 (5kD), S37 (20kD), S47 (40kD) or S64 (80kD) linkers

Kabat No.	Original amino acid	Conjugation efficiency of S13 (%)	Binding Activity after S13 (5kD) blocking	Binding Activity after S37 (20 kD) blocking	Binding Activity after S47 (40 kD) blocking	Binding Activity after S64 (80 kD) blocking
L3	V	99	A	A	A	A
L5	T	96	B	A	A	A
L6	Q	95	B	A	A	A
L7	S	87	B	A	A	A
L9	G	96	B	B	A	A
L10	T	95	B	B	B	A
L12	S	88	B	A	A	A
L14	S	88	B	A	A	A
L22	S	96	A	A	A	A
L36	Y	88	A	A	A	A
L38	Q	86	B	A	A	A
L39	K	89	B	B	A	A
L41	G	92	B	B	B	A
L42	Q	85	B	A	A	A
L49	Y	99	A	A	A	A
L58	I	95	A	A	A	A

L60	D	96	A	A	A	A
L61	R	86	B	A	A	A
L63	S	95	B	B	B	A
L64	G	95	B	B	B	A
L65	S	94	A	A	A	A
L67	S	88	A	A	A	A
L69	T	87	A	A	A	A
L70	D	94	A	A	A	A
L72	T	95	B	A	A	A
L74	T	95	B	A	A	A
L76	S	94	B	B	A	A
L77	R	96	B	B	B	A
L79	E	88	B	B	B	A
L81	E	85	B	B	B	A
L85	V	85	B	B	B	A
L98	F	87	B	B	B	A
L99	G	88	B	B	A	A
L101	G	86	B	B	A	A
L103	K	95	B	A	A	A
L105	E	95	B	A	A	A
L107	K	92	B	A	A	A
H2	V	95	A	A	A	A
H3	Q	86	A	A	A	A
H4	L	86	A	A	A	A
H5	V	88	A	A	A	A
H8	G	89	B	A	A	A
H9	G	85	B	A	A	A
H10	G	84	B	A	A	A
H13	Q	86	B	B	B	A
H14	P	85	B	B	B	A
H15	G	85	B	A	A	A
H16	R	86	B	A	A	A
H23	A	89	A	A	A	A
H25	S	86	A	A	A	A
H26	G	89	A	A	A	A

H27	F	88	A	A	A	A
H29	F	89	A	A	A	A
H30	S	89	A	A	A	A
H39	Q	89	B	A	A	A
H40	A	82	B	A	A	A
H42	G	96	B	B	A	A
H44	G	96	B	B	B	A
H45	L	93	B	A	A	A
H46	E	88	B	A	A	A
H68	T	95	A	A	A	A
H70	S	86	A	A	A	A
H72	D	83	A	A	A	A
H73	N	82	A	A	A	A
H74	S	85	A	A	A	A
H75	K	92	A	A	A	A
H76	N	98	A	A	A	A
H79	Y	95	A	A	A	A
H81	Q	95	B	A	A	A
H105	Q	92	B	A	A	A
H108	L	95	B	B	A	A
H109	V	88	B	B	A	A
H110	T	87	B	B	B	A
H112	S	90	B	B	B	A
H113	S	85	B	B	B	A

Improving binding affinity of R4 modified antibody by R4 library in framework (non-CDR) of a variable region of human CTLA-4 antibody and PD-1 antibody

Framework (non-CDR) region is a conservative sequence and has been used to link the CDR region to form different antibody. As in shown in Table 51 and Table 52, when the side chain of some amino acid changes, the binding are reduced (class B), indicating that side chain of amino acid may provide an interaction or a space to help antigen binding with CDR.

Normally, the interaction between framework and antigen is weaker than that between CDR region and antigen. By conjugation of the different R4 to the selected mutant position of PD-1 antibody (Nivolumab), we have the chance to obtain a higher affinity or to maintain the affinity of the chemical modified antibody, as compared to the wild type antibody.

Table 53: Binding activity of R4 conjugated with mutation biomolecular library in conservative FR sites (% of WT antibody) for PD-1 antibody (Nivolumab)

PD-1	R4-1	R4-2	R4-3	R4-4	R4-5	R4-6	R4-7	R4-8	R4-9
Gly41	62.6	45.6	1.6	33.6	24.6	34.4	32.7	31.7	58.7
Glu46	26.4	48.9	67.8	45.1	49.6	29.7	86.4	78.4	10.4
Tyr49	67.8	48.6	33.4	14.5	18.4	105.4	97.4	62.4	59.8
Arg61	67.4	108.4	48.6	78.4	64.1	89.1	91.5	57.9	48.5
Ser63	89.5	98.4	108.9	84.5	89.4	85.5	84.5	88.9	116.9
Thr72	48.9	47.6	115.9	67.4	48.5	75.8	68.7	12.4	34.9

5

The results showed that in FR region of different antibodies, conjugating different R4 to the conservative site can adjust the binding activity. In a representative Gly41, it also has a chance to recover 62.6% binding affinity by conjugation with R4-1. In conservative Gln3, Ser7, Ser26, Glu46, Thr68, Asp72 in VH, and Thr5, Tyr49, Arg61, Ser63, Ser65, Ser67, Thr72, Thr74, Ser76, Asp82 in framework, it can be screened out higher affinity binding conjugation. Because framework sequences (FR1, FR2 and FR3) are conservative in all kinds of human antibody, by conjugating different R4 to these conservative sites in FR region, any antibody has the chance to maintain or increase the binding affinity.

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15 **Analysis on the binding activity of mutants in a sequence of high homology in the framework (non-CDR) of a variable region of human germline antibody**

20

Human antibody consists of 4 peptide chains, including two identical light chains (LC) and two identical heavy chains (HC). The chains form a monomer by disulfide bond(s) and non-covalent bonds. There are two types of light chains, κ and λ , and five types of heavy chains, i.e., μ , δ , γ , ϵ and α . An antibody, as a whole, is divided into a constant region and a variable region. The variable region is located at the terminus of the two arms of the Y-shaped structure. Humanized or human antibodies have a certain generality, that is, they all contain 4 loops in heavy chain or light chain at the terminus of the two arms of the Y-shaped structure. Three loops are highly variable and directly anticipate in binding to an antigen. The regions in these loops are termed CDRs, wherein CDR1, CDR2 and CDR3 are present in these three loops, respectively.

25

Antibody was produced by recombination of genes of immunoglobulin superfamily *in vivo*. Some framework regions of antibodies against different antigens may be derived from a gene or

an amino acid sequence of a same germline antibody. Between the CDR, there are framework sequences (FR1, FR2, and FR3), which are conservative in all kinds of human antibody. All kinds of human antibody for variable region is shown in full from the start codon to the last nucleotide before the variable region gene exon in the case of the leader sequence, from the beginning of the V gene exon (Residue 1 in FR1) to the last nucleotide/amino acid before the heptamer recombination signal sequence in the case of VH, VK and VL. The 8 selected antibodies sequences are shown in Fig. 31.

We mutated other antibodies' heavy chain and light chain DNA sequences corresponding to the same conserved position for expression to compare the binding affinity with other sites in FR region and WT antibody. In the 5 antibody sequences, the conservative sites of Gln3, Ser7, Ser26, Glu46, Thr68 and Asp72 in VH, and Thr5, Tyr49, Arg61, Ser63, Ser65, Ser67, Thr72, Thr74, Ser76 and Asp82 in VL (the position of the amino acid is numbered according to Kabat numbering) were selected to conjugate with R4-7.

Table 54: Binding activity of R4-7 conjugated with mutation biomolecular library in conservative FR sites (% of WT antibody)

sites	41BB	Ada	PD-1-k	Ip1	Trast
Gln3	68.3	78.4	73.8	98.3	94.5
Ser7	94.5	87.4	105.6	110.4	110.6
Ser26	87.1	68.4	97.1	74.8	104.6
Glu46	97.4	78.4	69.4	67.8	78.4
Thr68	68.7	97.8	74.7	79.8	87.7
Thr5	86.4	97.4	84.1	67.4	69.7
Gly41	24.8	16.4	8.9	8.4	8.9
Tyr49	79.7	97.8	99.7	112.4	107.8
Arg61	87.9	78.9	67.8	79.8	94.1
Ser63	89.7	69.5	69.1	78.9	97.4
Ser65	69.4	78.9	70.8	69.7	97.8
Ser67	79.7	68.7	69.2	79.0	64.9
Thr72	68.5	66.4	72.8	70.9	79.8
Thr74	107.5	97.4	89.4	99.8	95.8
Ser76	69.4	78.9	69.1	78.4	78.4
Asp82	98.5	74.8	68.7	79.4	67.8

The results showed that in FR region of different antibodies, conjugating R4-6 to the

conservative sites of Gln3, Ser7, Ser26, Glu46, Thr68 or Asp72 in VH, and Thr5, Tyr49, Arg61, Ser63, Ser65, Ser67, Thr72, Thr74, Ser76 or Asp82 in VL can maintain the binding activity as compared to the wild type antibody or other positions, which was 60% or more of the original binding activity. In a representative negative control Gly41, it lost binding affinity in all antibody (<60% of WT antibody).

Example 14

CTLA-4 is on the T cell surface in tumor microenvironment. We mutated every amino acid in CDR region of its antibody to screen by different R4 group for maintaining or increasing the binding affinity. In some case, there are some R4 groups which could enhance the binding affinity, but we did not select them as drug candidates in our development. We prefer R-1, R4-7, R4-5, R4-8 and R4-12 for the large scale synthesis and stability in our drug development. The conjugated CTLA-4 antibody with R4 can recovery the binding >60% in some positions by chemical modified maturation of R4 library screening.

Table 55: Binding effect of mutants of anti-CTLA-4 antibody having mutation in CDR regions after conjugating to different R4 molecules

Region (Kabat)	Kabat No.	Original amino acid	R4	Binding activity
CDRL1	L24	R	R4-26	89%
	L25	A	R4-1	95%
	L26	S	R4-1	91%
	L27	Q	R4-11	88%
	L28	V	R4-7	78%
	L29	G	R4-1	95%
	L30	S	R4-1	81%
	L30	S	R4-2	104%
	L30	S	R4-3	96%
	L31	S	R4-1	99%
	L32	Y	R4-7	98%
	L32	Y	R4-8	107%
	L33	L	R4-1	95%
	L34	A	R4-1	92%
CDRL2	L50	G	R4-1	97%

	L50	G	R4-3	107%
	L51	A	R4-1	92%
	L52	F	R4-1	72%
	L53	S	R4-1	98%
	L54	R	R4-26	163%
	L55	A	R4-1	102%
	L55	A	R4-3	106%
	L56	T	R4-1	93%
CDRL3	L89	Q	R4-7	96%
	L90	Q	R4-7	75%
	L91	Y	R4-5	95%
	L91	Y	R4-7	105%
	L92	G	R4-1	99%
	L92	G	R4-3	104%
	L93	S	R4-1	92%
	L94	S	R4-1	95%
	L94	S	R4-11	142%
	L95	P	R4-1	62%
	L96	W	R4-4	72%
	L97	T	R4-1	91%
CDRH1	H31	S	R4-1	110%
	H32	Y	R4-7	92%
	H32	Y	R4-18	208%
	H33	T	R4-1	102%
	H34	M	R4-1	65%
	H35	H	R4-5	72%
CDRH2	H50	F	R4-4	73%
	H51	I	R4-4	72%
	H52	S	R4-1	95%
	H52	S	R4-3	107%
	H52A	Y	R4-7	93%
	H53	D	R4-11	91%
	H54	G	R4-1	95%
	H55	N	R4-11	96%

	H56	N	R4-11	91%
	H57	K	R4-7	88%
	H58	Y	R4-7	89%
	H59	Y	R4-4	92%
	H59	Y	R4-10	105%
	H60	A	R4-4	65%
	H61	D	R4-11	92%
	H62	S	R4-3	91%
	H63	V	R4-1	68%
	H64	K	R4-7	96%
	H64	K	R4-18	137%
	H65	G	R4-1	93%
CDRH3	H95	T	R4-3	92%
	H95	T	R4-1	152%
	H96	G	R4-1	65%
	H96	G	R4-2	85%
	H97	W	R4-8	92%
	H98	L	R4-3	84%
	H99	G	R4-1	94%
	H100	P	R4-1	69%
	H100A	F	R4-4	84%
	H101	D	R4-3	88%
	H102	Y	R4-7	95%
	H102	Y	R4-5	242%

According to result, after mutating G,A, S, L, T, I, F, E, K,D, N, Q, R or Y in the CDRs of anti-CTLA-4 antibody to C and binding to different R4, the mutants could retained a binding efficiency of >60%.

- 5 In some case, there are some R4 groups which could enhance the binding affinity. We selected the binding affinity between 60~100% of R4-s-R5 as drug candidates in our drug development. We prefer R-1, R4-7, R4-5, R4-8 and R4-12 for the large scale synthesis and stability in our drug development. S47 is cleaved by Legumain, and after cleaving the R4-7 chemical group is retained. After S47 conjugating to the amino acid of CDR, all these
- 10 conjugates can block CTLA-4 binding with decreased affintiy (activity <30% than WT

CTLA-4). So the positions in CDR regions or mutant can become a primary drug candidate for tumor microenvironment activated antibody.

Table 56: Blocking efficiency of CDR regions conjugated with S47 (40kD) linkers

Region (Kabat)	Kabat No.	Original amino acid	R4	Binding activity	Activity after S47(40kD) blocking
CDRL1	L24	R	R4-26	89%	28%
	L25	A	R4-1	95%	29%
	L26	S	R4-1	91%	18%
	L27	Q	R4-11	88%	8%
	L27A	S	R4-1	82%	24%
	L28	V	R4-7	78%	25%
	L29	G	R4-1	95%	19%
	L30	S	R4-1	81%	19%
	L31	S	R4-1	99%	12%
	L32	Y	R4-7	95%	13%
	L33	L	R4-1	95%	6%
	L34	A	R4-1	92%	11%
CDRL2	L50	G	R4-1	97%	19%
	L51	A	R4-1	92%	25%
	L52	F	R4-1	72%	16%
	L53	S	R4-1	98%	5%
	L54	R	R4-26	113%	17%
	L55	A	R4-1	102%	20%
	L56	T	R4-1	93%	5%
CDRL3	L89	Q	R4-7	96%	15%
	L90	Q	R4-7	75%	7%
	L91	Y	R4-7	95%	8%
	L92	G	R4-1	99%	16%
	L93	S	R4-1	92%	9%
	L94	S	R4-1	95%	12%
	L95	P	R4-1	62%	13%
	L96	W	R4-4	72%	12%

	L97	T	R4-1	91%	16%
CDRH1	H31	S	R4-1	110%	25%
	H32	Y	R4-7	92%	23%
	H33	T	R4-1	102%	26%
	H34	M	R4-1	65%	29%
	H35	H	R4-5	72%	28%
CDRH2	H50	F	R4-4	73%	25%
	H51	I	R4-4	72%	27%
	H52	S	R4-1	95%	5%
	H52A	Y	R4-7	93%	18%
	H53	D	R4-11	91%	11%
	H54	G	R4-1	95%	15%
	H55	N	R4-11	96%	22%
	H56	N	R4-11	91%	8%
	H57	K	R4-7	88%	11%
	H58	Y	R4-7	89%	7%
	H59	Y	R4-4	92%	15%
	H60	A	R4-4	65%	22%
	H61	D	R4-11	92%	19%
	H62	S	R4-3	91%	23%
	H63	V	R4-1	68%	22%
	H64	K	R4-7	96%	26%
	H65	G	R4-1	93%	28%
CDRH3	H95	T	R4-3	92%	18%
	H96	G	R4-1	65%	11%
	H97	W	R4-8	92%	9%
	H98	L	R4-3	84%	6%
	H99	G	R4-1	94%	12%
	H100	P	R4-1	69%	3%
	H100A	F	R4-4	84%	16%
	H101	D	R4-3	88%	23%
	H102	Y	R4-7	95%	16%

According to the results, after mutating native amino acid in the CDRs of anti-CTLA-4 antibody to cystine and chemically conjugating to different R4 (R4 library screening), the

mutants could retain a binding efficiency of >60%. After S47 conjugation reaction with amino acid of CDR, all these positions can block CTLA-4 binding with decrease affinity (<30% comparing with WT CTLA-4). Therefore, all S47 conjugates can become primary drug candidates for tumor microenvironment activated antibody.

- 5 To improve affinity of antibody, amino acid on CDR loop was mutated during affinity maturation. In fact, optimization of antibody affinity also can be achieved by conjugating to a suitable R4 (termed herein as chemical maturation of antibody). As shown in Fig. 32, by site-specially conjugating different R4 groups to the selected mutant site of antibody, we can selectively change the R4 group for screening. (1) Native amino acid has no H-bond or charge interaction with ligand. Chemical maturation can induce new interaction. (2) Native amino acid has weak H-bond or charge interaction with ligand for the distance. Chemical maturation can adjust distance (by R_{4-b}) to screen the best distance. (3) Native amino acid has weak H-bond or charge interaction. Chemical maturation can change interaction group to screen the best R_{4-c} group. (4) Chemical maturation also can increase charge by increasing NH₂⁺ or O⁻. As a result, there are chances to increase the interaction of H-bond or charge interaction as a kind of new affinity maturation.

We performed chemical maturation of antibody by conjugating R4 to three mutants having one or two mutations at a same CTLA-4 antibody. The results were shown in Table 57.

Table 57: Binding activity of conjugating R4 to three mutants

	Mutant 1	Mutant 2	Binding activity
R4-18	H32		208%
R4-18		H64	137%
R4-18	H32	H64	356%

Optimization of antibody affinity also can be achieved by chemical maturation in CDR loop of an antibody by conjugating to a suitable R4 group.

Example 15

Binding ELISA characterization of constructed CTLA-4 TMEAbodies

In drug development, we collected the sites with best blocking efficiency and restored activity for further development. After conjugation with S47, the human antibody become a tumor microenvironment activated antibody, and is named as TMEAbody.

To assess the recovery capability of TMEAbodies in binding against human CTLA-4

protein in a tumor microenvironment, the conjugated TMEAbodies were *in vitro* digested by Legumain and the digested product was used for evaluating recovered binding activity to human CTLA-4. To characterize the binding property of the constructed TMEAbodies to the human CTLA-4, 0.5 µg/ml CTLA-4 Fc fusion protein (R&D systems) was coated on the Maxisorp ELISA plate (Nunc) by incubation at 4°C overnight. Then the plate was washed three times with PBST and blocked by 2% BSA at room temperature for 2h. After washed by PBST for three times, the plate was incubated with serial concentration of conjugated TMEAbodies, TMEAbodies before conjugation (Cysteine mutant form), and control wild type (WT) Ipilimumab antibody at room temperature for 1h. The plate was then washed three times by PBST and incubated by goat anti-human IgG Fab fragment conjugated with HRP (Sigma) with 1:5000 dilution at room temperature for 1h. After washed by PBST three times, the plates were developed with tetramethylbenzidine (TMB, Solarbio) and ELISA stop buffer (Solarbio). Absorbance at 450 nm was then measured by ELISA plate reader (Biotek). Data was then analyzed by GraphPad Prism 5 software.

As shown in table 58, conjugation of chemical linker to different mutant sites can give rise to different degrees of blocking efficiency. Blocking efficiency can be calculated by the fold change the EC₅₀ value of binding curve. The conjugated TMEAbodies with blocking efficiency bigger than 10 fold and Restored (EC₅₀ value < 2 fold of WT) were considered as good candidates for further development.

Table 58: Ipilimumab TMEAbody candidates selected by blocking efficiency and restored activity after protease cleavage

Selected Mutation ID	Heavy or chain	Mutation Site	Position	Kabat No.	R4	Conjugation efficiency of S47	Blocking efficiency after conjugation with S47	Restored activity after protease cleavage
Ipi-se001	HC	Y	53	H52A	R4-7	95%	20 fold	Restored
Ipi-se002	HC	D	54	H53	R4-11	95%	9 fold	Restored
Ipi-se003	HC	Y	59	H58	R4-4	94%	15 fold	Restored
Ipi-se004	HC	D	73	H72	R4-11	94%	6 fold	Restored
Ipi-se005	HC	K	76	H75	R4-7	94%	8 fold	Restored
Ipi-se006	HC	P	104	H100	R4-1	96%	30 fold	Restored
Ipi-se007	LC	Q	27	L27	R4-11	95%	13 fold	Resorted
Ipi-se009	LC	S	54	L53	R4-1	95%	22 fold	Restored
Ipi-se053	LC	Y	50	L49	R4-7	98%	163 fold	Resorted

Ipi-se066	LC	S	68	L67	R4-7	97%	3 fold	Resorted
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The same ELISA method was performed as described above. As shown in Fig. 33, conjugating to different sites showed different degree of binding activity recovery. Some mutant sites showed comparable binding activity to the WT Ipilimumab after digestion (EC_{50} change fold < 2 , or in other words, the activity $> 50\%$ of WT).

Blocking of antigen binding of TMEAbodies resulted in decreased receptor blocking activity of Ipilimumab

Receptor blocking activity (RBA) assay was then employed to prove that the decreased binding of TMEAbodies to the CTLA-4 protein would also decrease the blocking efficacy of Ipilimumab for B7-1 CTLA-4 interaction. 0.5 $\mu\text{g/ml}$ human CTLA-4 Fc fusion protein (R&D systems) was absorbed on the Maxisorp ELISA plate (Nunc) and then the plate was blocked by 2% BSA. 0.02 $\mu\text{g/ml}$ biotinylated human B7-1 or B7-2 protein with different concentration of TMEAbodies or WT Ipilimumab were completely incubated with the plate and then the receptor blocking activity was measure with Streptavidin-HRP (ThermoFisher Scientific) incubation followed by TMB reaction like the procedure of standard ELISA.

As shown in Figure 34, the TMEAbodies with significant decreased binding activity to human CTLA-4 protein also showed dramatic decrease of receptor blocking activity. The fold change of IC_{50} could be used as quantitative parameter for evaluating the decrease of receptor blocking activity.

Data of other candidates on RBA assay was summarized in Table 59.

Table 59: RBA assay data summary of Ipilimumab TMEAbody candidates

Selected Mutation ID	Blocking efficiency (RBA)	Restored RBA activity after protease cleavage
Ipi-se001	15 fold	Restored
Ipi-se002	5 fold	Restored
Ipi-se003	8 fold	Resorted
Ipi-se004	7 fold	Resorted
Ipi-se005	10 fold	Resorted
Ipi-se006	25 fold	2.5 fold decreased
Ipi-se007	16 fold	Resorted
Ipi-se053	20 fold	Resorted
Ipi-se009	18 fold	Resorted
Ipi-se066	9 fold	3.5 fold decreased

Blocking of antigen binding of TMEAbodies resulted in decreased functional efficacy in SEB induced T cell activation assay

Next, to evaluate whether the decreased antigen binding activity and receptor blocking activity observed in TMEAbodies can contribute to decrease of functional efficacy of Ipilimumab, staphylococcal enterotoxin (SEB) induced T cell activation assay was performed. SEB is a superantigen which can strongly activate T lymphocyte and induce cytokine secretion. Whole PBMC cell from healthy donors (Allcells) were cultured as 1E5 cells per well in 1640 medium (GIBCO) with 10% FBS (GIBCO), 100ng/ml SEB (Toxin Technology) and different concentration of TMEAbodies, WT Ipilimumab, or isotype control human IgG, respectively. After 96 h of activation, supernatant were collected by centrifugation and IL2 release was measured by IL2 detection kit with ELISA method (R&D systems).

As shown in Figure 35, TMEAbody with decreased binding activity against CTLA-4 showed impairment of functional efficacy of T cell activation, and protease mediated activation can restore the activity of TMEAbody.

This assay was also carried out for other TMEAbody at single concentration (10ug/ml) and blocking efficiency was calculated $((\text{WT-TMEAbody})/(\text{WT-hIgG}) \times 100\%)$. Results were shown in Table 60.

Table 60: TMEAbody blocking efficiency SEB induced T cell activation

Selected Mutation ID	Blocking efficiency (SEB)	Restored RBA activity after protease cleavage
Ipi-se001	63%	Restored
Ipi-se002	45%	Restored
Ipi-se003	40%	Restored
Ipi-se004	43%	Restored
Ipi-se005	64%	Restored
Ipi-se006	90%	3 fold decreased
Ipi-se007	68%	Restored
Ipi-se053	70%	Restored
Ipi-se009	72%	Restored
Ipi-se066	56%	4 fold decreased

Ipilimumab TMEAbodies regulated Treg in tumor but not in periphery

Mechanism study was performed to see whether TMEAbodies were specifically activated in tumor microenvironment but not in the periphery lymph organs. One of the proposed

mechanism for Ipilimumab therapy is that it can down-regulate the population of Treg cells through antibody dependent cell mediated cytotoxicity (ADCC) effect, thus to activate the immune response against tumors. Treg population was analyzed with flow cytometry with CD4, CD25, and Foxp3 markers, respectively. As shown in Figure 36, Ipilimumab TMEAbodies significantly down regulated Treg population in the tumor, with the similar efficacy as WT Ipilimumab. Nevertheless, in the spleen or periphery blood, Ipilimumab TMEAbodies showed very weak or no modulation of Treg population. These results demonstrated that the Ipilimumab TMEAbodies showed specific activity in the tumor microenvironment but not in periphery lymph organs or blood.

TMEAbody are stable in human plasma

To evaluate the stability of TMEAbody in serum, 1 μ g CTLA-4 TEMAbody (Ipi053 with conjugation of S13) was put into 20 μ l mouse serum and kept in 37°C for 2h, 4h, 24h, 48h, and 96h, respectively. Then the sample was prepared for Western blot with anti-human Fab HRP antibody (Sigma). Gel intensity was analyzed with ImageJ software and the relative intensity was analyzed by GraphPad. As shown in the following Figure 37, TMEAbody showed high stability in mouse serum, without significant degradation after 96h at 37°C.

CTLA-4 TMEAbodies showed increased half-life and exposure by conjugation with S47 functional group comparing with WT-Ipilimumab and CTLA-4 probody

To evaluate the potential effect of chemical conjugation in modulating the pharmacokinetics property of TMEAbody, single IV dose of 1mg/kg WT Ipilimumab or IpilimumabTMEAbody was injected into Balb/c mice. After 0.5h, 2h, 4h, 8h, 1d, 2d, 5d, 10d, 15d, 20d, plasma was collected for ELISA test of total antibody and active antibody concentration determination. For total antibody concentration determination, anti-human Fc antibody (Invitrogen) was coated on the ELISA plate (NUNC) and injected antibody was detected by anti-human Fab HRP secondary antibody (Invitrogen). For active antibody concentration determination, human CTLA-4 protein (Sino Biological) was coated on the ELISA plate. Active antibody was then detected by anti-human Fc HRP secondary antibody (Invitrogen). Standard curve was drawn by serial dilution of WT Ipilimumab or IpilimumabTMEAbody and the standard binding curve was established through four-parameter fitting. The concentration of total antibody or active antibody was calculated through interpolate the Y value to the standard curve. As shown in Figure 38(a), the half life of TMEAbody was increased after conjugation with 40kd functional group, comparing with the WT Ipilimumab antibody or Ipilimumab probody (WO 2018/085555 A1 with MY11 as

masking peptide and 2011 as cleavage moiety). Moreover, CTLA-4TMEAbody showed less activation in plasma than Ipilimumab probody with the time, as shown in Figure 38(b).

***In vivo* characterization of TMEAbodies in mouse tumor model**

To further characterize the *in vivo* efficacy of TMEAbodies in treating tumor in animal model, Ipilimumab TMEAbodies, as well as WT Ipilimumab and control human IgG were administrated into MC38 colon adenocarcinoma tumor model in human CTLA-4 knock-in C57BL/6 mice. Human CTLA-4 knock-in C57BL/6 mice were subcutaneously injected with 2E6 MC38 cells into their left lower abdominal quadrant. After 7 days for tumor growth, animals were grouped to have similar mean tumor volume. Animals were administrated with indicated single dose of control human IgG, WT Ipilimumab or equimolar TMEAbodies (the concentration of antibody, n=6), respectively, and tumor volumes were monitored for each animal. As shown in Figure 39, Ipilimumab TMEAbodies control the tumor size with comparable efficacy as WT Ipilimumab, whereas control human IgG failed to show any efficacy. The tumor volume inhibition rate was summarized in the Table 61. This result implied that Ipilimumab TMEAbodies could be activated in the tumor microenvironment and inspired anti-tumor immune response.

Table 61: Tumor growth inhibition rate at day 17 after administration

Mutation ID	Dose	Cure rate	Dead
human IgG	5mg/kg	0	2
WT (Ipi)	5mg/kg	0	0
WT (Ipi)	20mg/kg	33.3	1
S47- Ipi-se053	5mg/kg	16.6%	0
S47- Ipi-se053	20mg/kg	83.3%	0
S47- Ipi-se066	5mg/kg	33.3%	0
S47- Ipi-se066	20mg/kg	83.3%	0

As shown in Table 61, inhibition on tumor growth and cure rate by S47-Ipi053 and S47-Ipi066 were greatly improved as compared with the groups treating by WT (Ipi) using the same molar concentration.

CTLA-4 TMEAbodies showed low immunogenicity in animals

To evaluate the immunogenicity of TMEAbody, three groups of Balb/c mice (each n=5) were immunized with 50μg Ipilimumab TMEAbody, WT Ipilimumab, or Ipilimumab Probody

(WO 2018/085555 A1 with MY11 as masking peptide and 2011 as cleavage moiety) with complete Freund's adjuvant (CFA). After 14 days of primary immunization, animals were boosted with 25 μ g Ipilimumab TMEAbody, WT Ipilimumab, or Ipilimumab Probody with incomplete Freund's adjuvant (IFA). Serum was obtained on the 7th day after boosting, and tested for antibody titer against Ipilimumab TMEAbody, WT Ipilimumab, or Ipilimumab Probody, respectively. 1% human serum was used in the serum dilution buffer to block any antibodies against constant region of human IgG. As shown in the Figure 40, TMEAbody caused very low immune response in the animals, with comparable or lower antibody titer than WT Ipilimumab. Nevertheless, Ipilimumab Probody caused dramatic increase of immunogenicity, which might be due to the foreign sequences included in the N terminal of light chain.

CTLA-4 TMEAbodies showed low toxicity in vivo

It is well known in the art, though combination of anti-PD-1 and anti-CTLA-4 antibody are effective (ORR) for treating melanoma, it was found in the current clinic research that combination exhibited 55% TRAEs grade 3-4 and 30% patient have to discontinue the therapy. We presume these TRAEs may be improved if the antibody is inhibited or blocked by a conjugate and is released after arriving at a local environment of tumor so as to reduce the exposed time or dose of active drug in a non-diseased environment. For this reason, experiments were conducted with mice suffered from type I diabetes mellitus (NOD). Diabetes mellitus of this kind of mice is an autoimmune disease, wherein self-activated T lymphocyte cells destroy pancreatic islet β cells, resulting in insufficient secretion of insulin. First, female NOD of 10 weeks old (Beijing Vital River Laboratory Animal Technology Co., Ltd.) were injected with control IgG, high (15 mpk) dose of anti-PD-1 and anti-CTLA-4 antibody or 15 mpk dose of anti-PD-1 and anti-CTLA-4 TMEAbody (S47-Ipi053), respectively at day 0. Indicators of diabetes mellitus, including glucose in urine and two blood sugar levels were observed every day for 12 days until no new indicator of urine glucose was observed.

Protection from autoimmunity by the conjugate was showed in Fig. 41. Results showed that protection of an immune system by a conjugate of anti-CTLA4 TMEAbody in combination therapy conjugate could reduce autoimmunity as compared to the primary antibody.

Example 16: Generation and characterization of PD-1 TMEAbody (Pembrolizumab)

As shown in example of Ipilimumab TMEAbody screening above, multiple sites of anti-PD-1 antibody (Pembrolizumab) were mutated into Cysteine for site specific conjugation. The mutation position in a heavy chain of the anti-PD-1 antibody (Pembrolizumab) is selected

from the group consisting of (Numbered sequentially from N terminal to C terminal without using the Kabat or other antibody numbering systems): Ser7, Gly8, Gly15, Ala16, Ser17, Ala24, Ser25, Gly26, Tyr27, Thr28, Thr30, Asn31, Tyr32, Tyr33, Tyr35, Ala40, Gly42, Gly44, Leu45, Gly49, Gly50, Ile51, Asn52, Ser54, Asn55, Gly56, Gly57, Thr58, Asn59, Lys63, Lys65, Thr69, Leu70, Thr71, Thr72, Asp73, Ser74, Ser75, Thr76, Thr77, Thr78, Ala79, Leu83, Ser85, Leu86, Thr91, Ala92, Arg99, Asp100, Tyr101, Arg102, Asp104, Gly106, Gly111, Gly113, Thr114, 115Thr, 117Thr, Ser119, Ser120, Ala121, Ser122, Thr123, Lys124, Gly125 and Ser127; the mutation position in a light chain is selected from the group consisting of: Ile2, Thr5, Ser7, Ala9, Thr10, Leu11, Ser12, Leu13, Ser14, Gly16, Ala19, Thr20, Ala25, Ser26, Lys27, Gly28, Ser30, Thr31, Ser32, Gly33, Tyr34, Ser35, Tyr36, Leu37, Gly45, Ala47, Leu50, Leu51, Ile52, Tyr53, Leu54, Ala55, Ser56, Tyr57, Leu58, Ser60, Gly61, Ala64, Ser67, Gly68, Ser69, Gly70, Ser71, Gly72, Thr73, Ala76, Thr78, Ser80, Ser81, Ser95, Arg96, Asp97, Leu98, Leu100, Thr101, Phe102, Gly104, Ile110, Lys111 and K130. ELISA characterization with human Fc tagged PD-1 protein (Sino Biological) was carried out to identify the candidate sites with good blocking efficiency and recovery efficiency. Sites with blocking efficiency >5 (in other words, the activity < 20%) fold and restored activity after enzyme digestion (EC50 change < 2 fold, or in other words, the activity > 50%) were selected for further development, as shown in Table 62.

Table 62: Selected anti-PD-1 TMEAbody (Pembrolizumab) candidates based on blocking and recovery efficiency

Selected Mutation ID	Heavy or light chain	Mutation site	R4	Conjugation efficiency	Blocking efficiency of S47	Restored activity after R4 conjugation
Pem-se001	HC	Tyr27	R4-7	89%	5 fold	Restored
Pem-se002	HC	Tyr32	R4-7	91%	6 fold	Restored
Pem-se003	HC	Asn55	R4-11	92%	5 fold	Restored
Pem-se004	HC	Lys65	R4-7	88%	7 fold	Restored
Pem-se005	HC	Arg102	R4-26	87%	6 fold	Restored
Pem-se006	LC	Lys27	R4-7	91%	6 fold	Restored
Pem-se007	LC	Gly28	R4-1	94%	8 fold	Restored
Pem-se008	LC	Tyr34	R4-7	90%	10 fold	Restored
Pem-se009	LC	Tyr36	R4-7	93%	18 fold	Restored
Pem-se010	LC	Tyr57	R4-7	95%	25 fold	Restored
Pem-se011	LC	Arg96	R4-26	90%	7 fold	Restored

Example 17: Functional characterization of anti-PD-1 TMEAbody (Pembrolizumab)

The human PBMC (Allcells) was inoculated in a 96-well plate in a concentration of 1×10^5 cells per well. The cells were stimulated with 0.1ug/ml SEB for three days. Different concentrations of WT anti-PD-1 antibody, TMEAbody, or activated TMEAbody were added and cultured at 37°C, 5% CO₂ for 4 days. Supernatant was collected and concentration of cytotoxic factor IFN- γ was detected by ELISA kit (R&D). The functional blocking efficiency and recovery rate was summarized in the Table 63.

Table 63: Functional characterization of anti-PD-1 TMEAbody (Pembrolizumab) candidates

Selected Mutation ID	Heavy or light chain	Mutation site	Blocking efficiency of S47 in IFN- γ assay	Restored activity after R4 conjugation
Pem-se001	HC	Tyr27	3 fold	Restored
Pem-se002	HC	Tyr32	4 fold	Restored
Pem-se003	HC	Asn55	6 fold	Restored
Pem-se004	HC	Lys65	7 fold	Restored
Pem-se005	HC	Arg102	5 fold	Restored
Pem-se006	LC	Lys27	7 fold	Restored
Pem-se007	LC	Gly28	10 fold	Restored
Pem-se008	LC	Tyr34	9 fold	Restored
Pem-se009	LC	Tyr36	19 fold	Restored
Pem-se010	LC	Tyr57	24 fold	Restored
Pem-se011	LC	Arg96	10 fold	Restored

Example 18: Generation and characterization of PD-1 TMEAbody (Nivolumab)

As shown in example of Ipilimumab and Pembrolizumab TMEAbody screening above, multiple sites of anti-PD-1 antibody (Nivolumab) were mutated into Cysteine for site specific conjugation. the mutation position in a heavy chain of the anti-PD-1 antibody (Nivolumab) is selected from the group consisting of: Gln3, Ser7, Gly8, Gly9, Gly10, Gly15, Ser17, Lys23, Ala24, Ser25, Gly26, Ile27, Asn31, Thr28, Ser30, Ser32, Gly33, Ala40, Gly42, Gly44, Leu45, Ala49, Ile51, Tyr53, Asp54, Gly55, Ser56, Lys57, Tyr59, Tyr60, Ala61, Asp62, Ser63, Lys65, Gly66, Thr69, Ile70, Ser71, Arg72, Asp73, Asn74, Ser75, Lys76, Asn77, Thr78, Leu79, Leu81, Ser85, Leu86, Ala88, Thr91, Ala92, Thr98, Asn99, Asp100, Asp101, Tyr102, Gly104, Gly106, Thr107, Leu108, Thr110, Ser112, Ser113, Ala114, Ser115, Thr116, Lys117, Gly118 and Ser120; the mutation position in a light chain is selected from the group consisting of: Ile2,

Leu4, Thr5, Ser7, Ala9, Thr10, Leu11, Ser12, Leu13, Ser14, Gly16, Ala19, Thr20, Leu21, Ala25, Ser26, Ser28, Ser30, Ser31, Tyr32, Leu33, Ala34, Tyr36, Gly41, Ala43, Leu46, Leu47, Ile48, Tyr49, Asp50, Ala51, Ser52, Asn53, Arg54, Ala55, Thr56, Gly57, Ile58, Ala60, Arg61, Ser63, Gly64, Ser65, Gly66, Ser67, Gly68, Thr69, Thr72, Leu73, Thr74, Ile75, Ser76, Ser77, Leu78, Ala84, Ser91, Ser92, Asn93, Arg96, Thr97, Phe98, Gly99, Gly101, Thr102, Ile106, Lys107, Thr109, Ala111, Ala112, Ser114, Ile117 and Ser121 Sites with blocking efficiency >5 fold and restored activity after enzyme digestion (EC₅₀ change < 2 fold) were selected for further development, as shown in Table 64.

Table 64: Selected anti-PD-1 TMEAbody (Nivolumab) candidates based on blocking and recovery efficiency

ID of selected Mutation	Heavy or light chain	Mutation site	R4	Conjugation efficiency	Blocking efficiency of S47	Restored activity after R4 conjugation
Niv-se001	HC	Lys23	R4-7	95%	15 fold	Increased, 432% of WT
Niv-se002	HC	Tyr53	R4-7	89%	5 fold	Restored
Niv-se003	HC	Lys57	R4-7	88%	6 fold	Restored
Niv-se004	LC	Tyr32	R4-7	92%	8 fold	Restored
Niv-se005	LC	Tyr49	R4-7	94%	85 fold	Increased, 125% of WT
Niv-se006	LC	Arg54	R4-26	93%	7 fold	Restored
Niv-se007	LC	Ser91	R4-1	95%	25 fold	Restored
Niv-se008	LC	Arg96	R4-26	93%	23 fold	Restored

As shown in the Figure 42, Niv-se001 showed increased activity after R4-7 conjugation or protease cleavage of 40kD conjugated TMEAbody, which is 432% of WT Nivolumab. This might be due to the R4-7 provided increased the binding activity than native Lysine residue.

Niv-se005 showed lost binding activity when the Try49 is mutated into Cysteine. However, after conjugation with R4-7, or after protease cleavage of Niv-se005 conjugated with 40kD functional group, the binding activity is restored with comparable level than WT Nivolumab (125% of WT), as shown in the Figure 43.

Example 19: Functional characterization of anti-PD-1 TMEAbody (Nivolumab)

The human PBMC (Allcells) was inoculated in a 96-well plate in a concentration of 1×10^5 cells per well. The cells were stimulated with 0.1ug/ml SEB for three days. Different

concentrations of WT anti-PD-1 antibody (Nivolumab), TMEAbody (Nivolumab), or activated TMEAbody (Nivolumab) were added and cultured at 37°C, 5% CO₂ for 4 days. Supernatant was collected and concentration of cytotoxic factor IFN-γ was detected by ELISA kit (R&D). The functional blocking efficiency and recovery rate was summarized in the Table 65.

5

Table 65: Functional characterization of anti-PD-1 TMEAbody (Nivolumab) candidates

Selected Mutation ID	Heavy chain or light chain	Mutation site	Blocking efficiency of S47 in IFN-γ assay	Restored activity after R4 conjugation
Niv-se001	HC	Lys23	18 fold	Increased, 332% of WT
Niv-se002	HC	Tyr53	3 fold	Restored
Niv-se003	HC	Lys57	7 fold	Restored
Niv-se004	LC	Tyr32	8 fold	Restored
Niv-se005	LC	Tyr49	25 fold	Restored. 122% of WT
Niv-se006	LC	Arg54	9 fold	Restored
Niv-se007	LC	Ser91	24 fold	Restored
Niv-se008	LC	Arg96	21 fold	Restored

Example 20: In vivo characterization of anti-PD-1 TMEAbody (Pembrolizumab and Nivolumab) in treating mouse tumor

10 To further characterize the *in vivo* efficacy of anti-PD-1 TMEAbodies in treating tumor in animal model, anti-PD-1 TMEAbodies (Pem-se010 TMEAbody based on Pembrolizumab and Niv-se007 TMEAbody based on Nivolumab), as well as WT PD-1 antibodies (Pembrolizumab and Nivolumab) and control human IgG were administrated into MC38 colon adenocarcinoma tumor model in human PD-1 knock-in C57BL/6 mice. Human PD-1 knock-in C57BL/6 mice
 15 were subcutaneously injected with 2E6 MC38 cells into their left lower abdominal quadrant. After 7 days for tumor growth, animals were grouped to have similar mean tumor volume. Animals were administrated with 10mg/kg single dose of PD-1 TMEAbodies (the concentration of antibody without PEG linker), WT PD-1 antibodies, or control human IgG and tumor volumes were monitored for each animal.

20 As shown in Figure 44, Pembrolizumab and Nivolumab TMEAbodies control the tumor size with comparable efficacy as WT Pembrolizumab or Nivolumab antibodies, whereas control human IgG failed to show any efficacy. Interestingly, Niv-se001 TMEAbody showed improved efficacy than WT Nivolumab in treating tumor, which might be due to its enhanced binding activity after protease cleavage. The results implied that these anti-PD-1 TMEAbodies could be

activated in the tumor microenvironment and inspire anti-tumor immune response.

Example 21: Generation of mouse anti-PD-1 antibody (J43v2) for efficacy study in mouse tumor model

Hamster anti-mouse PD-1 antibody sequences were disclosed in US 20170044259A1. This heavy chain of this antibody was re-formatted into mouse IgG2a to reduce the immunogenicity in mouse. As the screening method above, multiple sites were designed for screening of TMEAbody with high blocking efficiency. Finally Ser95 on LC was selected for TMEAbody generation due to its high efficiency blocking (35 fold in ELISA assay with mouse PD-1 protein). SN38 mouse tumor model was carried out with 10mg/kg single dose of WT J43v2 antibody or J43v2 TMEAbody (for each group n=8). At the days of 17 after administration, J43v2 TMEAbody showed 75% tumor inhibition, with comparable inhibition efficacy than WT J43v2 antibody (83%). This result indicated that the PD-1 antibody can be activated and played its anti-tumor activity *in vivo*.

Example 22: Generation of anti-mouse CTLA-4 antibody (9D9) for efficacy study in mouse tumor model

To generate mouse CTLA-4 surrogate TMEAbody for further functional and toxicity studies, we produced and purified anti-mouse CTLA-4 antibody and its mutant variants (9D9 clone, mIgG2b isotype, sequences shown in WO 2007/123737 A2). As the screening method above, multiple sites were designed for screening of TMEAbody with high blocking efficiency. Finally Tyr54 on LC was selected for TMEAbody generation due to its high efficiency blocking (26 fold in ELISA assay with mouse CTLA-4 protein). CT26 mouse tumor model was carried out with 10mg/kg single dose of WT 9D9 antibody or 9D9 TMEAbody (for each group n=8). At the days of 17 after administration, 9D9 TMEAbody showed 69% tumor inhibition, with comparable inhibition efficacy than WT 9D9 antibody (74%). This result indicated that the 9D9 TMEAbody can be activated and played its anti-tumor activity *in vivo*.

Example 23: Mouse PD-1 and CTLA-4 TMEAbody showed decreased toxicity than WT antibodies

It is well known in the art, though combination of anti-PD-1 and anti-CTLA-4 antibody are effective (ORR) for treating melanoma, it was found in the current clinic research that combination exhibited 55% TRAEs grade 3-4 and 30% patient have to discontinue the therapy . We presume these TRAEs may be improved if the antibody is inhibited or blocked by a conjugate and is released after arriving at a local environment of tumor so as to reduce the

exposed time or dose of active drug in a non-diseased environment. For this reason, experiments were conducted with mice suffered from type I diabetes mellitus (NOD). Diabetes mellitus of this kind of mice is an autoimmune disease, wherein self-activated T lymphocyte cells destroy pancreatic islet β cells, resulting in insufficient secretion of insulin. First, female NOD of 10 weeks old (Beijing Vital River Laboratory Animal Technology Co., Ltd.) were injected with control IgG, high (15 mpk) dose of anti-mouse PD-1 (J43v2) and anti-mouse CTLA-4 antibody (9D9), or one or both of these two antibodies were replaced with its TMEAbody form at day 0. Indicators of diabetes mellitus, including glucose in urine and two blood sugar levels were observed every day for 12 days until no new indicator of urine glucose was observed.

Protection from autoimmunity by the conjugate was showed in Fig. 45. Results showed that anti-CTLA4 or anti-PD1 TMEAbody in combination therapy could reduce autoimmunity as compared to the primary antibody. Moreover, combination of these two TMEAbodies resulted in very low toxicity with comparable level than control group.

Example 24: Generation and characterization of anti-PD-1 TMEAbody (patent WO 2017/124050 A1)

As the method above, anti-PD-1 antibody sequence was downloaded from patent WO 2017/124050 A1 and sites screening was performed to identify TMEAbody with good blocking efficiency. The mutation position in a heavy chain of the anti-PD-1 antibody is selected from the group consisting of: Ser28, Ser31, Tyr33, Asn36, Gly50, Tyr51, Ser53, Tyr54, Asp55, Ser57, Lys58, Asn59, Tyr60, Asn61, Lys65, Asn66, Thr69, Thr74, Gly100, Asp105, Tyr106; the mutation position in a light chain is selected from the group consisting of: Lys24, Gln27, Ser28, Asp31, Asp32, Asn33, Asn34, Gln35, Lys36, Asn37, Tyr38, Ser58, Arg60, Glu61, Ser62, Gly63, Gly70, Ser73, Thr75, Gln95, Gln96, Tyr98, Thr100, Tyr102. Binding ELISA was performed with Fc tagged human PD-1 protein (Sino Biological) and selected sites with good blocking efficiency (EC_{50} change > 5 fold) and recovery (EC_{50} change < 2 fold) was summarized in Table 66.

Table 66: Selected anti-PD-1 TMEAbody (WO 2017/124050 A1) candidates based on blocking and recovery efficiency

ID of selected Mutation	Heavy chain or light chain	Mutation site	R4	Conjugation efficiency	Blocking efficiency of S47	Restored activity after R4 conjugation
PD1-se001	HC	Tyr33	R4-7	99%	198 fold	Restored
PD1-se002	HC	Ser53	R4-1	95%	9 fold	Restored

PD1-se003	HC	Tyr60	R4-7	88%	6 fold	Restored
PD1-se004	LC	Tyr38	R4-7	94%	15 fold	Restored
PD1-se005	LC	Ser58	R4-1	93%	16 fold	Restored
PD1-se006	LC	Thr100	R4-3	91%	6 fold	Restored

Example 25: Generation and characterization of anti-4-1BB TMEAbody

4-1BB antibody sequence was downloaded from US 2018/0194851 A1 (clone MOR 7480.1). The mutation position in a heavy chain of the anti-4-1BB antibody is selected from the group consisting of: Thr31, Tyr32, Ser35, Lys50, Tyr52, Asp55, Ser56, Tyr57, Thr58, Asn59, Tyr60, Ser61, Gln65, Gly66, Gly99, Tyr100, Gly101, Asp104, Tyr105; the mutation position in a light chain is selected from the group consisting of: Ser23, Gly24, Asp25, Asn26, Gly28, Asp29, Gln30, Tyr31, Gln49, Asp50, Lys51, Asn52, Arg53, Ser55, Gly56, Thr89, Tyr90, Thr91, Gly92, Gly94, Ser95. Human 4-1BB protein was used for ELISA characterization to indentify mutant sites with good blocking efficiency (EC₅₀ change > 5 fold) as well as good recovery (EC₅₀ change < 2 fold) after protease digestion. The selected sites were summarized in Tables 67 and 68.

Table 67: Selected anti-4-1BB TMEAbody candidates based on blocking and recovery efficiency

ID of selected Mutation	Heavy chain or light chain	Mutation site	R4	Conjugation efficiency	Blocking efficiency of S47	Restored activity after R4 conjugation
4-1BB-se001	HC	Tyr32	R4-7	95%	23 fold	Restored
4-1BB -se002	HC	Lys50	R4-7	93%	16 fold	Restored
4-1BB -se003	HC	Tyr105	R4-7	94%	9 fold	Restored
4-1BB -se004	LC	Tyr31	R4-7	94%	18 fold	Restored
4-1BB -se005	LC	Lys51	R4-7	96%	6 fold	Restored
4-1BB -se006	LC	Tyr90	R4-7	95%	7 fold	Restored
4-1BB -se007	LC	Thr91	R4-1	91%	11 fold	Restored

Table 68: Selected anti-4-1BB TMEAbody candidates based on blocking and recovery efficiency

ID of selected Mutation	Heavy chain or light	Mutation site	R4	Blocking efficiency of S27	Blocking efficiency of S48	S48 Restored activity by Granzyme B	S27 Restored activity by
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	chain						pH6.0
4-1BB-se001	HC	Tyr32	R4-7	23 fold	20 fold	Restored	Restored
4-1BB -se002	HC	Lys50	R4-7	16 fold	12 fold	Restored	Restored
4-1BB -se003	HC	Tyr105	R4-7	9 fold	7fold	Restored	Restored
4-1BB -se004	LC	Tyr31	R4-7	18 fold	18 fold	Restored	Restored
4-1BB -se005	LC	Lys51	R4-7	6 fold	5 fold	Restored	Restored
4-1BB -se006	LC	Tyr90	R4-7	7 fold	6 fold	Restored	Restored
4-1BB -se007	LC	Thr91	R4-1	11 fold	10 fold	Restored	Restored

Example 26: Generation and characterization of anti-Her2 TMEAbody (Trastuzumab)

The heavy chain and light chain sequences of Trastuzumab was downloaded from Drug Bank (<https://www.drugbank.ca/drugs/DB00072>) and sites screening was performed to identify anti-Her2 TMEAbody with good blocking efficiency. The mutation position in a heavy chain of the anti-Her2 antibody (Trastuzumab) is selected from the group consisting of: Arg19, Lys30, Asp31, Tyr33, Arg50, Tyr62, Asn55, Tyr57, Arg59, Tyr60, Asp62, Lys65, Asp102, Tyr105; the mutation position in a light chain is selected from the group consisting of: Asp1, Gln3, Gln27, Asp28, Asn30, Tyr49, Tyr55, Arg66, Asp70, Tyr92. His tagged Her2 protein (Sino Biological) was used for ELISA. The selected sites with good blocking efficiency and good recovery were summarized in the Table 69.

Table 69: Selected anti-Her2 TMEAbody candidates based on blocking and recovery efficiency

ID of selected Mutation	Heavy chain or light chain	Mutation site	R4	Conjugation efficiency	Blocking efficiency of S47	Restored activity after R4 conjugation
Trast-se001	HC	Tyr33	R4-7	97%	13 fold	Restored
Trast-se002	HC	Lys65	R4-7	95%	8 fold	Restored
Trast-se003	HC	Tyr105	R4-7	90%	25 fold	Restored
Trast -se004	LC	Gln30	R4-11	89%	16 fold	Restored
Trast -se005	LC	Tyr49	R4-7	91%	20 fold	Restored
Trast -se006	LC	Asp70	R4-11	96%	7 fold	Restored

Example 27: Generation and characterization of anti-TNF α TMEAbody (Adalimumab)

The heavy chain and light chain sequences of Adalimumab was downloaded from Drug Bank (<https://www.drugbank.ca/drugs/DB00051>) and sites screening was performed to identify

anti-TNF α TMEAbody with good blocking efficiency. The mutation position in a heavy chain of the anti-TNF α antibody is selected from the group consisting of: Ser7, Gly8, Gly9, Gly10, Leu11, Gly15, Ser17, Leu18, Leu20, Ala24, Ser25, Gly26, Thr28, Asp30, Asp31, Tyr32, Ala33, Ala40, Gly42, Gly44, Leu45, Ser49, Ala50, Ile51, Thr52, Asn54, Ser55, Gly56, Ile58, Asp59, Tyr60, Ala61, Asp62, Ser63, Glu65, Gly66, Phe68, Thr69, Ile70, Ser71, Asp73, Asn74, Ala75, Lys76, Ser78, Leu79, Tyr80, Leu81, Ser85, Leu86, Ala88, Thr91, Ala92, Lys98, Ser100, Tyr101, Leu102, Ser103, Thr104, Ala105, Ser106, Ser107, Leu108, Asp109, Tyr110, Gly112, Gly114, Thr115, Leu116, Thr118, Ser120, Ser121, Ala122, Ser123 and Thr124; the mutation position in a light chain is selected from the group consisting of: Asp1, Thr5, Ser7, Ser9, Ser10, Leu11, Ser12, Ala13, Ser14, Gly16, Thr20, Ile21, Ala25, Ser26, Gln27, Gly28, Ile29, Arg30, Asn31, Tyr32, Leu33, Ala34, Tyr36, Lys39, Gly41, Lys42, Ala43, Leu48, Leu47, Ile48, Tyr49, Ala50, Ala51, Ser52, Thr53, Leu54, Gln55, Ser56, Gly57, Ser60, Ser63, Gly64, Ser65, Gly66, Ser67, Gly68, Thr69, Asp70, Thr72, Leu73, Thr74, Ile75, Ser76, Ser77, Leu78, Ala84, Thr85, Tyr91, Asn92, Arg93, Ala94, Tyr96, Thr97, Phe98, Gly99, Gly101, Thr102, Ile106, Lys107, Thr109 and Ala111. TNF α protein (Sino Biological) was used for ELISA characterization and selected sites with good blocking efficiency (EC₅₀ change > 5 fold) and good recovery (EC₅₀ change < 2 fold) were summarized in the Table 70.

Table 70: Selected anti-TNF α TMEAbody candidates based on blocking and recovery efficiency

ID of selected Mutation	Heavy chain or light chain	Mutation site	R4	Conjugation efficiency	Blocking efficiency of S47	Restored activity after R4 conjugation
Ada-se001	HC	Tyr32	R4-7	95%	11 fold	Restored
Ada-se002	HC	Asp59	R4-11	92%	7 fold	Restored
Ada-se003	HC	Tyr101	R4-7	93%	6 fold	Restored
Ada-se004	LC	Arg30	R4-26	96%	25 fold	Restored
Ada-se005	LC	Tyr32	R4-7	94%	28 fold	Restored
Ada-se006	LC	Gln55	R4-11	91%	32 fold	Restored
Ada-se007	LC	Tyr96	R4-7	92%	9 fold	Restored

Example 28: Generation and characterization of anti-PD-L1 TMEAbody (Atezolizumab)

The heavy chain and light chain sequences of anti-PD-L1 antibody (Atezolizumab) was downloaded from Drug Bank (<https://www.drugbank.ca/drugs/DB11595>) and sites screening was performed to identify anti-TNF α TMEAbody with good blocking efficiency. The mutation

position in a heavy chain of the anti-PD-L1 antibody (Atezolizumab) is selected from the group consisting of: Gln3, Asp31, Tyr54, Tyr59, Tyr60, Asp62, Lys65, Asp73, Lys76, Asn77, Arg99; the mutation position in a light chain is selected from the group consisting of: Arg24, Gln27, Asp28, Tyr49, Tyr55, Asp70, Gln89, Gln90, Tyr91, Tyr93. Fc tagged human PD-L1 protein (Sino Biological) was used for ELISA characterization and selected sites with good blocking efficiency (EC50 change > 5 fold) and good recovery (EC50 change < 2 fold) were summarized in the Table 71.

Table 71: Selected anti-PD-L1 TMEAbody candidates based on blocking and recovery efficiency

ID of selected Mutation	Heavy chain or light chain	Mutation site	R4	Conjugation efficiency	Blocking efficiency of S47	Restored activity after R4 conjugation
Ate-se001	HC	Tyr54	R4-7	98%	15 fold	Restored
Ate-se002	HC	Lys76	R4-7	93%	7 fold	Restored
Ate-se003	LC	Asp28	R4-11	91%	10 fold	Restored
Ate-se004	LC	Tyr49	R4-7	96%	13 fold	Restored
Ate-se005	LC	Tyr91	R4-7	92%	23 fold	Restored

Example 29: Generation and characterization of anti-CD28 TMEAbody

Anti-human CD28 antibody heavy chain and light chain sequences were downloaded from patent US008709414B2 and sites screening was performed to identify anti-CD28 TMEAbody with good blocking efficiency. The mutation position in a heavy chain of the anti-CD28 antibody is selected from the group consisting of: Ser7, Gly8, Gly15, Ala16, Ser17, Ser21, Ala24, Ser25, Gly26, Tyr27, Thr28, Thr30, Ser31, Tyr32, Ala40, Gly42, Gly44, Gly49, Tyr52, Gly54, Thr58, Ala68, Thr69, Thr71, Thr74, Ser75, Ser77, Thr78, Ala79, Ser84, Leu86, Ser88, Thr91, Ala92, Thr97, Ser99, Tyr101, Gly102, Leu103, Gly113, Thr114, Thr115, Thr117, Ser119, Ser120, Ala121, Ser122 and Thr123; the mutation position in a light chain is selected from the group consisting of: Thr5, Ser7, Ser9, Ser10, Ser11, Ser12, Ala13, Ser14, Gly16, Thr20, Thr22, Ala25, Ser26, Ser27, Ile29, Tyr30, Ala43, Leu46, Leu47, Tyr49, Lys50, Ala51, Ser52, Leu54, Thr56, Gly57, Ser60, Ser63, Gly64, Ser65, Gly66, Ser67, Gly68, Thr69, Asp70, Thr72, Thr74, Ser76, Ser77, Ala84, Thr85, Gly91, Thr93, Tyr94, Tyr96, Thr97, Phe98, Gly99, Gly100, Gly101, Thr102, Thr109 and Ala111. Fc tagged human CD28 protein (Sino Biological) was used for ELISA characterization and selected sites with good blocking efficiency (EC50 change > 5 fold) and good recovery (EC50 change < 2 fold) were summarized in the Table 72.

Table 72: Selected anti-CD28 TMEAbody candidates based on blocking and recovery efficiency

ID of selected Mutation	Heavy chain or light chain	Mutation site	R4	Conjugation efficiency	Blocking efficiency of S47	Restored activity after R4 conjugation
CD28-se001	HC	Tyr27	R4-7	95%	6 fold	Restored
CD28-se002	HC	Thr58	R4-1	93%	7 fold	Restored
CD28-se003	HC	Tyr101	R4-7	91%	6 fold	Restored
CD28-se004	LC	Tyr30	R4-7	91%	16 fold	Restored
CD28-se005	LC	Tyr49	R4-7	98%	32 fold	Restored
CD28-se006	LC	Lys50	R4-7	92%	27 fold	Restored
CD28-se007	LC	Tyr96	R4-7	90%	13 fold	Restored

Example 30: Tumor Microenvironment Activated IL-10 cytokine (IL-10 TMEAkine)

5 1. Expression and purification of the mutant IL-10 cytokine

The mutant IL-10 DNA sequence ligated to a modified pTT5 vector (Biovector) was optimized for expression in 293T cells and synthesized (GENEWIZ, Inc., Suzhou, China). Transfection of the mutant IL-10 DNA was performed and after incubation for 4-7 days, the supernatant containing mutant IL-10 was collected.

10 In *PichiaPastoris* expression, the expression vector pPICZ α A containing the mutant IL-10 genes was optimized and prepared (GENEWIZ, Inc., Suzhou, China). The amino acid sequence of the mutant IL-10 was described in SEQ ID NO.1. The expression vector pPICZ α A was transformed in E.coli (DH5 α) for plasmid purification. Then pPICZ α A was transformed into GS115 by electroporation. The transformed colony was selected by obtaining the growing colonies after growing on the 100, 300, 500, 1000, 1500, 2000 ug/mL ZeocinTM containing YPD plates. After finally selecting the transformant, the recombinant GS115 strain was grown in BMGY medium at 30°C, with vigorous shaking in baffled flasks to an OD600 of 2-6. The cells were then pelleted by centrifugation and suspended in BMMY to an OD600 of 1, to which was added 0.5% methanol daily in order to induce the heterologous protein expression. After a four-day induction, supernatant containing the secreted mutant IL-10 protein was collected by centrifugation. The total protein in the supernatant was concentrated by ultrafiltration using a 10-kDa molecular mass cutoff membrane. The concentrated protein was dialyzed with buffer A (20 mMBIS-TRIS, 0.065M NaCl, pH 6.5) for more than 24 h, then loaded onto a cation-exchange column equilibrated with buffer A. Mutant IL-10 was eluted from the column with gradient concentration of NaCl in the range of 0.065-0.4M and the eluent was collected and concentrated. The condensed sample was further purified on Sephacryl S-100 HRgel

filtration column using 10 mM Tris-HCl, pH 7.4, as the elution buffer.

2. Conjugate S48 to the mutant IL-10 and it was called as the name of IL-10 TMEAkine (Tumor Microenvironment Activated IL-1- cytokine).

5 Mutant IL-10 protein was generated and purified as described above. Purified mutant IL-10 was performed as the concentration of 0.5 mg/mL in 20 mM Tris buffer (pH 7.4) containing 2 mM EDTA. Add TCEP solution to mutant IL-10 as a ratio of 100:1 and incubate for 4 h at 4°C with gentle agitation. Then the mutant IL-10 solution was dialyzed with 20 mM Tris buffer (pH 7.4) containing 150 mM NaCl for 4 h at 4°C. Afterwards, immediately add
10 the S48 to mutant IL-10 solution as a ratio of 20:1 and incubate for 16 h at 25°C with gentle agitation. Terminate the reaction by removal of residual S48. Before the enzyme cleavage, change the IL-10 TMEAkine buffer to enzyme buffer through dialysis. For activation, enzyme was added to IL-10 TMEAkine solution and incubated for 16 h at 37°C.

15 3. Screening IL-10 TMEAkine that blocks the binding to IL-10R1 or R2, and recovers the binding activity after enzyme cleavage *in vitro*.

Dispense 60 µl PBS buffer containing 1 µg IL-10R1-Fc/ IL-10R2-Fc/His solution into the wells. Apply sealing tape to the top of the plate and incubate the plate overnight at 4°C. After incubation, remove the tape and aspirate each well. After three-time wash with PBST, block the
20 plate by dispensing 200 µl of PBS buffer containing 2% BSA into each well and incubate the plate for 2 h at room temperature. Wash the plate three times and add 60 µl of serial diluted samples to the appropriate wells. Incubate the plate for 1.5 h at room temperature. After three-time wash with PBST, dispense 60 µl of 2 µg/mL IL-10 biotinylated antibody solution to each well and incubate for 1 hour at room temperature. Wash the plate three times and dispense
25 60 µl of streptavidin solution to each well. Incubate for 30 minutes at room temperature. After washing three times, dispense 100 µl of the HRP substrate solution into each well and incubate for 15 minutes at 37°C. After color development, dispense 50 µl of stop solution into each well and immediately measure the absorbance of each well at a wavelength of 450 nm.

30 4. Summary of various IL-10 mutation sites

IL-10 receptors on the cell surface have two different forms: high-affinity receptor: IL-10R1 to IL-10 (K_d= 50-200 pM) and low-affinity receptor: IL-10R2 to IL-10. The conjugated IL-10 with R4 can recover the binding >80% in some positions by chemical modified maturation of R4 library screening. To select drug candidates, we also performed the screening
35 expression and S48 conjugation reaction with all amino acids of IL-10 in the domain of binding

IL-10R1 and IL-10R2. We acquired the possible drug candidates and results are shown in the following Table 73.

Table 73: Activity of mutated IL-10 and screening for linker arm

Position	Amino acid	Amino acid	Type of inhibitory ligand	R4	Binding recovery (>60%)	Decreased fold of binding to ligand after Conjugating with S48 (>3fold)
6	T	Thr	1	R4-7	101.2%	3-fold
8	S	Ser	1	R4-5	100.0%	3-fold
11	S	Ser	1	R4-7	132.9%	9-fold
13	T	Thr	1	R4-6	99.1%	7-fold
17	G	Gly	1	R4-1	90.2%	15-fold
18	N	Asn	1	R4-18	92.3%	12-fold
19	L	Leu	1	R4-7	95.3%	8-fold
21	N	Asn	1	R4-18	99.7%	7-fold
22	M	Met	1	R4-1	96.8%	5-fold
23	L	Leu	1	R4-6	101.2%	12-fold
24	R	Arg	1	R4-20	90.0%	33-fold
25	D	Asp	1	R4-12	97.1%	3-fold
26	L	Leu	1	R4-6	93.4%	3-fold
27	R	Arg	1	R4-20	86.6%	12-fold
28	D	Asp	1	R4-11	92.3%	30-fold
30	F	Phe	1	R4-8	97.1%	6-fold
31	S	Ser	1	R4-2	124.6%	39-fold
32	R	Arg	1	R4-7	90.4%	13-fold
34	K	Lys	1	R4-7	99.6%	50-fold
35	T	Thr	1	R4-6	90.1%	36-fold
38	Q	Gln	1	R4-19	87.0%	52-fold
39	M	Met	1	R4-1	88.0%	62-fold
41	D	Asp	1	R4-11	95.2%	13-fold
42	Q	Gln	1	R4-19	90.0%	18-fold
44	D	Asp	1	R4-11	92.1%	43-fold
45	N	Asn	1	R4-18	97.7%	7-fold
46	L	Leu	1	R4-6	89.6%	22-fold
48	L	Leu	1	R4-7	98.9%	3-fold

49	K	Lys	1	R4-5	114.0%	7-fold
50	E	Glu	1	R4-12	99.8%	4-fold
51	S	Ser	1	R4-7	96.7%	3-fold
53	L	Leu	1	R4-6	99.4%	3-fold
54	L	Leu	1	R4-6	98.4%	3-fold
56	Q	Gln	1	R4-19	95.1%	3-fold
57	E	Glu	1	R4-12	95.4%	10-fold
65	L	Leu	1	R4-6	92.3%	3-fold
69	I	Ile	1	R4-6	97.3%	3-fold
72	Y	Tyr	1	R4-4	109.0%	3-fold
75	V	Val	1	R4-5	98.6%	4-fold
90	H	His	1	R4-19	86.5%	8-fold
91	V	Val	1	R4-5	97.2%	3-fold
93	S	Asp	1	R4-2	100.1%	3-fold
94	L	Leu	1	R4-6	88.4%	5-fold
97	N	Asn	1	R4-18	94.5%	5-fold
100	T	Thr	1	R4-7	99.3%	4-fold
104	R	Arg	1	R4-20	98.5%	3-fold
105	L	Leu	1	R4-6	92.3%	5-fold
107	R	Arg	1	R4-20	99.5%	3-fold
111	F	Phe	1	R4-8	90.1%	12-fold
117	K	Lys	1	R4-7	116.6%	3-fold
118	S	Ser	1	R4-5	100.0%	3-fold
119	K	Lys	1	R4-7	99.0%	3-fold
130	K	Lys	1	R4-7	100.0%	3-fold
131	L	Leu	1	R4-6	98.5%	5-fold
134	K	Lys	1	R4-7	128.3%	5-fold
135	G	Gly	1	R4-1	99.5%	3-fold
137	Y	Tyr	1	R4-4	99.1%	5-fold
138	K	Lys	1	R4-7	97.6%	23-fold
140	M	Met	1	R4-1	89.0%	3-fold
141	S	Ser	1	R4-7	112.5%	19-fold
142	E	Glu	1	R4-12	78.0%	13-fold
143	F	Phe	1	R4-8	69.0%	7-fold
144	D	Asp	1	R4-11	90.5%	15-fold

148	N	Asn	1	R418	84.3%	10-fold
149	Y	Tyr	1	R4-4	115.3%	5-fold
151	E	Glu	1	R4-12	82.5%	13-fold
155	T	Thr	1	R4-7	98.1%	3-fold
157	K	Lys	1	R4-7	98.6%	3-fold
158	I	Ile	1	R4-6	81.7%	9-fold
159	R	Arg	1	R4-20	99.0%	3-fold
15	F	Phe	2	R4-8	90.0%	3-fold
18	N	Asn	2	R4-18	99.8%	4-fold
21	N	Asn	2	R4-18	85.3%	6-fold
22	M	Met	2	R4-1	80.0%	6-fold
24	R	Arg	2	R4-20	98.1%	9-fold
25	D	Asp	2	R4-11	91.5%	5-fold
28	R	Arg	2	R4-20	99.0%	6-fold
29	A	Ala	2	R4-5	78.0%	3-fold
31	S	Ser	2	R4-2	87.4%	7-fold
32	R	Arg	2	R4-7	89.6%	9-fold
33	V	Val	2	R4-7	91.4%	3-fold
34	K	Lys	2	R4-7	97.3%	5-fold
35	T	Thr	2	R4-5	123.0%	6-fold
36	F	Phe	2	R4-8	79.6%	3-fold
81	E	Glu	2	R4-12	93.6%	3-fold
86	D	Asp	2	R4-11	81.0%	3-fold
87	I	Ile	2	R4-6	95.7%	4-fold
88	K	Lys	2	R4-7	99.0%	3-fold
89	A	Ala	2	R4-1	78.7%	5-fold
90	H	His	2	R4-19	79.0%	7-fold
92	N	Asn	2	R4-18	86.4%	3-fold
96	E	Glu	2	R4-12	98.0%	3-fold
97	N	Asn	2	R4-18	80.7%	4-fold
99	K	Lys	2	R4-7	116.6%	3-fold
100	T	Thr	2	R4-7	90.7%	8-fold
101	L	Leu	2	R4-6	91.0%	3-fold
102	R	Arg	2	R4-20	99.0%	3-fold

103	L	Leu	2	R4-6	89.4%	3-fold
104	R	Arg	2	R4-20	96.6%	6-fold
106	R	Arg	2	R4-7	99.6%	3-fold
107	R	Arg	2	R4-20	84.0%	5-fold
119	K	Lys	2	R4-7	105.9%	4-fold
122	E	Glu	2	R4-12	99.8%	3-fold
123	Q	Gln	2	R4-19	96.5%	3-fold
126	N	Asn	2	R4-18	99.0%	3-fold
130	K	Lys	2	R4-7	99.7%	3-fold

5. Study on efficacy of IL10-K34C-S48 on the 4T1 tumor model in BALB/C mice model.

Test purpose: to investigate the anti-tumor efficacy of IL10-K34C-S48 in BALB/C mice for treatment of the 4T1 tumor model. Test drug: IL10-K34C-S48 and IL-10 injection, diluted to corresponding concentrations by physiological PBS when testing.

Method and results:

1. Animal: BALB/C mice of 5 weeks old, all female.

2. Production of tumor model

1) 4T1 cells were purchased from American type culture collection (ATCC) and identified according the specification provided by ATCC. Cells were cultivated in RPMI 1640 culture solution containing 10% fetal bovine serum at 37°C and 5% CO₂. The cells were passaged for every three days and cells within the 13th passage were used.

2) Production of tumor model. 4T1 cells were subcutaneously injected to the back of the BALB/C mice. Mice were randomly grouped after the tumor grew to about 100 mm³ and drug treatment began. Mice were killed after anesthesia on day 28.

3) Course of treatment. There were 3 groups with 6 animals in each group. Included were a control group treated daily, and two single agent groups (1 mg/kg IL-10 treated daily or IL-10-K34C-S48 daily (1 mg/kg IL-10 equivalents dose).

4) Grouping and test results are shown in Table 74.

5) Results and discussion. As shown in Table 74, comparing with IL-10, the complete regression on the 4T1 tumor of BALB/C mice was greatly improved after injection of IL10-K34C-S48, indicating that IL10-K34C-S48 exhibits a good anti-tumor efficacy on the 4T1 tumor model.

Table 74: Effects of IL10-K34C-S48 on the 4T1 tumor model in BALB/C mice model

Group	Number of animals	Complete Regression
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Control Group	6	0
IL10-K34C-S48	6	33.33%
IL-10	6	0

Tumor volumes were monitored 2-3 times a week and are presented in the Fig. 46.

Example 31: Activation efficiency of different R2 for targeted activation in different tissue.

Cleaving effect of conjugates in which R1 was S13, R3 was R3-5, R4 was R4-7 and R2 was each of the groups shown in Table 75 were evaluated in different tissues. The conjugates were each dissolved and diluted for ten times to a concentration of 0.1 mM/ml. At 37°C, conjugates were each added into 100µg different acidized human tumor tissue homogenates (pH6.0) in a concentration of 0.2mg/ml. The enzyme in tumor tissue homogenates could release R1. The released R1 was detected by HPLC, thereby comparing the activation efficiency of the linker. Results were showed in Table 75

Table 75: Activation ratio (%) of different R2 peptide in homogenates from different tumor tissues

Human tissues	Cells producing tumor	R2=LTPRLGPAAN	R2=GPAAN	R2=LSGRSDN	R2=AAN
Fibrosarcoma	HT-1080	66.4	47.8	19.8	45.9
Breast cancer	MDA-MB231	71.8	76.7	25.4	68.4
Liver cancer	Hepg2	68.9	58.4	21.8	55.7
Heart		1.6	1.1	4.3	0
Lung		1.7	1.5	5.9	0

According to the results, extended R2, which is activated by multiple enzymes, exhibits a higher activation than AAN. But activation with multiple enzymes may cause stability problem as shown in heart and lung tissue.

Example 32: Generation and characterization of anti-VEGF TMEAbody (Bevacizumab)

The heavy chain and light chain sequences of Bevacizumab were downloaded from Drug

Bank (<https://www.drugbank.ca/drugs/DB00112>) and site screening was performed to identify anti-VEGF TMEAbody with good blocking efficiency. The mutation position in a heavy chain of the anti-VEGF antibody (Bevacizumab) was selected from the group consisting of: Tyr32, Asn35, Tyr54, Tyr60, Lys65, Arg66, Tyr102, Tyr103 and Tyr109; and the mutation position in a light chain was selected from the group consisting of: Ser24, Ser26, Asp28, Tyr32, Tyr49, Thr51, Tyr91, Ser92, and Thr93. His tagged VEGF protein was used for ELISA. The selected sites with good blocking efficiency and good recovery were summarized in the Table 76 .

Table 76: Selected anti-VEGF TMEAbody candidates based on blocking and recovery efficiency

ID of selected Mutation	Heavy or light chain	Mutation site	R4	Conjugation efficiency	Blocking efficiency of S47	Restored activity after R4 conjugation
Beva-se001	HC	Lys65	R4-7	95%	9 fold	Restored
Beva-se002	LC	Ser26	R4-1	91%	16 fold	Restored
Beva-se003	LC	Tyr49	R4-7	95%	26 fold	Restored
Beva -se004	LC	Thr51	R4-1	87%	13fold	Restored

Example 33: Generation and characterization of anti-CD20 TMEAbody (Rituximab)

The heavy chain and light chain sequence of Bevacizumab was downloaded from Drug Bank (<https://www.drugbank.ca/drugs/DB00073>) and site screening was performed to identify anti-CD20 TMEAbody with good blocking efficiency. The mutation position in a heavy chain of the anti-CD20 antibody (Rituximab) was selected from the group consisting of: Tyr32, Asn33, Tyr52, Asn55, Lys63, Lys65, Tyr101, Tyr102 and Tyr107; and the mutation position in a light chain was selected from the group consisting of: Ser26, Ser28, Tyr31, Tyr48, Thr50, Asn52, Thr91 and Thr96. His tagged CD20 protein was used for ELISA. The selected sites with good blocking efficiency and good recovery were summarized in the Table 77.

Table 77: Selected anti-VEGF TMEAbody candidates based on blocking and recovery efficiency

ID of selected Mutation	Heavy or light chain	Mutation site	R4	Conjugation efficiency	Blocking efficiency of S47	Restored activity after R4 conjugation
Ritu-se001	HC	Tyr32	R4-7	92%	16 fold	Restored
Ritu-se002	HC	Lys63	R4-7	98%	8 fold	Restored
Ritu-se003	LC	Tyr31	R4-7	91%	13 fold	Restored
Ritu-se004	LC	Tyr48	R4-7	92%	15 fold	Restored

Ritu-se005	LC	Thr50	R4-1	89%	21 fold	Restored
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Example 34: Blocking and cleaving screening of different R4 and R5 for drug candidates.

Blocking and cleaving effect of conjugates in indicated conjugation were evaluated. The conjugates were each dissolved and diluted for ten times to a concentration of 0.1 mM/ml. At 37°C, conjugates were each added into 100μg MDA-MB231 human tumor tissue homogenates (pH7) in a concentration of 1mg/ml for 8hr. The conjugated and released biomolecule was detected binding by ELISA, thereby comparing the activation efficiency of the linker. Results were showed in Table 78.

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Table 78: Blocking and cleaving effect of S48 to different biomolecules

R1-R2-R3-R4	R5	Binding decrease by conjugation	Recovery binding after activation (%)
S48	SEQ ID:13	34-fold	96
S48	SEQ ID: 14	25-fold	95
S48	SEQ ID: 15	18-fold	105
S48	SEQ ID: 16	42-fold	96
S48	SEQ ID: 17	67-fold	95
S48	SEQ ID: 18	32-fold	96
S48	SEQ ID: 19	67-fold	85
S48	SEQ ID: 26	24-fold	94
S48	SEQ ID: 27	54-fold	94
S48	SEQ ID: 28	14-fold	107
S48	SEQ ID: 29	18-fold	106
S48	SEQ ID: 30	9-fold	91
S48	SEQ ID: 31	13-fold	89
S48	SEQ ID: 32	14-fold	93
S48	SEQ ID: 44	75-fold	99
S48	SEQ ID: 54	24-fold	96
S48	SEQ ID: 60	16-fold	96
S48	SEQ ID: 61	19-fold	107
S48	SEQ ID: 62	74-fold	96
S48	SEQ ID: 63	34-fold	93

Blocking and cleaving effect of conjugates in indicated conjugation in Table 79 were evaluated. The conjugates were each dissolved and diluted for ten times to a concentration of 0.1 mM/ml. At 37°C, conjugates were each added into MMP2 (pH6) in a concentration of 1mg/ml for 16hr. The conjugated and released biomolecule was detected binding by ELISA, thereby comparing the activation efficiency of the linker. Results were showed in Table 79.

Table 79: Blocking and cleaving effect of conjugations of S65 to different biomolecules

R1-R2-R3-R4	R5	Binding decrease by conjugation	Recovery binding after activation (%)
S65	SEQ ID:13	19-fold	96
S65	SEQ ID: 14	21-fold	99
S65	SEQ ID: 15	58-fold	95
S65	SEQ ID: 16	124-fold	97
S65	SEQ ID: 17	164-fold	99
S65	SEQ ID: 18	26-fold	95
S65	SEQ ID: 19	35-fold	89
S65	SEQ ID: 26	43-fold	94
S65	SEQ ID: 27	17-fold	95
S65	SEQ ID: 28	12-fold	99
S65	SEQ ID: 29	25-fold	105
S65	SEQ ID: 30	48-fold	101
S65	SEQ ID: 31	75-fold	97
S65	SEQ ID: 32	34-fold	93
S65	SEQ ID: 44	19-fold	96
S65	SEQ ID: 54	25-fold	94
S65	SEQ ID: 60	75-fold	97
S65	SEQ ID: 61	34-fold	93
S65	SEQ ID: 62	19-fold	96
S65	SEQ ID: 63	25-fold	94

Blocking and Cleaving effect of conjugates in indicated conjugation were evaluated. The conjugates were each dissolved and diluted for ten times to a concentration of 0.1 mM/ml. At 37°C, conjugates were each added into 100μg MDA-MB231 acidized human tumor tissue homogenates (pH6.5). The conjugated and released biomolecule was detected binding by ELISA, thereby comparing the activation efficiency of the linker. Results were showed in Table

80.

Table 80: Blocking and cleaving effect of conjugations of S27 to different biomolecules

R1-R2-R3-R4	R5	Binding decrease by conjugation	Recovery binding after activation (%)
S27	SEQ ID: 13	43-fold	91
S27	SEQ ID: 14	11-fold	95
S27	SEQ ID: 15	8-fold	98
S27	SEQ ID: 16	14-fold	95
S27	SEQ ID: 17	19-fold	98
S27	SEQ ID: 18	33-fold	95
S27	SEQ ID: 19	45-fold	96
S27	SEQ ID: 26	28-fold	99
S27	SEQ ID: 27	37-fold	95
S27	SEQ ID: 28	36-fold	97
S27	SEQ ID: 29	106-fold	106
S27	SEQ ID: 30	135-fold	102
S27	SEQ ID: 31	75-fold	107
S27	SEQ ID: 32	58-fold	97
S27	SEQ ID: 44	74-fold	99
S27	SEQ ID: 54	35-fold	97

According to the results, these drug candidates exhibited blocking effect and activation
 5 effect in the indicated activation conditions.

**Example 35: Study on efficacy of indicated S27, S39, S40, S47, S48 and S65
 conjugated with mouse CTLA-4 antibody in CT26 tumor immune model**

Test purpose: to investigate the anti-tumor efficacy of S27, S39, S40, S47, S48, S65
 10 conjugated with mouse CTLA-4 antibody for immune treatment.

Test drug: S27, S39, S40, S47, S48, S65 conjugated with mouse CTLA-4 antibody (9D9),
 all used in 20mg/kg (equimolar of CTLA-4 antibody) .

Production of tumor model:

1) CT26 tumor cells were purposed from ATCC. Cells were cultivated in DMEM culture
 15 solution containing 10% fetal bovine serum at 37°C and 5% CO₂. The cells were passaged for
 every three days and cells within the 15th passage were used.

2) Tumor immunization. 5×10^5 CT26 cancer cells (purchased from ATCC) which were
 killed by irradiation were intraperitoneally injected to mice. The mice were injected for 3 times,

once every two weeks. After immunization, mice were injected with tumor cells and the drugs were administered weekly for 4 weeks.

3) Production of tumor. At day 32, 10^6 live lung tumor cells were subcutaneously injected to the back of the C57 mice immunized by tumor. Treatment began when the tumor grew to 0.3-0.4cm.

4) Analysis on tumor CD8⁺ T cells. The tumor tissue was homogenated and individual cells in the tumor were filtered, separated and washed by buffer twice, then cultivated with the leucocyte common antigen CD45-PE and CD8-FITC marked antibodies for 1 hour at ambient temperature. The cells were washed by phosphate buffer containing 1% fetal bovine serum twice and then analyzed for the ratio of the T lymphocyte antigen (CD8) positive cells in the leucocyte common antigen (CD45) positive cells by flow cytometry. Increasement of the ratio indicates increased T lymphocyte cells and thus the animal immunity against the tumor was improved.

5) Grouping and test results are shown in Table 81.

Table 81: Effect on inhibition of tumor and immune activation of S27, S39, S40, S47, S48 and S65 conjugated with mouse CTLA-4 antibody and control

Group	Number of animal	Size of tumor (mm ³)	Cure number of mouse	CD8: CD45 (%)
		Day 28	Day 28	
PBS	8	1887.56±323.4	0	5.2
Mouse CTLA-4 antibody (WT)	8	74.46± 467.34	2 (Dead 2)	13.1
S27-Mouse CTLA-4 Antibody (9D9)	8	37.60 ±358.57	6	28.4
S39-Mouse CTLA-4 Antibody (9D9)	8	31.57± 124.45	5	29.7
S40-Mouse CTLA-4 Antibody (9D9)	8	57.63 ± 157.32	6	26.3
S47-Mouse CTLA-4 Antibody (9D9)	8	53 ± 216.47	7	38.4
S48-Mouse CTLA-4 Antibody (9D9)	8	75.78±32.74	4	23.6
S65-Mouse CTLA-4 Antibody (9D9)	8	74.28± 38.45	6	26.7

6) Results and discussion. Treatment effects of S27, S39, S40, S47, S48 and S65 conjugated with mouse CTLA-4 antibody were greatly improved as compared to the control group and the WT CTLA-4 antibody treatment groups. WT CTLA-4 antibody Causing 1 death in WT CTLA-4 antibody treatment may be caused by toxicity in high dose treatment.

- 5 Treatment effects of S27, S39, S40, S47, S48 and S65 conjugated with mouse CTLA-4 antibody show an excellent effect and promote CD8/CD45 T cell ratio in tumor tissue.

Example 36: Study on efficacy of Adalimumab (SEQ ID NO:29) conjugated with S27, S47, S48 or S65 in RA mouse model

- 10 The TgTC mice were generated using a human TNF/ β -globin (TNFglobin) recombinant gene construct, which contained a 2.8 kb fragment with the entire coding region and promoter of the hTNF α gene, fused to a 0.77 kb fragment with the 3' untranslated region (UTR) and polyadenylation site of human β -globin replacing that of the hTNF α gene. The fragment was then microinjected into pronuclei of FVB/J inbred strain fertilized eggs. Finally, the injected
15 fertilized eggs were implanted into the oviduct of 8-week-old female pseudo-pregnant ICR mice. Transgenic lineages were established by back-crossing the transgenic founder individuals to the FVB/J inbred strain. The genotyping was performed by PCR to screen for transgenic animals as well as routine tail genotyping. The transgene specific PCR primers were:

- 20 5'-GAACTCCCTCGATGTTAACCA-3' (forward primer, SEQ ID NO:87); and
5'-TTCAATCCCCAAATCCTAGCC-3' (reverse primer, SEQ ID NO:88).

The PCR reactions were performed as follows: 94°C for 4 min; 35 cycles at 95°C for 30 s, 57°C for 40 s, and 72°C for 40 s; 72°C for 10 min.

- Different anti-hTNF α antibody (Adalimumab 2mg/kg) and conjugated antibody (2mg/kg equimolar of Adalimumab) dissolved in saline were intraperitoneally administered (2mg/kg) to
25 TgTC mice weekly from three to ten weeks, with saline-treated TgTC mice serving as control. Clinical assessment Weekly body weight and arthritis scores in all four limbs were recorded after weaning. Clinical severity of arthritis for each paw (fingers, tarsus, and ankle) was quantified by attributing a score ranging from 0 to 3: 0, normal; 1, slight redness and/or swelling; 2, pronounced edematous swelling; 3, joint deformity and rigidity. The arthritis score
30 per mouse was an average of the four limbs. Grouping and test results are shown in Table 82.

Table 82: Effect on inhibition of arthritis by S27, S47, S48 and S65 conjugated with Adalimumab antibody

Group	Number of animal	The arthritis score	The arthritis score
		Day 5	Day 10

PBS	4	3	3
Adalimumab	4	3	2
S27-Adalimumab	4	1	1
S47-Adalimumab	4	2	1
S48-Adalimumab	4	1	1
S65-Adalimumab	4	1	1

Results showed that Adalimumab conjugated with S27, S47, S48 and S65 greatly reduced the arthritis scores.

5 Example 37: Generation and characterization of anti-Her2/anti-CD3 bispecific TMEAbody

The heavy chain and light chain sequences of Trastuzumab was downloaded from Drug Bank (<https://www.drugbank.ca/drugs/DB00072>) and sites screening was performed to identify scFv form of anti-Her2 with good blocking efficiency. The mutation position in a light chain is selected from the group consisting of: Asp1, Gln3, Gln27, Asp28, Asn30, Tyr49, Tyr55, Arg66, Asp70, and Tyr92. The mutation position in a light chain is selected from the group consisting of Arg19, Lys30, Asp31, Tyr33, Arg50, Tyr62, Asn55, Tyr57, Arg59, Tyr60, Asp62, Lys65, Asp102, and Tyr105. The scFv forms of above selected mutants were expressed in HEK293 with C Terminal 6His tag, purified with Ni-NTA column, and conjugated with corresponding chemical linkers. Binding ELISA was carried out with His-tagged human Her2 protein as antigen and anti-human kappa chain as secondary antibody. The blocking efficiency was summarized in the Table 84.

Table 83: Selected scFv form of anti-Her2 candidates based on blocking and recovery efficiency

ID of selected Mutation for scFv form	Single Heavy /Light chain	Mutation site	R4	Conjugation efficiency	Blocking efficiency of S47	Activity after R4 conjugation
Trast-se001scFv	Heavy chain	Tyr33	R4-7	95%	15 fold	Restored
Trast-se002scFv	Heavy chain	Lys65	R4-7	93%	6 fold	Restored
Trast-se003scFv	Heavy chain	Tyr105	R4-7	92%	32 fold	Restored
Trast -se004scFv	Light chain	Gln30	R4-11	83%	15 fold	Restored
Trast -se005scFv	Light chain	Tyr49	R4-7	93%	43 fold	Restored
Trast -se006scFv	Light chain	Asp70	R4-11	91%	13 fold	Restored

We fused anti-Her2 scFv with selected Cysteine mutation (Tyr49 in light chain) to anti-human CD3 scFv containing C terminal 6His tag to form bispecific antibody targeting to tumor and T cells. These Her2/CD3 bispecific antibodies were produced in HEK293 cells and purified with Ni-NTA column. These Her2/CD3 bispecific antibodies with mutant was further conjugated with S47 and a 38 fold decreased binding activity to human Her2 protein was obtained. After digestion with legumain, both binding activity were restored.

The single chain of Her2/CD3 TMEAbody were produced by conjugating S27, S47 or S48 to a fusion protein anti-Her2 scFV with anti-CD3 or its scFv, as shown in table 84.

Table 84: Selected scFv form of anti-Her2 candidates based on blocking and recovery efficiency

Bispecific TMEAbody	anti-Her2	anti-CD3	SEQ ID NO:	R1-R2-R3-R4
Her2/CD3 TMEAbody1	anti-Her2 scFv	anti-CD3 scFv	70	S27
Her2/CD3 TMEAbody2	anti-Her2 scFv	anti-CD3 scFv	70	S47
Her2/CD3 TMEAbody3	anti-Her2 scFv	anti-CD3 scFv	70	S48
Her2/CD3 TMEAbody4	anti-Her2 scFv	anti-CD3 scFv	82	S27
Her2/CD3 TMEAbody5	anti-Her2 scFv	anti-CD3 scFv	82	S47
Her2/CD3 TMEAbody6	anti-Her2 scFv	anti-CD3 scFv	82	S48
Her2/CD3 TMEAbody7	anti-Her2	anti-CD3	Anti-Her2: 71 and 72 Anti-CD3: 75	S27
Her2/CD3 TMEAbody8	anti-Her2	anti-CD3	Anti-Her2: 71 and 72 Anti-CD3:75	S47
Her2/CD3 TMEAbody9	anti-Her2	anti-CD3	Anti-Her2: 71 and 72 Anti-CD3: 75	S48

We fused PD-1 antibody or PD-L1 antibody with mutant IL-2 (IL2-S87C) to form targeting tumor associated antigen PD-L1/IL-2 TMEAkines or PD-1/IL-2 TMEAkines.

These TMEAkines with IL2-T41C mutant was further conjugated with S47. A >135 fold decreased binding activity to human IL-2R β was obtained. After digestion with legumain, both binding activity to IL-2R β were restored. The fused PD-L1 or PD-1 antibody with mutant IL-2 (mutant at binding IL-2R α S87C) were further conjugated with S47 to get legumain activation fusion TMEAkine as shown in table 85.

Table 85: Fusion protein candidates based on blocking and recovery efficiency

Fusion	Fusion protein sequece	R1-R2-R3-R4
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TMEAkine		
1	N-terminal-IL2(S87C)-GSGS-PD-1(SEQ ID NO:15)	S47
2	N-terminal-IL2(S87C)-GSGS-PD-1 (SEQ ID NO:17)	S47
3	N-terminal-IL2(S87C)-GSGS-PD-L1(SEQ ID NO:27)	S47
4	N-terminal- PD-1(SEQ ID NO:15) -GSGS-IL2(S87C)	S47
5	N-terminal- PD-1(SEQ ID NO:17) -GSGS-IL2(S87C)	S47
6	N-terminal- PD-L1(SEQ ID NO:27) -GSGS-IL2(S87C)	S47
7	N-terminal-EGFR(SEQ ID NO:78&79) -GSGS- -IL2(S87C)	S47
8	N-terminal- VEGFR (SEQ ID NO:80&81) -GSGS-IL2(S87C)	S47
9	N-terminal- Her2 (SEQ ID NO:82&83) -GSGS-IL2(S87C)	S47
10	PD-1/IL-2 fusion protein(SEQ ID NO:73)	S47
11	PD-L1/IL-2 fusion protein(SEQ ID NO:74)	S47

***In vivo* characterization of toxicity in Human PBMC-transferred mouse model**

Test purpose: to investigate the acute toxicity of the fusion TMEAbody via intravenous injection.

5 Animal: the first class SCID mouse, weighing 19-21 g and all mice being female.

Method and results: SCID mouse were randomly divided into 21 groups according to their body weights, with 10 mice in each group. As shown in Table 86, the mice were intravenously injected with D1, D7 and D14 for just one time in a dose of 30mg/kg (equimolar of antibody). Control tests were performed by injecting 30mg/kg human IgG. Animals were observed for 21 continuous days for presence or absence of the following behaviors on each day: pilo-erection, hair tousle and lackluster, lethargy, stoop and irritable reaction, and body weight and death were recorded as shown in Table 86.

Table 86: Comparison of mortality rates of test mice receiving different fusion TMEAbody injections

Group	injections	Number of animal	Number of dead animal (day21)	Mortality rate (%)
1	human IgG	10	0	0
2	Her2/CD3 scFv	10	4	40
3	Her2/CD3 antibody	10	3	30
4	Her2/CD3 TMEAbody1	10	0	0
5	Her2/CD3 TMEAbody2	10	0	0
6	Her2/CD3 TMEAbody3	10	0	0
7	Her2/CD3 TMEAbody4	10	0	0

8	Her2/CD3 TMEAbody5	10	0	0
9	Her2/CD3 TMEAbody6	10	0	0

Results and discussions: no pilo-erection, hair tousle and lackluster, lethargy, stoop, irritable reaction and death were observed in mice receiving 30 mg/kg of group 2, 3, 10, and 14. As shown in Table 86, The MTD of the fusion protein is less than 30 mg/kg, which can be observed toxicity and deaths.

***In vivo* characterization of toxicity in Human PBMC-transferred mouse model**

Test purpose: to investigate the acute toxicity of the fusion TMEAbody via intravenous injection.

10 Animal: the first class SCID mouse, weighing 19-21 g and all mice being female.

Method and results: SCID mouse were randomly divided into 21 groups according to their body weights, with 5 mice in each group. As shown in Table 88, the mice were intravenously injected with fusion protein and fusion protein conjugation with S47 at D1, D7 and D14 for just one time in a dose of 30mg/kg(equimolar of antibody). Control tests were performed by injecting 30mg/kg saline. Animals were observed for 21 continuous days for presence or absence of the following behaviors on each day: pilo-erection, hair tousle and lackluster, lethargy, stoop and irritable reaction, and body weight and death were recorded as shown in Table 87.

20 Table 87: Comparison of mortality rates of test mice receiving different fusion fusion TMEAkine injections

Fusion protein sequece	Death number	Death number after conjugating to S47
saline	0	0
N-teminal-IL2(S87C)-GSGS-PD-1(SEQ ID NO:15)	5	0
N-teminal-IL2(S87C)-GSGS-PD-1 (SEQ ID NO:17)	3	0
N-teminal-IL2(S87C)-GSGS-PD-L1(SEQ ID NO:27)	5	0
N-teminal- PD-1(SEQ ID NO:15) -GSGS-IL2(S87C)	2	0
N-teminal- PD-1(SEQ ID NO:17) -GSGS-IL2(S87C)	1	0
N-teminal- PD-L1(SEQ ID NO:27) -GSGS-IL2(S87C)	4	0
N-teminal-EGFR(SEQ ID NO:77&79) -GSGS- -IL2(S87C)	3	0
N-teminal- VEGFR (SEQ ID NO:80&81) -GSGS-IL2(S87C)	4	0
N-teminal- Her2 (SEQ ID NO:82&83) -GSGS-IL2(S87C)	5	1

PD-1/IL-2 fusion protein(SEQ ID NO:73)	4	0
PD-L1/IL-2 fusion protein(SEQ ID NO:74)	5	1

Results and discussions: no pilo-erection, hair tousle and lackluster, lethargy, stoop, irritable reaction and death were observed in mice receiving 30 mg/kg of all group fo fusion TMEAkine. But the toxicity was reduced after fusion with S47.

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***In vivo* characterization of single chain of CD3-Her2 TMEAbody in mouse tumor model**

To further characterize the *in vivo* efficacy of single chain of CD3-Her2 TMEAbody in treating tumor in animal model, single chain of CD3-Her2 TMEAbody, as well as single chain CD3-Her2 antibody were administrated into Tumor xenografts. To initiate tumor xenografts, 3×10^6 KPL-4 cells were implanted orthotopically into the right penultimate inguinal mammary fat pad of female severe combined immunodeficient (SCID) beige mice. Tumors were allowed to growth (20 d for KPL4) after implantation before initiation of treatment. Mice with KPL-4 tumors (100 mm^3) were treated with indicated drug (10 mg/kg weekly for 5 weeks) for the duration of the study. Tumor volumes and body weights were measured twice weekly. The tumor volume inhibition rate was summarized in the Table 88. Results implied that single chain of CD3-Her2 TMEAbody could be activated in the tumor microenvironment and enhance the efficacy of single chain of CD3-Her2 antibody.

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Table 88: Tumor growth inhibition rate at day 20 after administration

Group	Dose	Cure rate (%)	Dead
human IgG	10mg/kg	0	0
Her2/CD3 scFv	10mg/kg	16.7	0
Her2/CD3 antibody	10mg/kg	33.3	2
Her2/CD3 TMEAbody1	10 mg/kg	66.7%	0
Her2/CD3 TMEAbody2	10mg/kg	83.3%	0
Her2/CD3 TMEAbody3	10mg/kg	100%	0
Her2/CD3 TMEAbody4	10mg/kg	100%	0
Her2/CD3 TMEAbody5	10mg/kg	100%	0
CD3-Her2 TMEAbody6	10mg/kg	100%	0
PD-1/IL-2 fusion	10mg/kg	100%	2
Fusion TMEAkine1	10mg/kg	83.3%	0
Fusion TMEAkine 2	10mg/kg	100%	0

TMEAkine4	10mg/kg	100%	0
Fusion TMEAkine5	10mg/kg	100%	0
Fusion TMEAkine10	10mg/kg	100%	0
PD-L1/IL-2 fusion	10mg/kg	100%	2
FusionTMEAkine3	10mg/kg	83.3%	0
Fusion TMEAkine6	10mg/kg	100%	0
Fusion TMEAkine11	10mg/kg	83.3%	0

As shown in Table 88, inhibition on tumor growth and cure rate by Her2/CD3 TMEAbody were greatly improved as compared with the groups treating by Her2/CD3 scFv or Her2/CD3 antibody by using the same molar concentration. Inhibition on tumor growth and cure rate by

5 PD-L1/IL-2 TMEAkine or antibody show efficacy and cure the mice. But in the PD-L1/IL-2 fusion antibody group, the toxicity caused some mice death.

Claims

1. A conjugate of biomolecule having the following structure:

5



wherein,

R5 represents a biomolecule with one or more cysteine residues introduced by mutation;

cys represents the cysteine residue(s) contained in R5;

10

S represents sulfur atom(s) of the cysteine residue(s);

R1 is a group that prevents R5 from binding to its antigen, ligand or receptor;

R2 is absent, or R2 is a cleavable linker arm capable of being activated by one or more proteolytic enzymes or a chemical bond capable of being acidically activated in a pathologic microenvironment;

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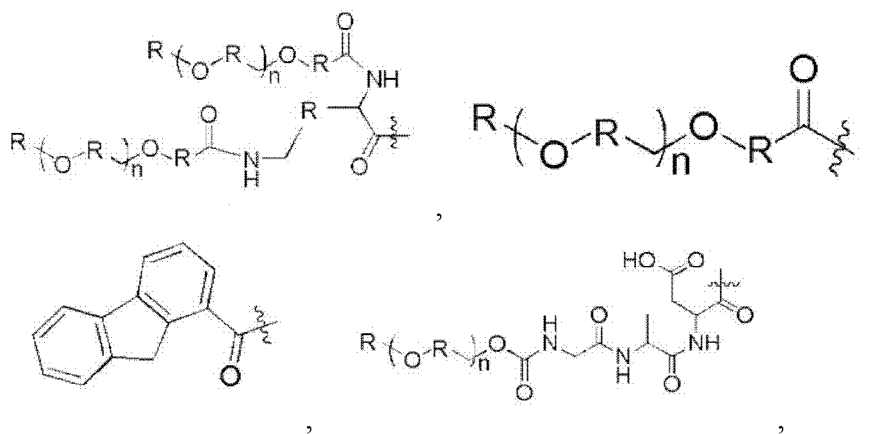
R3 is absent, or R3 is a linker arm capable of automatically shedding after R2 is cleaved or a chemical bond capable of being acidically activated in a pathologic microenvironment; with the proviso that when R2 is absent, R3 is the chemical bond capable of being acidically activated in a pathologic microenvironment; and

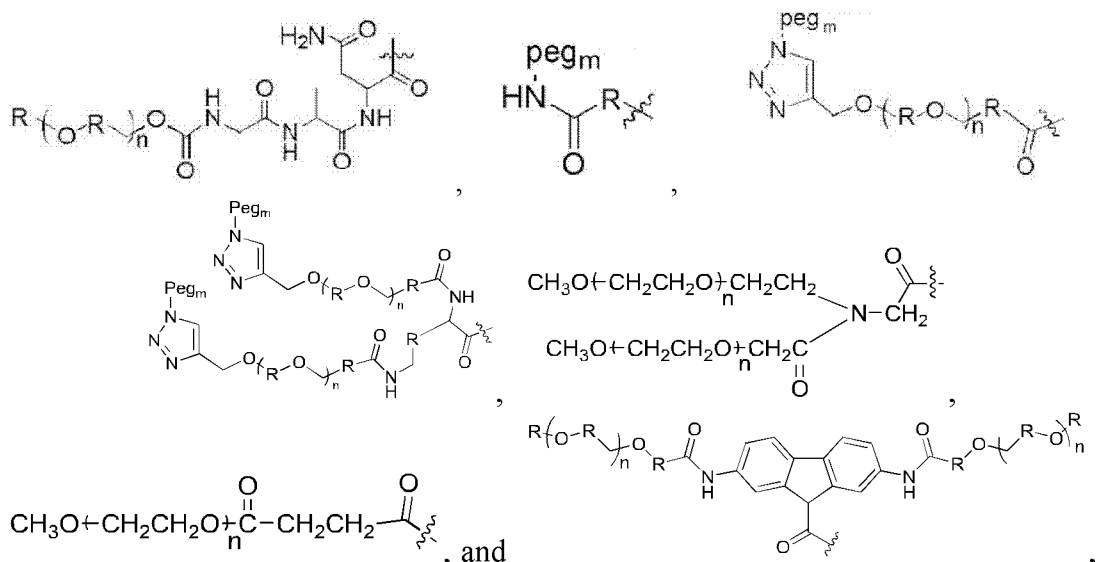
R4 is a group covalently linked to R5 via the sulfur atom(s) of the cysteine residue(s) contained in R5 that recovers, maintains or promotes the binding capacity of R5 to its antigen, ligand or receptor after the moiety R1-R2-R3 is cleaved.

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2. The conjugate of biomolecule according to claim 1, wherein R1 is selected from the group consisting of polyethylene glycol-C₁₋₅ alkylcarbonyl,

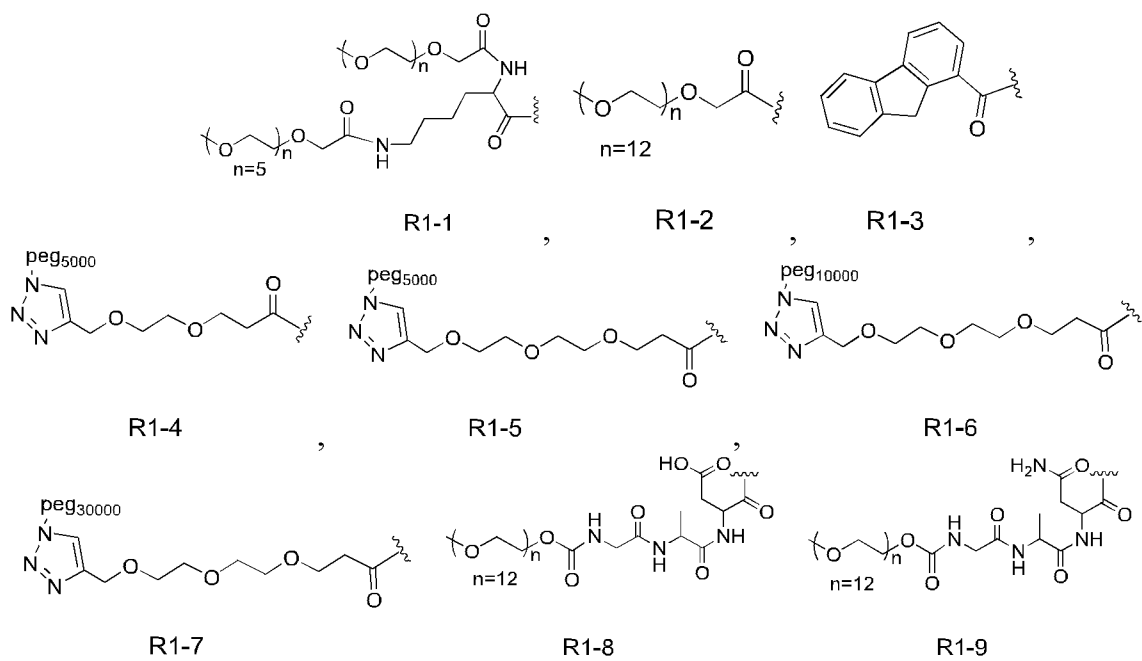
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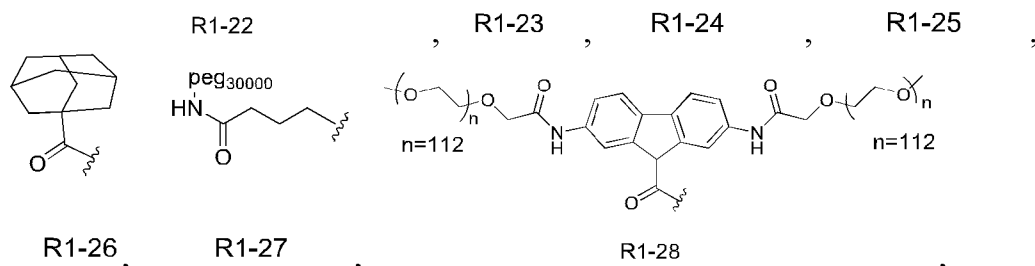
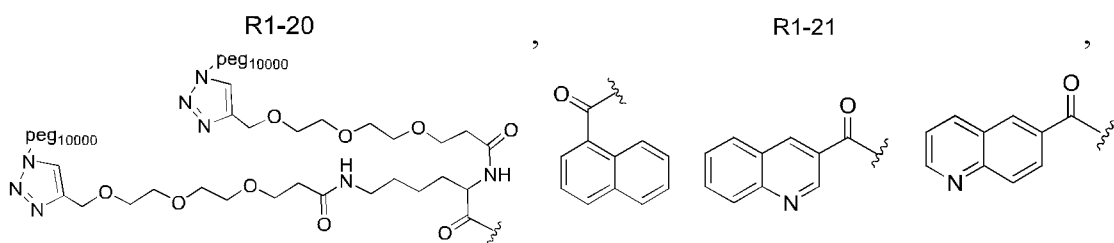
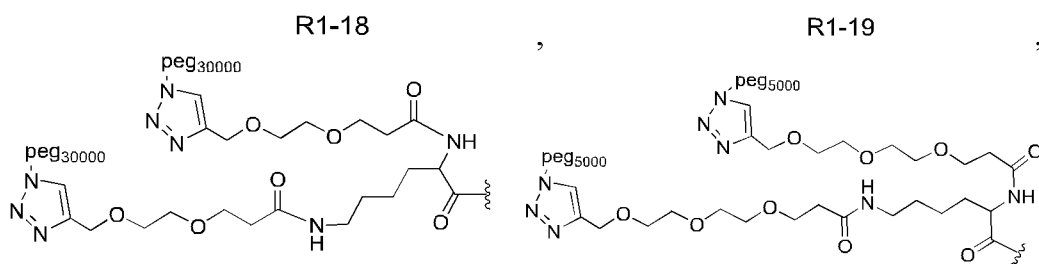
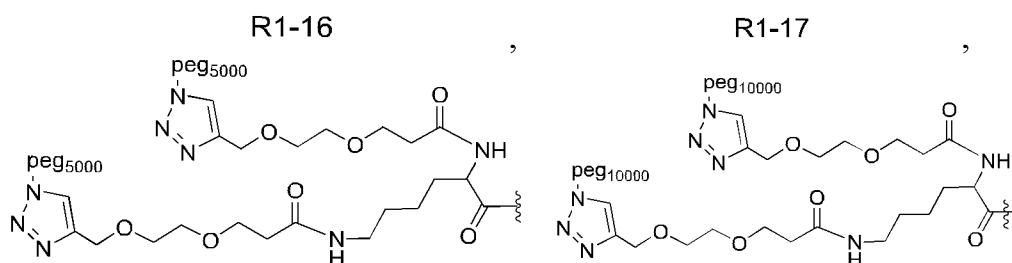
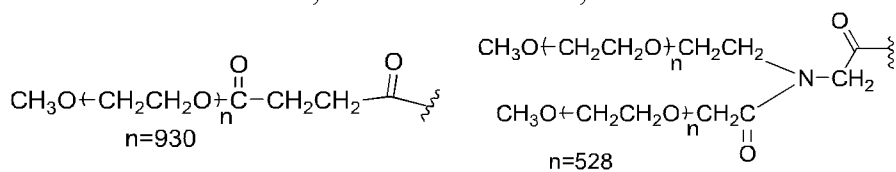
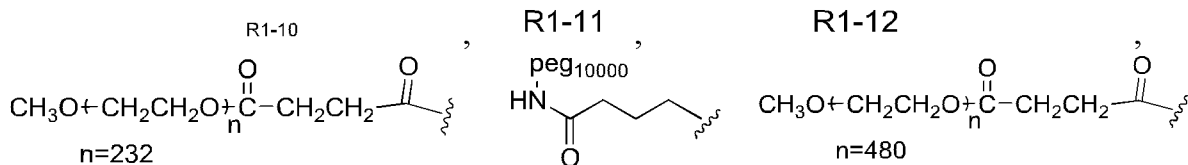
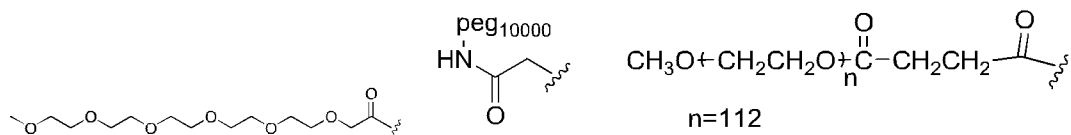


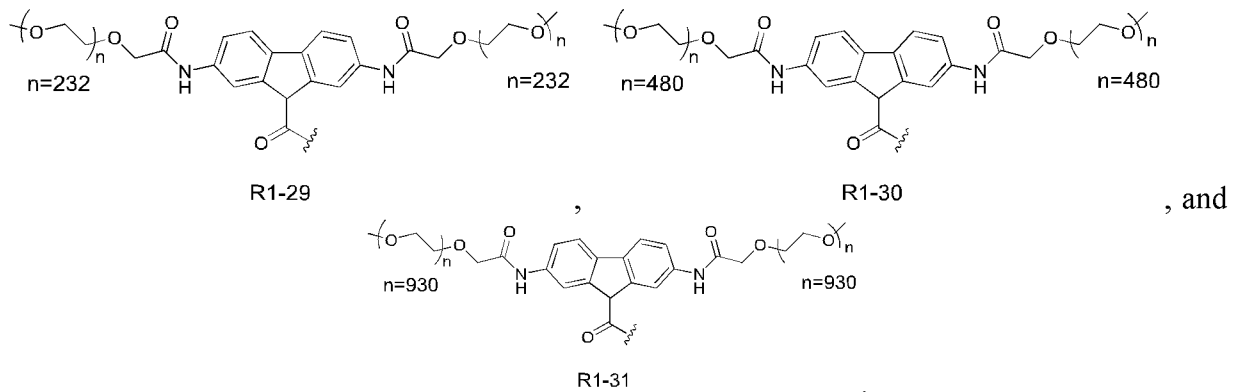


wherein each R is independently C1-4 alkyl; each n is independently an integer in a range of 1 to 30000, such as 1-150, 1-50, 1-20 or 3-12; the polyethylene glycol or peg_m is a polyethylene glycol having a molecular weight in a range of 44 to 132000, such as a polyethylene glycol having a molecular weight in a range of 1000 to 50000 or 10000 to 30000; m represents the molecular weight of the polyethylene glycol; and the wave line indicates the position of R1 linking to R2.

3. The conjugate of biomolecule according to claim 1, wherein R1 is selected from the group consisting of:







4. The conjugate of biomolecule according to claim 5, wherein R2 is a peptide capable of being activated or cleaved by one or multiple proteolytic enzyme, protease or peptidase, wherein the protease is selected from the group consisting of cysteine protease, asparagines protease, aspartate protease, glutamic acid protease, threonine protease, gelatinase, metallopro-teinase, or asparagine peptide lyase;

preferably, R2 is a peptide capable of being cleaved by at least one of enzymes selected from the group consisting of Legumain, granzyme, Cathepsin B, Cathepsin C, Cathepsin D, Cathepsin E, Cathepsin K, Cathepsin L, kallikrein, hK1, hK10, hK15, plasmin, collagenase, Type IV collagenase, astromelysin, Factor Xa, chymotrypsin-like protease, trypsin-like protease, elastase-like protease, subtilisin-like protease, actinidain, bromelain, calpain, caspase, caspase-3, Mirl-CP, papain, HIV-1 protease, HSV protease, CMV protease, chymosin, pepsin, matriptase, plasmepsin, nepenthesin, metalloexopeptidase, metalloendopeptidase, matrix metalloprotease (MMP), MMP1, MMP2, MMP3, MMP8, MMP9, MMP10, MMP11, MMP12, MMP13, MMP14, ADAM10, ADAM12, urokinase plasminogen activator (uPA), nenterokinase, prostate-specific antigen (PSA, hK3), interleukin-113 converting enzyme, thrombin, FAP (FAP-a), meprin, dipeptidyl peptidase, and dipeptidyl peptidase IV (DPPIV/CD26).

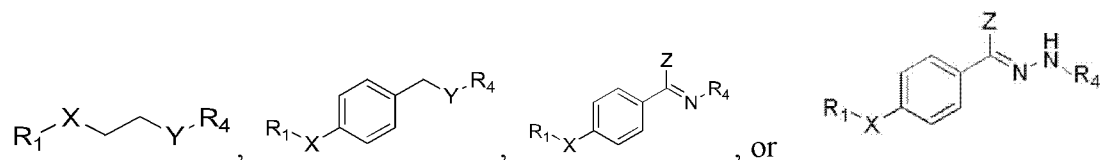
5. The conjugate of biomolecule according to claim 1, wherein R2 is a peptide represented by -R2a-, -R2b-, -R2a-N-, -R2a-D-, -R2a-AAN-, -R2a-AAD-, or -R2a-R2b-; wherein R2a is a peptide capable of being cleaved at amide bond by one or more proteolytic enzymes; R2b is a peptide with its nitrogen in side chain forming a carbamate with R3, wherein the carbamate is capable of being cleaved by one or more proteolytic enzymes; A is alanine; N is asparagine with its nitrogen in side chain forming a carbamate with R3, wherein the carbamate is capable of being cleaved by Legumain; D is aspartic acid with its nitrogen in side chain forming a carbamate with R3 and the carbamate can be cleaved by Granzyme B;

preferably, R2 is a tripeptide, wherein the first amino acid residue of the tripeptide linked to R1 is selected from the group consisting of Ala, Thr, Val and Ile, the middle second amino

acid residue is selected from the group consisting of Ala, Thr, Val and Asn, and the third amino acid residue linked to R3 is selected from the group consisting of Asn and Asp; and wherein R2 links to R1 via an amino group of the first amino acid residue in a linkage manner of amide, ester, carbamate, urea or hydrazone bond, and to R3 via a carboxyl group of the third amino acid residue in a linkage manner of amide, ester, carbamate, urea or hydrazone bond; preferably, the tripeptide is selected from the group consisting of Ala-Ala-Asn and Ala-Ala-Asp.

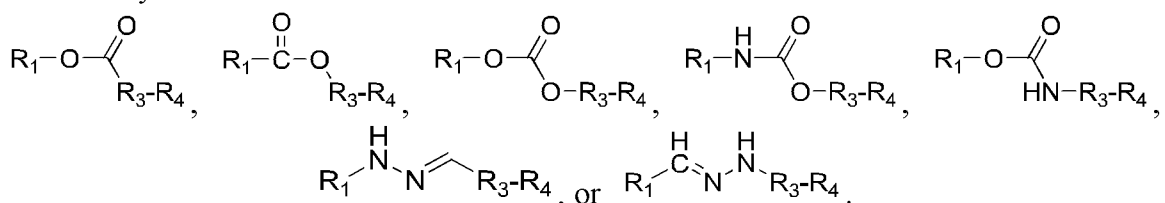
6. The conjugate of biomolecule according to claim 1, wherein R2 is a bond cleavable in an acidic condition of a pathologic microenvironment and is selected from the group consisting of amide, ester, carbamate, urea and hydrazone bonds;

preferably, wherein the structure of R1-R2-R3-R4 is represented by:

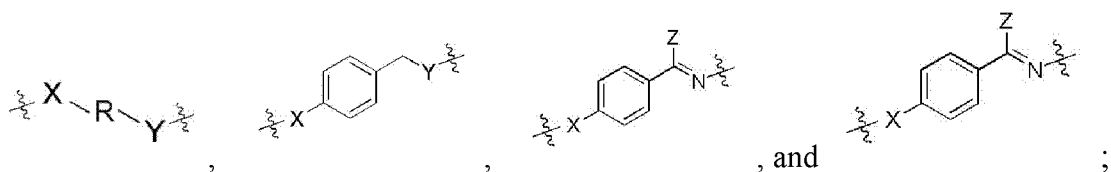


wherein X and Y are each independently NR' or O, Z is H or C1-10 alkyl, and R' is H or C₁₋₄alkyl; R3 links to R1 and R4 via X and Y respectively in a linkage manner of amide, ester, carbamate, urea or hydrazone bond.

7. The conjugate of biomolecule according to claim 1, wherein R2 is a bond cleavable in an acidic condition of a pathologic microenvironment and is selected from the group consisting of amide, ester, carbamate, urea and hydrazone bonds, and the structure of R1-R2-R3-R4 is represented by:



8. The conjugate of biomolecule according to claim 1, wherein R3 is selected from the group consisting of:



wherein X and Y are each independently NR' or O, Z is H or C₁₋₁₀ alkyl, R is C₁₋₄ alkyl, and R' is H or C₁₋₄alkyl; and wherein R4 links to R3 via the Y or N in the above formulae in a linkage manner of amide, ester, carbamate, urea or hydrazone bond.

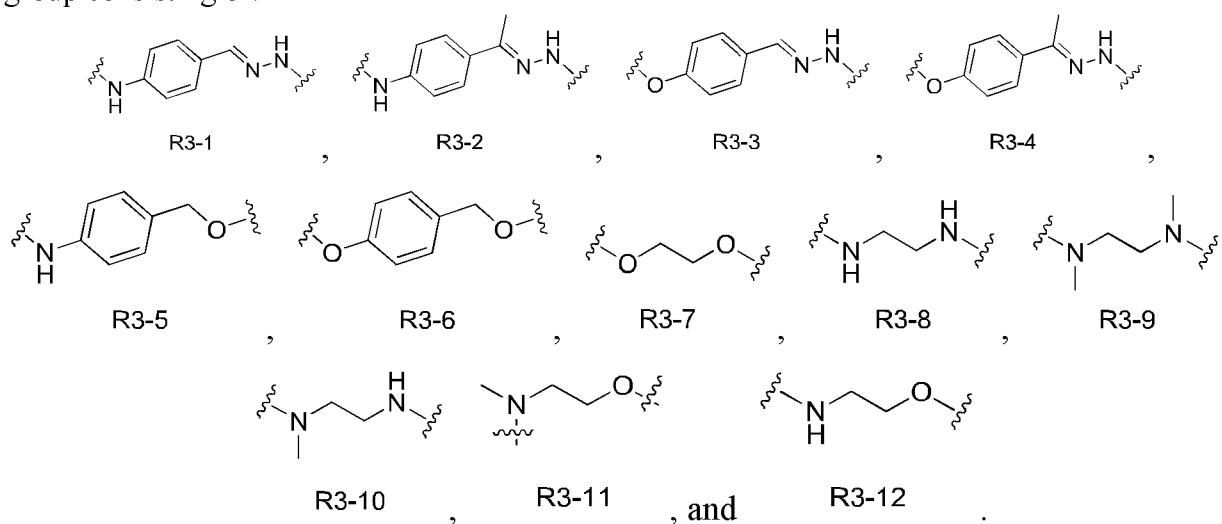
9. The conjugate of biomolecule according to claim 8, wherein R3 is selected from the group consisting of -NH-phenyl-CH₂O-, -NH-phenyl-CH=N-, -NH-phenyl-C(CH₃)=N-, -O-phenyl-CH=N- and -O-phenyl-C(CH₃)=N-.

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10. The conjugate of biomolecule according to claim 1, wherein R3 is selected from the group consisting of amide, ester, carbamate, urea and hydrazone bonds.

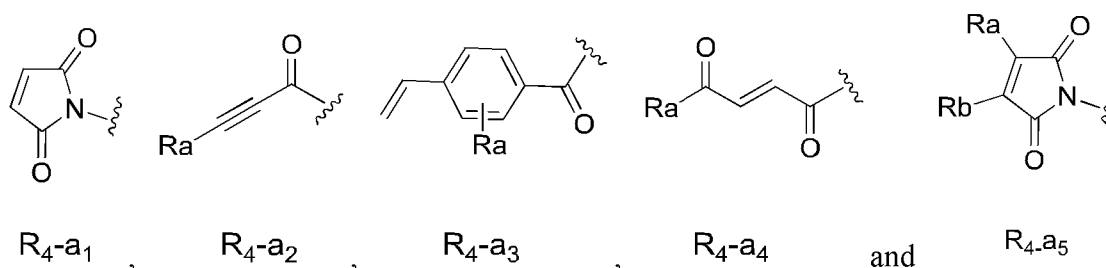
11. The conjugate of biomolecule according to claim 1, wherein R3 is selected from the group consisting of:

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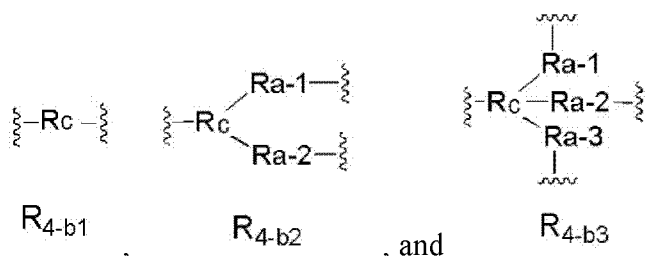
12. The conjugate of biomolecule according to claim 1, wherein R4 is represented by -R_{4-a}-R_{4-b}-R_{4-c}-, wherein R_{4-a} is selected from the group consisting of:



wherein Ra and Rb are each independently selected from the group consisting of H and C₁₋₆ alkyl or C₁₋₆ alkoxy;

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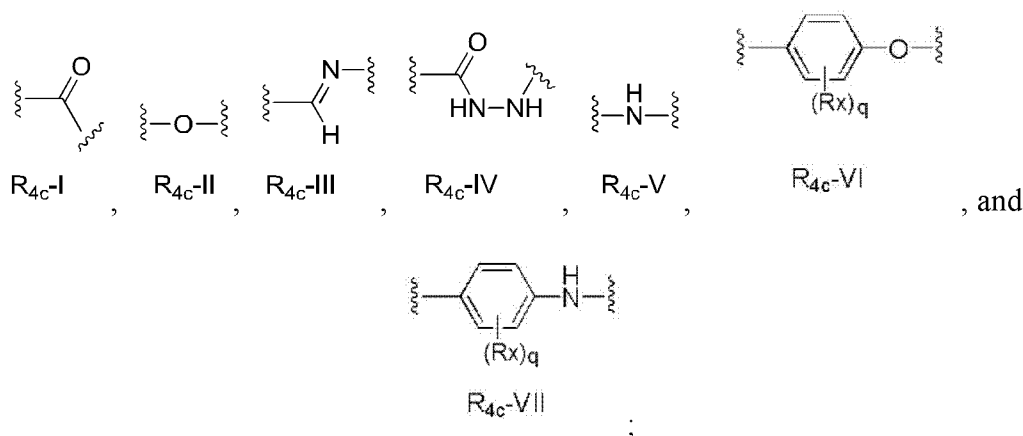
R_{4-b} is selected from the group consisting of:



wherein in formula R4-b1, Rc is absent, or is selected from the group consisting of C₁₋₁₂ alkyl, C₁₋₁₂alkoxy-C₁₋₁₂alkyl, C₁₋₁₂alkyl-C₃₋₈cycloalkyl, (C₁₋₄alkyl-O)_p-C₁₋₁₂alkyl, C₁₋₁₂alkylcarbonylamino-(C₁₋₄alkyl-O)_p-C₁₋₁₂alkyl, phenyl-C₁₋₁₂alkyl, C₃₋₈cycloalkyl,

- 5 C₁₋₁₂alkyl-C₃₋₈cycloalkyl-C₁₋₁₂alkyl, and C₁₋₁₂alkyl-phenyl-C₁₋₁₂alkyl; in formula R4-b2, Rc is a C₁₋₁₂alkylamino with Ra-1 and Ra-2 substituted at N atom of the amino group, and in formula R4-b3, Rc is a C₁₋₁₂alkyl with the last C atom at the end of the alkyl being substituted by Ra-1, Ra-2 and Ra-3, wherein Ra-1, Ra-2 and Ra-3 are each independently selected from the group consisting of C₁₋₁₂alkyl, C₁₋₁₂alkyl-OH, and C₁₋₁₂alkyl-NR''R''', wherein R'' and R''' are each
- 10 independently selected from the group consisting of H and C₁₋₁₂alkyl; wherein in formulae R4-b2 and R4-b3, R4-b links to R4-c via at least one of the Ra-1, Ra-2 and Ra-3;

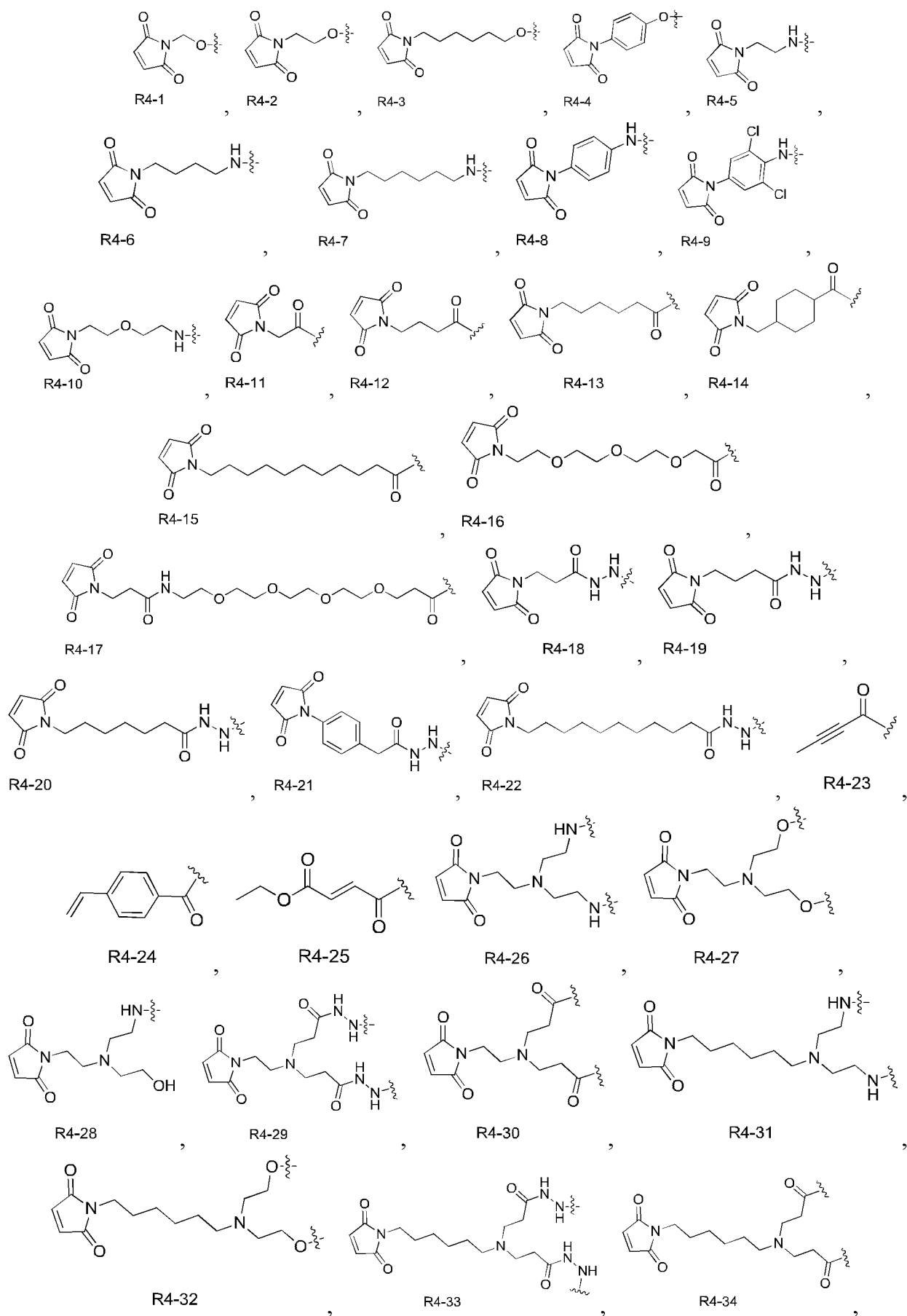
R_{4-c} is selected from the group consisting of:



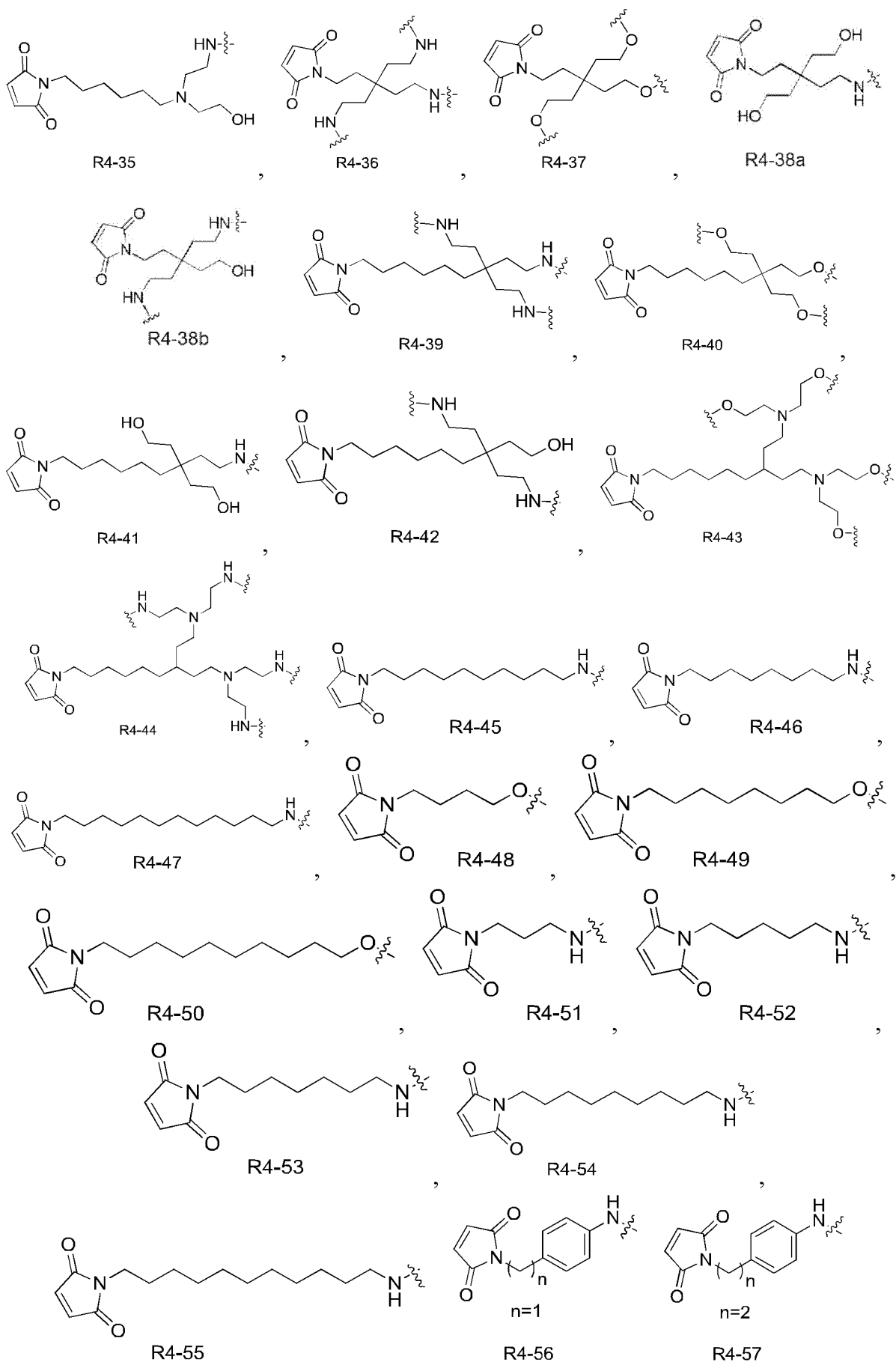
wherein Rx is selected from the group consisting of H, halo and C₁₋₄alkyl; p is an integer in a range of 1 to 10, such as an integer in a range of 1 to 5; and q is an integer in a range of 1 to 4, such as an integer in a range of 1 to 2; with the proviso that R4-c is absent when R4-a is selected from the group consisting of formulae R4-a2, R4-a3 and R4-a4;

- 20 wherein R3 links to R4 via the R_{4-c} of R4, and the wave line shown in each formula of R4-a indicates the position at which R4-a links to R4-b.

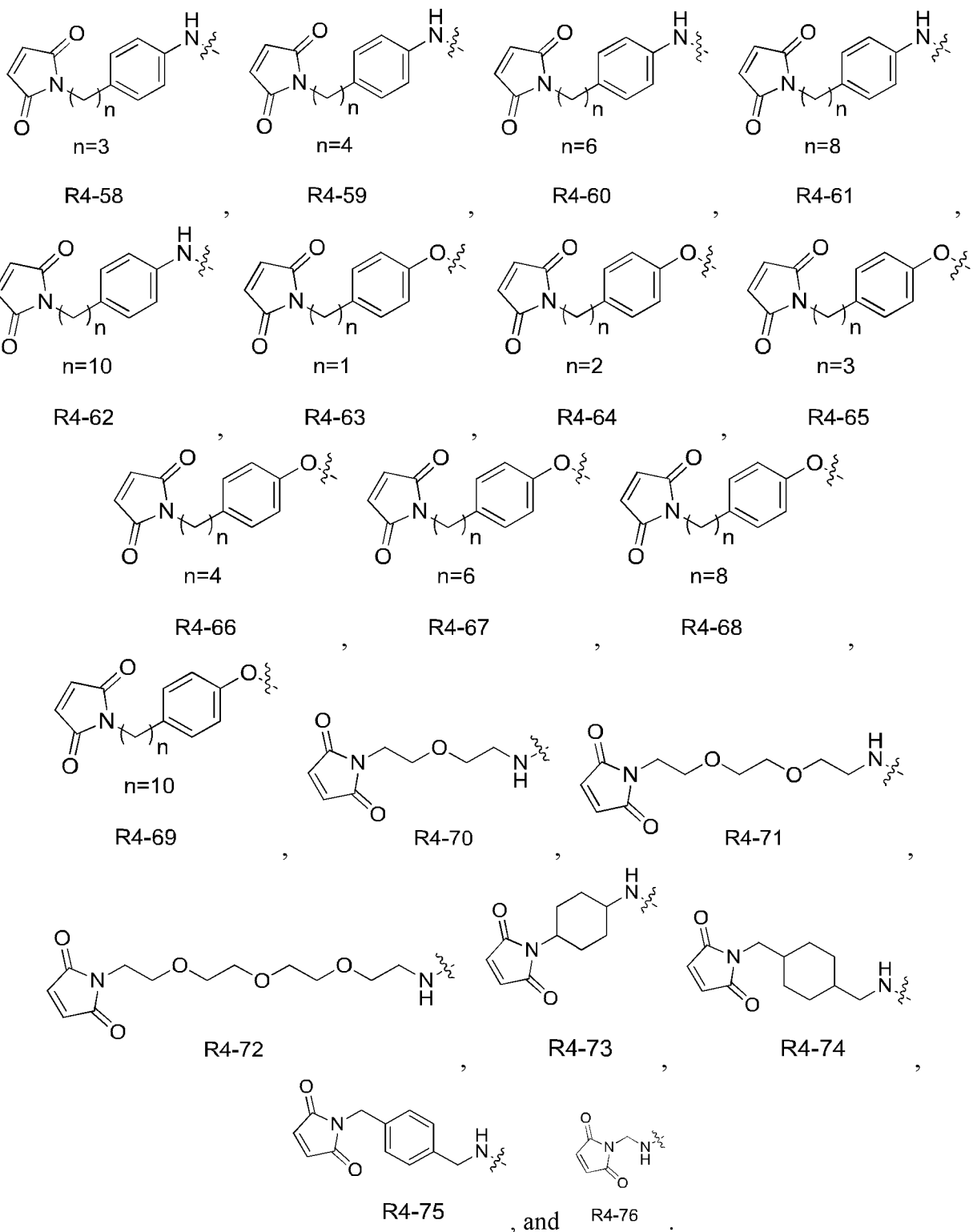
13. The conjugate of biomolecule according to claim 1, wherein R4 is selected from the group consisting of:



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14. The conjugate of biomolecule according to claim 1, wherein R5 is a protein with one or more of its amino acids being mutated to cysteine, and R5 links to R4 via the thiol group of the cysteine;

preferably, R5 is an antibody with one or more of its amino acids being mutated to cysteine.

15. The conjugate of biomolecule according to claim 1, wherein R5 is an antibody and the mutations in a non-complementarity determining region of a variable region of the antibody molecule; preferably, the conjugate of biomolecule eventually obtained molecular weight increasing via conjugation more than 200.

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16. The conjugate of biomolecule according to claim 14, wherein R5 represents a cytokine with one or more of its amino acids in its protein sequence mutated to cysteine.

17. The conjugate of biomolecule according to claim 14, wherein R5 represents an antibody with one or more mutations in a complementarity determining region of a variable region of the antibody molecule, preferably, R5 represents an antibody with one or more of amino acid residues selected from the group consisting of G, A, S, T, L, I, F, E, K, D and Y in the complementarity determining region mutated to cysteine.

18. The conjugate of biomolecule according to claim 14, wherein R5 represents an antibody with one or more mutations in a non-complementarity determining region of a variable region of the antibody, preferably, R5 represents an antibody with one or more of amino acid residues selected from the group consisting of G, A, S, T, L, I, F, E, K, D and Y in the non-complementarity determining region mutated to cysteine.

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19. The conjugate of biomolecule according to claim 16, wherein the biomolecule is a cytokine with one or more of amino acid residues selected from the group consisting of G, A, S, T, L, I, F, E, K, D and Y mutated to cysteine.

20. The conjugate of biomolecule according to claim 14, wherein R5 represents an antibody with one or more mutations in a non-complementarity determining region of a variable region of the antibody, preferably, with one or more of Gln3, Ser7, Ser26, Glu46, Thr68, Asp72 in non-complementarity determining region of VH and Thr5, Tyr49, Arg61, Ser63, Ser65, Ser67, Thr72, Thr74, Ser76, Asp82 in non-complementarity determining region of VL mutated to cysteine.

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21. The conjugate of biomolecule according to claim 14, wherein before mutation, R5 corresponds to a biomolecule selected from the group consisting of IL-2, IL-7, IL-10, IL-11, IL-12, IL-15, IL-21, IFN- α , IFN- β , IFN- γ , G-CSF, GM-CSF, TNF- α , TRAP and TRAIL; R5 corresponds to an antibody or functional fragment thereof selected from the group

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consisting of anti-Her2 antibody, anti-EGFR antibody, anti-VEGFR antibody, anti-CD20 antibody, anti-CD33 antibody, anti-PD-L1 antibody, anti-PD-1 antibody, anti-CTLA-4 antibody, anti-TNF α antibody, anti-CD28 antibody, anti-4-1BB antibody, anti-OX40 antibody, anti-GITR antibody, anti-CD27 antibody, anti-CD40 antibody, or anti-ICOS antibody, anti-CD25 antibody, anti-CD30 antibody, anti-CD3 antibody, anti-CD22 antibody, anti-CCR4 antibody, anti-CD38 antibody, anti-CD52 antibody, anti-Complement C5 antibody, anti-F protein of RSV, anti-GD2 antibody, anti-GITR antibody, anti-Glycoprotein receptor lib/Illa antibody, anti-ICOS antibody, anti-IL2R antibody, anti-LAG3 antibody, anti-Integrin α 4 antibody, anti-IgE antibody, anti-PDGFR α antibody, anti-RANKL antibody, anti-SLAMF7 antibody, anti-LTIGIT antibody, anti-TIM-3 antibody, anti-VEGFR2 antibody, anti-VISTA antibody; or

R5 corresponds to an antibody or functional fragment thereof selected from the group consisting of Utomilumab, Urelumab, ADG106, PoteligeoTM (Mogamulizumab), PoteligeoTM (Mogamulizumab), BexxarTM (tositumomab), ZevalinTM (ibritumomab tiuxetan), RituxanTM (rituximab), ArzerraTM (Ofatumumab), GazyvaTM (Obinutuzumab), BesponsaTM (Inotuzumab ozogamicin), ZenapaxTM (daclizumab), Varlilumab, Theralizumab, AdcetrisTM (Brentuximab vedotin), MyelotargTM (gemtuzumab), DarzalexTM (Daratumumab), CDX-1140, SEA-CD40, RO7009789, JNJ-64457107, APX-005M, Chi Lob 7/4, CampathTM (alemtuzumab), RaptivaTM (efalizumab), SolirisTM (eculizumab), YervoyTM (ipilimumab), tremelimumab, ErbituxTM (cetuximab), VectibixTM (panitumumab), PortrazzaTM (Necitumumab), TheraCIMTM (Nimotuzumab), SynagisTM (palivizumab), UnituxinTM (Dinutuximab), TRX-518, MK-4166, MK-1248, GWN-323, INCAGN0186, BMS-986156, AMG-228, ReoProTM (abixicimab), HerceptinTM (trastuzumab), PerjetaTM (Pertuzumab), KadcylaTM (Ado-trastuzumab emtansine), GSK-3359609, JTX-2011, SimulectTM (basiliximab), TysabriTM (natalizumab), BMS-986016, REGN3767, LAG525, XolairTM (omalizumab), Tavolimab, PF-04518600, BMS-986178, MOXR-0916, GSK-3174998, INCAGN01949, IBI-101, KeytrudaTM (Pembrolizumab), OpdivoTM (Nivolumab), LartruvoTM (Olaratumab), TencentriqTM (Atezolizumab), BMS-936559, BavencioTM (Avelumab), ImfinziTM (Duralumab), ProliaTM (Denosumab), EmplicitiTM (Elotuzumab), MTIG7192A, TSR-022, MBG-453, RemicadeTM (infiximab), HumiraTM (adalimumab), AvastinTM (bevacizumab), LucentisTM (ranibizumab), CyramzaTM (Ramucirumab), and JNJ-61610588.

22. The conjugate of biomolecule according to claim 21, wherein:

the mutation position in IL2 is selected from the group consisting of: Lys32, Lys35, Thr37, Met39, Thr41, Lys43, Tyr45, Lys48, Lys49, Lys64, Leu72, Ala73, Ser75, Lys76, Leu94, Thr101, Thr102, Tyr107, Ala108, Thr111, Ala112, Leu12, His16, Leu19, Met23,

Gly27, Ser75, Arg81, Leu85, Ser87 and Asn88; or

the mutation position in IL10 is selected from the group consisting of: Thr6, Ser8, Ser11, Thr13, Gly17, Arg24, Ser31, Arg32, Lys34, Thr35, Lys40, Leu46, Lys49, Ser51, Lys57, Gly58, Ser66, Tyr72, Lys88, His90, Ser93, Lys99, Thr100, Arg104, Lys117, Ser118, Lys119, Lys125, Lys130, Lys134, Gly135, Tyr137, Tyr149, Thr155, Lys157 and Arg159; or

the mutation position in a heavy chain of the anti-PD-1 antibody Pembrolizumab is selected from the group consisting of: Ser7, Gly8, Gly15, Ala16, Ser17, Ala24, Ser25, Gly26, Tyr27, Thr28, Thr30, Asn31, Tyr32, Tyr33, Tyr35, Ala40, Gly42, Gly44, Leu45, Gly49, Gly50, Ile51, Asn52, Ser54, Asn55, Gly56, Gly57, Thr58, Asn59, Lys63, Lys65, Thr69, Leu70, Thr71, Thr72, Asp73, Ser74, Ser75, Thr76, Thr77, Thr78, Ala79, Leu83, Ser85, Leu86, Thr91, Ala92, Arg99, Asp100, Tyr101, Arg102, Asp104, Gly106, Gly111, Gly113, Thr114, 115Thr, 117Thr, Ser119, Ser120, Ala121, Ser122, Thr123, Lys124, Gly125 and Ser127; the mutation position in a light chain is selected from the group consisting of: Ile2, Thr5, Ser7, Ala9, Thr10, Leu11, Ser12, Leu13, Ser14, Gly16, Ala19, Thr20, Ala25, Ser26, Lys27, Gly28, Ser30, Thr31, Ser32, Gly33, Tyr34, Ser35, Tyr36, Leu37, Gly45, Ala47, Leu50, Leu51, Ile52, Tyr53, Leu54, Ala55, Ser56, Tyr57, Leu58, Ser60, Gly61, Ala64, Ser67, Gly68, Ser69, Gly70, Ser71, Gly72, Thr73, Ala76, Thr78, Ser80, Ser81, Ser95, Arg96, Asp97, Leu98, Leu100, Thr101, Phe102, Gly104, Ile110, Lys111 and K130;; or

the mutation position in a heavy chain of the anti-PD-1 antibody Nivolumab is selected from the group consisting of: Gln3, Ser7, Gly8, Gly9, Gly10, Gly15, Ser17, Lys23, Ala24, Ser25, Gly26, Ile27, Asn31, Thr28, Ser30, Ser32, Gly33, Ala40, Gly42, Gly44, Leu45, Ala49, Ile51, Tyr53, Asp54, Gly55, Ser56, Lys57, Tyr59, Tyr60, Ala61, Asp62, Ser63, Lys65, Gly66, Thr69, Ile70, Ser71, Arg72, Asp73, Asn74, Ser75, Lys76, Asn77, Thr78, Leu79, Leu81, Ser85, Leu86, Ala88, Thr91, Ala92, Thr98, Asn99, Asp100, Asp101, Tyr102, Gly104, Gly106, Thr107, Leu108, Thr110, Ser112, Ser113, Ala114, Ser115, Thr116, Lys117, Gly118 and Ser120; the mutation position in a light chain is selected from the group consisting of: Ile2, Leu4, Thr5, Ser7, Ala9, Thr10, Leu11, Ser12, Leu13, Ser14, Gly16, Ala19, Thr20, Leu21, Ala25, Ser26, Ser28, Ser30, Ser31, Tyr32, Leu33, Ala34, Tyr36, Gly41, Ala43, Leu46, Leu47, Ile48, Tyr49, Asp50, Ala51, Ser52, Asn53, Arg54, Ala55, Thr56, Gly57, Ile58, Ala60, Arg61, Ser63, Gly64, Ser65, Gly66, Ser67, Gly68, Thr69, Thr72, Leu73, Thr74, Ile75, Ser76, Ser77, Leu78, Ala84, Ser91, Ser92, Asn93, Arg96, Thr97, Phe98, Gly99, Gly101, Thr102, Ile106, Lys107, Thr109, Ala111, Ala112, Ser114, Ile117 and Ser121;

the mutation position in a heavy chain of the anti-CTLA-4 antibody Ipilimumab is selected from the group consisting of: Gln3, Arg19, Leu20, Ser25, Gly26, Phe27, Thr28, Phe29, Ser30, Ser31, Tyr32, Thr33, Met34, His35, Gly44, Phe50, Ile51, Ser52, Tyr53, Asp54,

Gly55, Asn56, Asn57, Lys58, Tyr59, Tyr60, Thr69, Ser71, Arg72, Asp73, Asn74, Ser75, Lys76, Asn77, Thr99, Gly100, Trp101, Leu102, Gly103 and Pro104; the mutation position in a light chain is selected from the group consisting of: Gln6, Arg24, Ala25, Ser26, Gln27, Ser28, Val29, Gly30, Ser31, Ser32, Tyr33, Ile49, Tyr50, Gly51, Ala52, Phe53, Ser54, Arg55, Ala56, Phe53, Ser54, Arg55, Ala56, Thr57, Gly58, Ile59, Pro60, Asp61, Arg62, Ser68, Gly69, Thr70, Gln90, Gln91, Tyr92, Gly93, Ser94, Ser95, Pro96 and Trp 97;

the mutation position in a heavy chain of the anti-TNF α antibody is selected from the group consisting of: Ser7, Gly8, Gly9, Gly10, Leu11, Gly15, Ser17, Leu18, Leu20, Ala24, Ser25, Gly26, Thr28, Asp30, Asp31, Tyr32, Ala33, Ala40, Gly42, Gly44, Leu45, Ser49, Ala50, Ile51, Thr52, Asn54, Ser55, Gly56, Ile58, Asp59, Tyr60, Ala61, Asp62, Ser63, Glu65, Gly66, Phe68, Thr69, Ile70, Ser71, Asp73, Asn74, Ala75, Lys76, Ser78, Leu79, Tyr80, Leu81, Ser85, Leu86, Ala88, Thr91, Ala92, Lys98, Ser100, Tyr101, Leu102, Ser103, Thr104, Ala105, Ser106, Ser107, Leu108, Asp109, Tyr110, Gly112, Gly114, Thr115, Leu116, Thr118, Ser120, Ser121, Ala122, Ser123 and Thr124; the mutation position in a light chain is selected from the group consisting of: Asp1, Thr5, Ser7, Ser9, Ser10, Leu11, Ser12, Ala13, Ser14, Gly16, Thr20, Ile21, Ala25, Ser26, Gln27, Gly28, Ile29, Arg30, Asn31, Tyr32, Leu33, Ala34, Tyr36, Lys39, Gly41, Lys42, Ala43, Leu48, Leu47, Ile48, Tyr49, Ala50, Ala51, Ser52, Thr53, Leu54, Gln55, Ser56, Gly57, Ser60, Ser63, Gly64, Ser65, Gly66, Ser67, Gly68, Thr69, Asp70, Thr72, Leu73, Thr74, Ile75, Ser76, Ser77, Leu78, Ala84, Thr85, Tyr91, Asn92, Arg93, Ala94, Tyr96, Thr97, Phe98, Gly99, Gly101, Thr102, Ile106, Lys107, Thr109 and Ala111;

the mutation position in a heavy chain of the anti-CD28 antibody is selected from the group consisting of: Ser7, Gly8, Gly15, Ala16, Ser17, Ser21, Ala24, Ser25, Gly26, Tyr27, Thr28, Thr30, Ser31, Tyr32, Ala40, Gly42, Gly44, Gly49, Tyr52, Gly54, Thr58, Ala68, Thr69, Thr71, Thr74, Ser75, Ser77, Thr78, Ala79, Ser84, Leu86, Ser88, Thr91, Ala92, Thr97, Ser99, Tyr101, Gly102, Leu103, Gly113, Thr114, Thr115, Thr117, Ser119, Ser120, Ala121, Ser122 and Thr123; the mutation position in a light chain is selected from the group consisting of: Thr5, Ser7, Ser9, Ser10, Ser11, Ser12, Ala13, Ser14, Gly16, Thr20, Thr22, Ala25, Ser26, Ser27, Ile29, Tyr30, Ala43, Leu46, Leu47, Tyr49, Lys50, Ala51, Ser52, Leu54, Thr56, Gly57, Ser60, Ser63, Gly64, Ser65, Gly66, Ser67, Gly68, Thr69, Asp70, Thr72, Thr74, Ser76, Ser77, Ala84, Thr85, Gly91, Thr93, Tyr94, Tyr96, Thr97, Phe98, Gly99, Gly100, Gly101, Thr102, Thr109 and Ala111;

the mutation position in a heavy chain of the anti-4-1BB antibody is selected from the group consisting of: Thr31, Tyr32, Ser35, Lys50, Tyr52, Asp55, Ser56, Tyr57, Thr58, Asn59, Tyr60, Ser61, Gln65, Gly66, Gly99, Tyr100, Gly101, Asp104 and Tyr105; the mutation position in a light chain is selected from the group consisting of: Ser23, Gly24, Asp25, Asn26,

Gly28, Asp29, Gln30, Tyr31, Gln49, Asp50, Lys51, Asn52, Arg53, Ser55, Gly56, Thr89, Tyr90, Thr91, Gly92, Gly94 and Ser95; or

the mutation position in a heavy chain of the anti-Her2 antibody is selected from the group consisting of: Arg19, Lys30, Asp31, Tyr33, Arg50, Tyr62, Asn55, Tyr57, Arg59, Tyr60, Asp62, Lys65, Asp102 and Tyr105; the mutation position in a light chain is selected from the group consisting of: Asp1, Gln3, Gln27, Asp28, Asn30, Tyr49, Tyr55, Arg66, Asp70 and Tyr92.

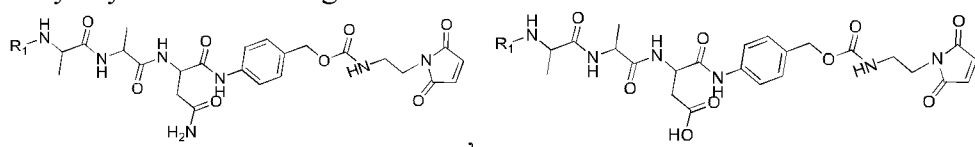
the mutation position in a heavy chain of the anti-PD-L1 antibody Atezolizumab is selected from the group consisting of: Gln3, Asp31, Tyr54, Tyr59, Tyr60, Asp62, Lys65, Asp73, Lys76, Asn77 and Arg99; the mutation position in a light chain is selected from the group consisting of: Arg24, Gln27, Asp28, Tyr49, Tyr55, Asp70, Gln89, Gln90, Tyr91 and Tyr93.

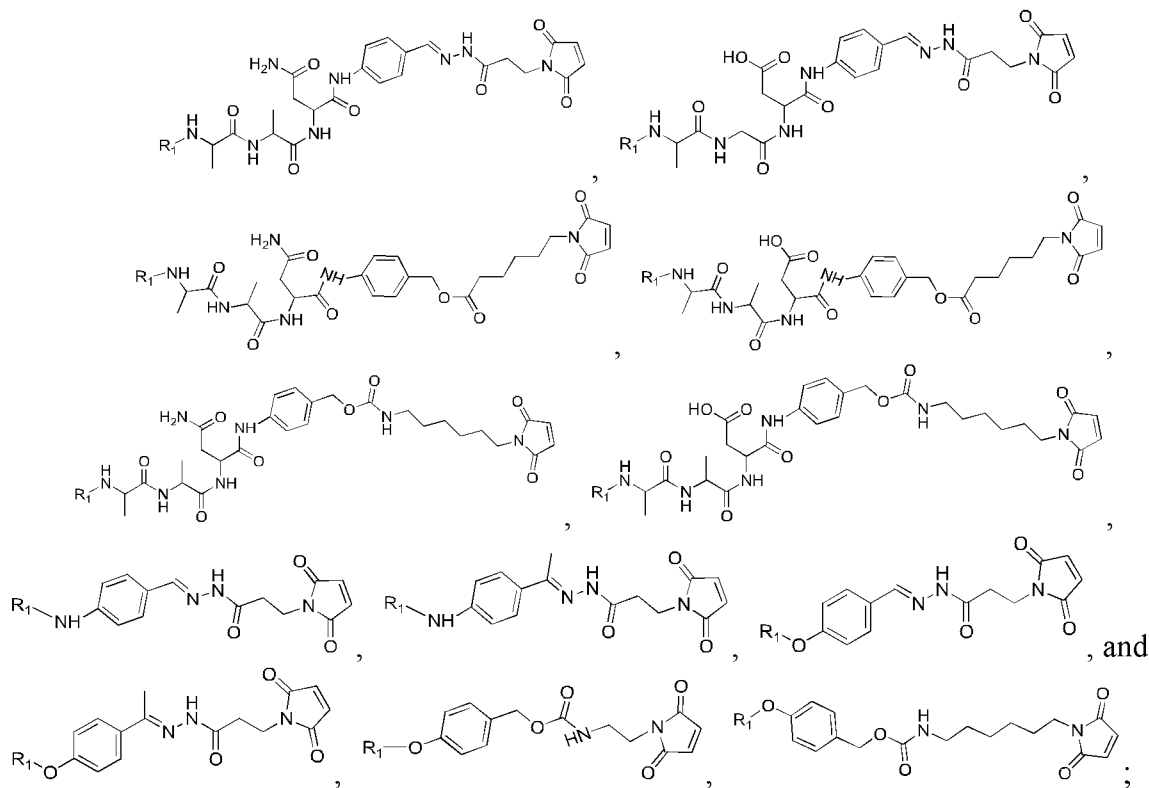
23. The conjugate of biomolecule according to claim 1, wherein R5 is a fusion protein containing one or more amino acid residues being mutated to cysteine;

preferably, the fusion protein containing an antigen binding domain of an antibody and optionally a cytokine; preferably, the fusion protein is a bispecific antibody containing an antigen binding domain to an antigen selected from the group consisting of HER2, CD19, EGFR, CD22, CD3, TROP2, Glycoprotein NMB, Guanylyl cyclase C, CEA, CD79b, PSMA, ENPP3, Mesothelin, CD138, NaPi2b, CD56, CD74, FOLR1, DLL3, CEACAM5, CD142, SLAMF7, CD25, SLTRK6, CD37, CD70, AGS-22, C4.4A, FGFR2, Ly6E, MUC16, BCMA, pCadherin, Ephrin-A, LAMP1, MUC1, CD19, PDL1, HER2, NY-ESO-1, BCMA, WT1, MUC1, CD20, CD23, ROR1, CD123, CD33, CD44v6, CD174, CD30, CD133, cMet, EGFR, FAP, EphA2, GD2, GPC3, IL-13Ra2, LewisY, Mesothelin, SS1, CEA, CD171, EGFR, EGFRvIII, VEGFR2, NY-ESO-1, MUC-1 and MAGE-A3; preferably, the bispecific antibody is a single chain bispecific antibody;

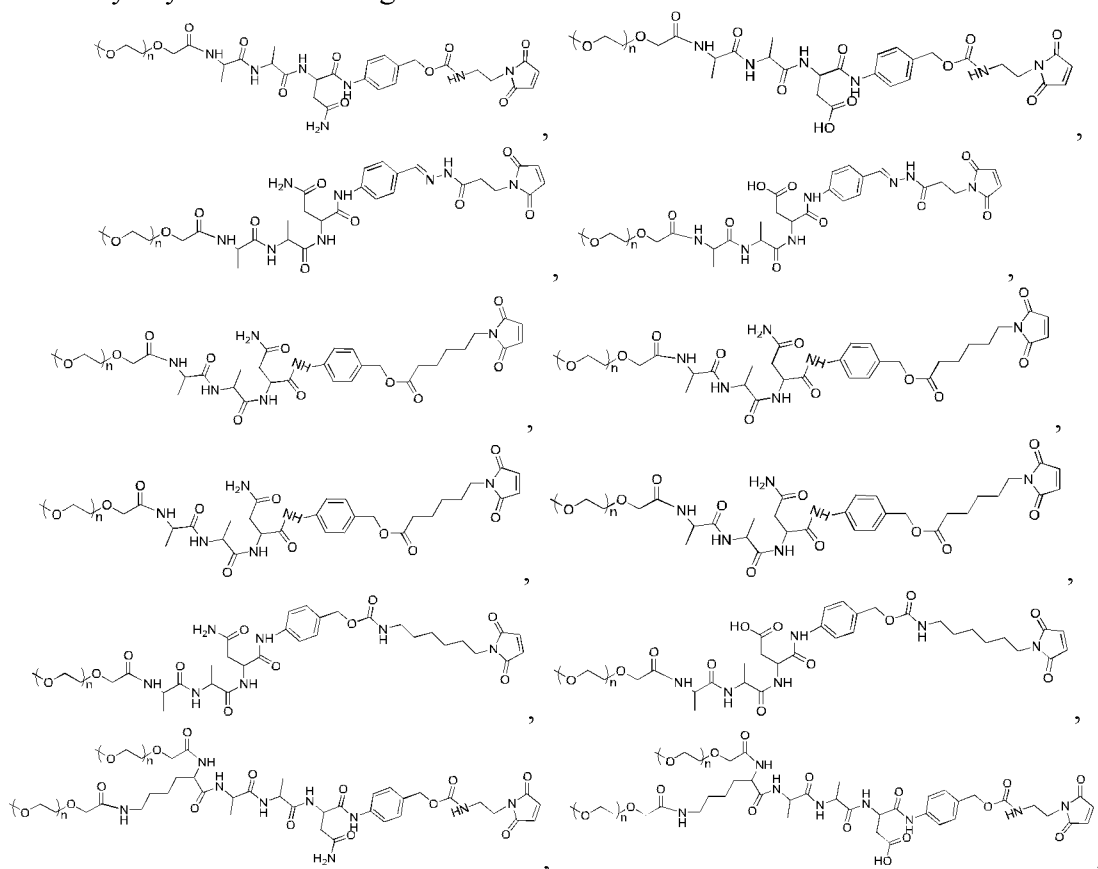
preferably, the fusion protein contains an antigen binding domain of an antibody and a cytokine selected from the group consisting of IL-2, IL-7, IL-10, IL-11, IL-12, IL-15, IL-21, IFN- α , IFN- β , IFN- γ , G-CSF, GM-CSF, TNF- α , TRAP, and TRAIL; preferably, the fusion protein is a fusion protein of anti-PD-1 antibody and IL2.

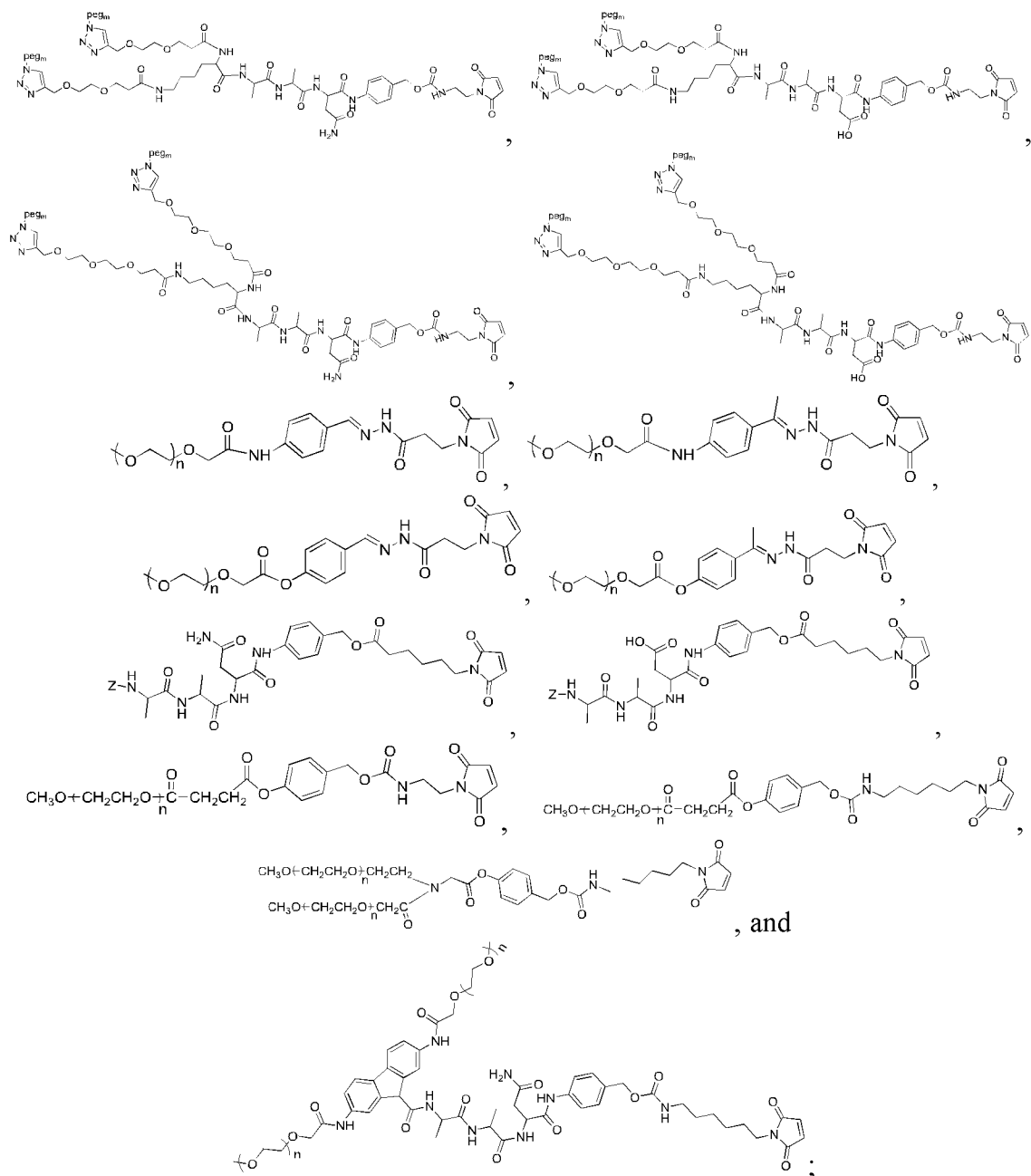
24. The conjugate of biomolecule according to claim 1, wherein R1-R2-R3-R4 is represented by any of the following structures:

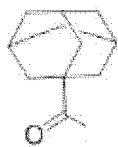




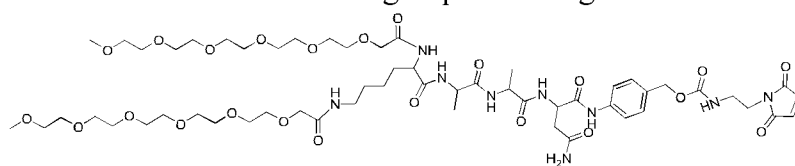
25. The conjugate of biomolecule according to claim 1, wherein R1-R2-R3-R4 is represented by any of the following structures:



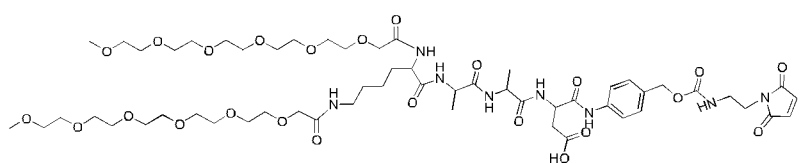




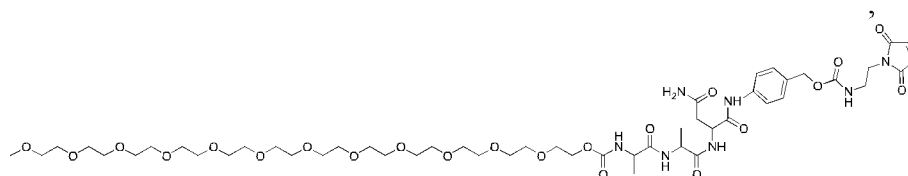
26. The conjugate of biomolecule according to claim 1, wherein in the conjugate of biomolecule, R1-R2-R3-R4 is selected from the group consisting of:



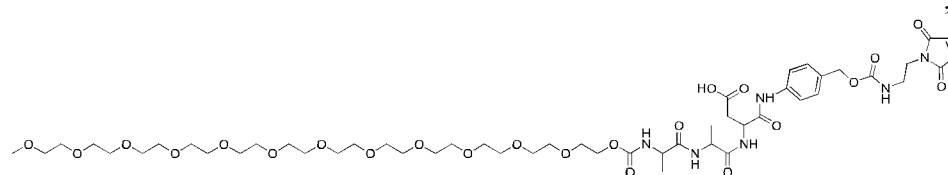
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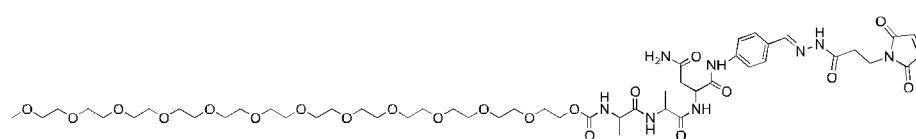
S2



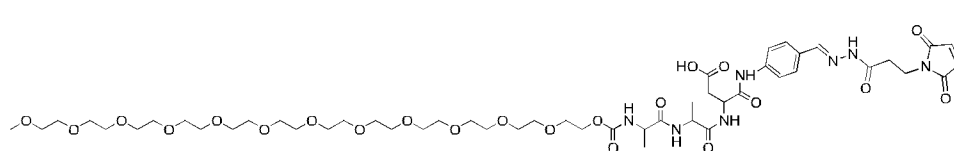
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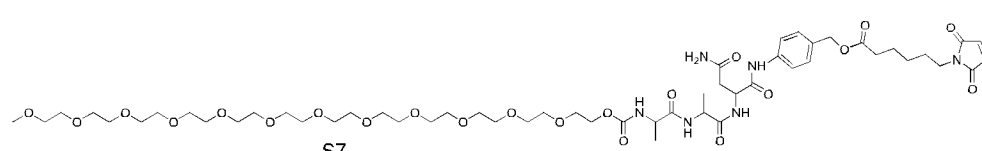
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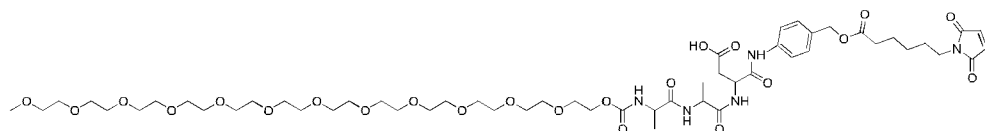
S5



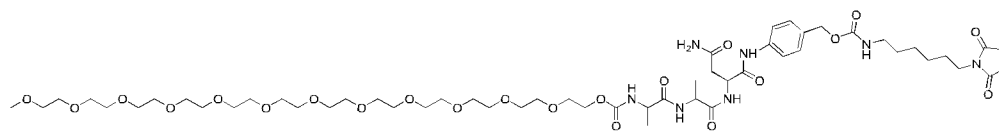
S6



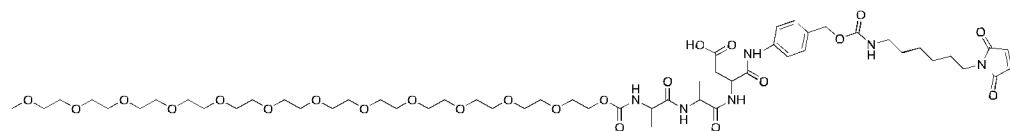
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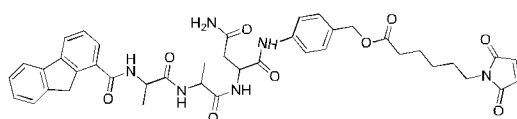
S8



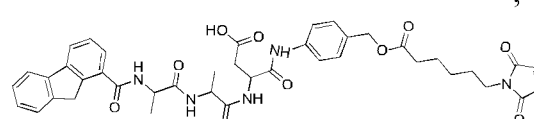
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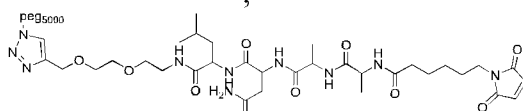
S10



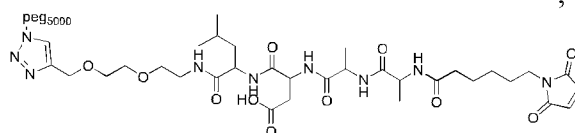
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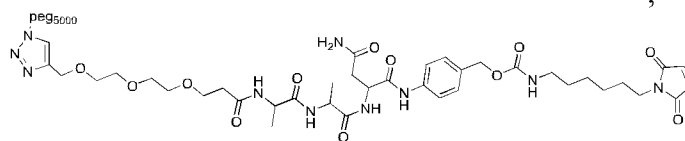
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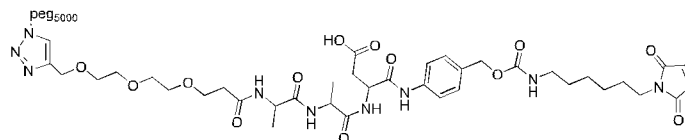
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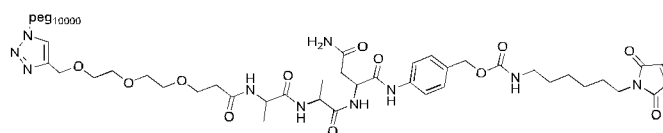
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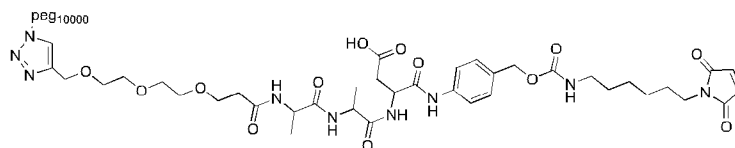
S15



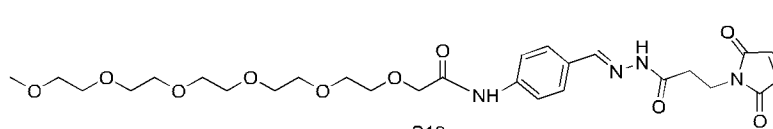
S16



S17



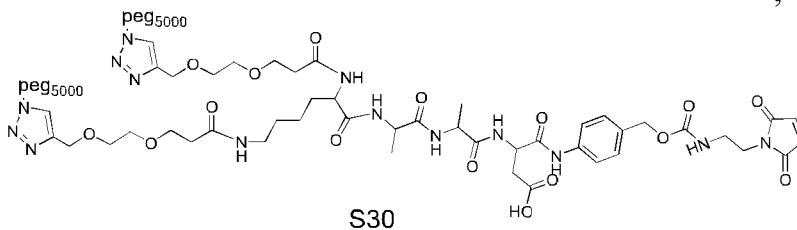
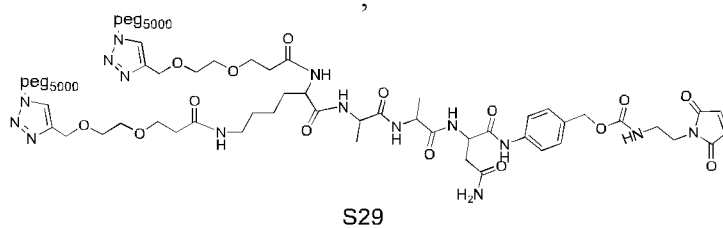
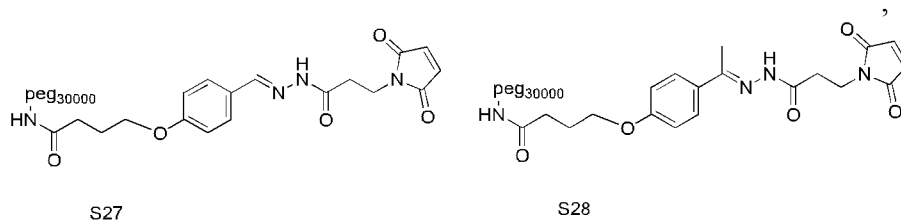
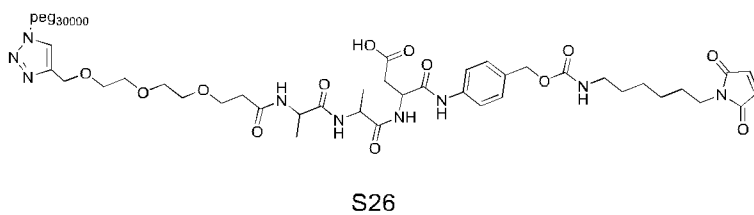
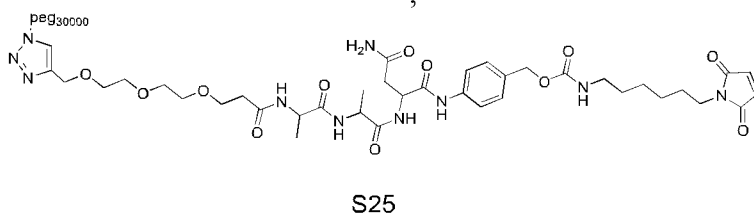
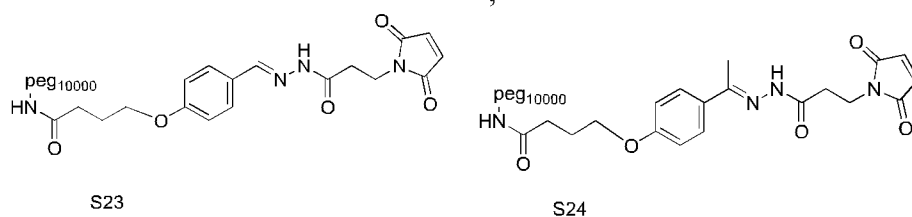
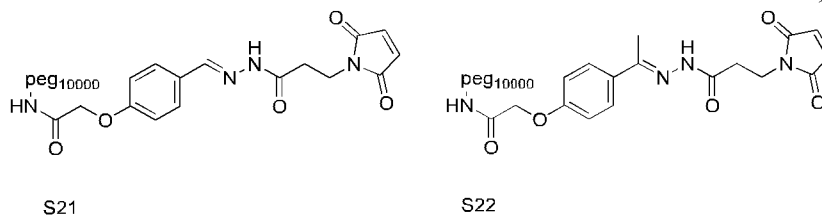
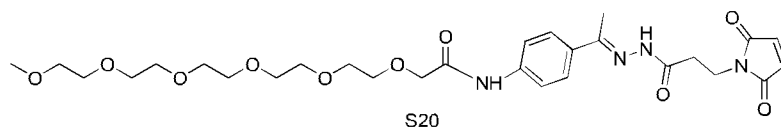
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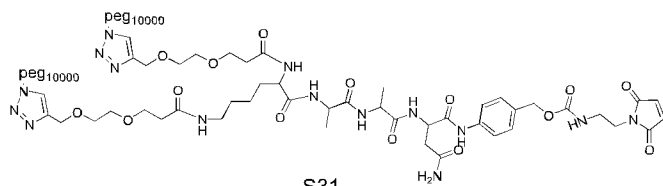


S19

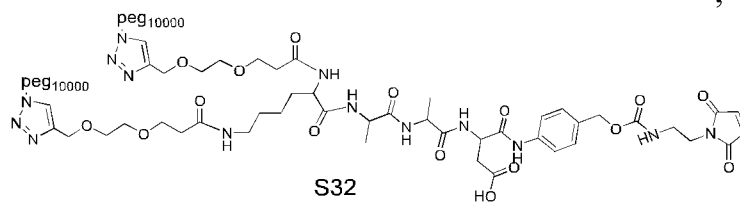
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10

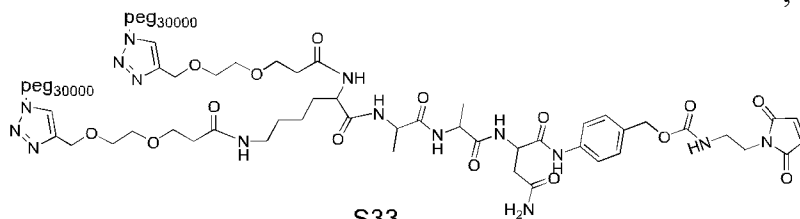




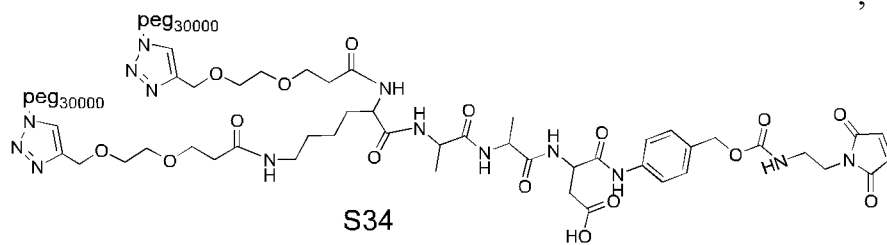
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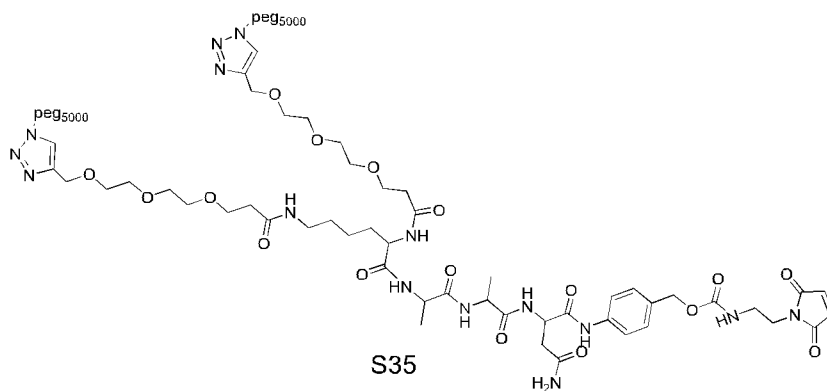
S32



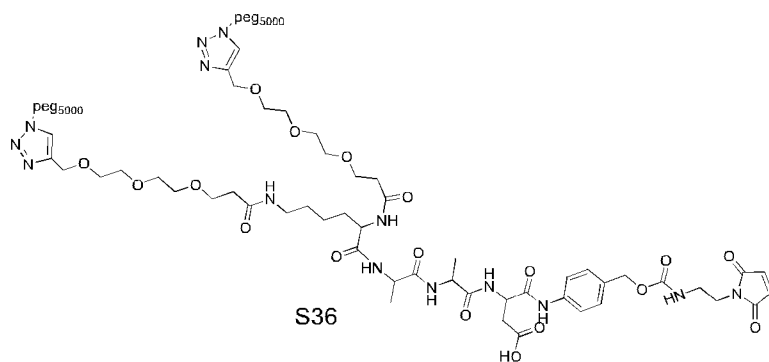
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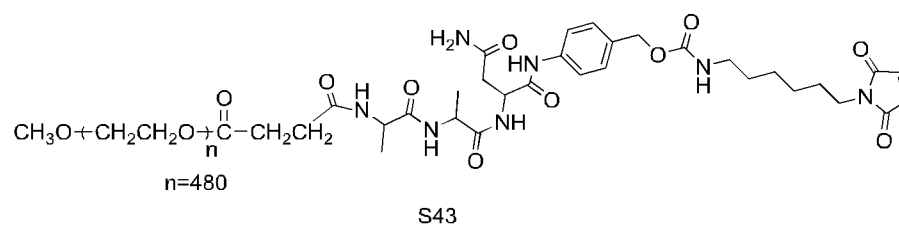
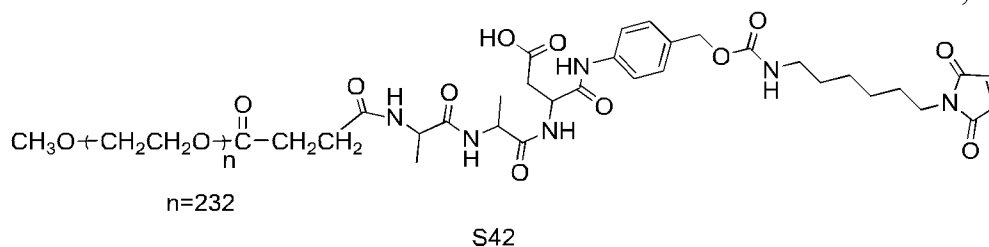
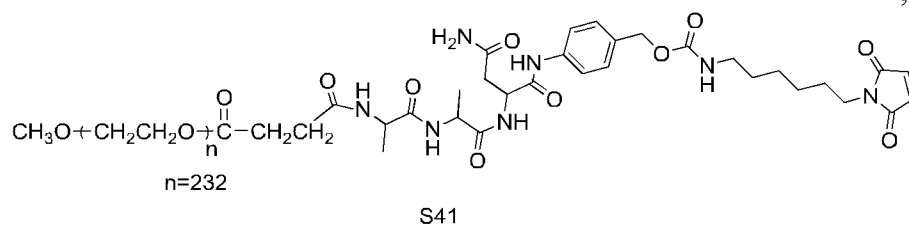
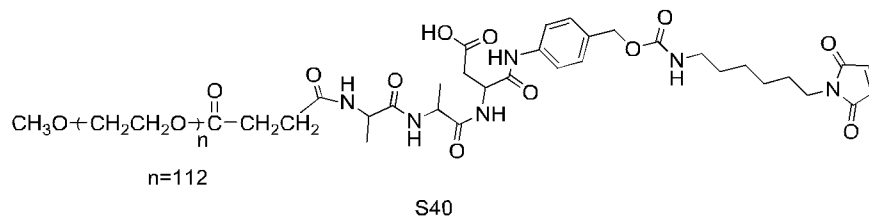
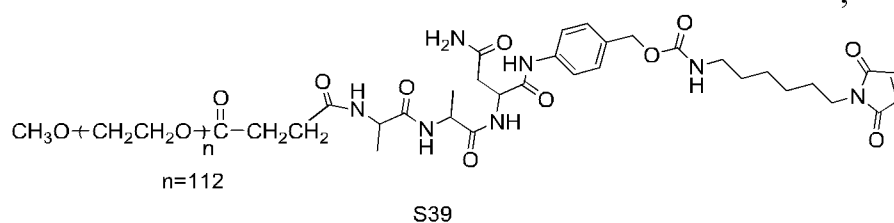
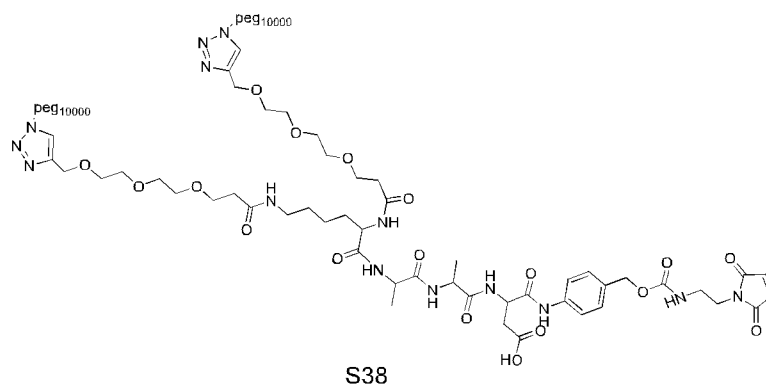
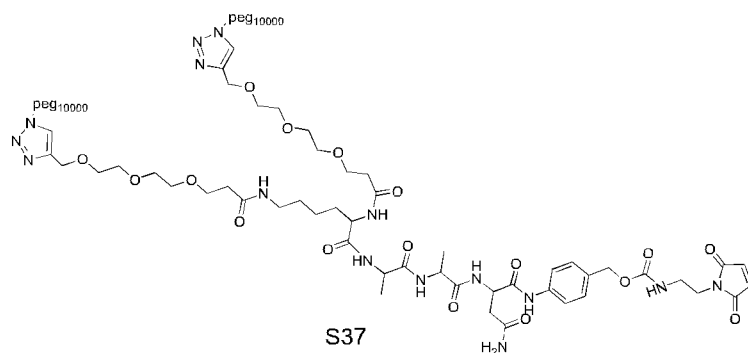
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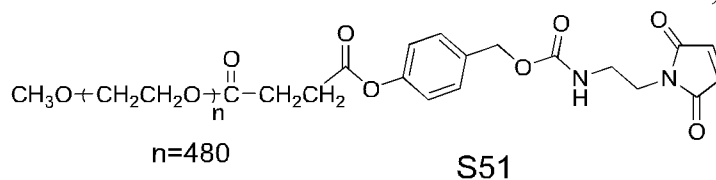
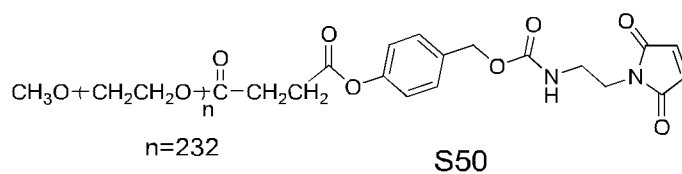
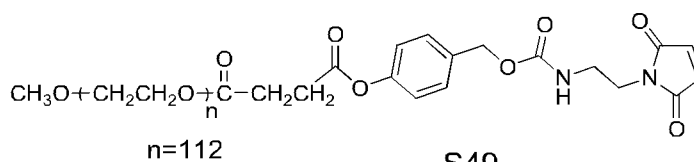
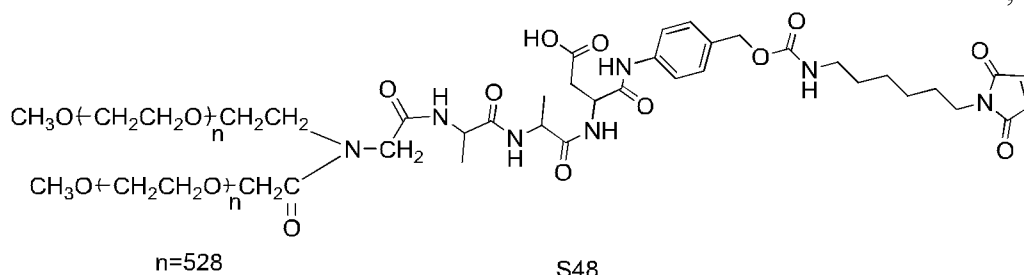
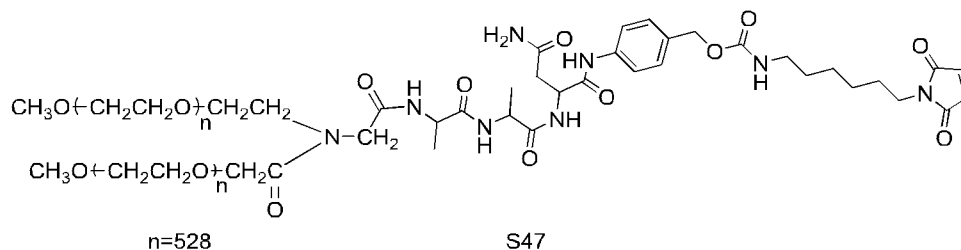
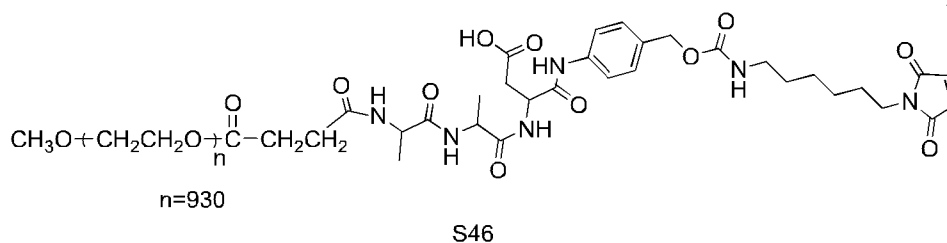
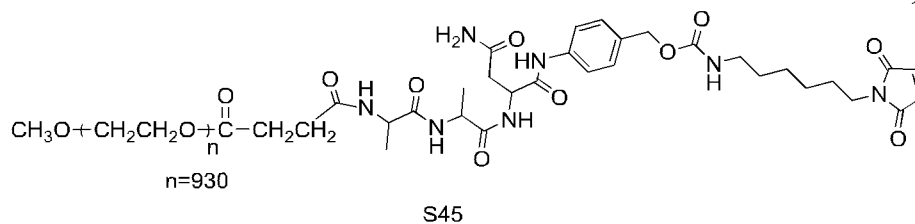
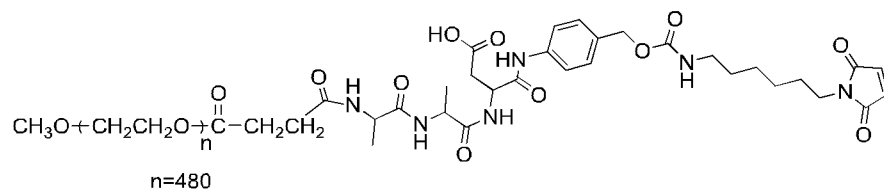


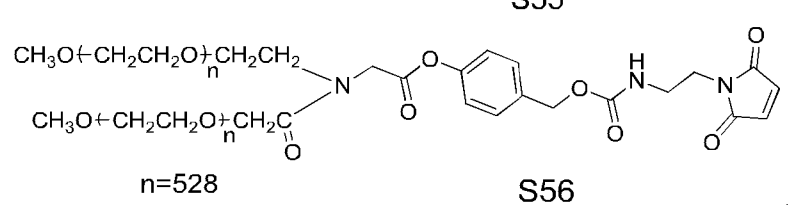
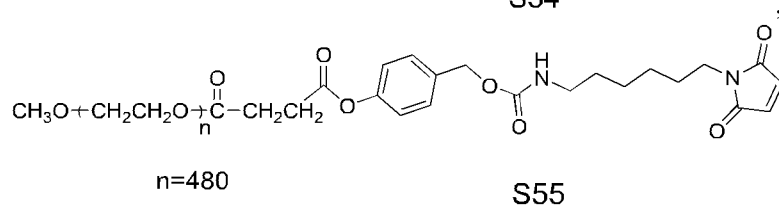
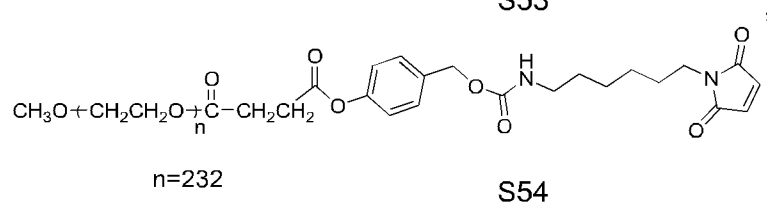
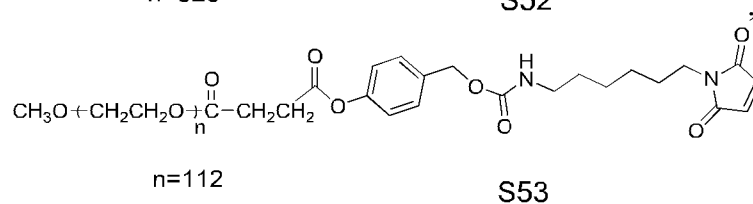
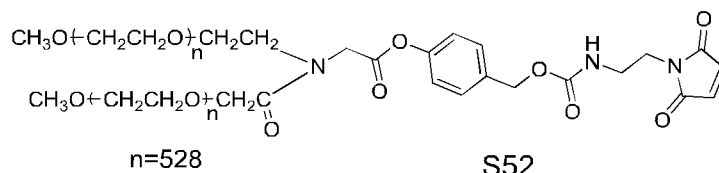
S35



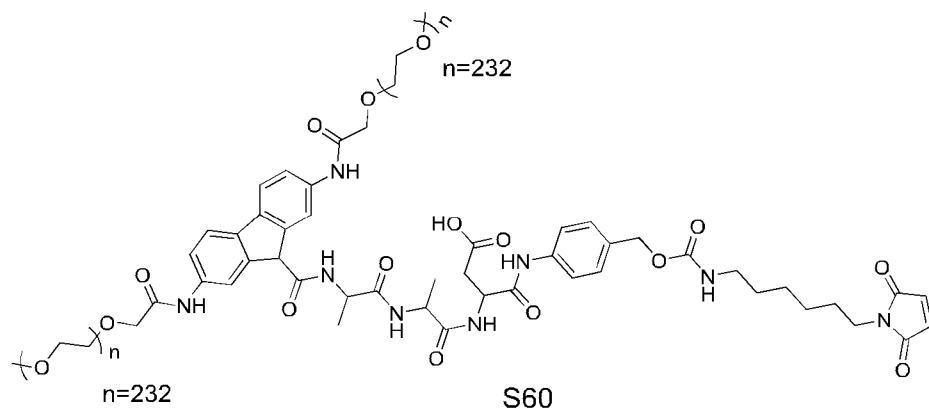
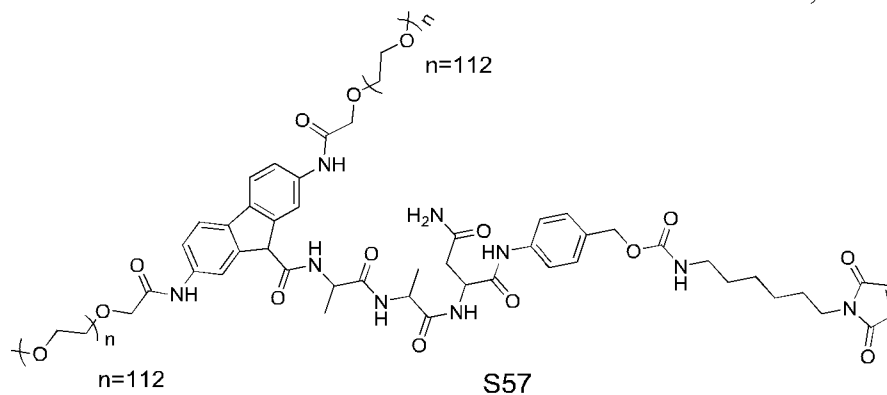
S36

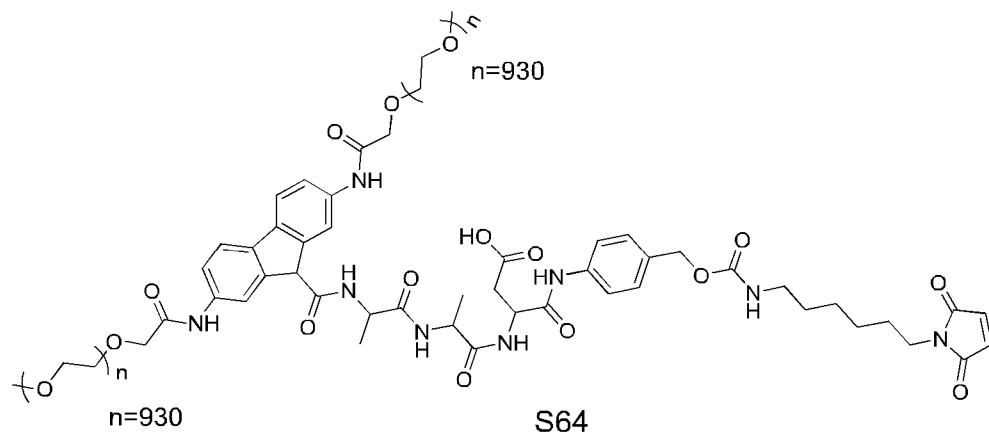
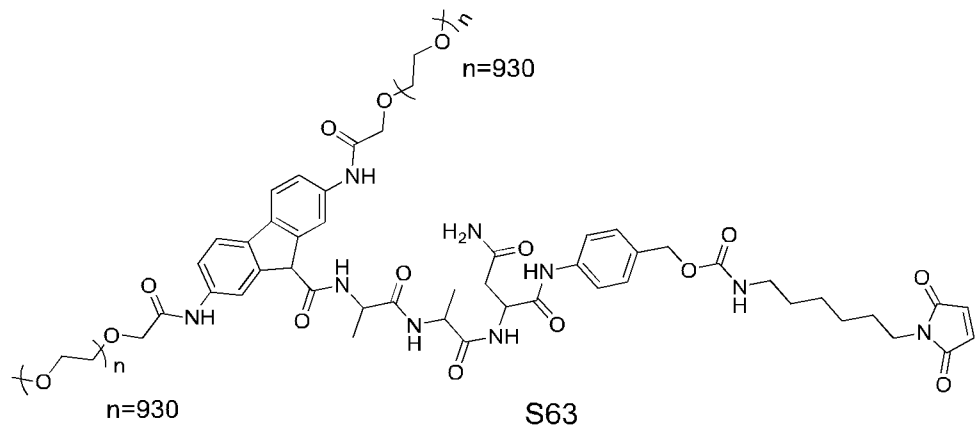
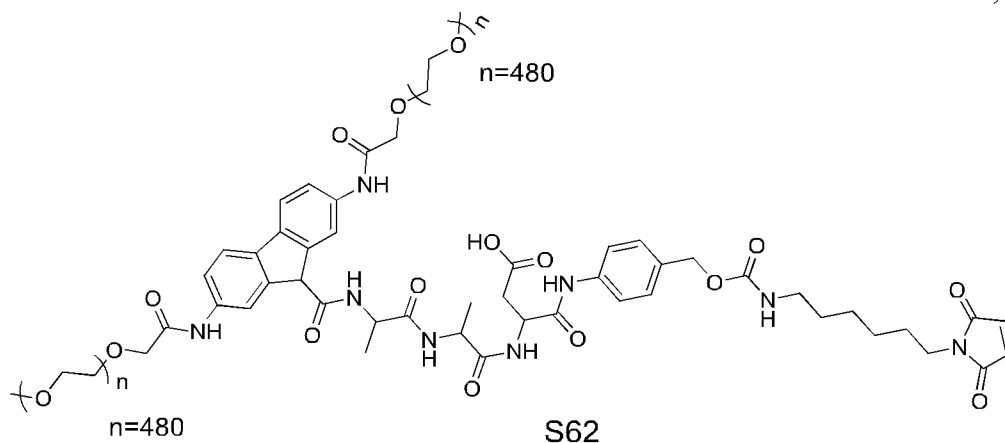
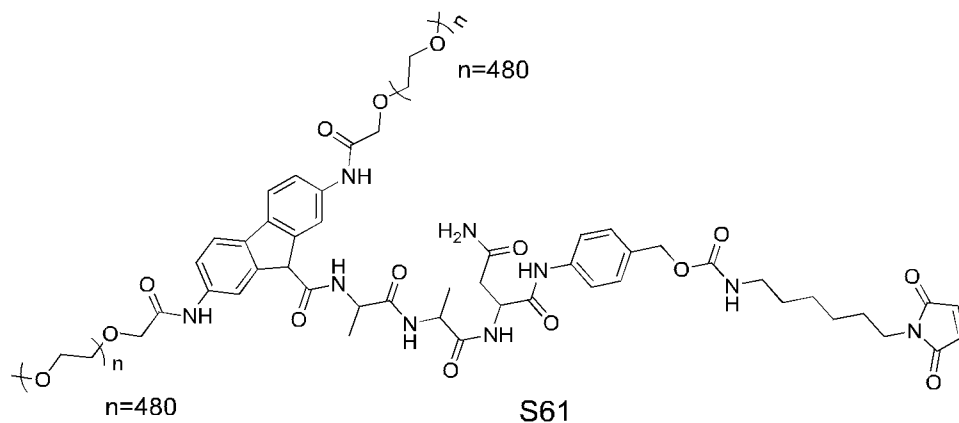






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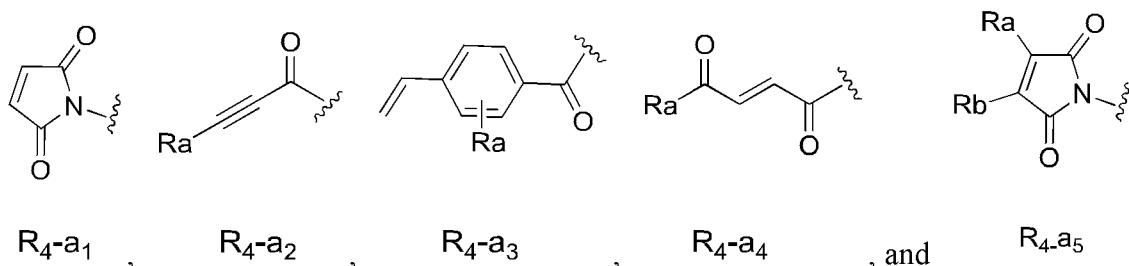
, and

27. A conjugate having the following structure:

R4-S-cys-R5

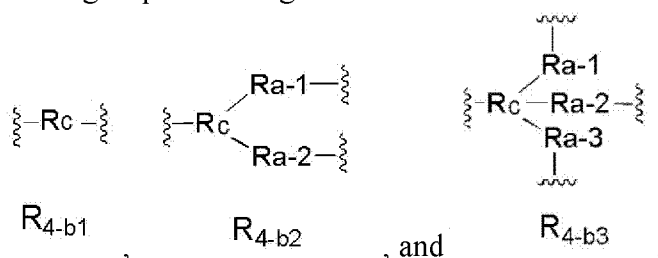
wherein:

- 5 R4 is represented by -R_{4-a}-R_{4-b}-R_{4-c}, wherein R_{4-a} is selected from the group consisting of:



wherein Ra and Rb are each independently selected from the group consisting of H and C₁₋₆ alkyl or C₁₋₆ alkoxy;

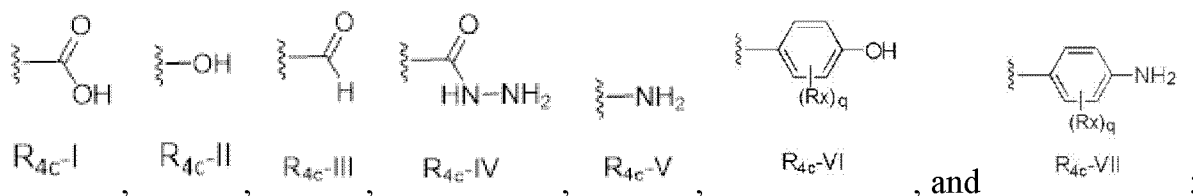
- 10 R_{4-b} is selected from the group consisting of:



wherein in formula R_{4-b1}, R_c is absent, or is selected from the group consisting of C₁₋₁₂ alkyl, C₁₋₁₂alkoxy-C₁₋₁₂alkyl, C₁₋₁₂alkyl-C₃₋₈cycloalkyl, (C₁₋₄alkyl-O)_p-C₁₋₁₂alkyl, C₁₋₁₂alkylcarbonylamino-(C₁₋₄alkyl-O)_p-C₁₋₁₂alkyl, phenyl-C₁₋₁₂alkyl, C₃₋₈cycloalkyl,

- 15 C₁₋₁₂alkyl-C₃₋₈cycloalkyl-C₁₋₁₂alkyl, and C₁₋₁₂alkyl-phenyl-C₁₋₁₂alkyl; in formula R_{4-b2}, R_c is a C₁₋₁₂alkylamino with Ra-1 and Ra-2 substituted at N atom of the amino group, and in formula R_{4-b3}, R_c is a C₁₋₁₂alkyl with the last C atom at the end of the alkyl being substituted by Ra-1, Ra-2 and Ra-3, wherein Ra-1, Ra-2 and Ra-3 are each independently selected from the group consisting of C₁₋₁₂alkyl, C₁₋₁₂alkyl-OH, and C₁₋₁₂alkyl-NR''R''', wherein R'' and R''' are each
- 20 independently selected from the group consisting of H and C₁₋₁₂alkyl; wherein in formulae R_{4-b2} and R_{4-b3}, R_{4-b} links to R_{4-c} via at least one of the Ra-1, Ra-2 and Ra-3;

R_{4-c} is selected from the group consisting of:

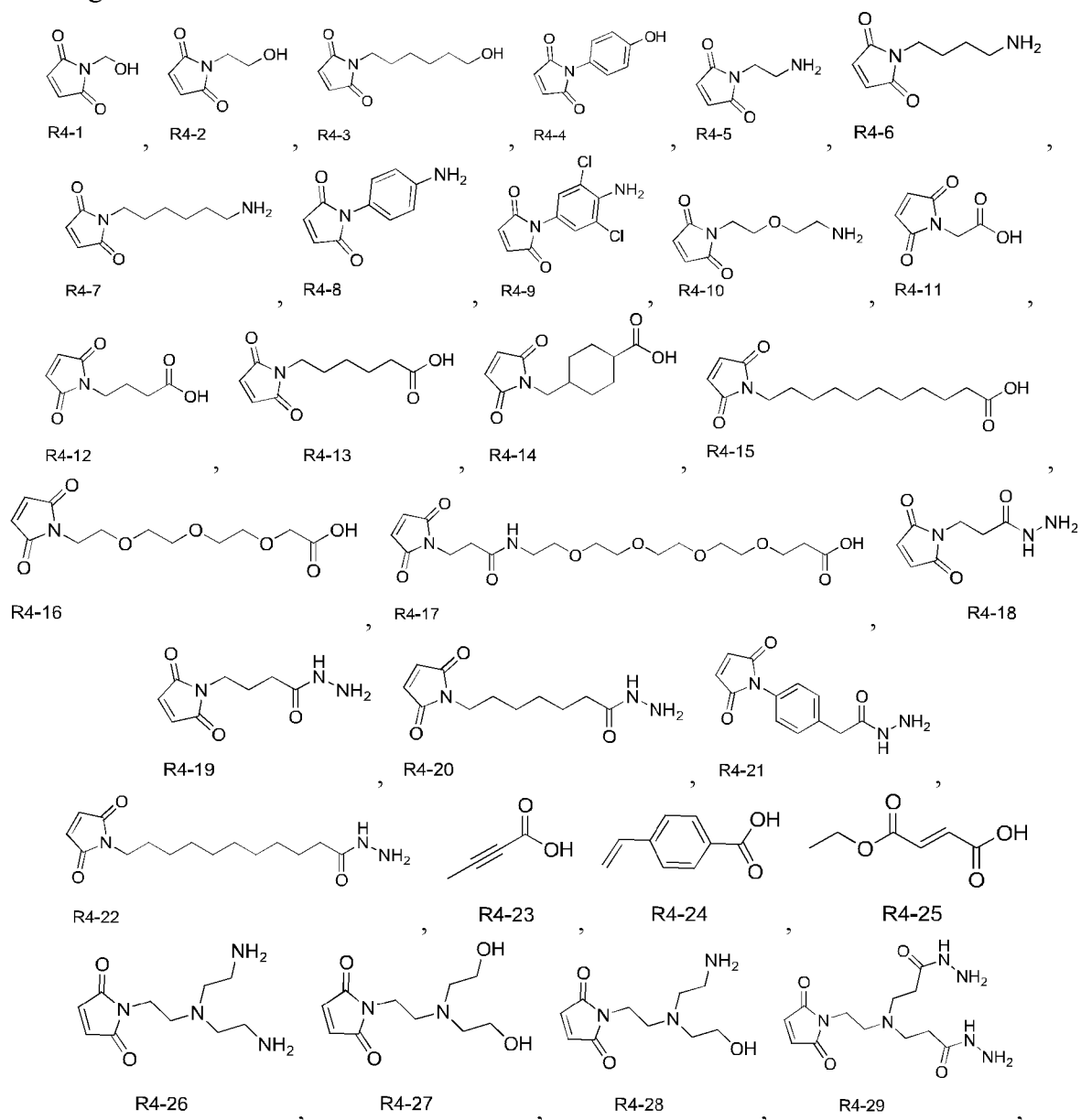


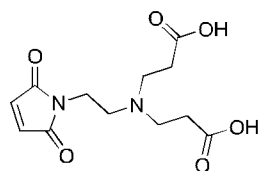
- 25 wherein Rx is selected from the group consisting of H, halo and C₁₋₄alkyl; p is an integer in a

range of 1 to 10, such as an integer in a range of 1 to 5; and q is an integer in a range of 1 to 4, such as an integer in a range of 1 to 2; with the proviso that R4-c is absent when R4-a is selected from the group consisting of formulae R4-a2, R4-a3 and R4-a4;

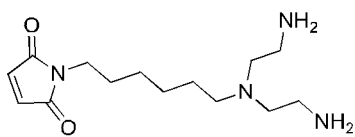
wherein the wave line shown in each formula of R4-a indicates the position at which R4-a links to R4-b, and the wave line shown in each formula of R4-c indicates the position at which R4-c links to R4-b.

28. The compound according to claim 27, wherein R4 is selected from the group consisting of:

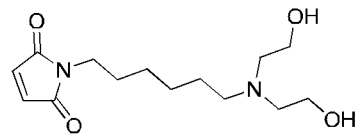




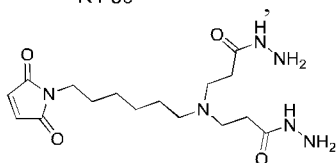
R4-30



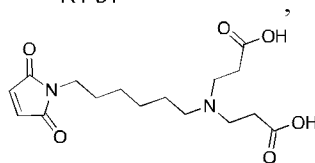
R4-31



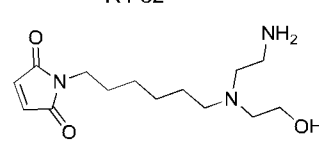
R4-32



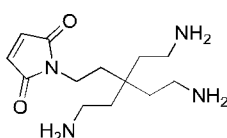
R4-33



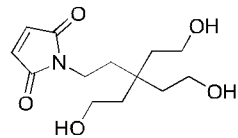
R4-34



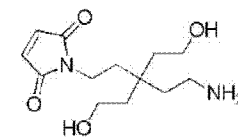
R4-35



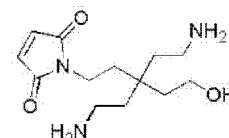
R4-36



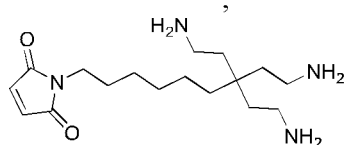
R4-37



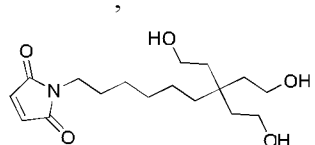
4-38a



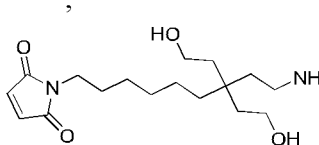
4-38b



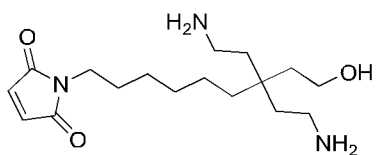
R4-39



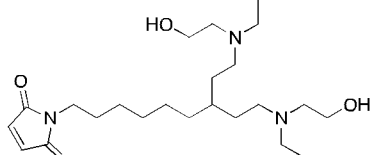
R4-40



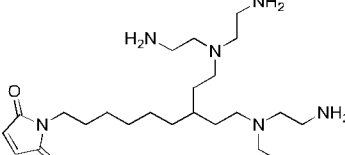
R4-41



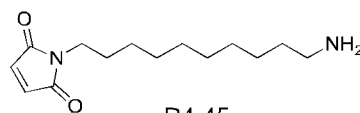
R4-42



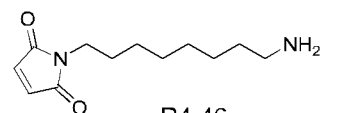
R4-43



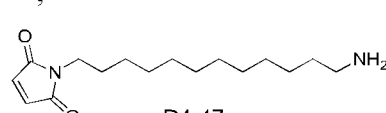
R4-44



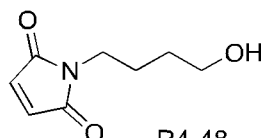
R4-45



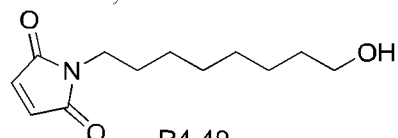
R4-46



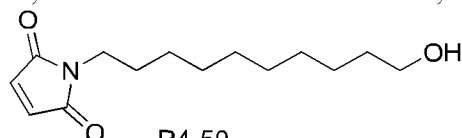
R4-47



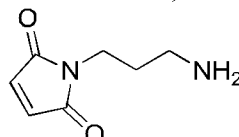
R4-48



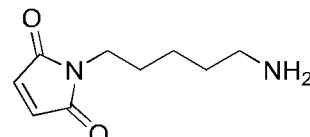
R4-49



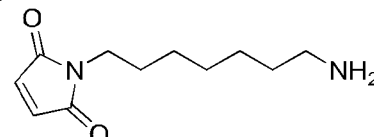
R4-50



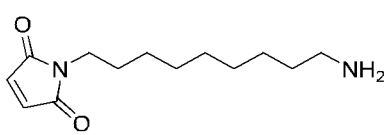
R4-51



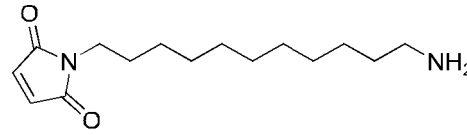
R4-52



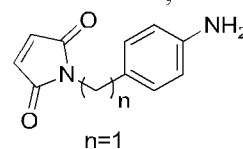
R4-53



R4-54

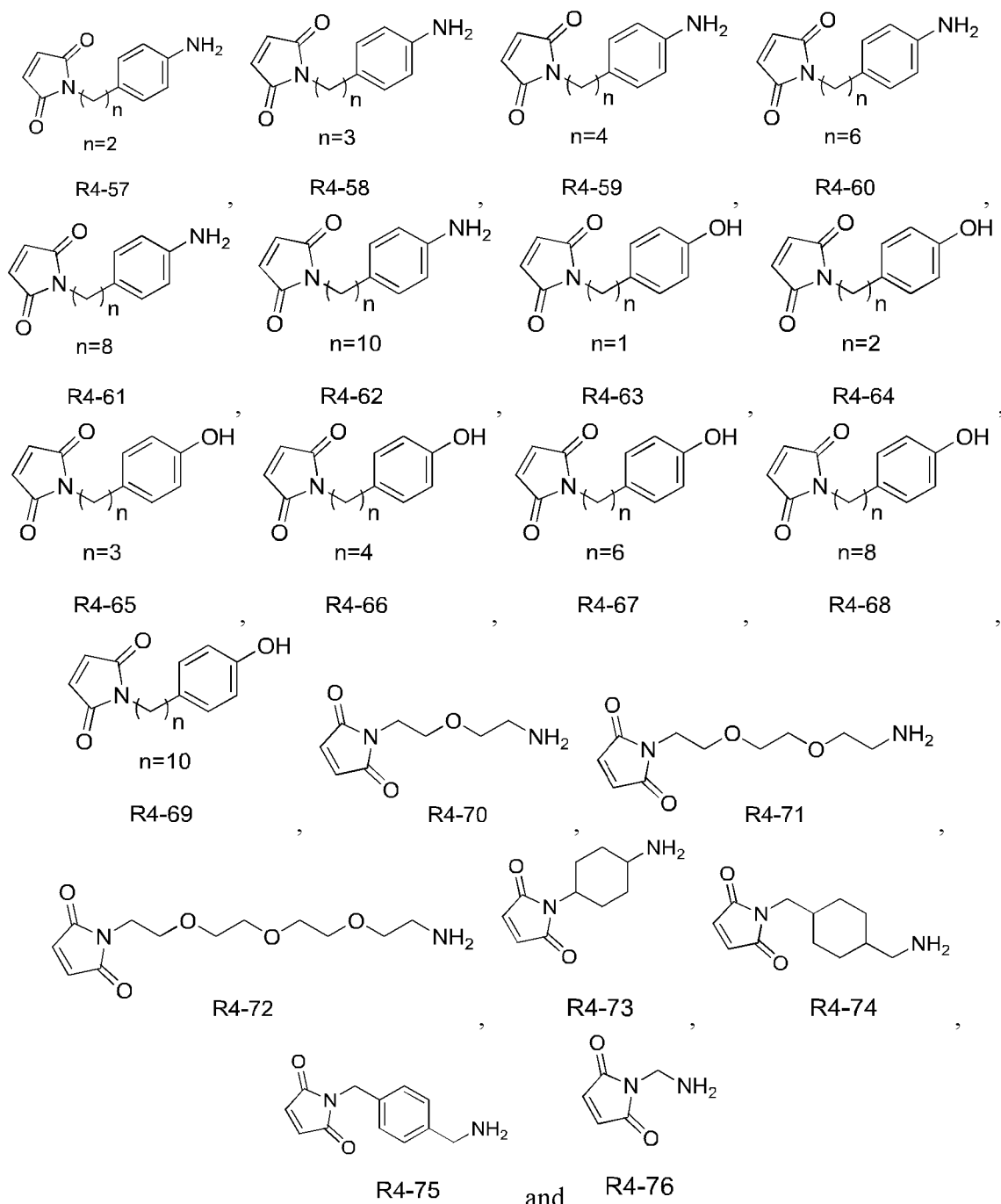


R4-55



R4-56

5



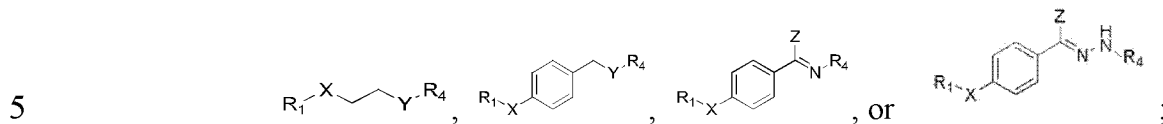
29. A method for treating tumor or autoimmune disease, comprising providing to a subject in need thereof a therapeutically effective amount of the conjugate of biomolecule of any of claims 1-26 or a compound of claim 27 or 28.

30. The method according to claim 29, wherein the tumor is a cancer in bladder, brain, breast, cervix, colon-rectum, esophagus, kidney, liver, lung, nasopharynx, pancreas, prostate, skin, stomach, uterus, ovary, testiculus or blood.

31. A compound represented by R1-R2-R3-R4, wherein R1 is H or is defined in any of

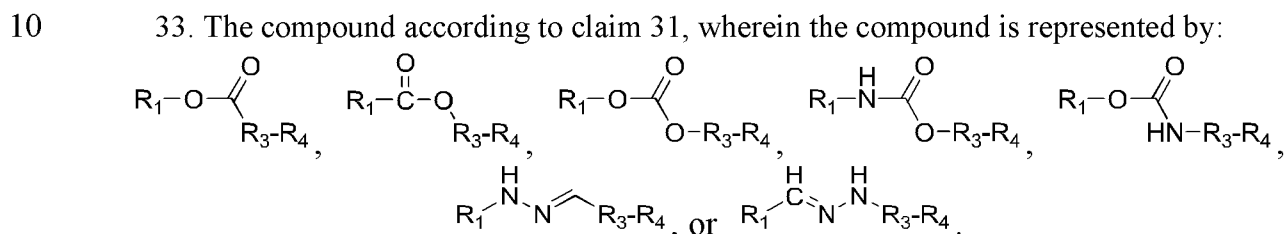
claims 1-3; R₂ is defined in any of claims 1 and 4-7; R₃ is defined in any of claims 1 and 8-11; and R₄ is defined in any of claims 1 and 12-13.

32. The compound according to claim 31, wherein the compound is represented by:

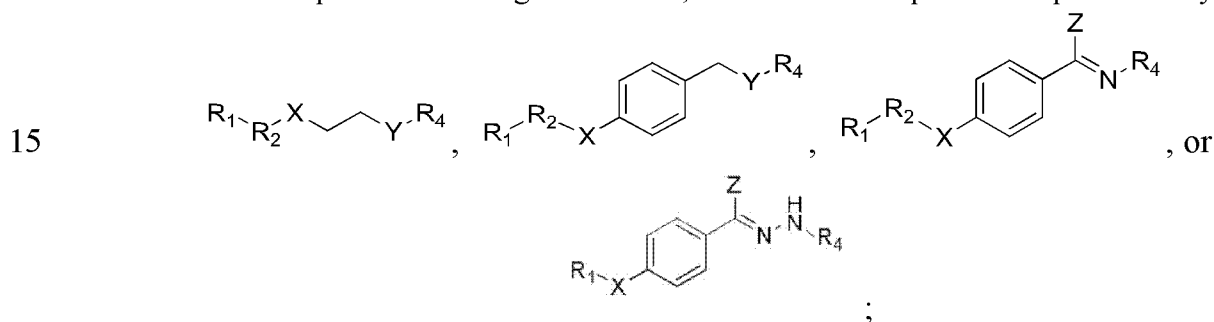


wherein X and Y are each independently NR' or O, Z is H or C₁₋₁₀ alkyl, and R' is H or C₁₋₄alkyl; and wherein R₁ links to X and R₄ links to Y or N respectively in a linkage manner of amide, ester, carbamate, urea or hydrazone bond.

33. The compound according to claim 31, wherein the compound is represented by:

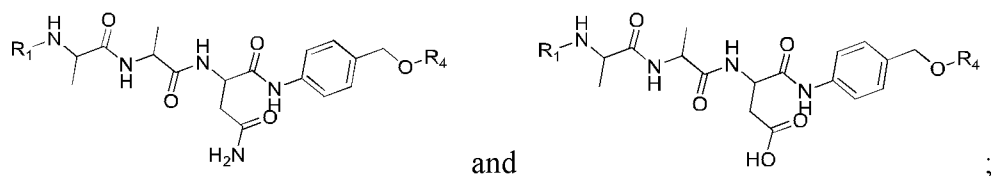


34. The compound according to claim 31, wherein the compound is represented by:



wherein X and Y are each independently NR' or O, Z is H or C₁₋₁₀ alkyl, and R' is H or C₁₋₄alkyl; and wherein R₂ links to X and R₄ links to Y or N respectively in a linkage manner of amide, ester, carbamate, urea or hydrazone bond.

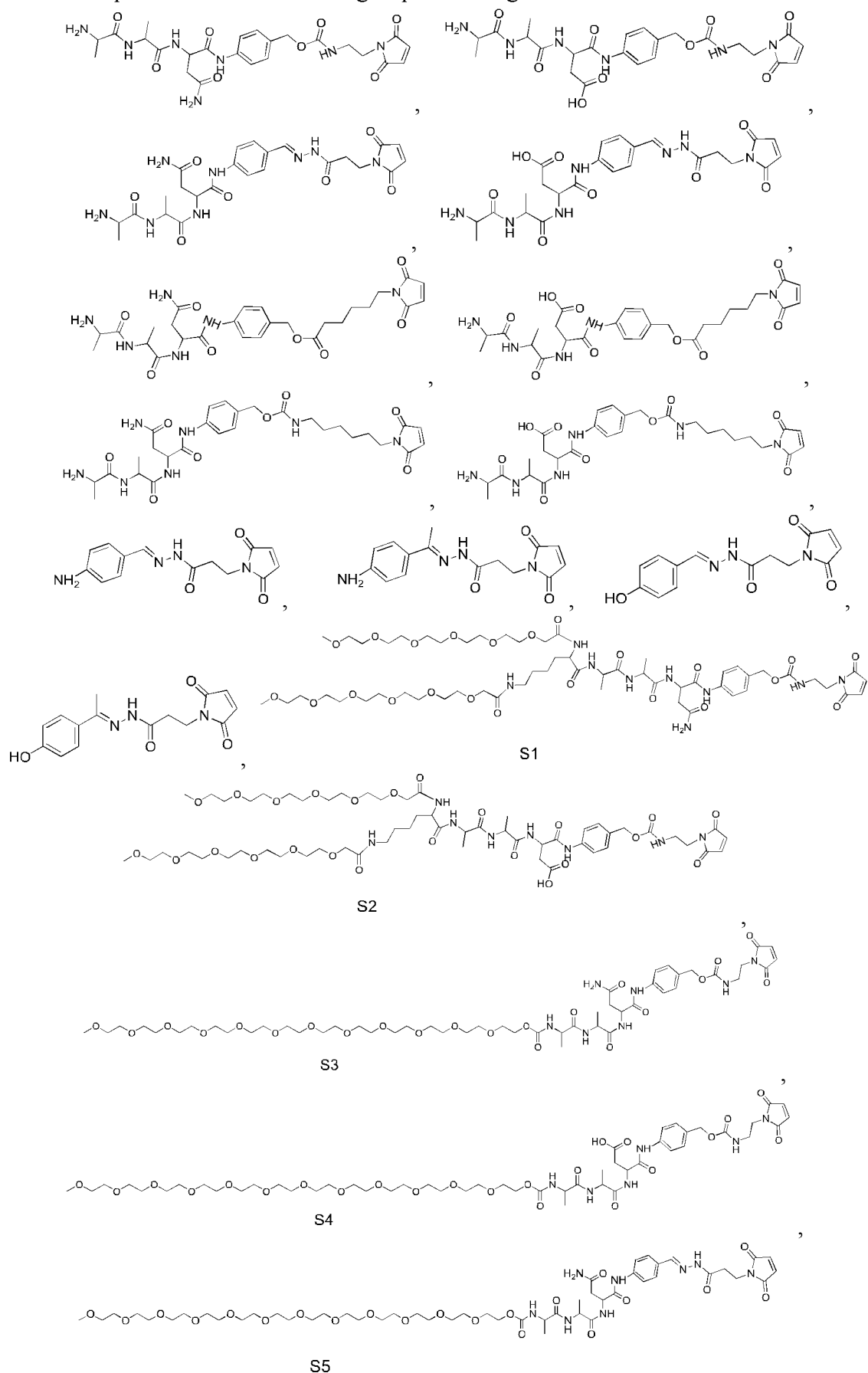
35. A compound selected from the group consisting of:

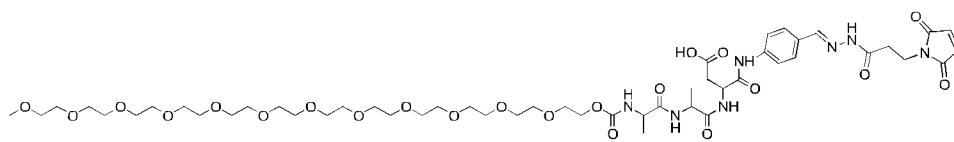


wherein,

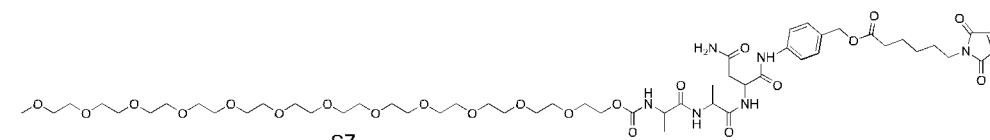
R₁ is H, or as defined in claim 2 or 3; and
R₄ is defined as in any of claims 12-13.

36. A compound selected from the group consisting of:

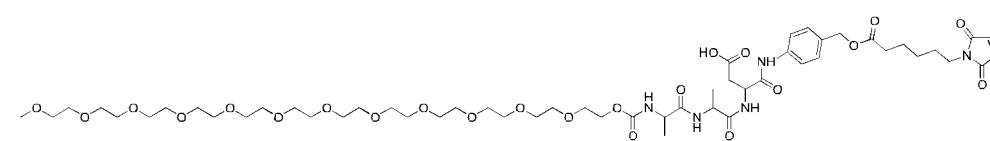




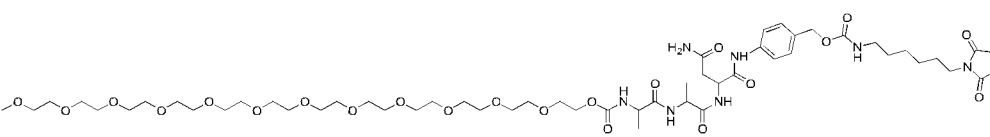
S6



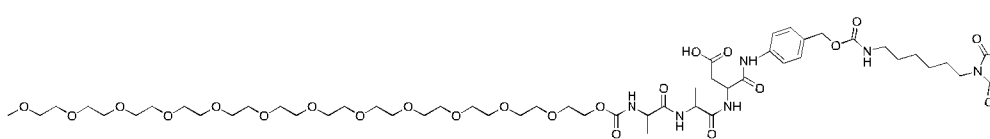
S7



S8

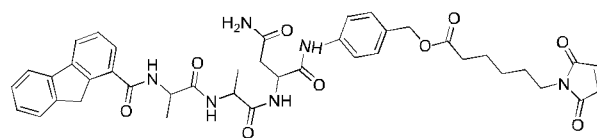


S9

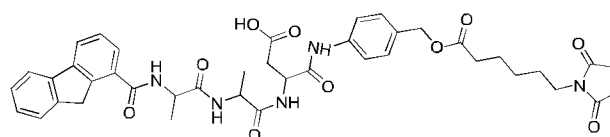


S10

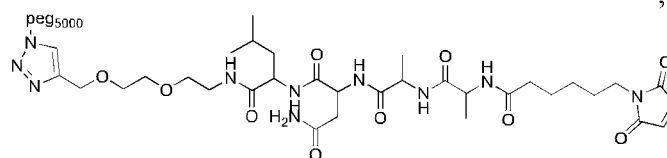
5



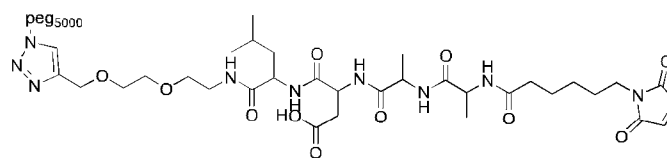
S11



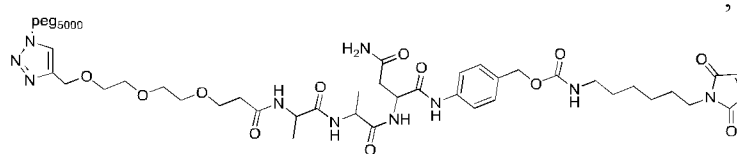
S12



S13

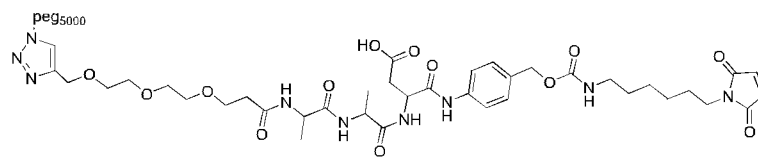


S14

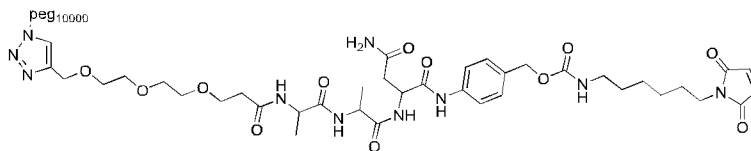


S15

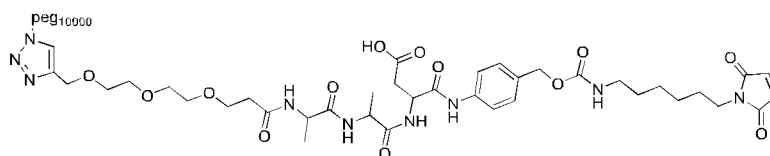
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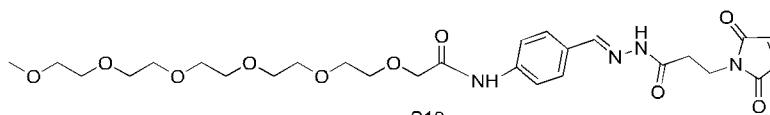
S16



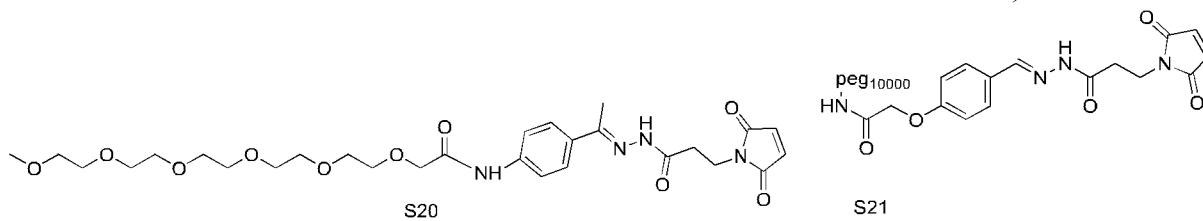
S17



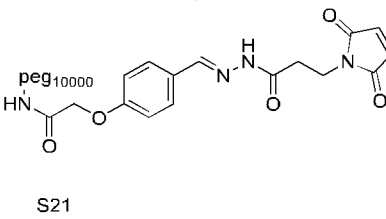
S18



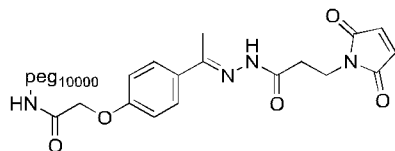
S19



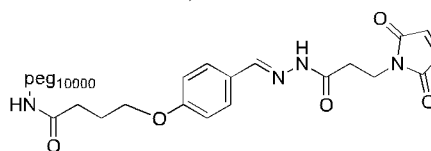
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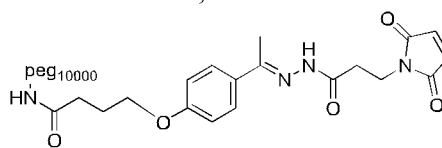
S21



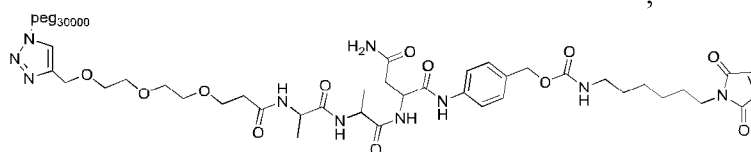
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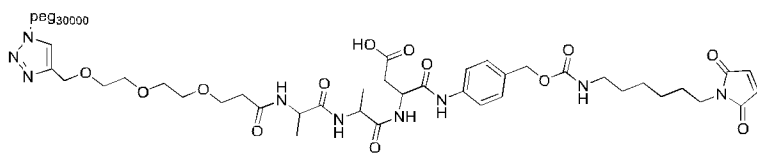
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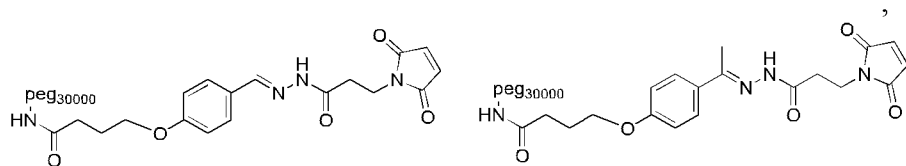
S24



S25

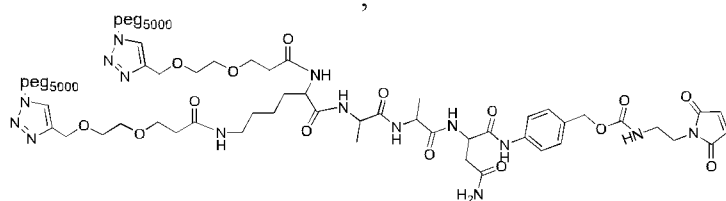


S26

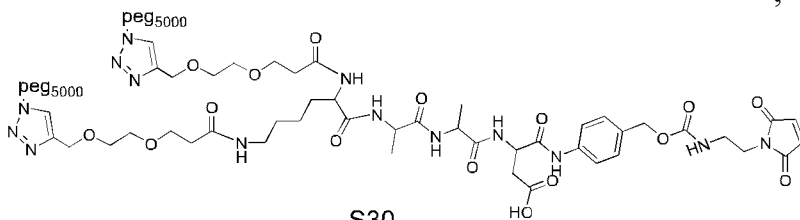


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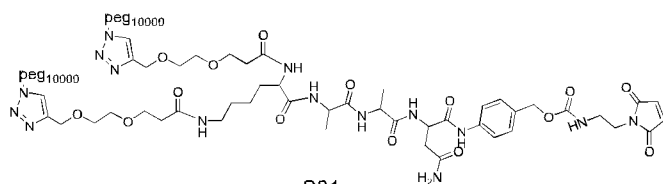
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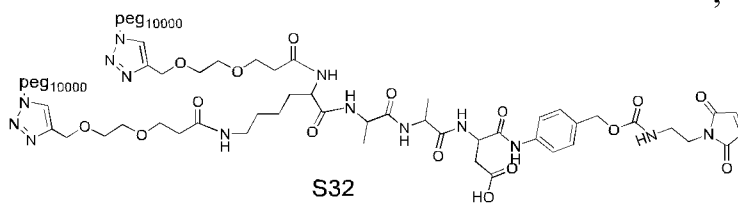
S29



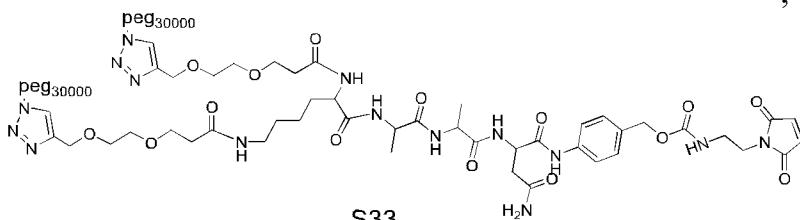
S30



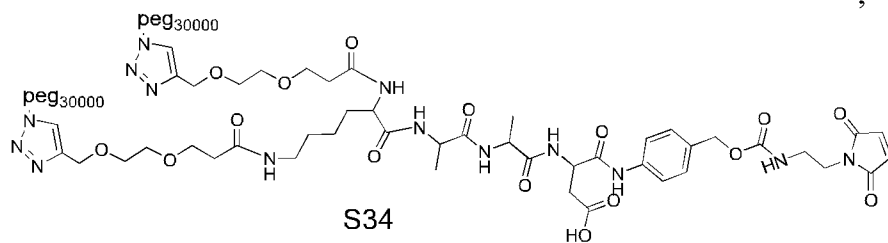
S31



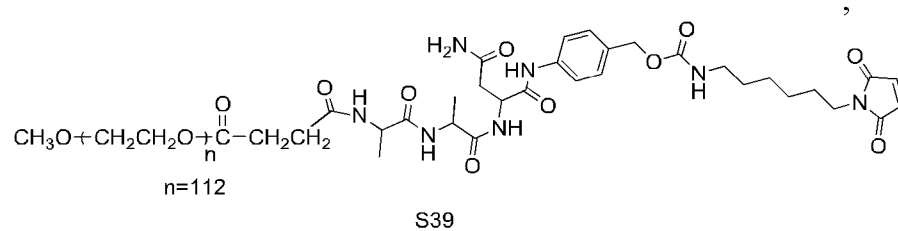
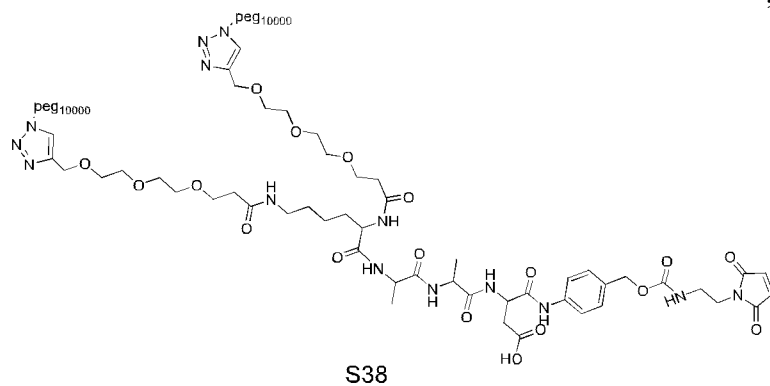
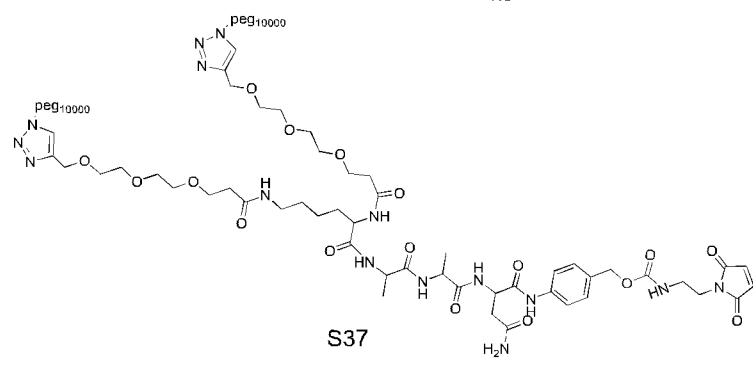
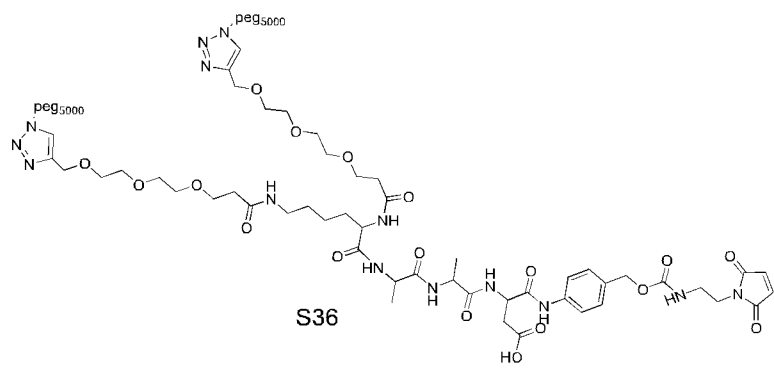
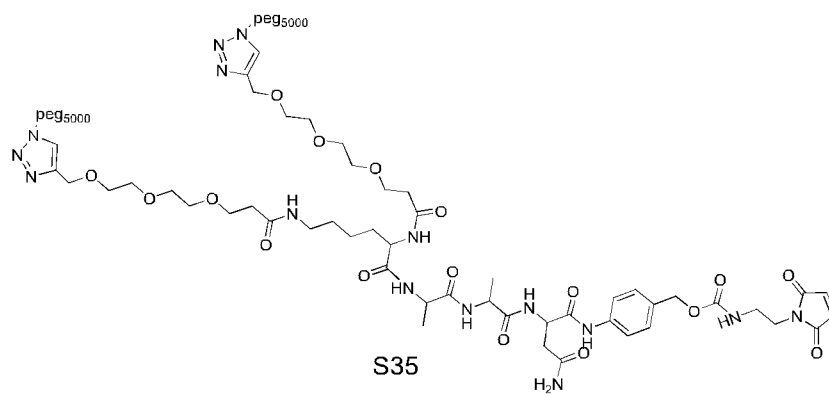
S32

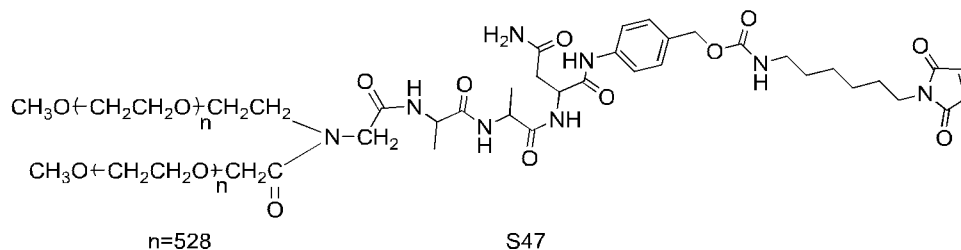
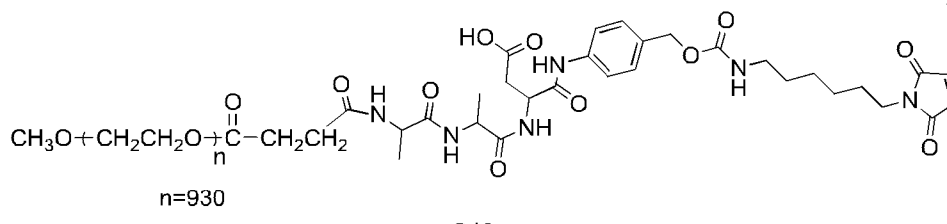
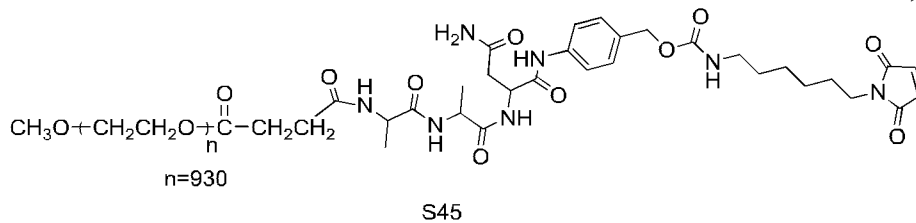
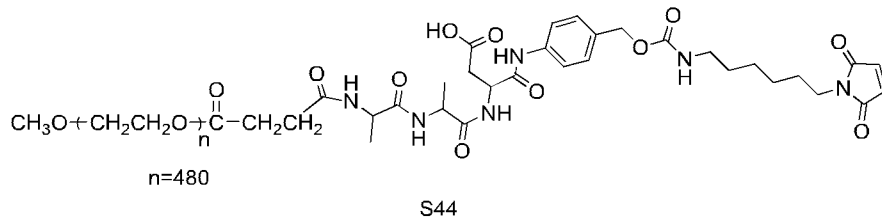
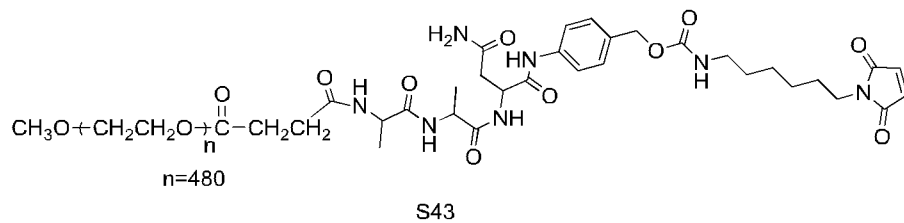
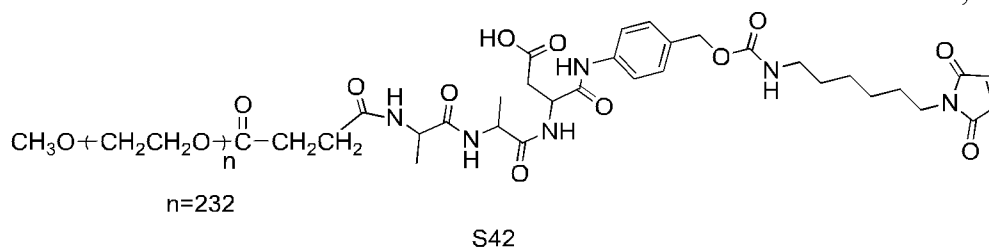
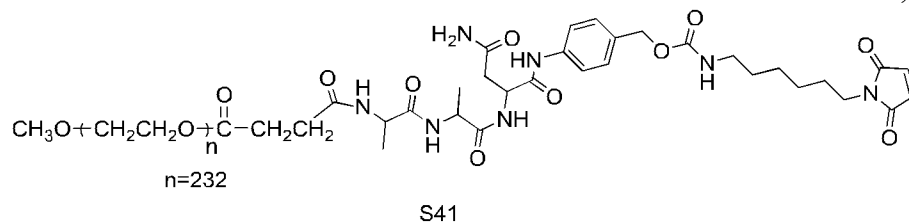
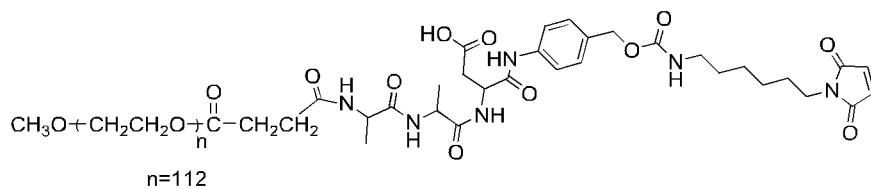


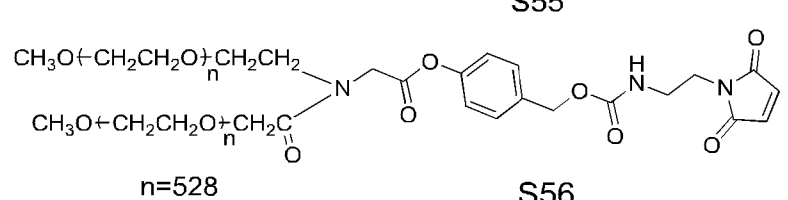
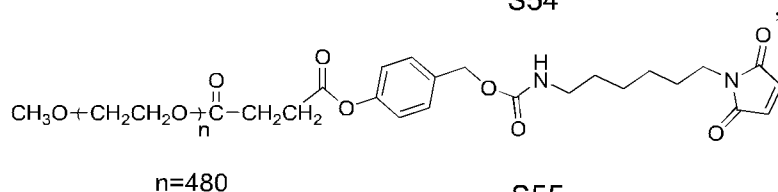
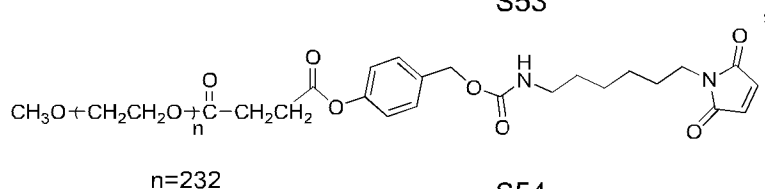
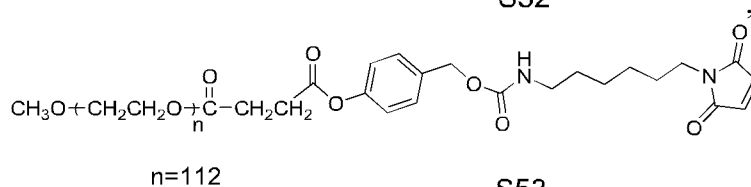
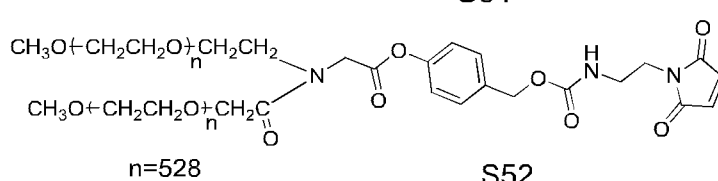
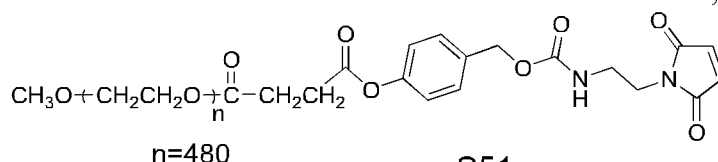
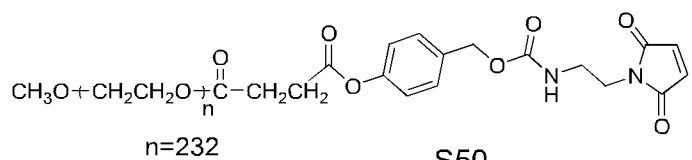
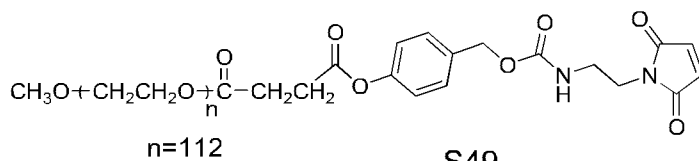
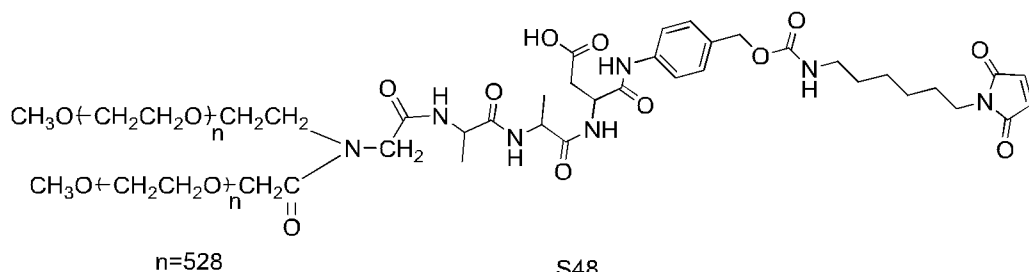
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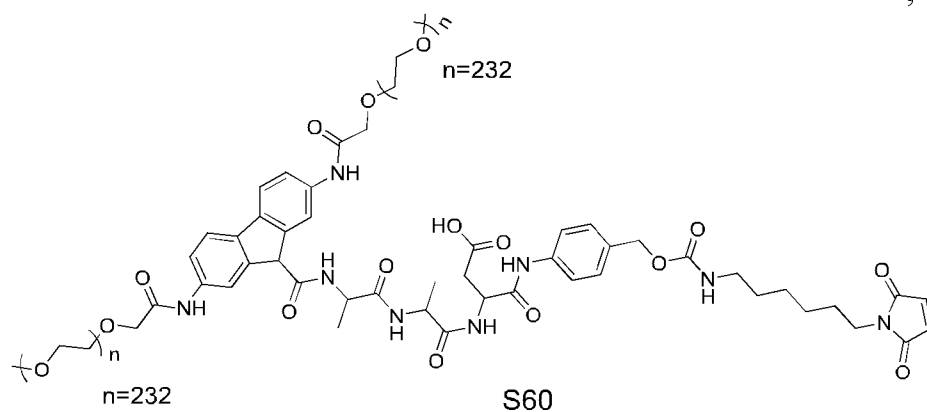
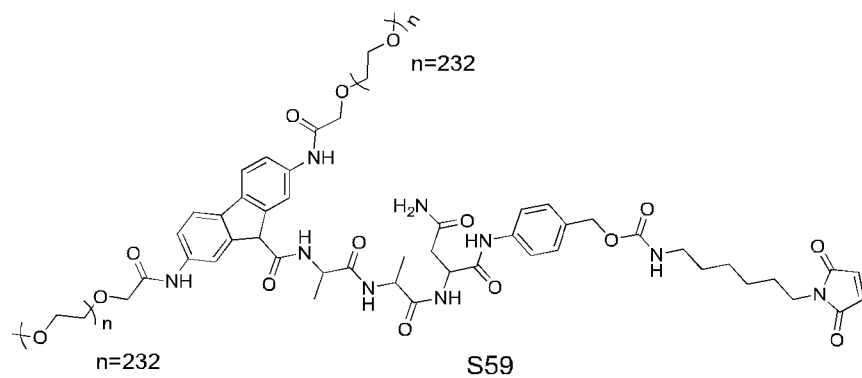
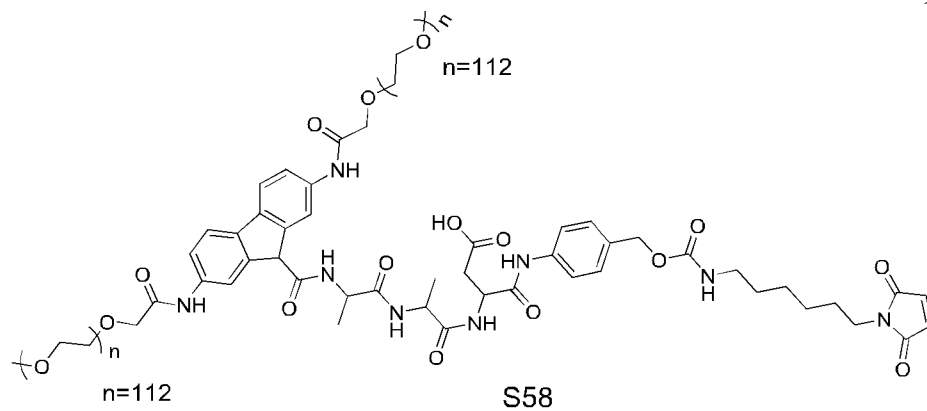
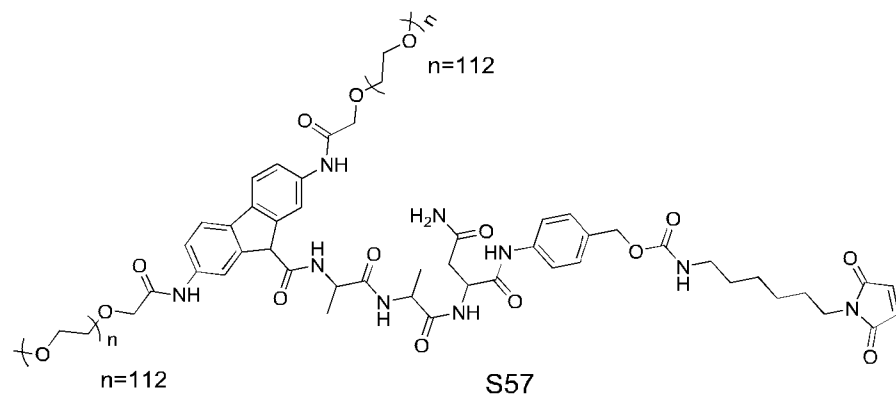


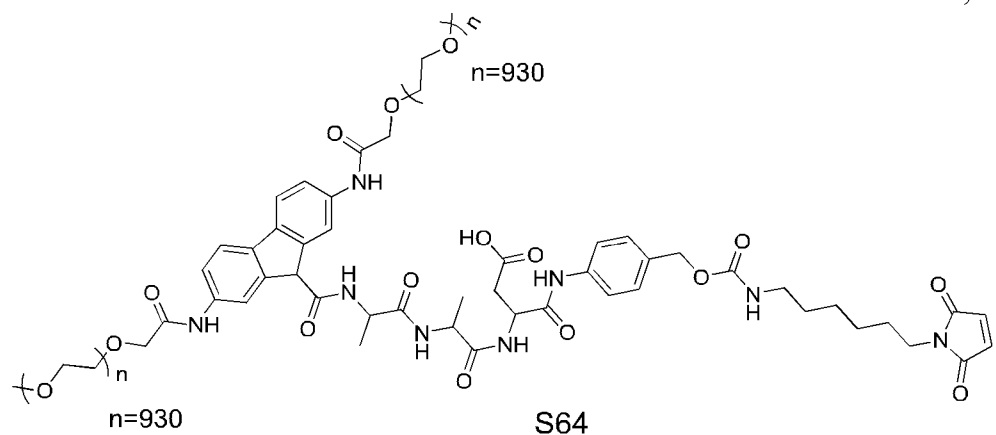
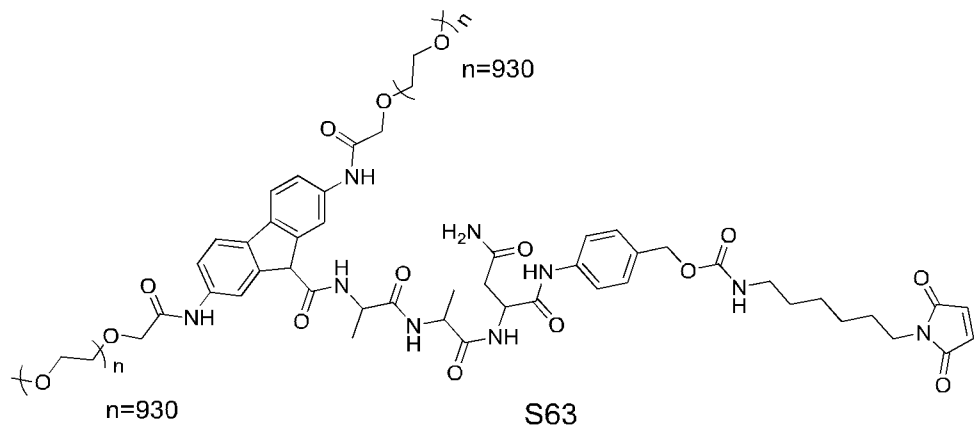
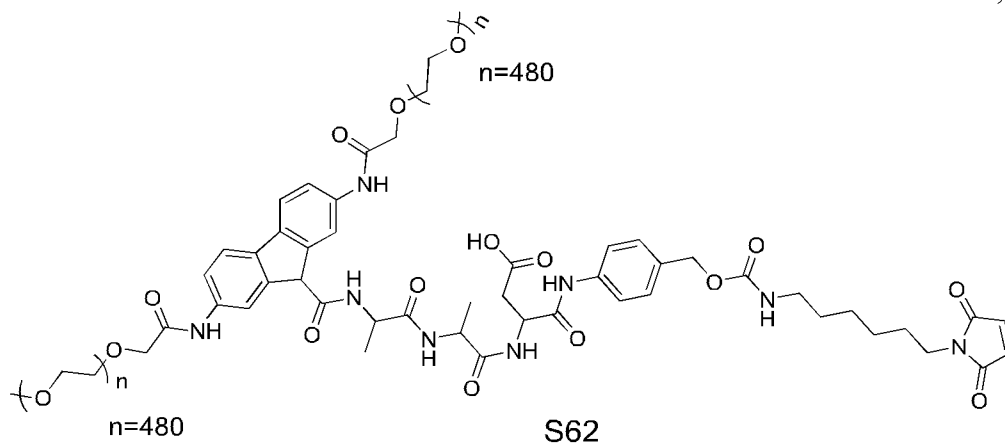
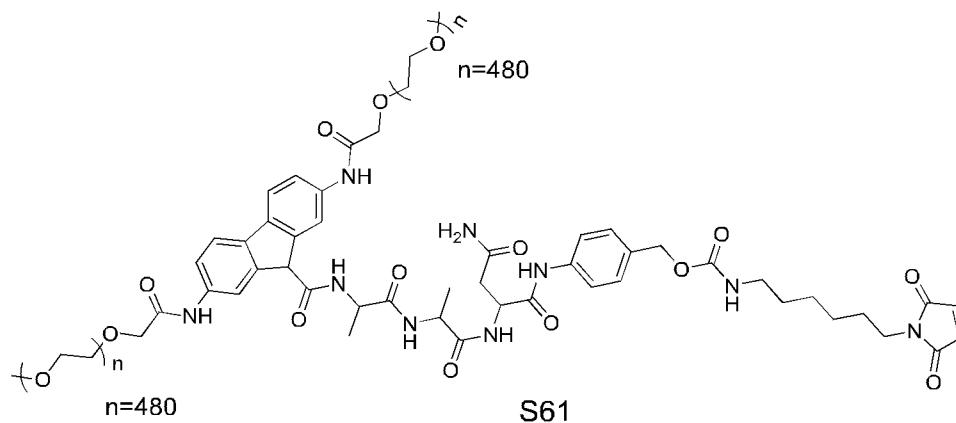
S34











, and

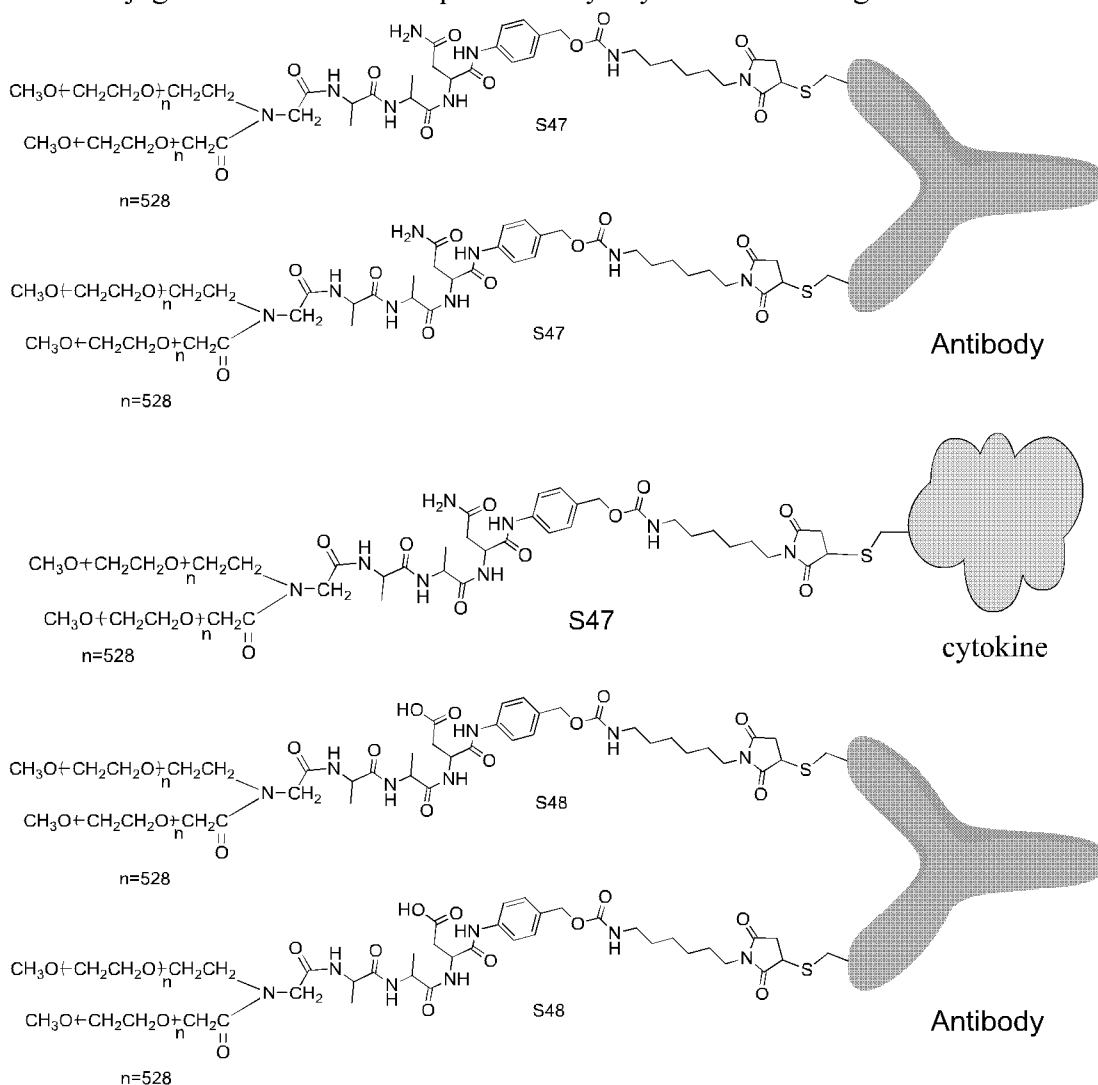
selected from the group consisting of G, A, S, T, L, I, F, E, K, D and Y in the complementarity determining region and/or one or more of amino acid residues selected from the group consisting of G, A, S, T, L, I, F, E, K, D and Y in the non-complementarity determining region mutated to cysteine;

- 5 preferably, the antibody is selected from the group consisting of anti-Her2 antibody, anti-EGFR antibody, anti-VEGFR antibody, anti-CD20 antibody, anti-CD33 antibody, anti-PD-L1 antibody, anti-PD-1 antibody, anti-CTLA-4 antibody, anti-TNF α antibody, anti-CD28 antibody, anti-4-1BB antibody, anti-OX40 antibody, anti-GITR antibody, anti-CD27 antibody, anti-CD40 antibody, or anti-ICOS antibody, anti-CD25 antibody, anti-CD30 antibody, anti-CD3 antibody, anti-CD22 antibody, anti-CCR4 antibody, anti-CD38 antibody, 10 anti-CD52 antibody, anti-Complement C5 antibody, anti-F protein of RSV, anti-GD2 antibody, anti-GITR antibody, anti-Glycoprotein receptor lib/Illa antibody, anti-ICOS antibody, anti-IL2R antibody, anti-LAG3 antibody, anti-Integrin α 4 antibody, anti-IgE antibody, anti-PDGFR α antibody, anti-RANKL antibody, anti-SLAMF7 antibody, anti-LTIGIT antibody, 15 anti-TIM-3 antibody, anti-VEGFR2 antibody, anti-VISTA antibody; or
- more preferably, the antibody is selected from the group consisting of Utomilumab, Urelumab, ADG106, PoteligeoTM (Mogamulizumab), PoteligeoTM (Mogamulizumab), BexxarTM (tositumomab), ZevalinTM (ibritumomab tiuxetan), RituxanTM (rituximab), ArzerraTM (Ofatumumab), GazyvaTM (Obinutuzumab), BesponsaTM (Inotuzumab 20 ozogamicin), ZenapaxTM (daclizumab), Varlilumab, Theralizumab, AdcetrisTM (Brentuximab vedotin), MyelotargTM (gemtuzumab), DarzalexTM (Daratumumab), CDX-1140, SEA-CD40, RO7009789, JNJ-64457107, APX-005M, Chi Lob 7/4, CampathTM (alemtuzumab), RaptivaTM (efalizumab), SolirisTM (eculizumab), YervoyTM (ipilimumab), tremelimumab, ErbituxTM (cetuximab), VectibixTM (panitumumab), PortrazzaTM (Necitumumab), 25 TheraCIMTM (Nimotuzumab), SynagisTM (palivizumab), UnituxinTM (Dinutuximab), TRX-518, MK-4166, MK-1248, GWN-323, INCAGN0186, BMS-986156, AMG-228, ReoProTM (abiximab), HerceptinTM (trastuzumab), PerjetaTM (Pertuzumab), KadcylaTM (Ado-trastuzumab emtansine), GSK-3359609, JTX-2011, SimulectTM (basiliximab), TysabriTM (natalizumab), BMS-986016, REGN3767, LAG525, XolairTM (omalizumab), Tavolimab, 30 PF-04518600, BMS-986178, MOXR-0916, GSK-3174998, INCAGN01949, IBI-101, KeytrudaTM (Pembrolizumab), OpdivoTM (Nivolumab), LartruvoTM (Olaratumab), TencentriqTM (Atezolizumab), BMS-936559, BavencioTM (Avelumab), ImfinziTM (Duralumab), ProliaTM (Denosumab), EmplicitiTM (Elotuzumab), MTIG7192A, TSR-022, MBG-453, RemicadeTM (infliximab), HumiraTM (adalimumab), AvastinTM (bevacizumab), LucentisTM (ranibizumab), 35 CyramzaTM (Ramucirumab), and JNJ-61610588;

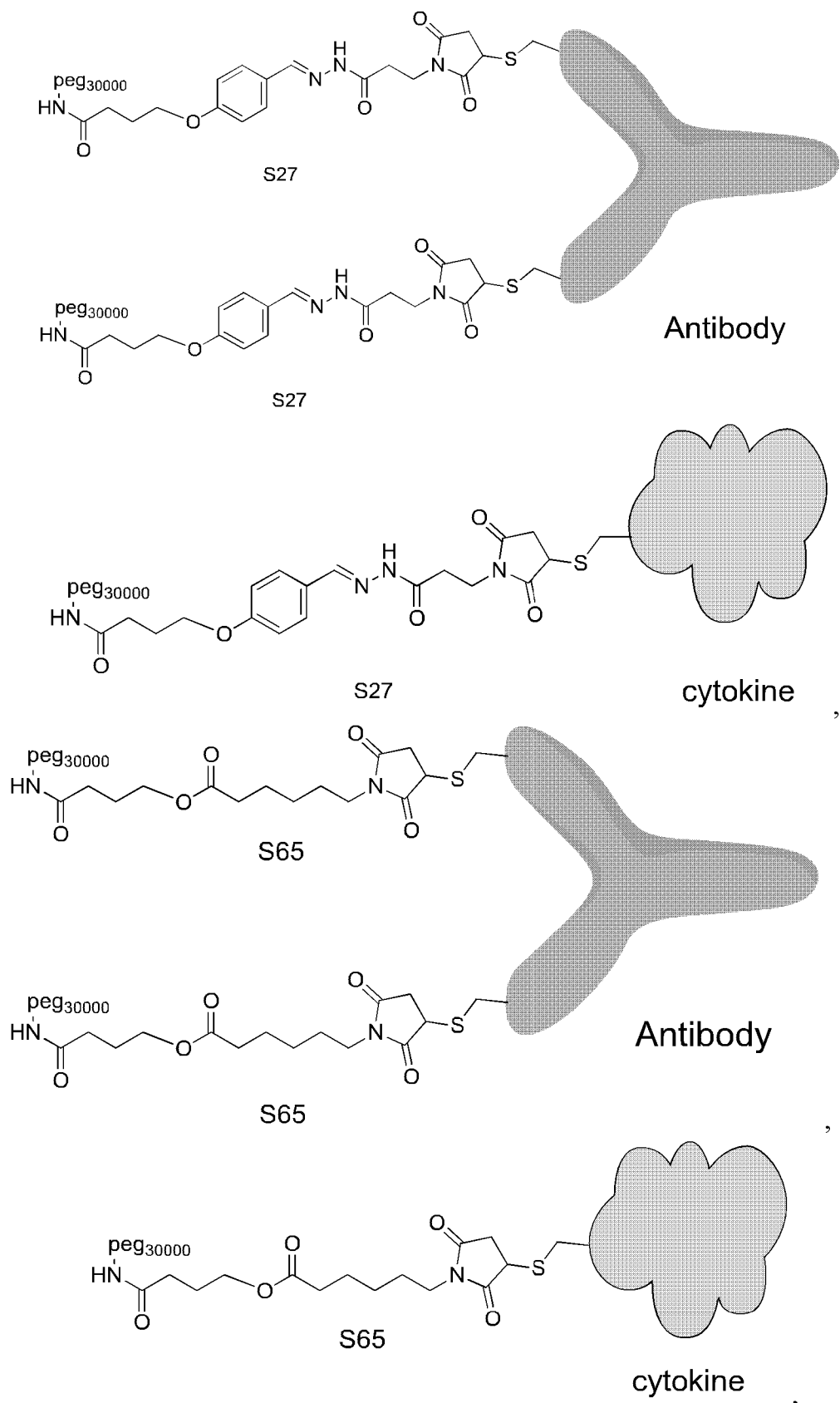
more preferably, the antibody or its functional fragment contains one or more of the mutations as defined in claim 22.

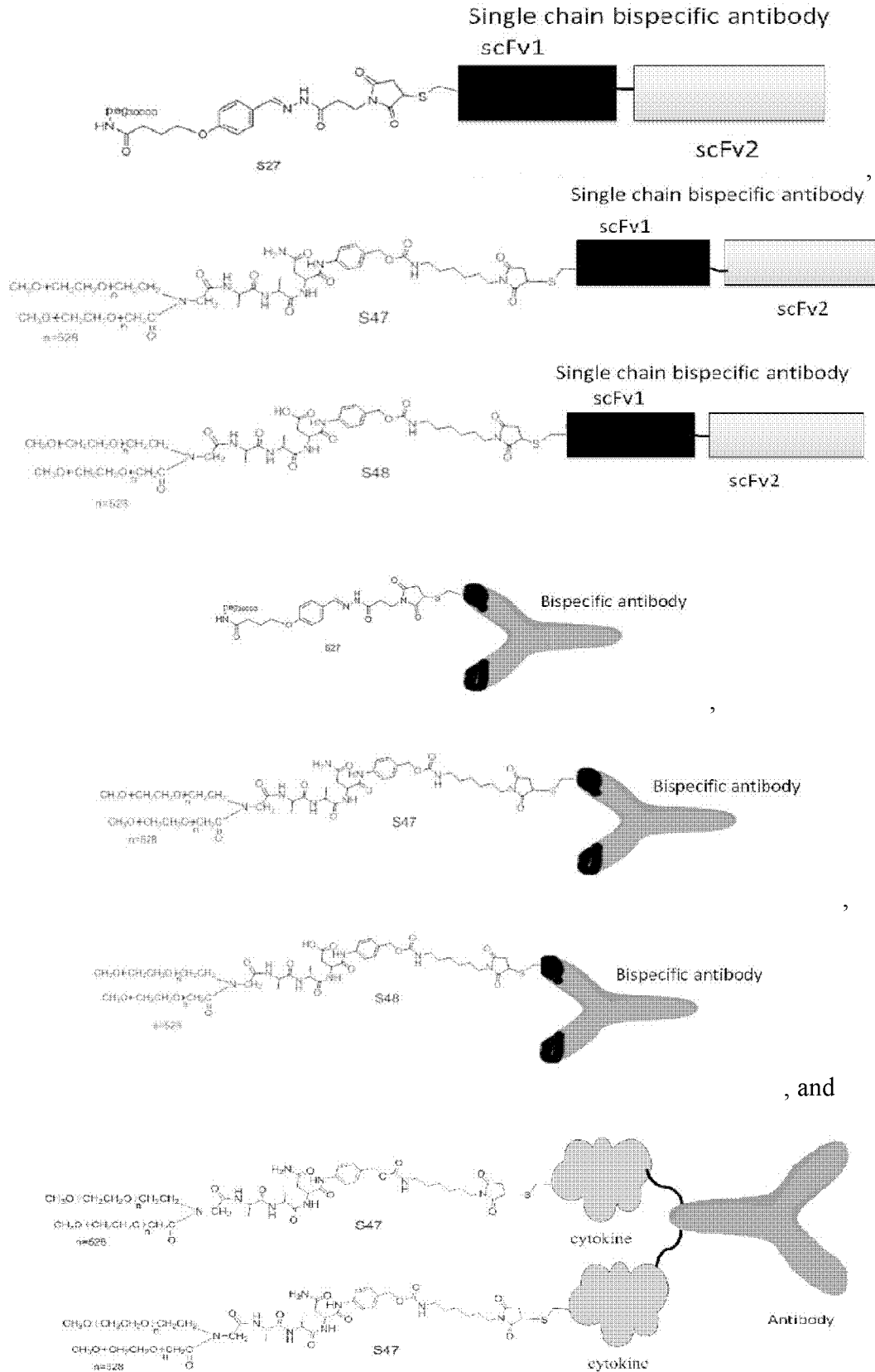
38. A cytokine with one or more amino acid residues selected from the group consisting of G, A, S, T, L, I, F, E, K, D and Y mutated to cysteine;
preferably, the cytokine is selected from the group consisting of IL-2, IL-7, IL-10, IL-11, IL-12, IL-15, IL-21, IFN- α , IFN- β , IFN- γ , G-CSF, GM-CSF, TNF- α , TRAP and TRAIL;
more preferably, the cytokine contains one or more of the mutations as defined in claim 22.

39. A conjugate of biomolecule represented by any of the following structures:









wherein the cytokine and antibody is defined as in any of claims 15-23, preferably, the antibody and cytokine is selected from the group consisting of any of SEQ ID NO: 1-83.

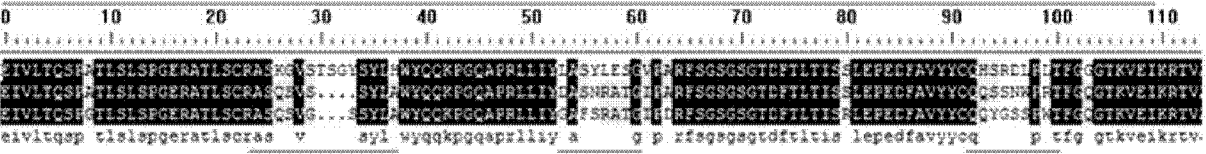


Fig. 1

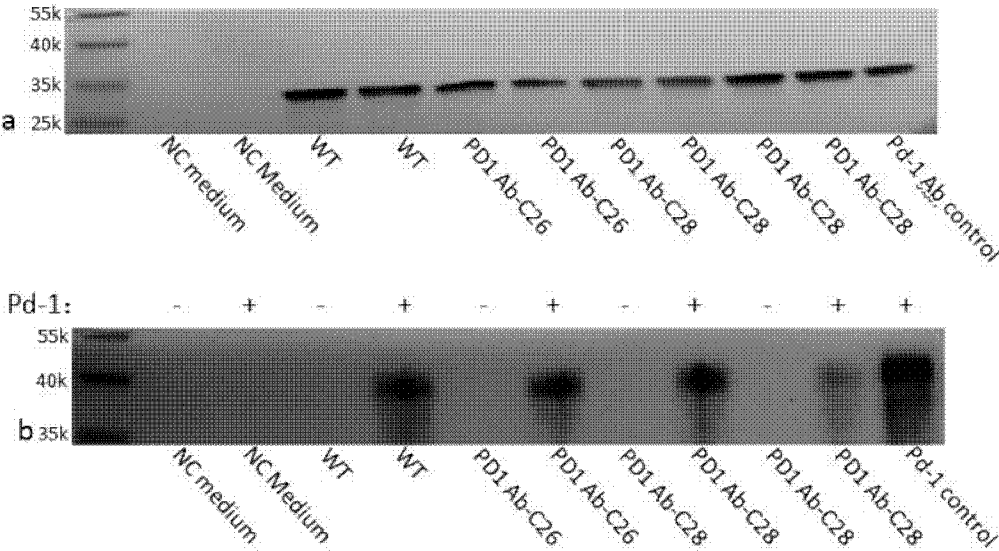


Fig. 2

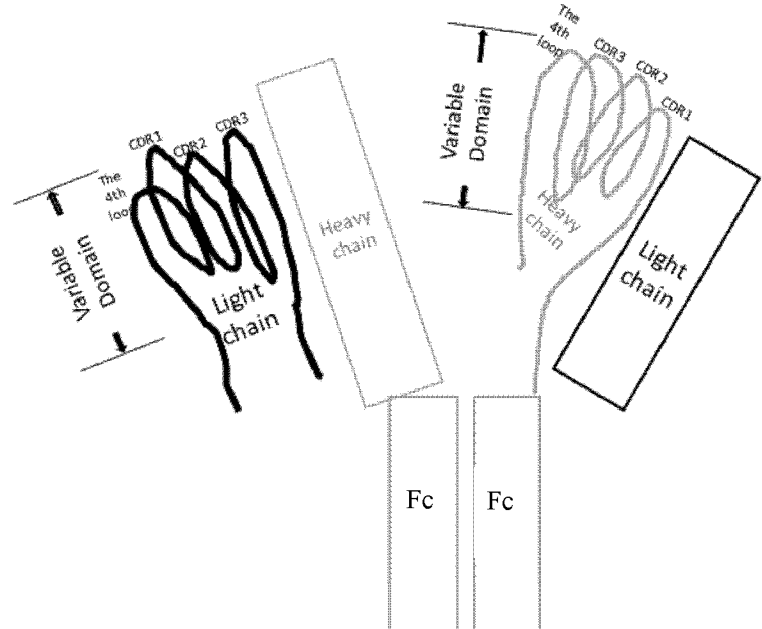


Fig. 3

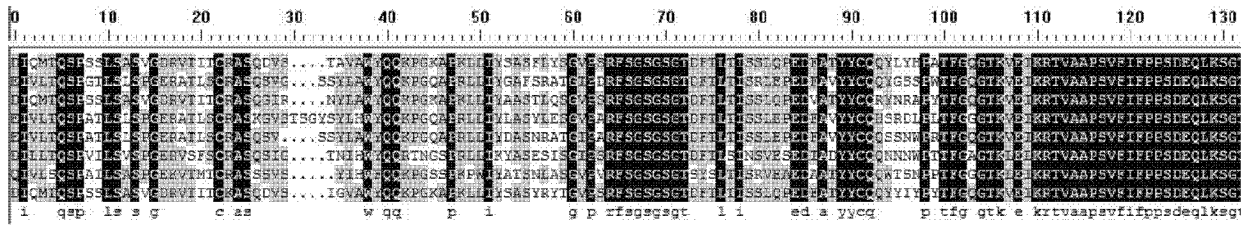


Fig. 4

Opdivo VL(upper):Keytruda VL(lower)



Tecentrig VH(upper):Herceptin VH(lower)



Fig. 5

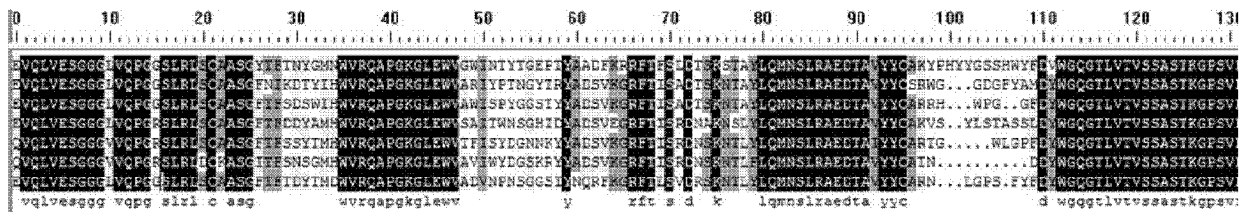


Fig. 6

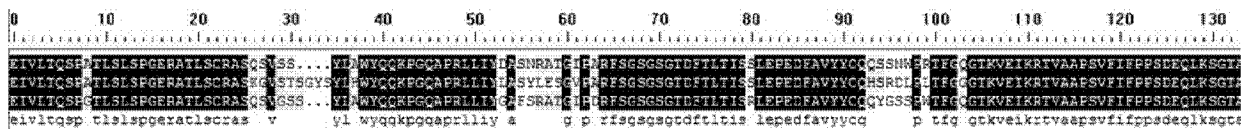


Fig. 7

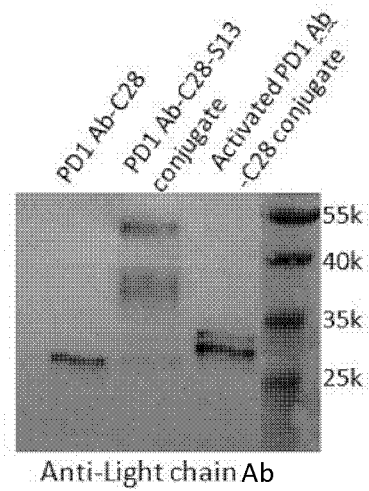


Fig. 8

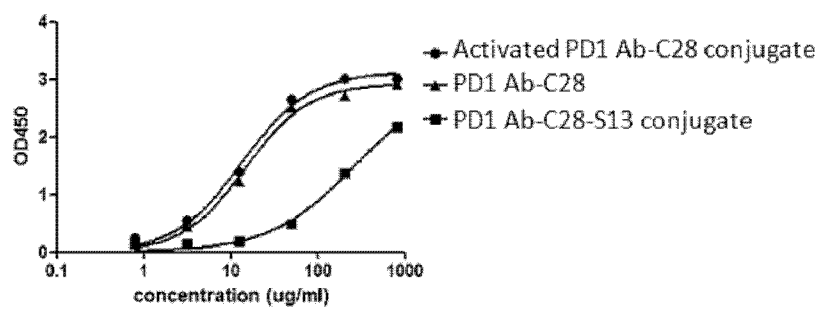


Fig. 9

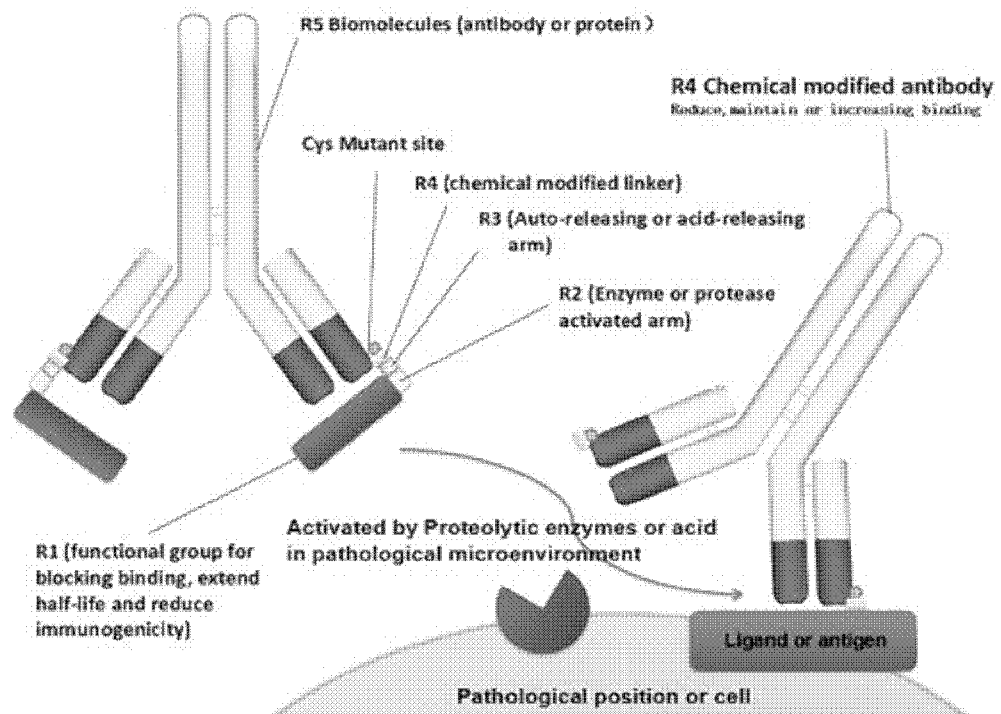


Fig. 10

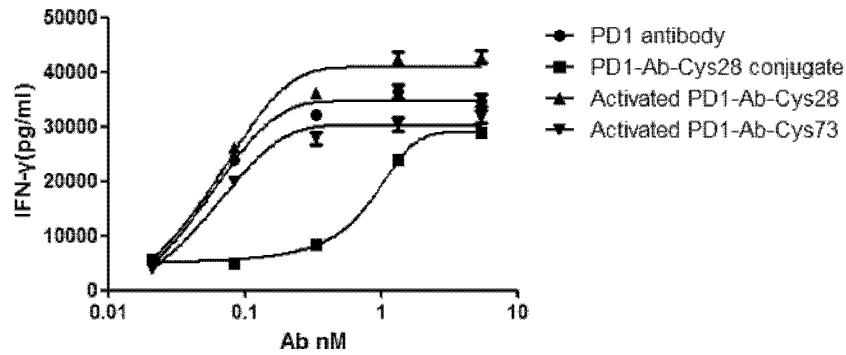
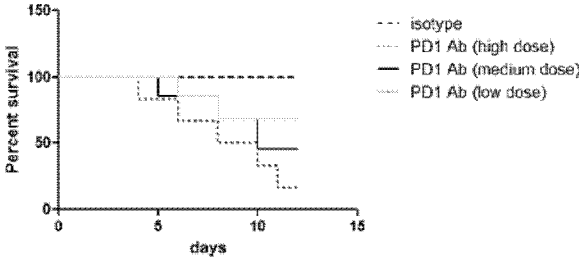


Fig. 11

Survival of Two groups:Survival proportions



Survival of Two groups:Survival proportions

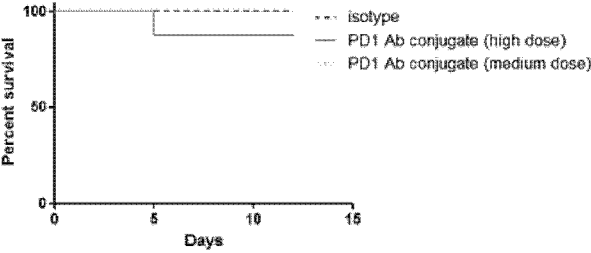
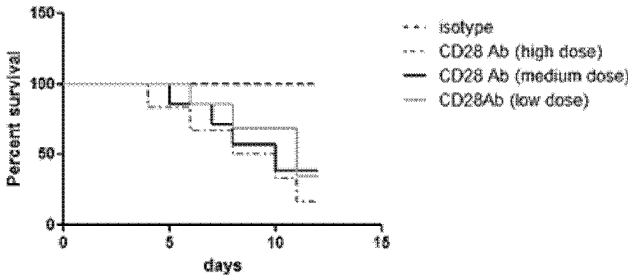


Fig. 12

Survival of Two groups:Survival proportions



Survival of Three groups:Survival proportions

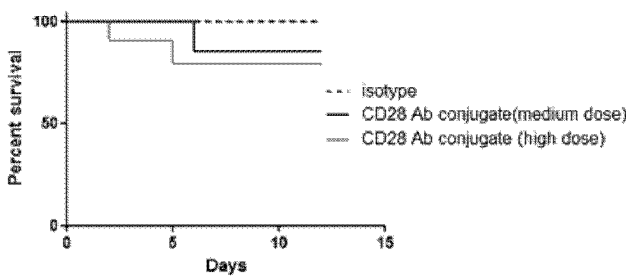


Fig. 13

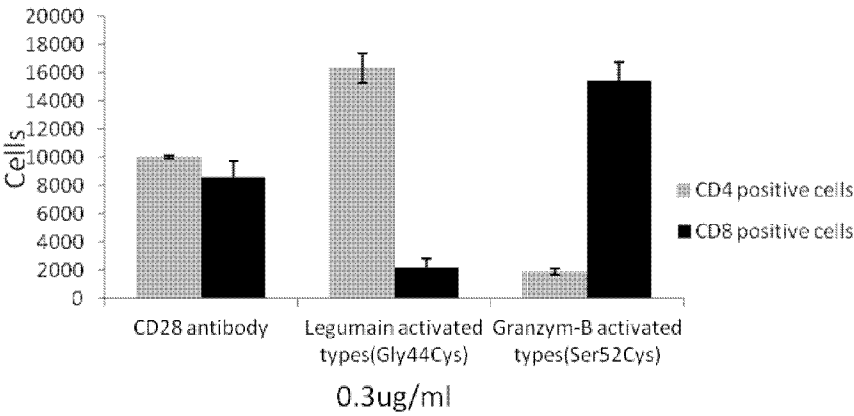


Fig. 14

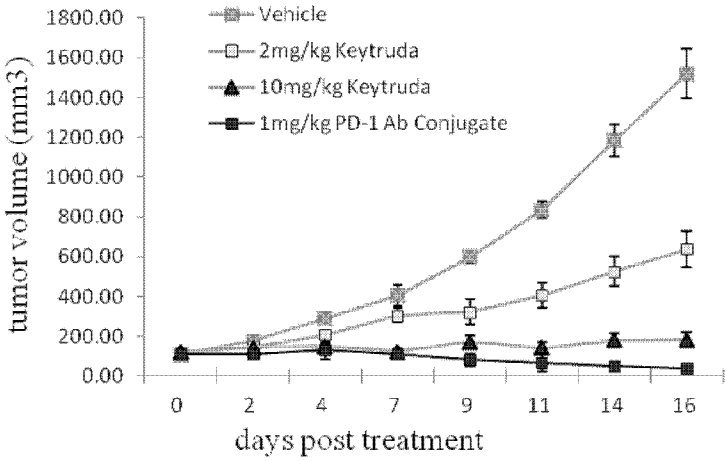


Fig. 15

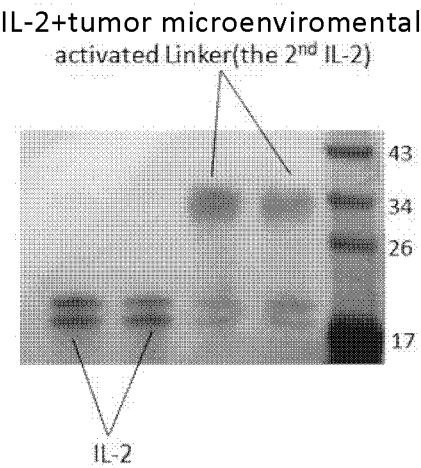


Fig. 16

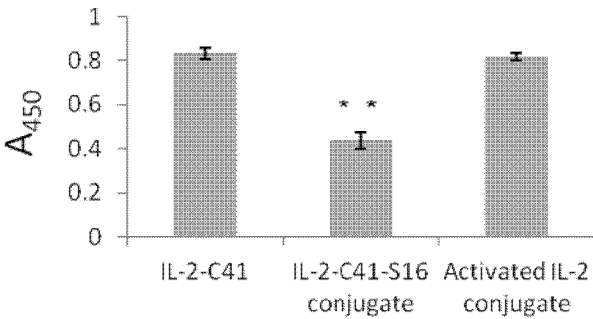


Fig. 17

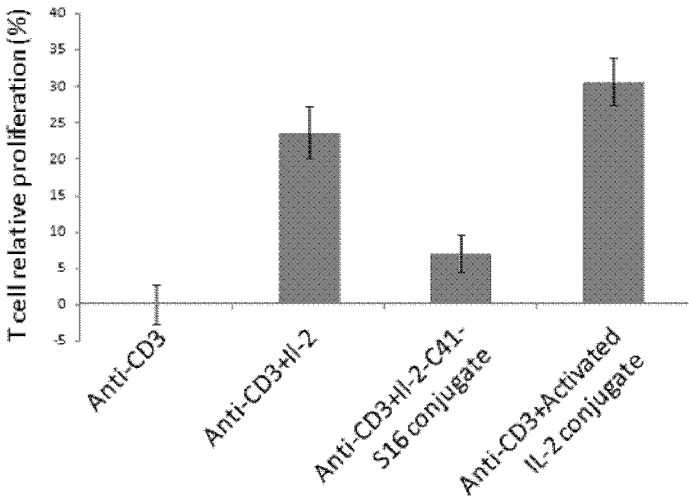


Fig. 18

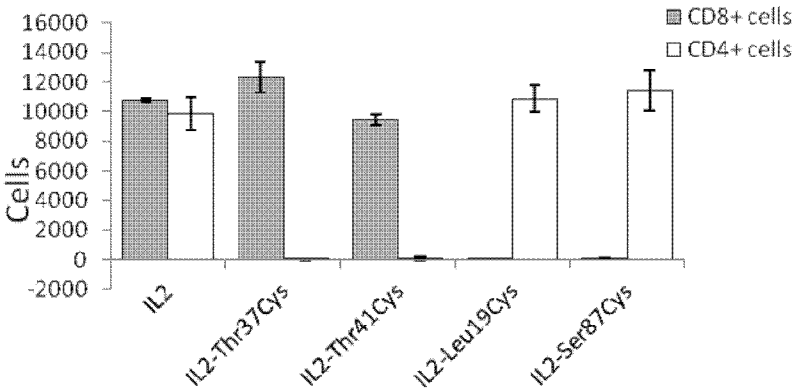


Fig. 19

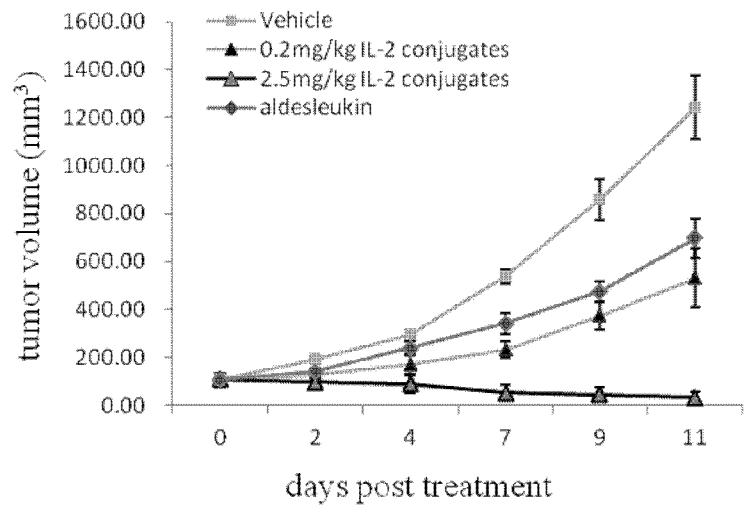


Fig. 20

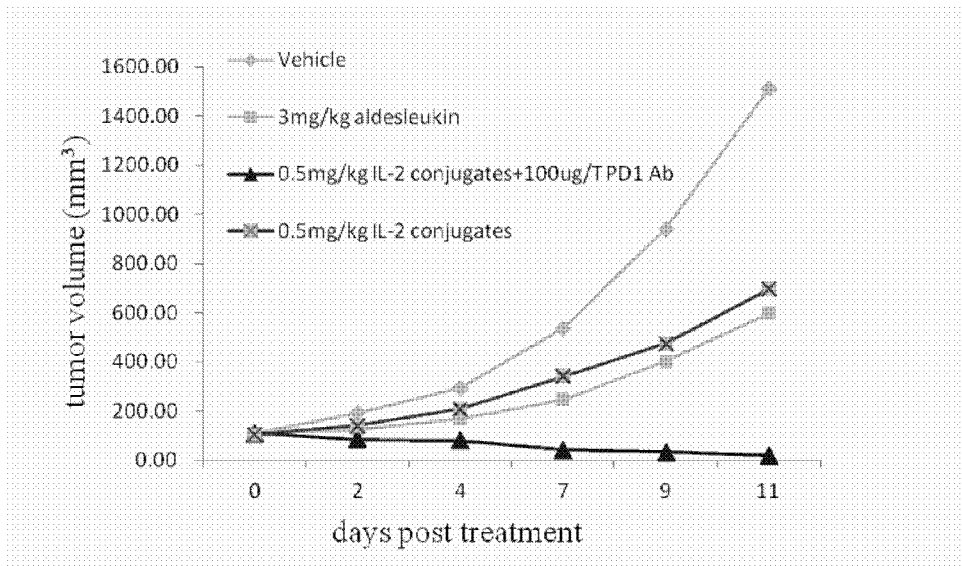


Fig. 21

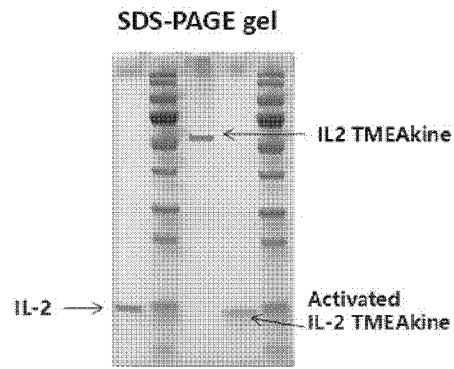


Fig. 22

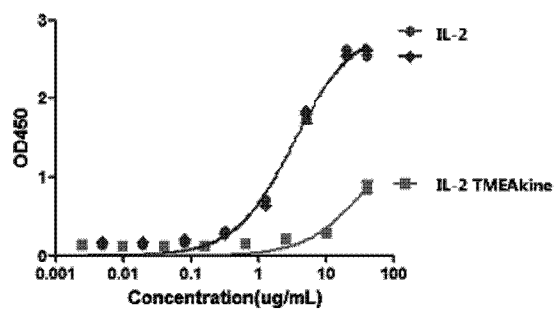
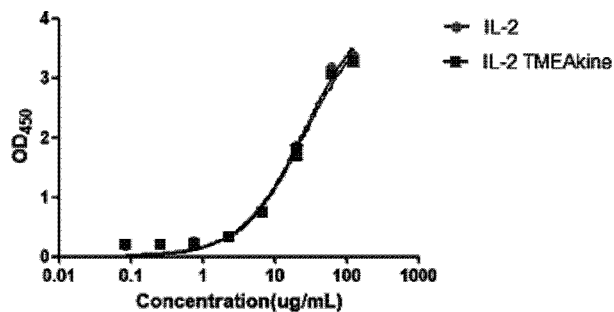
Binding Assay to IL-2 receptors α (IL-2R α)**Binding Assay to IL-2 receptor β (IL-2R β)**

Fig. 23

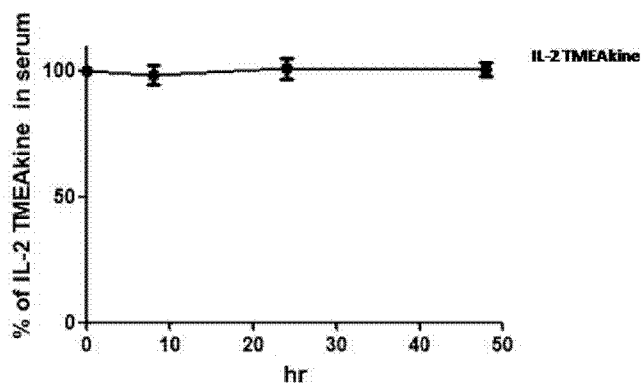


Fig. 24

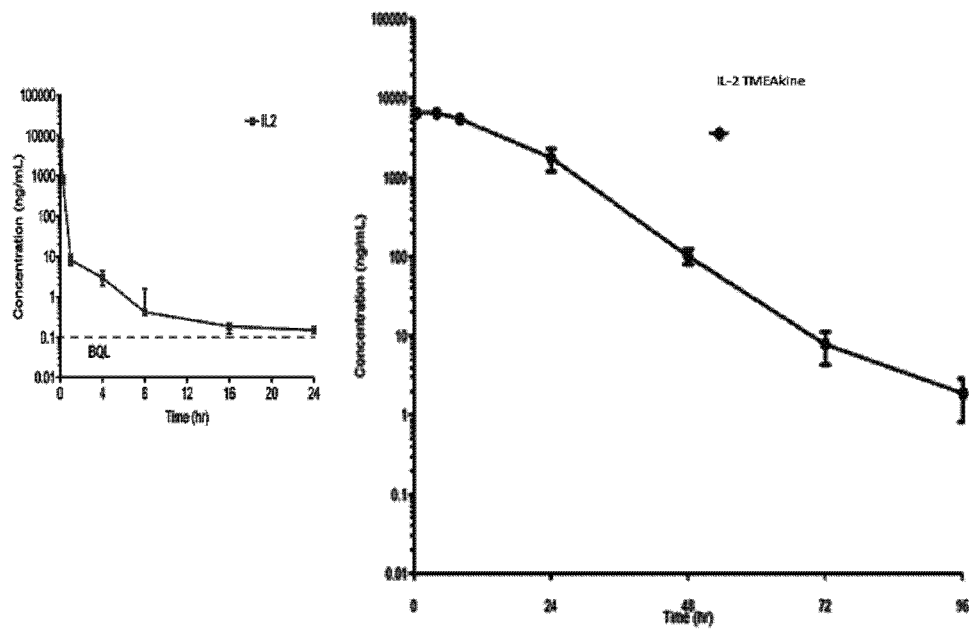


Fig. 25

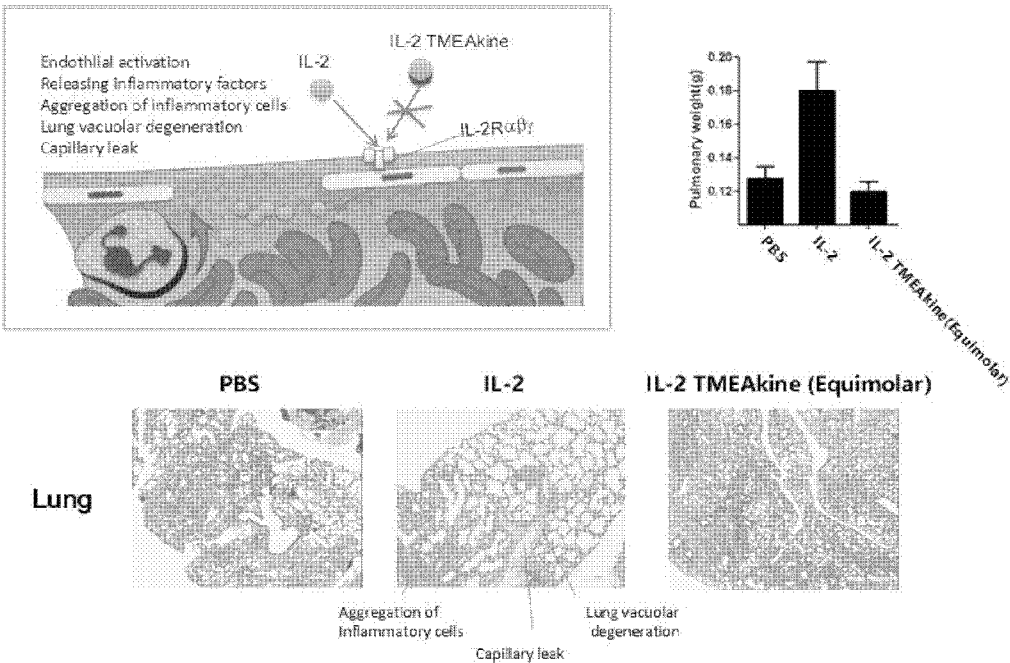


Fig. 26

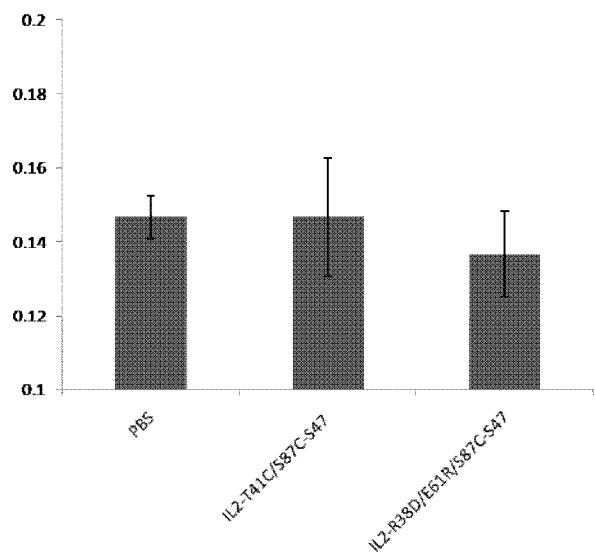


Fig. 27

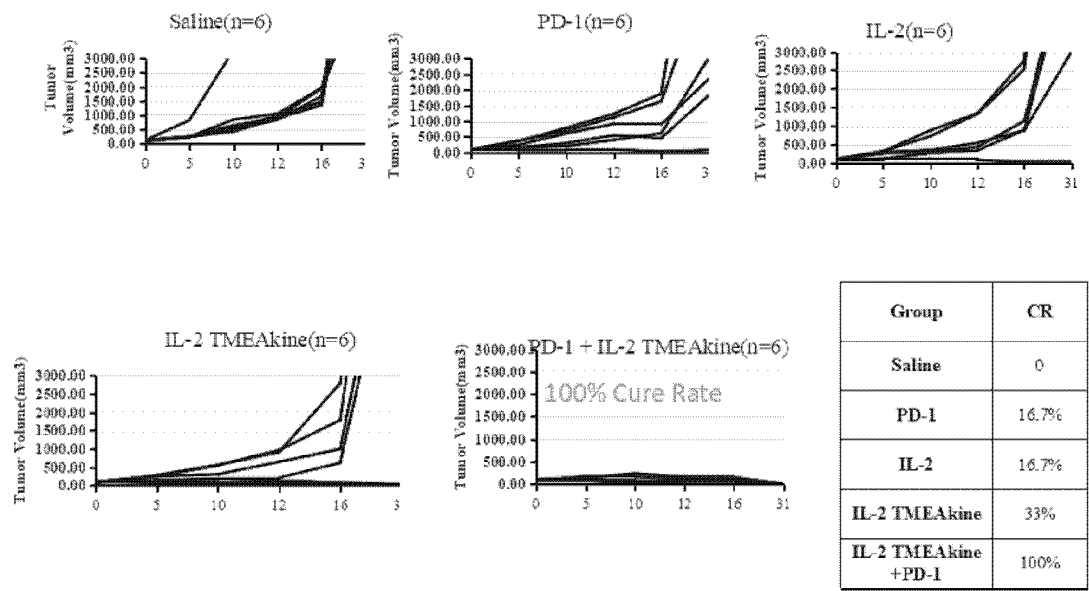


Fig. 28

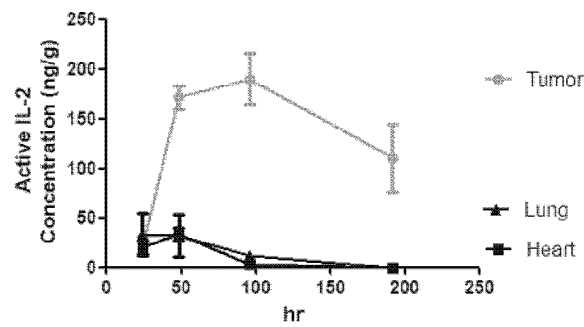


Fig. 29

Conjugation to different mutant site

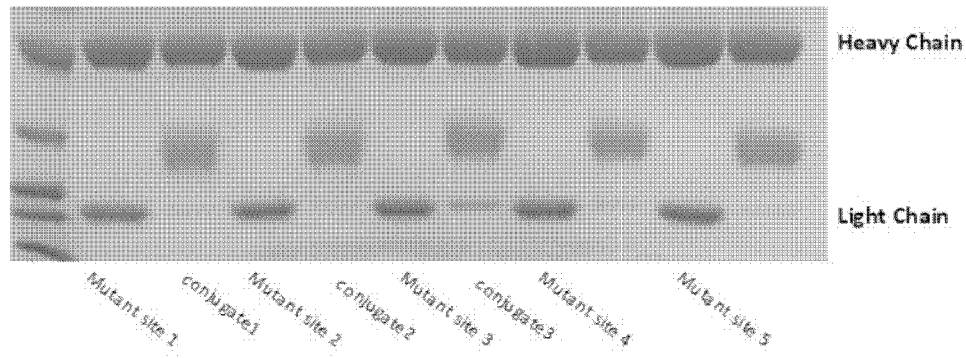


Fig. 30

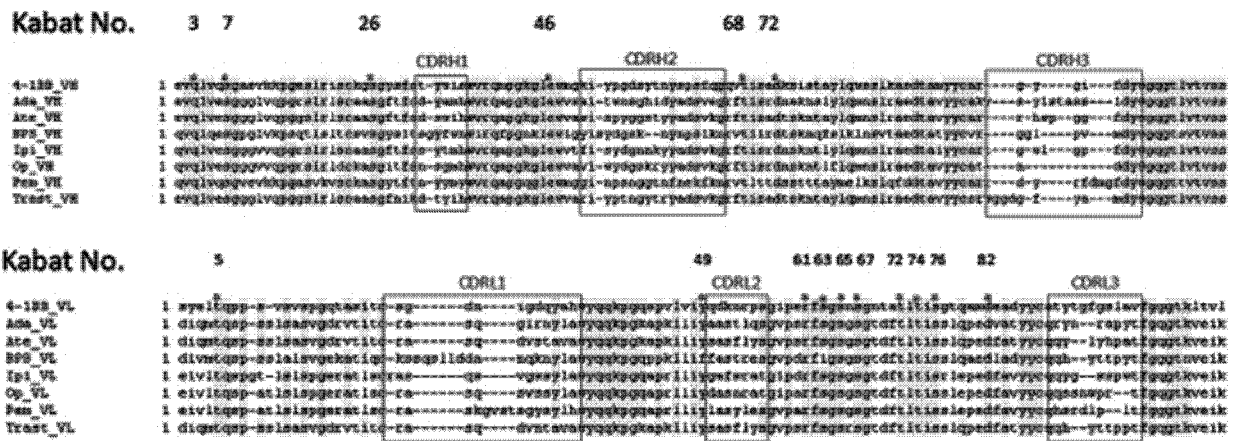


Fig. 31

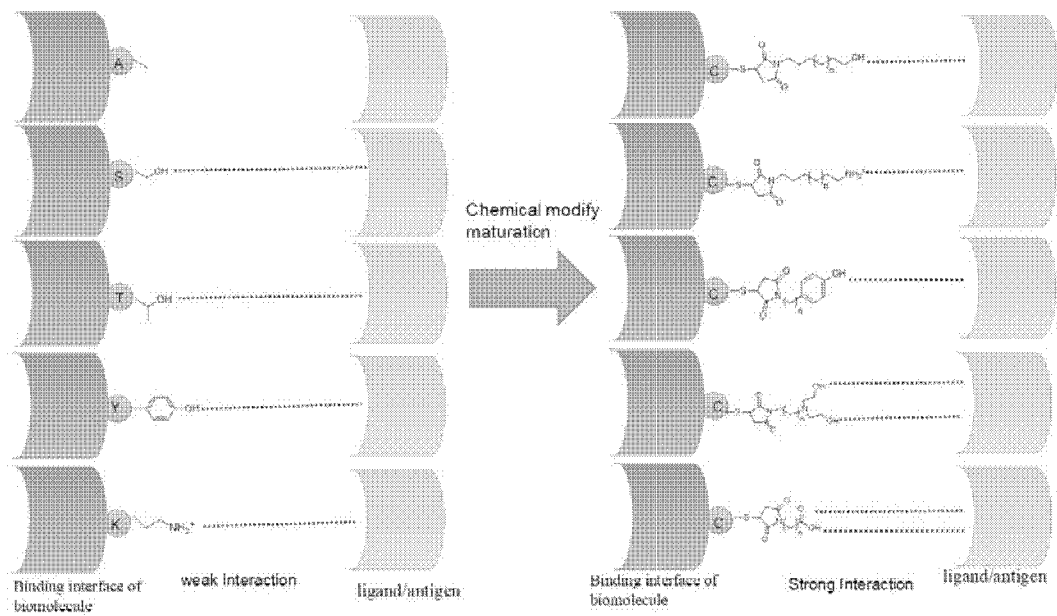


Fig. 32

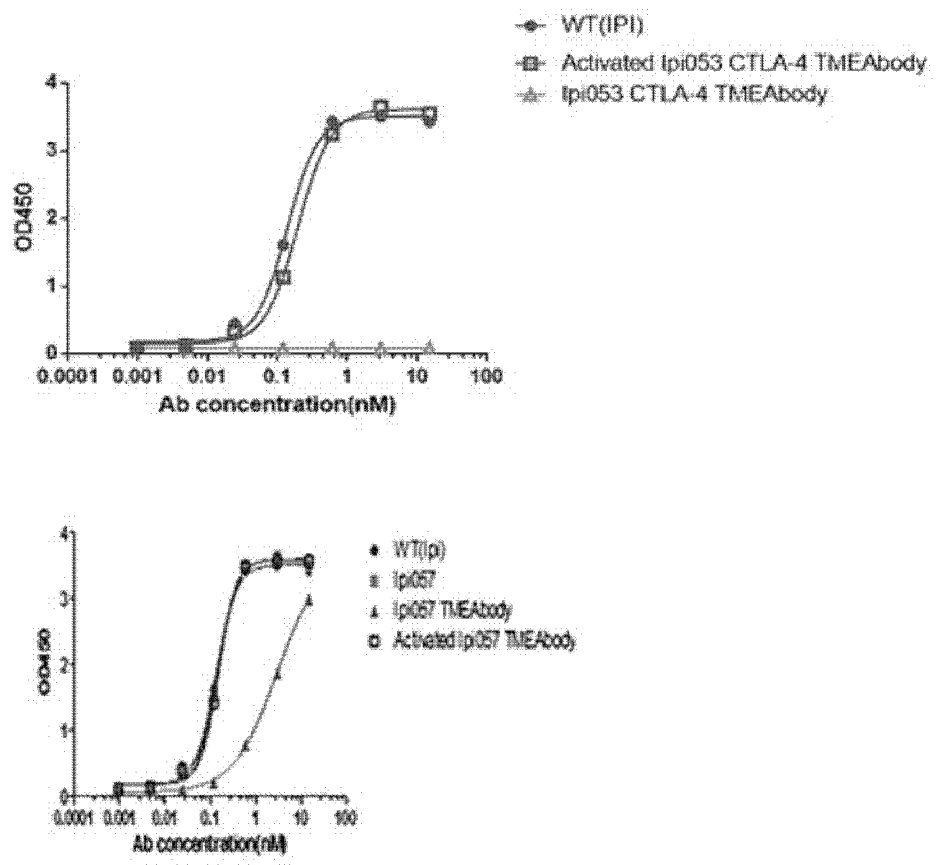


Fig. 33

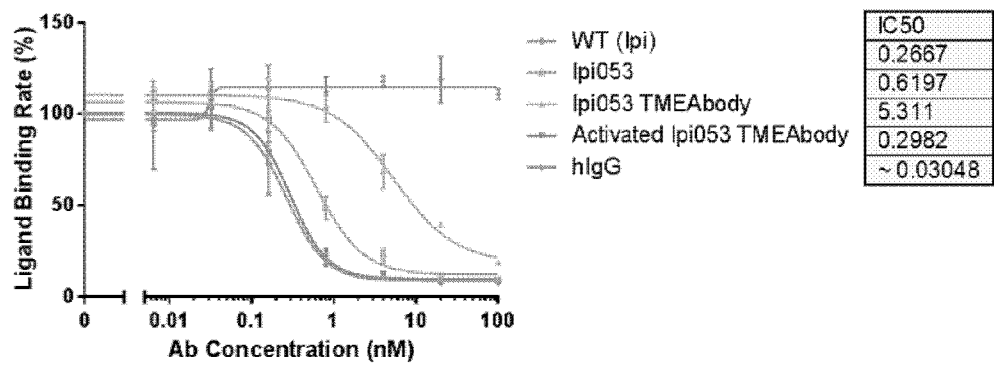


Fig. 34

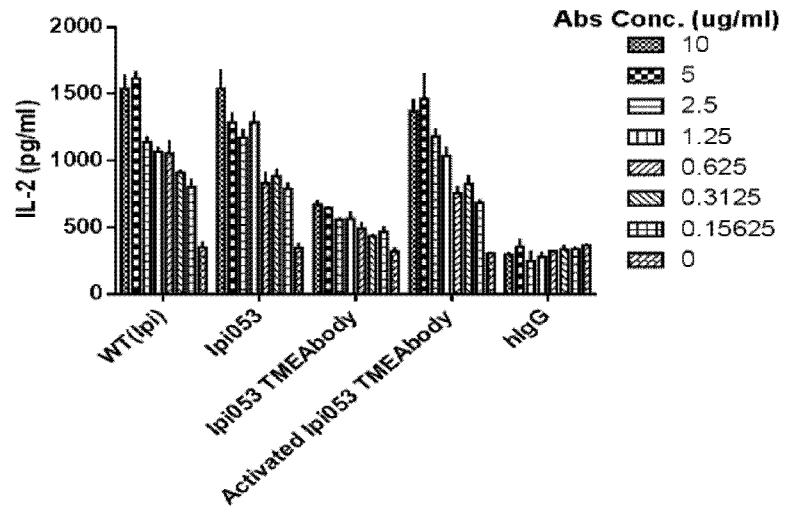


Fig. 35

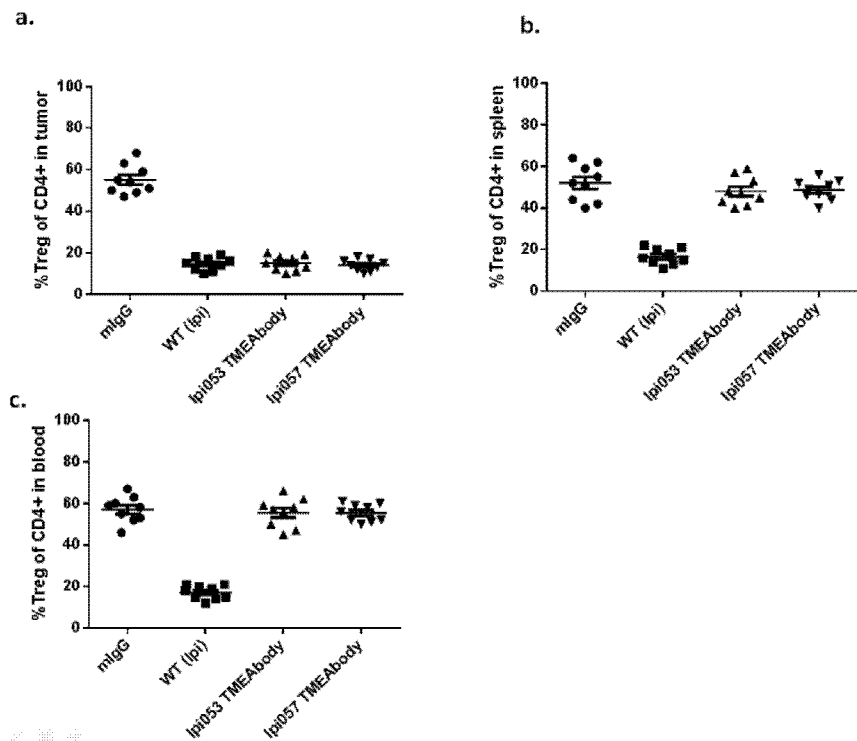


Fig. 36

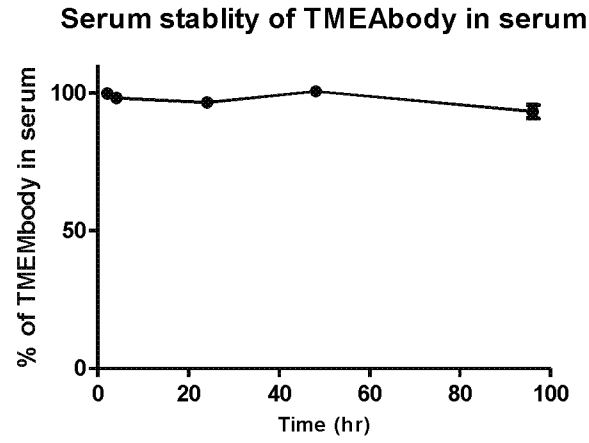
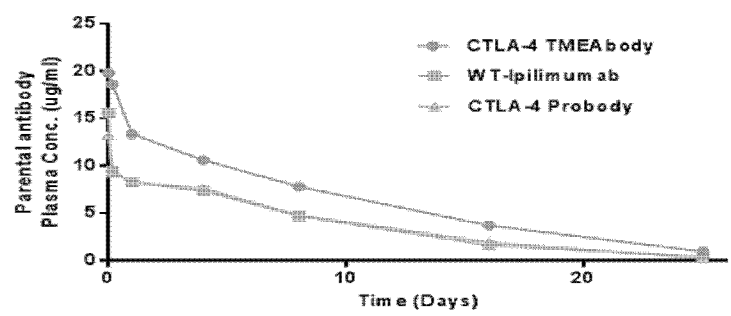


Fig. 37

a.



b.

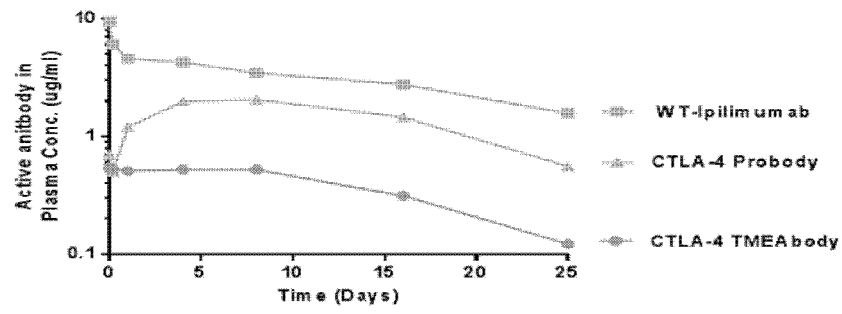


Fig. 38

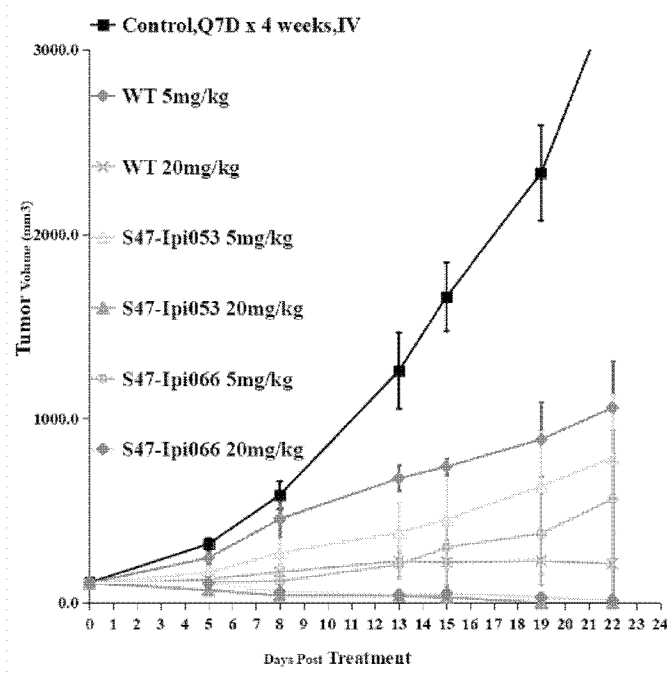


Fig. 39

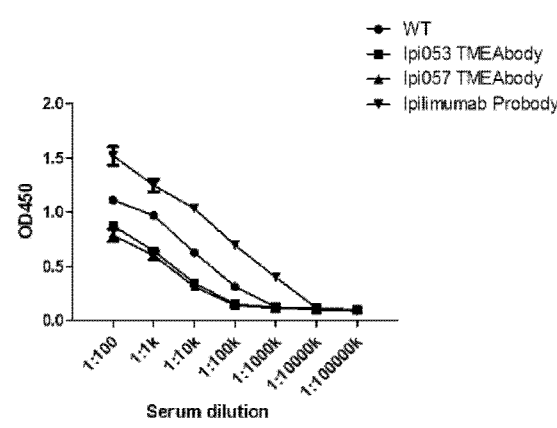


Fig. 40

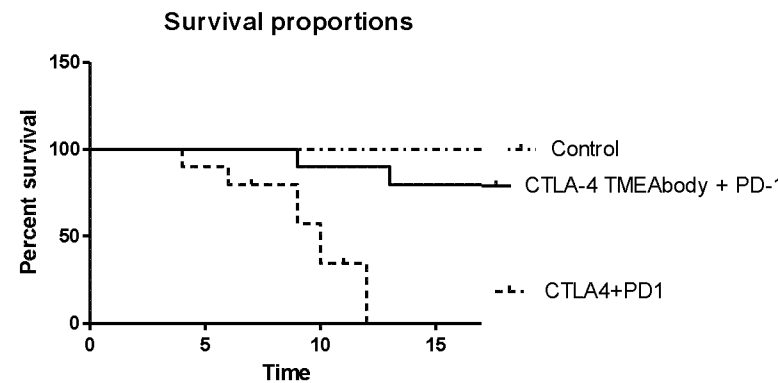


Fig. 41

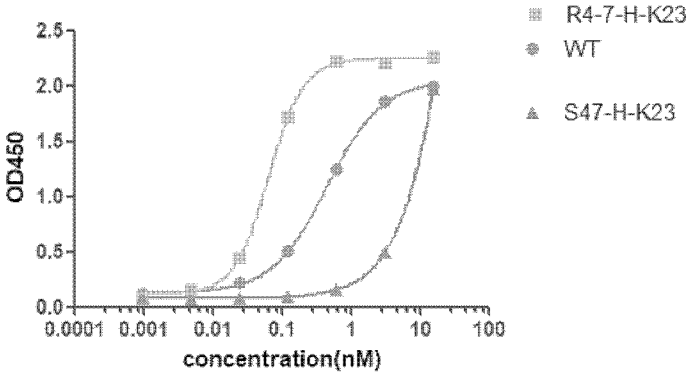


Fig. 42

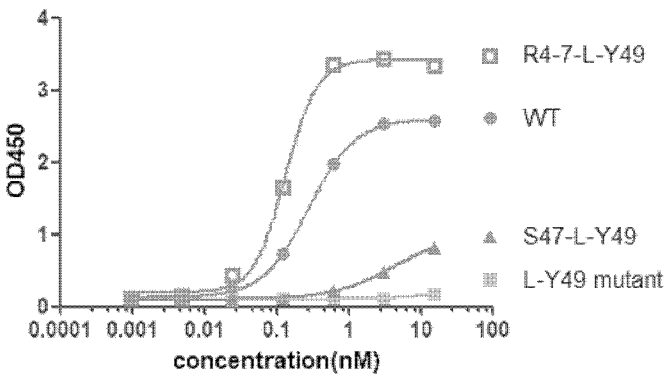


Fig. 43

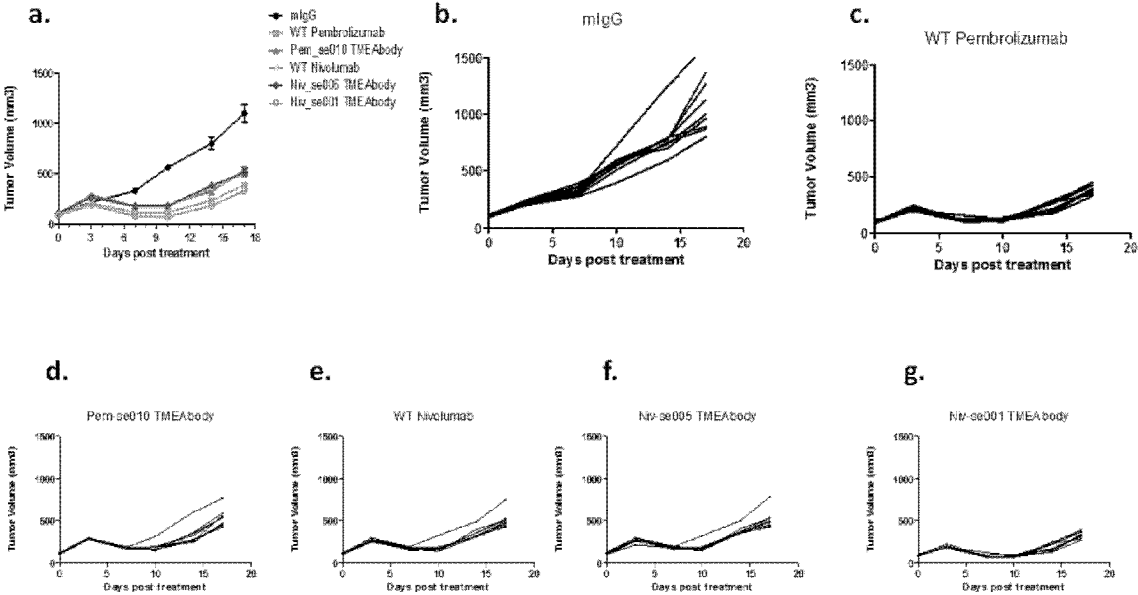


Fig. 44

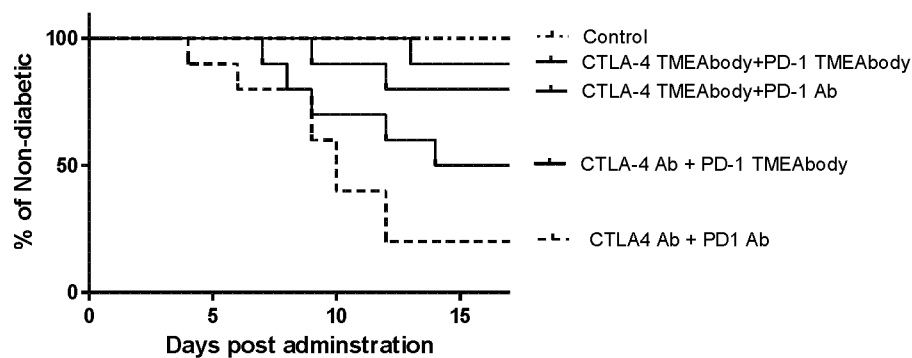


Fig. 45

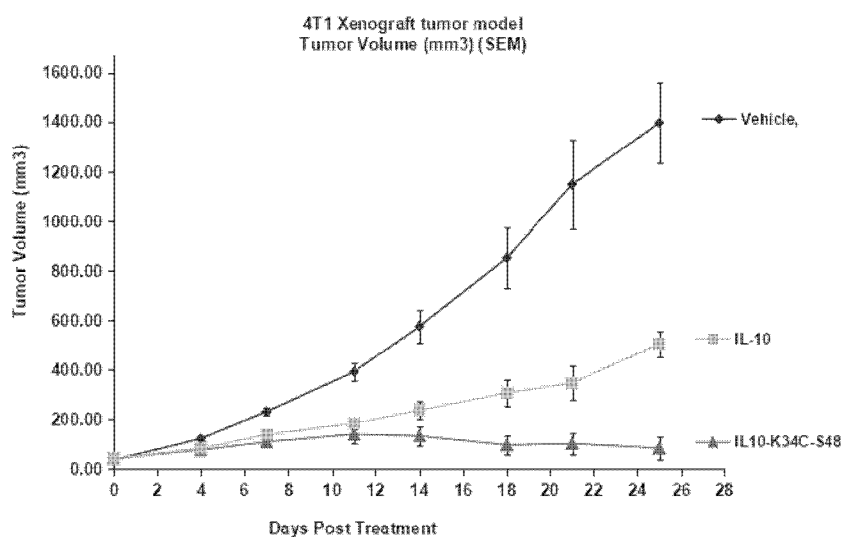


Fig. 46

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2018/114266

A. CLASSIFICATION OF SUBJECT MATTER

A61K 47/60(2017.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K

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

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

CNXTX; CNABS; CPRSABS; DWPI; SIPOABS; VEN; USTXT; WOTXT; KRTXT; JPTXT; CATXT; EPTXT; ISI
 WEB OF KNOWLEDGE(Biosis, medline, Embase) ; CNKI; PUBMED;Google,STN; YAFE-N, C07K+, PEG, A61K39,
 cys, R1, conjugate, linker,YAFEI SHANGHAI, R1-R2-R3-R4, A61K38+, R1-R2-R3, R2, R1-R2-R3-R4-S-cys-R5,
 modification, R3, peptide.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2012007880 A2 (ABLYNX NV) 19 January 2012 (2012-01-19) Description Pages 5-25	1, 14, 16, 21, 29-30, 38
X	WO 2006121168 A1 (ONO PHARMACEUTICAL CO., LTD. ET AL.) 16 November 2006 (2006-11-16) Description Pages 2-76	37
Y	WO 2012007880 A2 (ABLYNX NV) 19 January 2012 (2012-01-19) Description Pages 5-25	1-36, 39
Y	CN 104147612 A (YAFEISHANGHAIBIOLOGY MEDICINE SCIENCE & TECHNOLOGY CO., LTD.) 19 November 2014 (2014-11-19) Description Pages 1-17	1-36, 39
Y	CN 102321239 A (HEBEI UNIVERSITY OF SCIENCE AND TECHNOLOGY) 18 January 2012 (2012-01-18) Description Pages 2-14	1-36, 39



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

“A” document defining the general state of the art which is not considered to be of particular relevance

“E” earlier application or patent but published on or after the international filing date

“L” document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

“O” document referring to an oral disclosure, use, exhibition or other means

“P” document published prior to the international filing date but later than the priority date claimed

“T” later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

“X” document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

“Y” document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

“&” document member of the same patent family

Date of the actual completion of the international search

21 January 2019

Date of mailing of the international search report

12 February 2019

Name and mailing address of the ISA/CN

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 6, Xitucheng Rd., Jimen Bridge, Haidian District, Beijing
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Telephone No. 86-(10)-53961962

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2018/114266**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2006121168 A1 (ONO PHARMACEUTICAL CO., LTD. ET AL.) 16 November 2006 (2006-11-16) Description Pages 2-76	1-36, 39
A	CN 104177474 A (YAFEISHANGHAIBIOLOGY MEDICINE SCIENCE & TECHNOLOGY CO., LTD.) 03 December 2014 (2014-12-03) the whole document	1-39
A	WO 2007056083 A2 (AMBRX INC.) 18 May 2007 (2007-05-18) the whole document	1-39

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2018/114266

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

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

1. ☒ Claims Nos.: **29-30**
because they relate to subject matter not required to be searched by this Authority, namely:

[1] Although claims 29-30 directed to a method of treating tumor or autoimmune disease, the search has been carried out and based on the use of the conjugate of biomolecule for the manufacturing of a medicament for treating disorder.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2018/114266

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2018/114266

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