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(54) Title: A NOVEL THIOL REDUCTANT, PREPARATION METHOD AND USE THEREOF

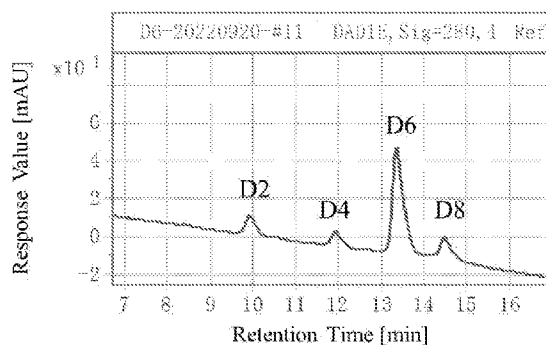


Figure 1

(57) Abstract: The present disclosure relates to a novel thiol reductant having the formula (I), the preparation and the use in antibody modification.

A NOVEL THIOL REDUCTANT, PREPARATION METHOD AND USE THEREOF**CROSS-REFERENCE TO RELATED APPLICATIONS**

This application claims the priority to PCT Application No. PCT/CN2022/113992, filed on August 22, 2022, PCT Application No. PCT/CN2022/119999, filed on September 20, 2022, PCT Application No. PCT/CN2022/119955, filed on September 20, 2022, PCT Application No. PCT/CN2022/131521, filed on November 11, 2022, and PCT Application No. PCT/CN2023/073070, filed on January 19, 2023. The contents of the prior PCT applications are considered as a part of the present disclosure and is incorporated herein in its entirety.

TECHNICAL FIELD

The disclosure relates to a novel thiol reductant, preparation method and use thereof. The thiol reductant could be used in antibody modification.

BACKGROUND

The statements in this section merely provide background information related to the present disclosure and do not necessarily constitute prior art.

Antibody-drug conjugates (ADCs) are innovative biopharmaceutical products in which a monoclonal antibody is linked to a small molecule drug with a stable linker. ADCs ideally combine the specificity of antibodies and high potency of cytotoxic drugs by delivering potent cytotoxic drugs to antigen-expressing cells, thereby enhancing their targeted cytotoxic activity.

Generally, antibody conjugation to cytotoxic agents commonly involves conjugation to exposed residues including lysines or reduction of disulfide bonds to expose free interchain cysteines on a therapeutic IgG (Immunoglobulin G) antibody. There are other, more recent approaches that introduce conjugation sites to the mAb such as site-specific glycan conjugation, cysteine engineering, incorporation of unnatural amino acids and coupling short peptide tags to drug-linkers. There are typically 80 lysine residues on an antibody; however, less than ten residues are chemically accessible for conjugation. Cysteine conjugation eventuates in the reduction of four interchain disulfide bonds. These bonds are reduced under specific conditions and subsequently result in two, four, six or eight exposed sulfhydryl groups. Both Cys and Lys conjugation methods result in heterogeneous mixtures.

(“Advances and Limitations of Antibody Drug Conjugates for Cancer”. *Biomedicines*. 2021 Aug; 9(8): 872.).

The drug-antibody ratio (DAR), or number of drug molecules conjugated to a single ADC, is very important for the determination of efficacy of ADCs. DAR widely varies and depends on other ADC variables. The DAR values are also dependent on the site of conjugation and the use of light or heavy conjugated chains. The DAR value influences the effectiveness of the medicine due to the depression in potency caused by low drug loading, while elevated drug loading can impact toxicity and pharmacokinetics (“Introduction to Antibody-Drug Conjugates”. *Antibodies (Basel)*. 2021 Dec; 10(4): 42.). The conventional non-specific conjugation and conjugate distribution are largely influenced by factors such as pH, concentration, salt concentration, and co-solvents, so establishing a robust conjugation process always is challenging.

A number of methods have been developed to improve the homogeneity of ADCs. For example, Genentech’s THIOMAB technology is developed based on improve the homogeneity of ADCs through antibody engineering, by introducing cysteine in the primary sequence of the antibody and realizing site-directed coupling to improve the uniformity of the product (“Cysteine-Based Coupling: Challenges and Solutions”. *Bioconjug Chem*. 2021 Aug 18;32(8):1525-1534.).

US20210040145 discloses a 14-amino acid peptide Tub-tagf used to the C-terminus of any POI and catalyzes the addition of a variety of different tyrosine derivatives. Taking advantage of this enzyme, Tub-tag technology repurposed tubulin-tyrosine ligase for the attachment of functional moieties at the C-terminus of antibody to homogeneously generate antibody conjugates with DAR 2.

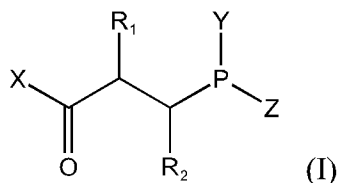
However, those technologies involve protein engineering and/or enzyme catalysis, so that those technologies suffer from several drawbacks, such as lower level of antibody expression, immunogenicity risk, complicated purification, and/or high cost.

Therefore, antibody-drug conjugates with improved homogeneity, could provide benefits in terms of better stability and lower immunogenicity, and further result in therapeutic benefits, for example, better efficacy and lower toxicity. So, novel reductant and processes for preparing ADCs with high homogeneity are highly desirable and long-term pursuit.

SUMMARY

For the above-mentioned purpose, provided herein is a reductant having the following formula

(I):



or a salt, solvate, stereoisomer thereof, which characterized in that,

R_1 is H, $-NH_2$, $-C(O)(R_3R_4)$, optionally substituted C_1 - C_5 alkyl group, optionally substituted C_1 - C_5 hydroxyalkyl group, or optionally substituted aryl group;

R_3 is N, NH or O;

R_4 is H, optionally substituted C_1 - C_5 alkyl group, optionally substituted C_1 - C_5 hydroxyalkyl group, or optionally substituted aryl group;

R_2 is H, optionally substituted C_1 - C_5 alkyl group, or optionally substituted C_1 - C_5 hydroxyalkyl group;

X is OH, optionally substituted C_1 - C_5 alkoxy group or $-NR_5R_6$,

R_5 and R_6 independently are H, C_0 - C_5 hydroxyalkyl group, optionally substituted C_1 - C_5 alkyl group, optionally substituted C_2 - C_8 carboxy alkyl group, optionally substituted C_1 - C_5 alkoxy group, optionally substituted heteroaryl alkyl group, optionally substituted aryl alkoxy group, optionally substituted arylalkyl group, optionally substituted aryl group, C_1 - C_5 alkyl sulfonyl group, or $-(CH_2)n^1(OCH_2CH_2O)n^2CH(R_8)CO(R_7)$,

R_7 is C_0 - C_5 hydroxyalkyl group, $-NHOH$,

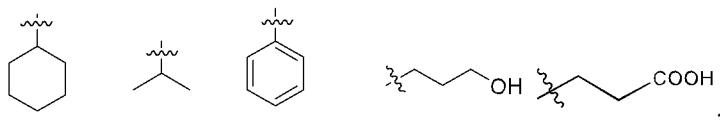
R_8 is H, optionally substituted arylalkyl group,

n^1 and n^2 independently are the number 0, 1, 2, 3, 4,

Y is the same as X, or Y is an ester or amide of X,

Z is the same as X or Y, or

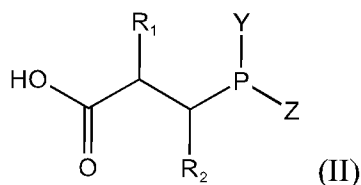
Y and Z independently are selected from the group consisting of



X, Y and Z are not at the same time.

In one aspect, provided herein is a composition comprising a reductant described above and transition metal ions. The transition metal ions are Zn^{2+} , Cd^{2+} , Hg^{2+} , Ni^{2+} , Co^{2+} or the combination thereof.

In one aspect, provided herein is a method of preparing the reductant described above, which characterized in that, at least one X' is connected to a compound of formula II by introducing a condensation reagent under an inert atmosphere,



R₁ is H, -NH₂, -C(O)(R₃R₄), optionally substituted C₁-C₅ alkyl group, optionally substituted C₁-C₅ hydroxyalkyl group, or optionally substituted aryl group;

R₃ is N, NH or O;

R₄ is H, optionally substituted C₁-C₅ alkyl group, optionally substituted C₁-C₅ hydroxyalkyl group, or optionally substituted aryl group;

R₂ is H, optionally substituted C₁-C₅ alkyl group, or optionally substituted C₁-C₅ hydroxyalkyl group;

X' is optionally substituted C₁-C₅ alkyl alcohol or NR₅R₆,

R₅ and R₆ independently are H, C₀-C₅ hydroxyalkyl group, optionally substituted C₁-C₅ alkyl group, optionally substituted C₂-C₈ carboxy alkyl group, optionally substituted C₁-C₅ alkoxy group, optionally substituted heteroaryl alkyl group, optionally substituted aryl alkoxy group, optionally substituted arylalkyl group, optionally substituted aryl group, C₁-C₅ alkyl sulfonyl group, or - (CH₂)^{n¹}(OCH₂CH₂O)^{n²}CH(R₈)CO(R₇),

R₇ is C₀-C₅ hydroxyalkyl group, -NHOH,

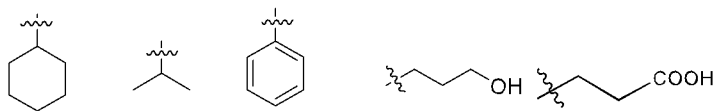
R₈ is H, optionally substituted arylalkyl group,

n¹ and n² independently are the number 0, 1, 2, 3, 4,

Y is the same as X, or Y is an ester or amide of X,

Z is the same as X or Y, or

Y and Z independently are selected from the group consisting of



In one aspect, provided herein is use of the reductant described above or the composition described above in reducing the interchain disulfide bonds of an antibody.

In one aspect, provided herein is a method of preparing an antibody with site-specific modification, comprising steps of

(A1) incubating a reductant described above as a first reductant and the transition metal ions in the presence of an antibody in a buffer system to selectively reduce interchain disulfide bonds within the antibody to afford the antibody bearing reduced thiol groups.

In some embodiments, two interchain disulfide bonds in Fab region of the antibody and one interchain disulfide bonds in hinge region of the antibody are reduced.

In some embodiments, the method further comprising step of

(A2) introducing oxidant to selectively re-oxidize the reduced thiol groups resulted from step (A1), optionally re-oxidize the reduced thiol groups in Fab region of the antibody.

In some embodiments, the method further comprising step of

(A3) incubating a second reductant in a buffer system to selectively reduce the interchain disulfide bonds resulted from step (A2), optionally reduce the interchain disulfide bonds in the hinge region of the antibody.

In some embodiments, the method further comprising the following steps,

(B1) introducing metal chelators and first payload units to react with the reduced thiol groups resulted from step (A1), step (A2) or step (A3), wherein, the first payload unit is an end capping reagent, a first linker-payload or a first thio-bridging reagent, optionally, the first thio-bridging reagent bears the first linker-payload or reactive groups.

In some embodiments, the method further comprising step of

(B2) incubating a second reductant in a buffer system to reduce the interchain disulfide bonds resulted from step (B1), optionally, introducing the transition metal ions; and

(B3) introducing second payload units to react with the reduced thiol groups resulted from step (B2), optionally, introducing the metal chelators, wherein, the second payload unit is a second linker-payload or a second thio-bridging reagent, optionally, the second thio-bridging reagent bears the second linker-payload or reactive groups.

In one aspect, provided herein is a modified antibody prepared by the method described above.

In some embodiments, the modified antibody is the antibody with site-specific modification, optionally, the modified antibody comprises the ADC with D2, the ADC with D4, the ADC with D1, the ADC with D6, the ADC with D3, the ADC with D1+D6, the ADC with D1+D2, the ADC with D1+D4, the ADC with D2+D4, the ADC with D6+D2, the ADC with D6+D1, the ADC with D3+D1, the ADC with D3+D2, the ADC with D0+D6, or the ADC with D0+D2.

In one aspect, provided herein is a pharmaceutical composition comprising an antibody with site-specific modification prepared by the method described above, and at least one pharmaceutically acceptable ingredient.

In one aspect, provided herein is use of the antibody with site-specific modification prepared by the method described above or the pharmaceutical composition described above in the manufacture of a therapeutic agent for preventing, diagnosing or treating a disease.

In one aspect, provided herein is a method of preventing or treating a disease in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of an antibody with site-specific modification prepared by the method described above.

BRIEF DESCRIPTION OF THE DRAWINGS

The following is a brief description of the drawings, which are presented for the purposes of illustrating the exemplary embodiments disclosed herein and not for the purposes of limiting the same.

Figure 1 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Example 32. HIC-HPLC is short for Hydrophobic interaction chromatography-High performance liquid chromatography.

Figure 2 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Example 33.

Figure 3 A-H show HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Examples 34-41, wherein, the reductant is TCEP-1, TCEP-2, TCEP-3, TCEP-4, TCEP-5, TCEP-6, TCEP-7 and TCEP-8.

Figure 4 A-H show HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Examples 42-49, wherein, the reductant is TCEP-9, TCEP-10, TCEP-15, TCEP-18, TCEP-19, TCEP-20, TCEP-23 and TCEP-24.

Figure 5 A-H show HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Examples 50-57, wherein, the reductant is TCEP-25, TCEP-26, TCEP-28, TCEP-A, TCEP-A, TCEP-30, TCEP-31 and TCEP-33.

Figure 6 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Comparative example 5, wherein, the reductant is TCEP.

Figure 7 A-B show HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of examples 58-59, wherein, the molar ratio of the reductant and the antibody is 2.8:1, 3.5:1.

Figure 8 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Example 60.

Figure 9 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Example 61.

Figure 10 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Example 62.

Figure 11 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Example 63.

Figure 12 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Example 64.

Figure 13 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Example 65.

Figure 14 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Example 66.

Figure 15 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Example 67.

Figure 16 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Example 68.

Figure 17 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Example 69.

Figure 18 A-C show HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of examples 70-72, wherein, the molar ratio of the reductant and the antibody is 5:1, 6:1, 7:1.

Figure 19 A-F show HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of examples 73-78, wherein, the molar ratio of the reductant and the antibody is 8:1, 9:1, 10:1, 11:1, 12:1, 13:1.

Figure 20 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Comparative example 1, wherein, the molar ratio of the transition metal ions and the reductant is 0:1.

Figure 21 A-C show HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Comparative examples 2-4, wherein, the molar ratio of the transition metal ions and the reductant is 0:1.

Figure 22 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Example 79.

Figure 23 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Example 80.

Figure 24 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Example 81.

Figure 25 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Example 82.

Figure 26 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Example 83.

Figure 27 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Example 84.

Figure 28 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Example 85.

Figure 29 A-E show HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of examples 86-90, wherein, the molar ratio of the transition metal ions and the reductant is 7.5:1, 15:1, 30:1, 0.22:1, 3.33:1.

Figure 30 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Example 91.

Figure 31 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Example 92.

Figure 32 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Example 93.

Figure 33 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Example 94.

Figure 34 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Example 95.

Figure 35 A-G show HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Examples 96-102, wherein, the buffer system is different.

Figure 36 A-E show HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Examples 103-107, wherein, the buffer system is different.

Figure 37 A-C show HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Examples 108-110, wherein, the concentration of the buffer system is different.

Figure 38 A-F show HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Examples 111-116, wherein, the incubation time in step (1) is different.

Figure 39 A-C show HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate prepared of Examples 117-119, wherein, the incubation time and temperature in step (1) is different.

Figure 40 shows HIC-HPLC of Trastuzumab-[Bismaleimide-DBCO]₃ conjugate of example 120.

Figure 41 A shows HIC-HPLC of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate of example 121; B shows HIC-HPLC of Trastuzumab-[MC-VC-PAB-MMAE]₆ [MC-GGFG-DXd]₂ conjugate of example 121.

Figure 42 A shows HIC-HPLC of Trastuzumab-[MC-GGFG-DXd]₆ conjugate of example 122; B shows HIC-HPLC of Trastuzumab-[MC-GGFG-DXd]₆ [Maleimide-PEG4-N3-DBCO-Cy3]₁ conjugate of example 122.

Figure 43 A shows HIC-HPLC of Trastuzumab-[Maleimide]₆ conjugate of example 123; B shows HIC-HPLC of Trastuzumab-[Maleimide]₆ [MC-VC-PAB-MMAE]₂ conjugate of example 123.

Figure 44 shows HIC-HPLC of Trastuzumab-[Maleimide]₆[Maleimide-PEG4-N3-DBCO-Cy3]₁ conjugate of example 124.

Figure 45 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₂ conjugate prepared of Example 125.

Figure 46 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₂ conjugate prepared of Example 126.

Figure 47 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₂ conjugate prepared of Example 127.

Figure 48 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₂ conjugate prepared of Example 128.

Figure 49 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₂ conjugate prepared of Example 129.

Figure 50 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₂ conjugate prepared of Example 130.

Figure 51 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₂ conjugate prepared of Example 131.

Figure 52 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₂ conjugate prepared of Example 132.

Figure 53 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₂ conjugate prepared of Example 133.

Figure 54 A-H show HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₂ conjugate prepared of Examples 134-141, wherein, the molar ratio of DHAA and the antibody, the molar ratio of the reductant and the antibody, and/or the incubation time in step (1) is different.

Figure 55 A-F show HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₂ conjugate prepared of Examples 142-147, wherein, the molar ratio of DHAA and the antibody and/or the molar ratio of the reductant and the antibody is different.

Figure 56 A-C show HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₂ conjugate prepared of Examples 148-150, wherein, the oxidation temperature and time are different.

Figure 57 A-H show HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₂ conjugate prepared of Examples 151-158, wherein, the buffer system is different.

Figure 58 A-H show HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₂ conjugate prepared of Examples 159-166, wherein, the buffer system is different.

Figure 59 shows HIC-HPLC chromatogram of Trastuzumab-[MC-VC-PAB-MMAE]₂ conjugate prepared of Comparative example 6.

Figure 60 shows HIC-HPLC of Trastuzumab-[Maleimide-PEG4-N3-DBCO-Cy3]₁ conjugate of example 167.

Figure 61 shows HIC-HPLC of Trastuzumab-[Maleimide-PEG4-N3-DBCO-Cy3]₁[MC-VC-PAB-MMAE]₆ conjugate of example 168.

Figure 62 shows HIC-HPLC of Trastuzumab-[Maleimide-PEG4-N3-DBCO-Cy3]₁[MC-VC-PAB-MMAE]₂ conjugate of example 169.

Figure 63 A shows HIC-HPLC of Trastuzumab-[Maleimide-PEG4-N3-DBCO-Cy3]₁ conjugate of example 170; B-C show HIC-HPLC of Trastuzumab-[Maleimide-PEG4-N3-DBCO-Cy3]₁[MC-VC-PAB-MMAE]₄ conjugate of examples 170-171.

Figure 64 A shows HIC-HPLC of Trastuzumab-[MC-GGFG-DXd]₂ conjugate of example 172; B shows HIC-HPLC of Trastuzumab-[MC-GGFG-DXd]₂[MC-VC-PAB-MMAE]₄ conjugate of example 172.

Figure 65 A shows HIC-HPLC of Trastuzumab-[MC-VC-PAB-MMAE]₂ conjugate of example 173; B-C show HIC-HPLC of Trastuzumab-[MC-VC-PAB-MMAE]₂[MC-GGFG-DXd]₄ conjugate of examples 173-174.

DETAILED DESCRIPTION

The present disclosure is explained in greater detail below. This description is not intended to be a detailed catalog of all the different ways in which the invention may be implemented, or all the features that may be added to the instant invention. For example, features illustrated with respect to one embodiment may be incorporated into other embodiments, and features illustrated with respect to a particular embodiment may be deleted from that embodiment. In addition, numerous variations and additions to the various embodiments suggested herein will be apparent to those skilled in the art in

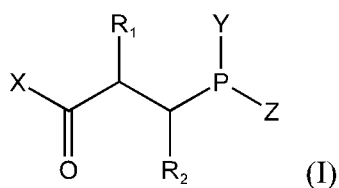
light of the instant disclosure which do not depart from the instant invention. Hence, the following description is intended to illustrate some particular embodiments of the invention, and not to exhaustively specify all permutations, combinations and variations thereof.

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 the disclosure pertains. Although any methods and materials similar or equivalent to those described herein may be used in the practice for testing of the present disclosure, the preferred materials and methods are described herein. In describing and claiming the present disclosure, the following terminology will be used.

Reductant

The present disclosure provides examples of reductant when preparing antibody-drug conjugates (ADCs).

Provided herein is a reductant having the following formula (I):



or a salt, solvate, stereoisomer thereof, which characterized in that,

R_1 is H, $-NH_2$, $-C(O)(R_3R_4)$, optionally substituted C_1 - C_5 alkyl group, optionally substituted C_1 - C_5 hydroxyalkyl group, or optionally substituted aryl group;

R_3 is N, NH or O;

R_4 is H, optionally substituted C_1 - C_5 alkyl group, optionally substituted C_1 - C_5 hydroxyalkyl group, or optionally substituted aryl group;

R_2 is H, optionally substituted C_1 - C_5 alkyl group, or optionally substituted C_1 - C_5 hydroxyalkyl group;

X is OH, optionally substituted C_1 - C_5 alkoxy group or $-NR_5R_6$,

R_5 and R_6 independently are H, C_0 - C_5 hydroxyalkyl group, optionally substituted C_1 - C_5 alkyl group, optionally substituted C_2 - C_8 carboxy alkyl group, or optionally substituted C_1 - C_5 alkoxy group, optionally substituted heteroaryl alkyl group, optionally substituted aryl alkoxy group, optionally substituted arylalkyl group, optionally substituted aryl group, C_1 - C_5 alkyl sulfonyl group, $-(CH_2)n^1(OCH_2CH_2O)n^2CH(R_8)CO(R_7)$,

R_7 is C_0 - C_5 hydroxyalkyl group, $-NHOH$,

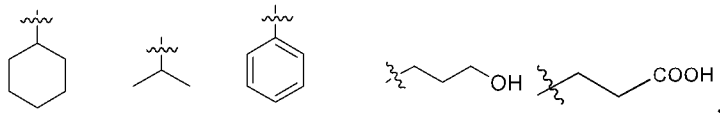
R_8 is H, optionally substituted arylalkyl group,

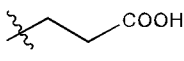
n^1 and n^2 independently are the number 0, 1, 2, 3, 4,

Y is the same as X, or Y is an ester or amide of X,

Z is the same as X or Y, or

Y and Z independently are selected from the group consisting of



X, Y and Z are not  at the same time.

The term “C₁-C₅ alkyl group” refers to an aliphatic hydrocarbon group which having 1 to 5 carbon atoms in the chain or cyclic. Exemplary alkyl groups include methyl, ethyl, n-propyl and i-propyl.

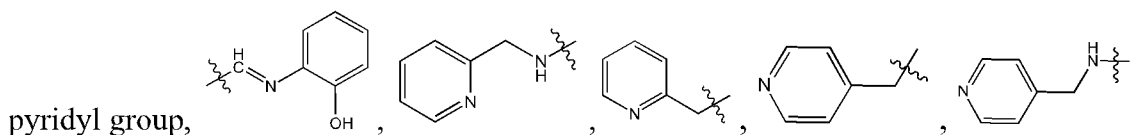
The term “C₀-C₅ hydroxyalkyl group” refers to hydroxy group or C₁-C₅ alkyl group, wherein one or several H atoms are substituted with one, two or three hydroxy groups. Exemplary C₁-C₅ hydroxyalkyl group is hydroxy methyl group, 2-hydroxy ethyl group, 3-hydroxy propyl group.

The term “C₂-C₈ carboxy alkyl group” refers to a C₂-C₈ alkyl group which is substituted with one, two, three, four, five, six or seven carboxy groups. Exemplary C₂-C₈ carboxy alkyl group is -COOH, -CH₂COOH, -CH₂CH₂COOH, -CH₂(CH₂)₂COOH, -CH₂(CH₂)₃COOH, -CH₂(CH₂)₄COOH, -CH₂(CH₂)₅COOH, -CH₂(CH₂)₆COOH or -CH₂(CH₃)COOH.

The term “C₁-C₅ alkyl sulfonyl group” refers to a C₁-C₅ alkyl group, wherein one or several H atoms are substituted with one, two or three sulfonyl group. Exemplary C₁-C₅ alkyl sulfonyl group is -CH₂S(O)₂OH, -CH₂CH₂S(O)₂OH or -CH₂(CH₂)₂S(O)₂OH.

The term “aryl group” refers to an aromatic or hetero aromatic group, composed of one or several rings, comprising three to fourteen carbon atoms, preferentially six to ten carbon atoms. Exemplary aryl group is phenyl group.

The term “aryl group” also refers to an aromatic group, wherein one or several H atoms are replaced independently by other group, such as F, Cl, Br, I, hydroxy, carboxy, sulfonyl, amino, methoxy or ethoxy, N-hydroxy formamide group, N-hydroxy acetamido group, 4-pyridyl group, 2-



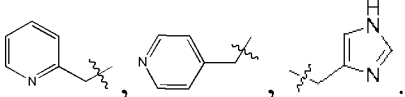
The term “heteroaryl group” refers to one or several carbon on aromatic group, preferentially one, two, three or four carbon atoms are replaced by O, N, Si, Se, P or S, preferentially by O, S, N.

Exemplary heteroaryl group is imidazolyl group, pyridyl group, bipyridyl group, quinolinyl group, iso-quinolinyl group.

The term “heteroaryl group” also refers to hetero aromatic group, wherein one or several H atoms are replaced independently by other group, such as F, Cl, Br, I, hydroxy, carboxy, amino, hydroxyalkyl group, carboxy alkyl group, N-hydroxy amide alkyl group, heteroaryl group.

The term “arylalkyl group” refers to a liner, branched or cycloalkyl which is linked to at least one aryl group. Preferable the number of carbon atoms in the chain or cyclic is 1-4. Exemplary arylalkyl group is $-\text{CH}_2\text{C}_6\text{H}_5$, $-\text{CH}_2\text{CH}_2\text{C}_6\text{H}_5$, $-\text{CH}_2\text{CH}_2\text{CH}_2\text{C}_6\text{H}_5$, $-\text{CH}_2(\text{CH}_3)\text{CH}_2\text{C}_6\text{H}_5$, $-\text{CH}_2(\text{CH}_3)\text{CH}_2\text{CH}_2\text{C}_6\text{H}_5$.

The term “heteroaryl alkyl group” refers to a liner, branched or cycloalkyl which is linked to at least one heteroaryl group. Preferable the number of carbon atoms in the chain or cyclic is 1-4.

Exemplary heteroaryl alkyl group is .

The term “aryl alkoxy group” refers to an aromatic group, wherein one or several H atoms are replaced by alkoxy group. Exemplary phenyl-O-CH₂-, phenyl-O-(CH₂)₂-, phenyl-O-(CH₂)₃-, phenyl-O-(CH₂)₄-, phenyl-O-(CH₂)₅-.

The term “C₁-C₅ alkoxy group” refers to an oxygen atom attached to C₁-C₅ alkyl group. Exemplary C₁-C₅ alkoxy group is -OCH₃, -OCH₂CH₃, -OCH₂(CH₃)₂, -OCH₂CH₂CH₃.

The term “halogen” refers to F, Cl, Br or I.

The term “Alkenyl” refers to a straight or branched chain unsaturated hydrocarbon containing 2-12 carbon atoms. The “alkenyl” group contains at least one double bond in the chain. The double bond of an alkenyl group can be unconjugated or conjugated to another unsaturated group. Examples of alkenyl groups include ethenyl, propenyl, n-butenyl, iso-butenyl, pentenyl, or hexenyl. An alkenyl group can be unsubstituted or substituted and may be straight or branched.

The term “Cyano” refers to a substituent having a carbon atom joined to a nitrogen atom by a triple bond, e.g., -CN.

In some embodiments, R₁ is H, and R₂ is H.

In some embodiments, X is -OCH₃, -OCH₂CH₃, or -O(CH₃)₂.

In some embodiments, X is $-\text{NR}_5\text{R}_6$, R_5 is H, and

R_6 is H, $\text{C}_0\text{-C}_5$ hydroxyalkyl group, $\text{C}_1\text{-C}_5$ alkoxy group, optionally substituted heteroaryl alkyl group, optionally substituted aryl alkoxy group, optionally substituted aryl group, optionally substituted arylalkyl group, $\text{C}_1\text{-C}_5$ alkyl sulfonyl group or $-(\text{CH}_2)^{n^1}(\text{OCH}_2\text{CH}_2\text{O})^{n^2}\text{CH}(\text{R}_8)\text{CO}(\text{R}_7)$,

R_7 is $\text{C}_0\text{-C}_3$ hydroxyalkyl group or $-\text{NHOH}$,

R_8 is H or optionally substituted arylalkyl group,

n^1 and n^2 independently are the number 0.

In some embodiments, R_6 is H, $\text{C}_0\text{-C}_2$ hydroxyalkyl group, $\text{C}_1\text{-C}_3$ alkoxy group, $\text{C}_1\text{-C}_3$ alkyl sulfonyl group, bipyridyl group, benzyl group, aryl alkoxy group, phenyl group which is optionally substituted with OH, carboxy or pyridyl group, or $-\text{CH}(\text{R}_8)\text{CO}(\text{R}_7)$,

R_7 is OH or $-\text{NHOH}$,

R_8 is H or benzyl group which is optionally substituted with OH, halogen, cyano group or nitro group.

In some embodiments, X is $-\text{NR}_5\text{R}_6$, R_5 is H, and

R_6 is H, OH, C_1 hydroxyalkyl group, C_2 hydroxyalkyl group, C_3 hydroxyalkyl group, C_4 hydroxyalkyl group, C_5 hydroxyalkyl group, C_1 alkoxy group, C_2 alkoxy group, C_3 alkoxy group, C_4 alkoxy group, C_5 alkoxy group, heteroaryl alkyl group optionally substituted with heteroaryl group, aryl methoxy group, aryl ethoxy group, aryl propoxy group, aryl butoxy group, aryl group optionally substituted with OH, carboxy or pyridyl group, benzyl group, aryl ethyl group, aryl propyl group, C_1 alkyl sulfonyl group, C_2 alkyl sulfonyl group, C_3 alkyl sulfonyl group, C_5 alkyl sulfonyl group, C_5 alkyl sulfonyl group or $-(\text{CH}_2)^{n^1}(\text{OCH}_2\text{CH}_2\text{O})^{n^2}\text{CH}(\text{R}_8)\text{CO}(\text{R}_7)$,

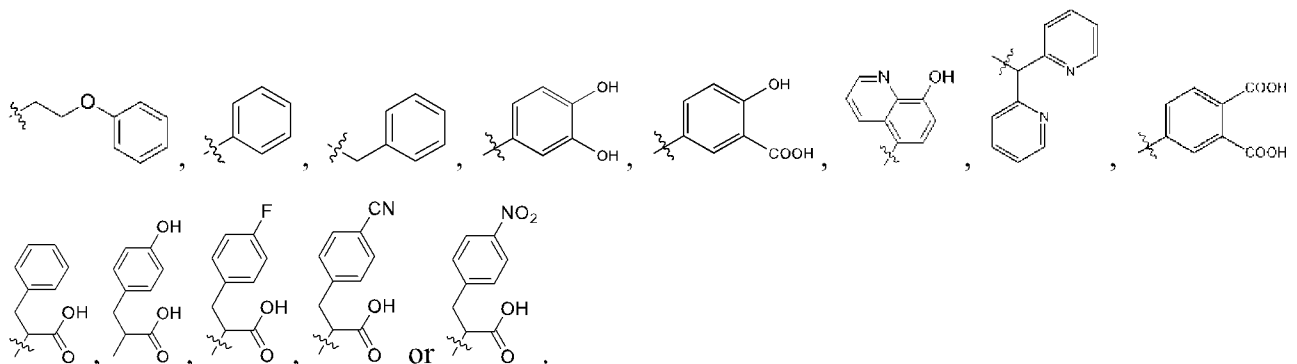
R_7 is OH, C_1 hydroxyalkyl group, C_2 hydroxyalkyl group, C_3 hydroxyalkyl group or $-\text{NHOH}$,

R_8 is H or arylalkyl group which is optionally substituted with OH, halogen, cyano group or nitro group, wherein, halogen is selected from F, Cl, Br or I,

n^1 and n^2 independently are the number 0.

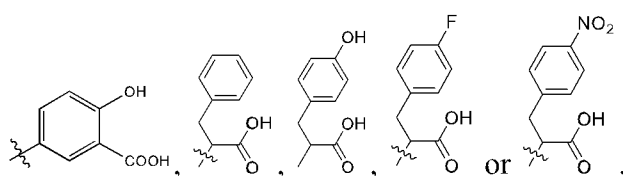
In some embodiments, X is $-\text{NR}_5\text{R}_6$, R_5 is H, and

R_6 is H, OH, $-\text{CH}_2\text{OH}$, $-(\text{CH}_2)_2\text{OH}$, $-\text{CH}_3$, $-\text{CH}_2\text{CH}_3$, $-\text{CH}_2\text{COOH}$, $-(\text{CH}_2)_2\text{COOH}$, $-(\text{CH}_2)_3\text{COOH}$, $-(\text{CH}_2)_4\text{COOH}$, $-(\text{CH}_2)_5\text{COOH}$, $-\text{OCH}_3$, $-\text{OCH}_2\text{CH}_3$, $-\text{CH}_2\text{CONHOH}$, $-\text{OC}(\text{C}_6\text{H}_5)_3$, $-(\text{CH}_2)_3\text{S}(\text{O})_2\text{OH}$,



In some embodiments, X is $-NR_5R_6$, R_5 is H, and

R_6 is H, OH, $-CH_2COOH$, $-CH_2CONHOH$, $-OC(C_6H_5)_3$,



In some embodiments, X is $-NR_5R_6$, R_5 is H, and R_6 is OH, $-CH_2COOH$, $-(CH_2)_2COOH$, $-(CH_2)_3COOH$, $-(CH_2)_4COOH$, $-(CH_2)_5COOH$, $-OCH_3$, $-OCH_2CH_3$, or $-OC(C_6H_5)_3$.

In some embodiments, X is $-NR_5R_6$, R_5 is OH,

R_6 is C_1 - C_5 alkyl group, optionally substituted heteroaryl alkyl group, optionally substituted arylalkyl group, optionally substituted aryl group, or $-(CH_2)n^1(OCH_2CH_2O)n^2CH(R_8)CO(R_7)$,

R_7 is C_0 - C_5 hydroxyalkyl group,

R_8 is H,

n^1 and n^2 independently are the number 0, 1, 2, 3, 4.

In some embodiments, R_6 is C_1 - C_3 alkyl group, heteroaryl alkyl group which comprises a heteroatom N, optionally substituted benzyl group, optionally substituted phenyl group, or $-CH(R_8)CO(R_7)$,

R_7 is C_0 - C_3 hydroxyalkyl group,

R_8 is H.

In some embodiments, R_6 is C_1 alkyl group, C_2 alkyl group, C_3 alkyl group, C_4 alkyl group, C_5 alkyl group, heteroaryl methyl group, heteroaryl ethyl group, heteroaryl propyl group, benzyl group, aryl ethyl group, aryl propyl group, or $-CH(R_8)CO(R_7)$,

R_7 is hydroxy, C_1 hydroxyalkyl group, C_2 hydroxyalkyl group, C_3 hydroxyalkyl group, C_4 hydroxyalkyl group or C_5 hydroxyalkyl group,

R₈ is H.

In some embodiments, R₆ is -CH₃, -CH₂COCH₃, -CH₂COOH, ,  or .

In some embodiments, R₆ is -CH₂COOH.

In some embodiments, X is -NR₅R₆,

R₅ and R₆ independently are C₁-C₅ alkyl group, C₀-C₅ hydroxyalkyl group, optionally substituted heteroaryl alkyl group or -(CH₂)^{n¹}(OCH₂CH₂O)^{n²}CH(R₈)CO(R₇),

R₇ is C₀₋₅ hydroxyalkyl group or -NHOH,

R₈ is H,

n¹ and n² independently are the number 0, 1, 2, 3, 4.

In some embodiments, X is -NR₅R₆,

R₅ and R₆ independently are C₁ alkyl group, C₂ alkyl group, C₃ alkyl group, C₄ alkyl group, C₅ alkyl group, OH, C₁ hydroxyalkyl group, C₂ hydroxyalkyl group, C₃ hydroxyalkyl group, C₄ hydroxyalkyl group, C₅ hydroxyalkyl group, heteroaryl methyl group, heteroaryl ethyl group, heteroaryl propyl group or -(CH₂)^{n¹}(OCH₂CH₂O)^{n²}CH(R₈)CO(R₇),

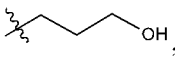
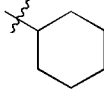
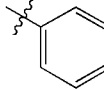
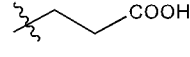
R₇ is hydroxy, C₁ hydroxyalkyl group, C₂ hydroxyalkyl group, C₃ hydroxyalkyl group, C₄ hydroxyalkyl group, C₅ hydroxyalkyl group or -NHOH,

R₈ is H,

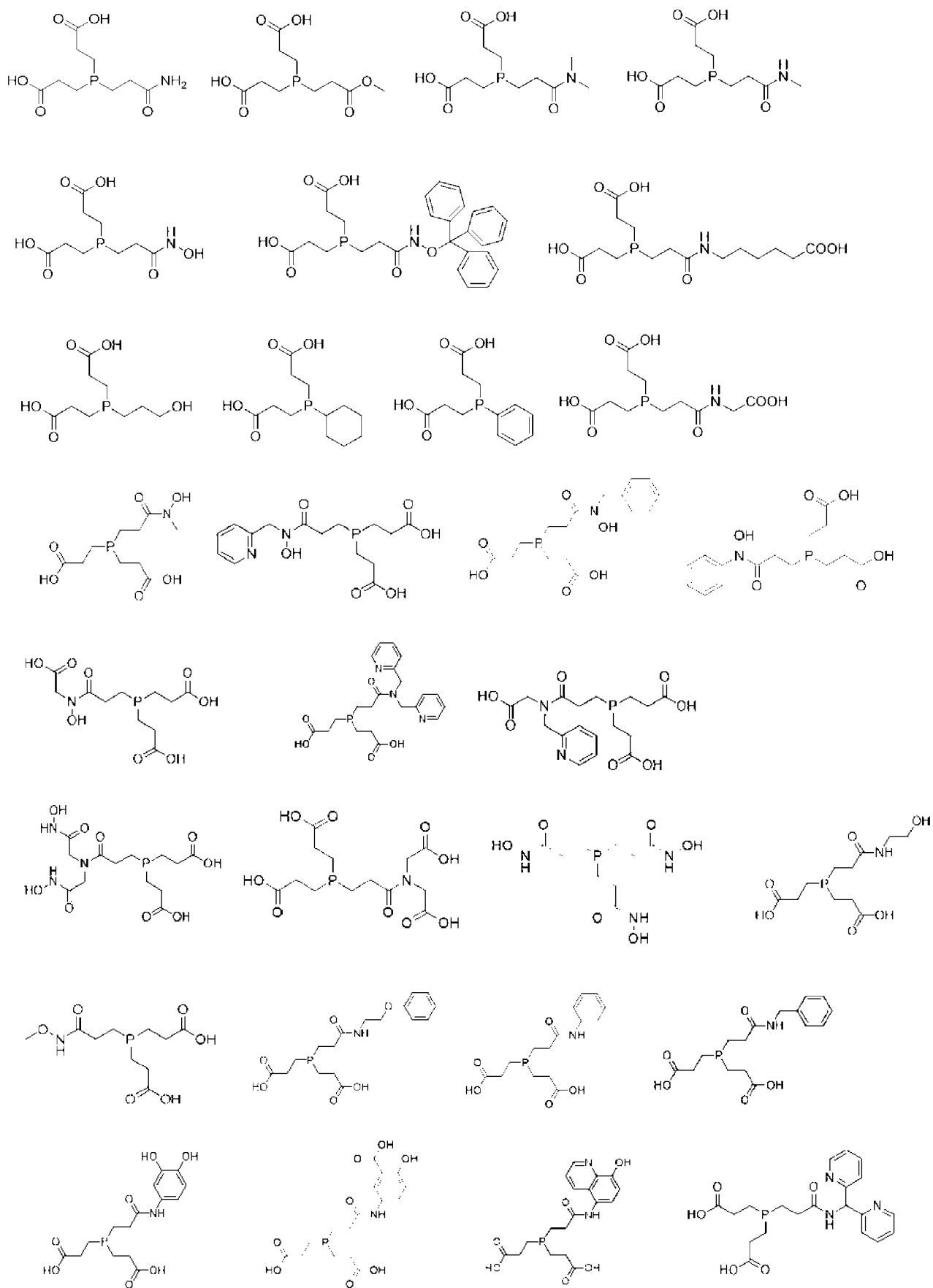
n¹ and n² independently are the number 0, 1, 2, 3, 4.

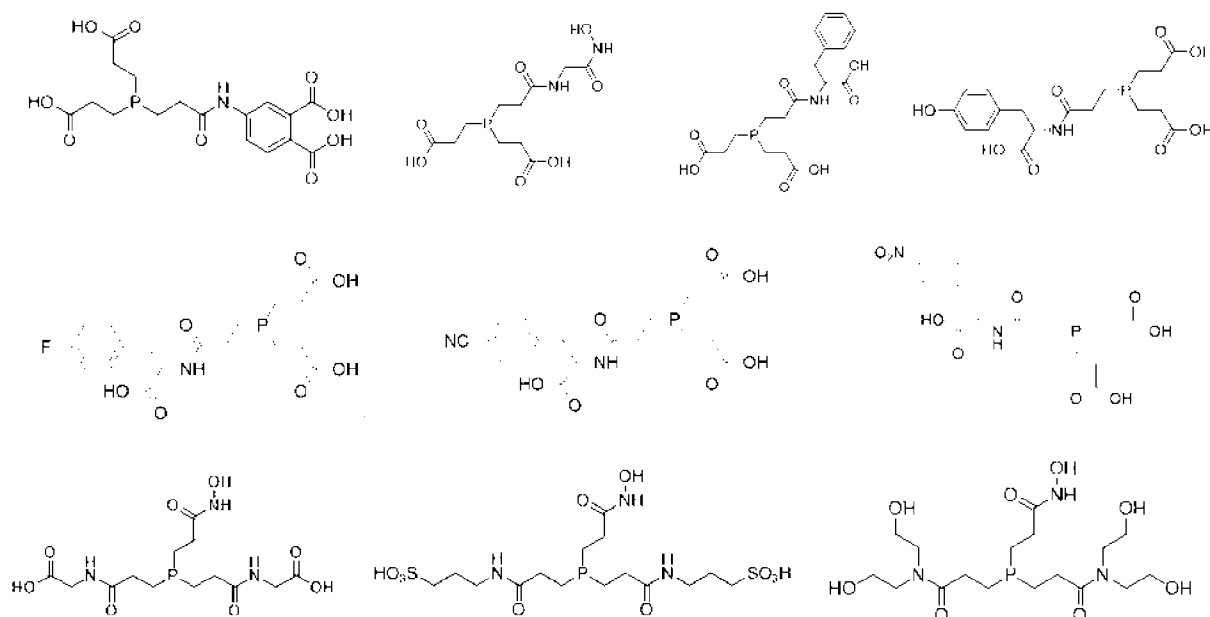
In some embodiments, X is -NR₅R₆, R₅ and R₆ independently are methyl, ethyl group, -(CH₂)₂OH,

-CH₂COOH, -CH₂CONHOH or .

In some embodiments, Y is ,  or ; Z is .

In some embodiments, the reductant is selected from the group consisting of





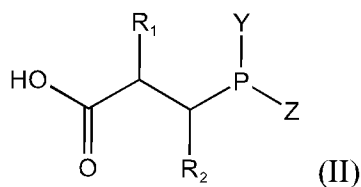
The disclosure provides a composition including a reductant described above and transition metal ions.

In some embodiments, the transition metal ions are Zn^{2+} , Cd^{2+} , Hg^{2+} , Ni^{2+} , Co^{2+} or the combination thereof. In some embodiments, the transition metal ions are Zn^{2+} .

In some embodiments, the molar ratio of the transition metal ions and the first reductant described above is 0.05:1 to 40:1, 0.25:1 to 30:1, 0.25:1 to 15:1, 0.1:1 to 10:1, 0.25:1 to 9:1, 0.2:1 to 7.5:1, 0.2:1 to 6:1, 0.2:1 to 5:1, 0.25:1 to 4:1, 0.5:1 to 7.5:1, 1:1 to 7.5:1, or 2:1 to 4:1.

The reductant having formula (I) described above could be prepared as the following steps:

at least one X' is connected to a compound of formula II by introducing a condensation reagent under an inert atmosphere,



R_1 is H, $-\text{NH}_2$, $-\text{C}(\text{O})(\text{R}_3\text{R}_4)$, optionally substituted C_1 - C_5 alkyl group, optionally substituted C_1 - C_5 hydroxyalkyl group, or optionally substituted aryl group;

R_3 is N, NH or O;

R_4 is H, optionally substituted C_1 - C_5 alkyl group, optionally substituted C_1 - C_5 hydroxyalkyl group, or optionally substituted aryl group;

R₂ is H, optionally substituted C₁-C₅ alkyl group, or optionally substituted C₁-C₅ hydroxyalkyl group;

X' is optionally substituted C₁-C₅ alkyl alcohol or NR₅R₆,

R₅ and R₆ independently are H, C₀-C₅ hydroxyalkyl group, optionally substituted C₁-C₅ alkyl group, optionally substituted C₂-C₈ carboxy alkyl group, optionally substituted C₁-C₅ alkoxy group, optionally substituted heteroaryl alkyl group, optionally substituted aryl alkoxy group, optionally substituted arylalkyl group, optionally substituted aryl group, C₁-C₅ alkyl sulfonyl group, - (CH₂)^{n¹}(OCH₂CH₂O)^{n²}CH(R₈)CO(R₇),

R₇ is C₀-C₅ hydroxyalkyl group, -NHOH,

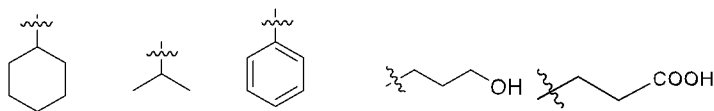
R₈ is H, optionally substituted arylalkyl group,

n¹ and n² independently are the number 0, 1, 2, 3, 4,

Y is the same as X, or Y is an ester or amide of X,

Z is the same as X or Y, or

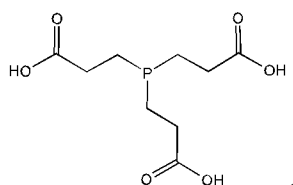
Y and Z independently are selected from the group consisting of



The term “condensation reagent” refers to a condensation reaction reagent, which helps two molecules (functional groups) combine covalently to form one single molecule. Condensation reagent includes, but not limited to 1-Hydroxybenzotriazole (HOBt), O-Benzotriazole-N,N,N',N'-tetramethyluronium-hexafluorophosphate (HBTU), and O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU).

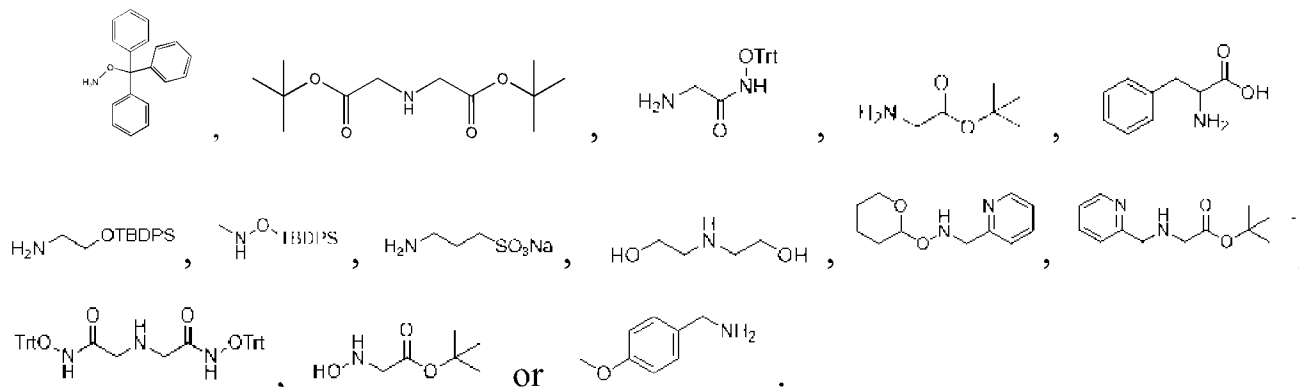
The term “inert atmosphere” refers to the chemically inactive atmosphere, such as nitrogen, carbon dioxide, helium.

In some embodiments, the compound of formula II is



In some embodiments, characterized in that, the X' is 2-phenoxy-ethylamine, Phenylamine, Benzylamine, 4-Aminobenzene-1,2-diol, 5-Amino-2-hydroxybenzoic acid, Bis(pyridin-2-ylmethyl)amine, 5-Amino-8-hydroxyquinoline, Bis(pyridin-2-yl) methanamine, 4-Aminophthalic

acid, tert-Butyl L-tyrosinate, DL-3-(4-Fluorophenyl)alanine, DL-4-Cyanophenylalanine, DL-4-nitrophenylalanine, N-Benzylhydroxylamine hydrochloride, N-Phenylhydroxylamine,



Use of Reductant

The reductant having formula (I) provided above has reducibility and could reduce the disulfide bond of an antibody, thus the reductant could be used to modify protein or antibody.

As used herein, the term “disulfide bond” refers to a covalent bond with the structure R-S-S-R'. The amino acid cysteine includes a thiol group that can form a disulfide bond with a second thiol group, for example from another cysteine residue. The disulfide bond can be formed between the thiol groups of two cysteine residues residing respectively on the two polypeptide chains, thereby forming an interchain bridge or interchain bond.

In some embodiments, the reductant having formula (I) could reduce the interchain disulfide bonds of an antibody.

In some embodiments, the reductant having formula (I) could selectively reduce three of the interchain disulfide bonds of the antibody.

In some embodiments, the reductant having formula (I) could selectively reduce the interchain disulfide bonds, thus the antibody is selectively modified.

In some embodiments, provided herein is the use of the reductant having formula (I) provided above and the composition described above in reducing the interchain bonds of an antibody, optionally, provided herein is the use of the reductant having formula (I) provided above and the composition described above in reducing three of the interchain bonds of an antibody.

In some embodiments, the use of the reductant having formula (I) in the preparation of an antibody with site-specific modification, optionally, the antibody with site-specific modification is antibody drug conjugate (ADCs).

A mixture of antibody-drug conjugates will be generated by the conventional conjugation processes or the bio-conjugation process of the present disclosure. In general, one antibody molecule belonging to IgG1 or IgG4 subclass has 4 inter-chain disulfide bonds, each of which is formed with two -SH groups. The antibody molecule can be subjected to partial or complete reduction of one or more interchain disulfide bonds to form $2n$ (n is an integer selected from 1, 2, 3 or 4) reactive -SH groups, and thus, the number of drugs (or payloads) coupling to a single antibody molecule is 1, 2, 3, 4, 5, 6, 7, or 8. In accordance with the number of drugs coupling to a single antibody molecule, the different conjugates containing different number of drug molecules are denominated as D0, D2, D4, D6, D8, D3, D1, D6+D1, D6+D2, D3+D1, D3+D2, D0+D1, D0+D2, D1+D6, D1+D2, D1+D4 or D2+D4. And thus, the “homogeneity” of antibody-drug conjugates is used to describe the property of dominance of one specific type of antibody-drug conjugate (i.e., one type selected from D0, D2, D4, D6, D8, D3, D1, D6+D1, D6+D2, D3+D1, D3+D2, D0+D1, D0+D2, D1+D6, D1+D2, D1+D4 or D2+D4 conjugates) in one given mixture of antibody-drug conjugates.

Drug to Antibody Ratio (DAR) of ADC is the average number of drugs linked to each antibody. DAR is a key property used to measure the quality of ADC because it can significantly affect ADC efficacy. The DAR distribution (D0, D2, D4, D6, D8) could reflect the homogeneity of the ADC.

Drug loading is represented by the number of drug moieties per antibody in a molecule of ADC. For some antibody-drug conjugates, the drug loading may be limited by the number of attachment sites on the antibody. For example, where the attachment is a cysteine thiol, as in certain exemplary embodiments described herein, the drug loading may range from 0 to 8 drug moieties per antibody. In certain embodiments, the average drug loading for an antibody-drug conjugate ranges from 1 to about 8; from about 2 to about 6; or from about 3 to about 5.

As used herein, the term “D0” or “the ADC with D0” refers to the ADC in which the average number of drugs coupling to a single antibody molecule is about zero.

As used herein, the term “D2” or “the ADC with D2” refers to DAR about 2, it means about two drug molecules (e.g., 1.5, 2.0, 2.5 molecules) are coupled to one single antibody molecule on average. Drug molecules may be coupled to -SH groups generated by reduction of disulfide bond between heavy and light chains or heavy and heavy chains via linkers.

As used herein, the term “D4” or “the ADC with D4” refers to the ADC in which about four drug molecules (e.g., 3.5, 4.0, 4.5 molecules) are coupled to one single antibody molecule on average, where the drug molecules may be coupled to four -SH groups generated by reduction of two interchain disulfide bonds or intrachain disulfide bonds.

As used herein, the term “D6” or “the ADC with D6” refers to the ADC in which about six drug molecules (e.g., 5.5, 6.0, 6.5 molecules) are coupled to one single antibody molecule on average, where the drug molecules may be coupled to six -SH groups generated by reduction of three disulfide bond.

As used herein, the term “D8” or “the ADC with D8” refers to the ADC in which about eight drug molecules (e.g., 7.5, 8.0, 8.5 molecules) are coupled to one single antibody molecule on average, where the drug molecules may be coupled to eight-SH groups generated by reduction of four disulfide bond.

As used herein, the term “D1” or “the ADC with D1” refers to the ADC in which one of the first thio-bridging group bearing the first linker-payload re-bridges two thiol groups of one single antibody molecule.

As used herein, the term “D3” or “the ADC with D3” refers to the ADC in which three of the first thio-bridging group bearing the first linker-payload re-bridges six thiol groups of one single antibody molecule.

As used herein, the term “D6+D1” or “the bi-payload ADC with D6+D1” refers to the ADC in which six of the first linker-payloads and one of the second thio-bridging groups bearing the second linker-payload are coupled to one single antibody molecule.

As used herein, the term “D6+D2” or “the bi-payload ADC with D6+D2” refers to the ADC in which six of the first linker-payloads and two of the second linker-payloads are coupled to one single antibody molecule.

As used herein, the term “D3+D1” or “the bi-payload ADC with D3+D1” refers to the ADC in which three of the first thio-bridging group bearing the first linker-payload and one of the second thio-bridging group bearing the second linker-payload re-bridge eight thiol groups of one single antibody molecule.

As used herein, the term “D3+D2” or “the bi-payload ADC with D3+D2” refers to the ADC in which three of the first thio-bridging group bearing the first linker-payload re-bridge six thiol groups and two of the second linker-payloads are coupled to one single antibody molecule.

As used herein, the term “D0+D2” or “the ADC with D0+D2” refers to the ADC in which one, two or three of the first thio-bridging group re-bridge six thiol groups and two of the second linker-payloads are coupled to one single antibody molecule, or refers to the ADC in which two, four or six of the end capping reagents and two of the second linker-payloads are coupled to one single antibody molecule.

As used herein, the term “D0+D1” or “the ADC with D0+D1” refers to the ADC in which three of the first thio-bridging group re-bridges six thiol groups and one of the second thio-bridging group bearing the linker-payload re-bridge two thiol groups of one single antibody molecule, or refers to the ADC in which six of the end capping reagents react with six thiol groups and one of the second thio-bridging group bearing the linker-payload re-bridge two thiol groups of one single antibody molecule.

As used herein, the term “D1+D6” or “the bi-payload ADC with D1+D6” refers to the ADC in which one of the first thio-bridging group bearing the first linker-payload re-bridging two thiol groups and six of the second linker-payloads are coupled to one single antibody molecule, wherein, the first linker-payload and the second linker-payload may be same or different.

As used herein, the term “D1+D2” or “the bi-payload ADC with D1+D2” refers to the ADC in which one of the first thio-bridging group bearing the first linker-payload re-bridging two thiol groups and two of the second linker-payloads are coupled to one single antibody molecule, wherein, the first linker-payload and the second linker-payload may be same or different.

As used herein, the term “D1+D4” or “the bi-payload ADC with D1+D4” refers to the ADC in which one of the first thio-bridging group bearing the first linker-payload re-bridging two thiol groups and four of the second linker-payloads are coupled to one single antibody molecule, wherein, the first linker-payload and the second linker-payload may be same or different.

As used herein, the term “D2+D4” or “the bi-payload ADC with D2+D4” refers to the ADC in which two of the first linker-payloads and four of the second linker-payloads are coupled to one single antibody molecule.

As used herein, the term “about” refers to a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that varies by as much as 30, 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1% to a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length. In particular embodiments, the terms “about” when preceding a numerical value indicates the value plus or minus a range of 50%, 30%, 15%, 10%, 5%, or 1%.

In some embodiments, by conjugating two different linker-payloads to a single antibody to form a bi-payload ADC. According to the DAR value of each linker-payload, the bi-payload ADC may be with two DAR values, such as D6 for first linker-payload and D2 for second linker-payload. The DAR value of di-payload ADC in present disclosure is referred to as DN+DM, of which N denotes the average number of the first linker-payload coupled to one single antibody molecule on average, and M denotes the average number of the second linker-payload coupled to one single antibody molecule on average.

In some embodiments, the reductant having formula (I) provided above or the composition provided above could be used to prepare ADC with improved homogeneity.

In some embodiments, the disclosure provides the use of reductant having formula (I) or the composition provided above in the preparation of ADC, optionally, in the preparation of the ADC with D2, the ADC with D4, the ADC with D1, the ADC with D6, the ADC with D3, the ADC with D1+D6, the ADC with D1+D2, the ADC with D1+D4, the ADC with D2+D4, the ADC with D6+D2, the ADC with D6+D1, the ADC with D3+D1, the ADC with D3+D2, the ADC with D0+D6, or the ADC with D0+D2.

In some embodiments, the ADC includes D6 in a content at least up to 50% of the total weight of D0, D2, D4, D6 and D8 combined. In some embodiments, the ADC includes D6 in a content up to 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95% or 96% of the total weight of D0, D2, D4, D6 and D8 combined.

In some embodiments, the ADC includes D2 in a content at least up to 70% of the total weight of D0, D2, D4, D6 and D8 combined. In some embodiments, the ADC includes D6 in a content up to 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, or 95% of the total weight of D0, D2, D4, D6 and D8 combined.

In some embodiments, the homogeneity of the ADC with D3 is up to 82%, 83% or 85%.

In some embodiments, the homogeneity of the ADC with D6+D2 is up to 80%, 81%, 82%, 83%, 84%, 85% or 86%.

In some embodiments, the homogeneity of the ADC with D6+D1 is up to 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91% or 92%.

In some embodiments, the homogeneity of the ADC with D0+D2 is up to 65%, 67%, 69%, 70%, 71% or 73%.

In some embodiments, the homogeneity of the ADC with D0+D1 is up to 70%, 71%, 73%, 75%, 77%, 79%, 80% or 83%.

In some embodiments, the homogeneity of the ADC with D1 is up to 77%, 79%, 80%, 83% or 85%.

In some embodiments, the homogeneity of the ADC with D1+D6 is up to 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89% or 90%.

In some embodiments, the homogeneity of the ADC with D1+D2 is up to 65%, 67%, 69%, 70%, 71%, 73%, 75% or 77%.

In some embodiments, the homogeneity of the ADC with D1+D4 is up to 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89% or 90%.

In some embodiments, the homogeneity of the ADC with D2+D4 is up to 60%, 77%, 79%, 80%, 83% or 85%.

Antibody with site-specific modification and preparation thereof

The present disclosure also provides a method of preparing an antibody with site-specific modification by using the reductant of formula (I) and the modification antibody thereof. Optionally, the present disclosure provides a method of preparing ADCs with improved homogeneity by using the reductant of formula (I) or a salt, solvate, stereoisomer thereof.

In some embodiments, the method of preparing an antibody with site-specific modification includes the following steps:

(A1) incubating a reductant of formula (I) or a salt, solvate, stereoisomer thereof as a first reductant and the transition metal ions in the presence of an antibody in a buffer system to selectively reduce interchain disulfide bonds within the antibody to afford the antibody bearing reduced thiol groups.

In some embodiments, two interchain disulfide bonds in Fab region of the antibody and one interchain disulfide bonds in hinge region of the antibody are reduced in step (A1).

In some embodiments, the method of preparing the antibody with site-specific modification further includes the step:

(A2) introducing oxidant to selectively re-oxidize the reduced thiol groups resulted from step (A1).

In some embodiments, the oxidant in step (A2) re-oxidizes the reduced thiol groups in Fab region of the antibody. Optionally, four of the reduced thiol groups are re-oxidized to form two disulfide bonds.

In some embodiments, the resulting product of step (A2) also performs purification after oxidation. The purification could conduct by, include but not limited to, a desalting column, ultrafiltration (UF) and diafiltration (DF).

In some embodiments, the method of preparing the antibody with site-specific modification further includes the step,

(A3) incubating a second reductant in a buffer system to reduce the interchain disulfide bonds resulted from step (A2).

In some embodiments, the second reductant reduce the interchain disulfide bonds of the antibody in step (A3), optionally, one interchain disulfide bond in hinge region is reduced.

In some embodiments, the method of preparing the antibody with site-specific modification further comprising the following step of,

(B1) introducing metal chelators and first payload units to react with the reduced thiol groups resulted from step (A1), step (A2) or step (A3), wherein, the first payload unit is an end capping reagent, a first linker-payload or a first thio-bridging reagent, optionally, the first thio-bridging reagent bears the first linker-payload or reactive groups.

In some embodiments, when the first payload units are the first thio-bridging reagent bearing reactive groups, the step (B1) further comprising step of

incubating the metal chelators and the first linker-payloads in the buffer system to react with the reactive groups of the first thio-bridging reagent bearing reactive groups.

In some embodiments, the method of preparing the antibody with site-specific modification further includes the steps:

(B2) incubating a second reductant in a buffer system to reduce interchain disulfide bonds resulted from step (B1), optionally, introducing the transition metal ions; and

(B3) introducing second payload units to react with the reduced thiol groups resulted from step (B2), optionally, introducing the metal chelators, wherein, the second payload unit is a second linker-payload or a second thio-bridging reagent, optionally, the second thio-bridging reagent bears the second linker-payload or reactive groups.

In some embodiments, without the transition metal ions, the second reductant reduces all the interchain disulfide bonds of the antibody in step (B2). In some embodiments, with step (A1), (B1) and (B2), one interchain disulfide bond in the hinge region of the antibody is reduced. In some embodiments, with step (A1), (A2), (B1) and (B2), three interchain disulfide bonds are reduced. In some embodiments, with step(A1), (A2), (A3), (B1) and (B2), two interchain disulfide bonds are reduced.

In some embodiments, with the transition metal ions, the second reductant selective reduces the interchain disulfide bonds of the antibody, optionally, one or two interchain disulfide bonds are reduced. In some embodiments, with step (A1), (A2), (B1) and (B2), the second reductant and the transition metal ions selective reduce one or two interchain disulfide bonds of the antibody.

In some embodiments, when the second payload units are the second thio-bridging reagent bearing reactive groups, the step (B3) further comprising step of

incubating the second linker-payloads in the buffer system to react with the reactive groups of the second thio-bridging reagent bearing reactive groups, optionally, introducing the metal chelators.

In some embodiments, when introducing the transition metal ions in step (B2), introducing the metal chelators to trap the excess transition metal ions in step (B3).

In some embodiments, the molar ratio of the reductant of formula (I) or the second reductant and the antibody in step (A1), (A3) and (B2) independently is 1:1 to 20:1, 1:1 to 10:1, 1:1 to 8:1, 1:1 to 5:1, 2:1 to 5:1, 3:1 to 5:1, 1:1 to 3:1, 1:1 to 2:1, 2:1 to 4:1, or 3:1 to 4:1.

In some embodiments, the molar ratio of the first reductant and the antibody in step (A1) is 2.8:1 to 13:1, optionally, the molar ratio of the first reductant and the antibody is 3.5:1 to 5:1, 4:1 to 10:1 or 5:1 to 13:1, 3:1 to 5:1, 4:1 to 5:1, or 3.8:1 to 4.6:1. In some embodiments, the molar ratio of the first reductant and the antibody in step (A1) is 2.8:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, 15:1, 16:1, 17:1, 18:1, 19:1 or 20:1.

In some embodiments, the incubation temperature in step (A1), (A3) and (B2) independently is 0°C to 37°C, 0°C to 25°C, 0°C to 15°C, 0°C to 10°C, or 0°C to 5°C.

In some embodiments, the incubation temperature in step (A1) is 37°C, 35°C, 33°C, 30°C, 28°C, 24°C, 20°C, 18°C, 15°C, 13°C, 10°C, 8°C, 4°C or 0°C.

In some embodiments, the incubation time in step (A1) is 2h to 24h, 14h to 24h, 16h to 20h, or 16h to 18h. In some embodiments, the incubation time in step (A1) is 2h, 4h, 6h, 8h, 10h, 12h, 14h, 16h, 18h, 20h, 22h or 24h.

In some embodiments, in step (A1), the molar ratio of the first reductant and the antibody is 2.8:1 to 3:1, the incubation time is 10h to 24h. In some embodiments, the incubation time in step (A1), the molar ratio of the first reductant and the antibody is 2.8:1, 2.9:1 or 3:1, the incubation time is 10h, 12h, 16h, 18h, 20h, 22h or 24h.

In some embodiments, the incubation time in step (A1) is shortened with increasing the molar ratio of the first reductant and the antibody. In some embodiments, in step (A1), the molar ratio of the first reductant and the antibody is 4:1 to 20:1, the incubation time is 1h to 24h. In some embodiments, in step (A1), the molar ratio of the first reductant and the antibody is 4:1 to 10:1, the incubation time is 2h to 16h. In some embodiments, in step (A1), the molar ratio of the first reductant and the antibody is 4:1, 4.5:1, 5:1, 5:1, 7:1, 8:1, 9:1 or 10:1, the incubation time is 1h, 2h, 4h, 5h, 6h, 7h, 8h, 9h, 10, 11h or 12h.

In some embodiments, in step (A1), the molar ratio of the first reductant and the antibody is 5:1 to 20:1, the incubation time is 3h to 24h. In some embodiments, in step (A1), the molar ratio of the first reductant and the antibody is 6:1 to 13:1, the incubation time is 4h to 16h. In some embodiments, in step (A1), the molar ratio of the first reductant and the antibody is 4.5:1, 5:1, 5:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1 or 13:1, the incubation time is 4h, 5h, 6h, 7h, 8h, 9h, 10, 11h, 12h, 13h, 14h, 15h or 16h.

In some embodiments, the molar ratio of the transition metal ions and the first reductant in step (A1) is 0.05:1 to 40:1, 0.08:1 to 30:1, 0.1:1 to 20:1, 0.2:1 to 8:1, or 0.25:1 to 7.5:1.

In some embodiments, the molar ratio of the transition metal ions and the first reductant in step (A1) is 0.1:1 to 20:1, 0.2:1 to 20:1, 0.1:1 to 10:1, 0.1:1 to 8:1, 0.2:1 to 8:1, 0.25:1 to 7.5:1, or 0.4:1 to 1:1.

In some embodiments, the molar ratio of the transition metal ions and the first reductant in step (A1) is 0.08:1, 0.2:1, 0.5:1, 0.8:1, 1:1, 2:1, 4:1, 8:1, 10:1, 12:1, 14:1, 16:1, 18:1, 20:1, 23:1, 25:1, 27:1, 29:1, 32:1, 34:1, 36:1, 38:1 or 40:1.

In some embodiments, the molar ratio of the transition metal ions and the antibody in step (A1) is 1:1 to 50:1, 1:1 to 30:1, 1:1 to 20:1, 1:1 to 15:1, 8:1 to 30:1, 12:1 to 30:1, 12:1 to 30:1, 8:1 to 16:1,

4:1 to 30:1, 4:1 to 16:1, 8:1 to 16:1, 1:1 to 10:1, 1:1 to 8:1, 1:1 to 6:1, 1:1 to 5:1, 1:1 to 4:1, 1:1 to 3:1, 1:1 to 2:1, 2:1 to 6:1, 2:1 to 4:1.

The term “transition metal ions” refers to the elements of groups 4-12, justified by their typical chemistry, i.e., a large range of complex ions in various oxidation states, colored complexes, and catalytic properties either as the element or as ions (or both). Sc and Y in Group 3 are also generally recognized as transition metals.

In some embodiments, the transition metal ions are selected from the group consisting of Zn^{2+} , Cd^{2+} , Hg^{2+} , Ni^{2+} , Co^{2+} , or the combination thereof.

In some embodiments, the transition metal ions are Zn^{2+} .

In some embodiments, there is no specific limitation to the salts of the transition metal ions, as long as the transition metal ions are soluble in the reaction solution so that free transition metal ions can be released in the reaction solution. In some embodiments of the present application, the salts of the transition metal ions are chloride, nitrate, sulfate, acetate, iodide, bromine, formate or tetrafluoroborate.

In some embodiments, the salts of Zn^{2+} are ZnCl_2 , $\text{Zn}(\text{NO}_3)_2$, ZnSO_4 , $\text{Zn}(\text{CH}_3\text{COO})_2$, ZnI_2 , ZnBr_2 , Zinc formate, or zinc tetrafluoroborate. In some embodiments, the salts of Zn^{2+} are ZnCl_2 .

In some embodiments, there is no specific limitation to the concentration of the first reductant, as long as scaling up or down the concentration of the transition metal ions and the antibody in equal proportions. In some embodiments, the concentration of the first reductant is 0.01 mM to 0.2 mM. In some embodiments, the concentration of the first reductant is 0.02 mM to 0.15 mM. In some embodiments, the concentration of the first reductant is 0.05 mM to 0.1 mM. In some embodiments, the concentration of the first reductant is 0.01 mM, 0.02 mM, 0.03 mM, 0.04 mM, 0.05 mM, 0.06 mM, 0.07 mM, 0.08 mM, 0.09 mM, 0.10 mM, 0.11 mM, 0.12 mM, 0.13 mM, 0.14 mM, 0.15 mM, 0.16 mM, 0.17 mM, 0.18 mM, 0.19 mM or 0.20 mM.

In some embodiments, there is no specific limitation to the concentration of the transition metal ions in step (A1), as long as scaling up or down the concentration of the first reductant and the antibody in equal proportions.

In some embodiment, there is no specific limitation to the concentration of the antibody in step (A1), as long as scaling up or down the concentration of the first reductant and the transition metal ions in equal proportions.

In some embodiments, there is no specific limitation to the oxidant of step (A2), as long as the oxidant can re-oxidize the reduced thiol groups. In some embodiments, the oxidant is Dehydroascorbic acid (DHAA).

In some embodiments, in step (A2), the molar ratio of the oxidant and the antibody is 2:1 to 25:1. In some embodiments, in step (A2), the molar ratio of the oxidant and the antibody is 4:1 to 22:1 or 3:1 to 15:1. In some embodiments, in step (A2), the molar ratio of the oxidant and the antibody is 2:1 to 15:1, 3:1 to 15:1, 6:1 to 15:1, 8:1 to 14:1, 6:1 to 10:1, 8:1 to 12:1, 6:1 to 10:1, 6:1 to 12:1, 3:1 to 8:1, 3:1 to 6:1, 5:1 to 15:1, 5:1 to 10:1, 5:1 to 8:1, 2:1 to 7:1, 4:1 to 9:1, 1:1 to 5:1, 2:1 to 4:1, or 2:1 to 6:1 in step (A2).

In some embodiments, in step (A2), the oxidation temperature is 0°C to 37°C, and/or the oxidation time is 1h to 48h, optionally, in step (A2) the oxidation temperature is 0°C to 30°C, and/or the oxidation time is 1h to 5h.

In some embodiments, in step (A2), the oxidation temperature is 0°C to 37°C, 0°C to 30°C, 0°C to 25°C, 0°C to 20°C, 0°C to 15°C, 0°C to 10°C, or 0°C to 5°C, 5°C to 30°C, 10°C to 30°C, 15°C to 30°C, 20°C to 30°C, 0°C to 25°C, 0°C to 20°C, 10°C to 25°C, 15°C to 30°C, 5°C to 25°C, 10°C to 20°C or 10°C to 15°C. In some embodiments, in step (A2) the oxidation temperature is 0°C, 3°C, 6°C, 8°C, 10°C, 12°C, 15°C, 18°C, 20°C, 22°C, 25°C, 28°C, 30°C, 32°C, 35°C or 37°C. In some embodiments, in step (A2) the oxidation temperature is room temperature.

In some embodiments, in step (A2), the oxidation time is 0.5h to 15h, 1h to 10h, 1h to 5h, 0.5h to 5h, 0.5h to 3h, 1h to 3h, 2h to 5h, 2h to 4h or 2h to 3h. In some embodiments, in step (A2), the oxidation time is 1h, 3h, 5h, 7h, 9h, 11h, 13h, 15h, 18h, 20h, 23h, 25h, 27h, 30h, 33h, 35h, 37h, 40h, 43h, 45h or 48h.

In some embodiments, in step (A2), the oxidation reaction is in darkness.

In some embodiments, in step (A2), it is significant to improve the content of the ADC with D2, the ADC with D4 and the ADC with D1 that removing the excessive oxidant to purify the oxidized products.

In some embodiments, there is no specific limitation to the second reductant, as long as the second reductant could reduce the interchain disulfide bonds within the antibody. In some embodiments, the second reductant in step (A3) is the same as the first reductant in step (A1). In some embodiments, the second reductant in step (A3) is different with the first reductant in step (A1). In some embodiments, the second reductant in step (A3) independently is tris (2-carboxyethyl) phosphine (TCEP).

In some embodiments, in step (A3), introducing the metal chelators and the second reductant.

In some embodiments, in step (A3), the molar ratio of the metal chelators and the antibody is 2:1 to 120:1, 2:1 to 100:1, 2:1 to 80:1, 5:1 to 60:1, 10:1 to 60:1, 20:1 to 60:1, 30:1 to 60:1, 40:1 to 60:1 or 50:1 to 60:1.

In some embodiments, the molar ratio of the second reductant and the antibody in step (A3) is 10:1 to 25:1, 10:1 to 23:1, 10:1 to 20:1, 10:1 to 19:1, 10:1 to 18:1, 15:1 to 25:1, 15:1 to 20:1, 1:1 to 8:1, 1:1 to 5:1, 1:1 to 3:1, 1:1 to 2:1.

In some embodiments, in step (A3), the reduction temperature is 0°C to 37°C, 5°C to 25°C, 10°C to 20°C or 10°C to 15°C. In some embodiments, the incubation temperature in step (A3) is room temperature.

As used herein, the term “room temperature” refers to 23°C±2°C, 25°C±5°C or 20°C±5°C.

In some embodiments, incubation time in step (A3) is 0.5h to 12h, 1h to 10h, 1h to 8h, 1h to 5h, 1h to 3h, 2h to 4h, 1h to 4h, or 2h to 5h.

In some embodiments, the reaction temperature is 0°C to 40°C in step (B1) and/or (B3). In some embodiments, the reaction time is 0.5 h to 10h in step (B1) and/or (B3).

In some embodiments, the reaction temperature is 4°C to 40°C, 10°C to 40°C, 10°C to 35°C, 10°C to 30°C, 10°C to 25°C, 15°C to 35°C, 20°C to 30°C in step (B1) and/or (B3). In some embodiments, the reaction time is 0.5 h to 5h, 0.5 h to 4h, 0.5 h to 3 h, 0.5 h to 2 h, 0.5 h to 1 h, 1 h to 4h, 1 h to 3 h, 1 h to 2 h, or 2 h to 4 h in step (B1) and/or (B3).

In some embodiments, the reaction is performing at room temperature in step (B1) and/or (B3).

In some embodiments, the reaction temperature is 15°C to 25°C in step (B1) and/or (B3), the reaction time is 1 h to 3 h in step (B1) and/or (B3).

In some embodiments, in step (B1) and/or in step (B3), the temperature of reaction with the reduced thiol groups is 4°C to 37°C, the time of reaction with the reduced thiol groups is 0.5 h to 6 h.

In some embodiments, in step (B1) and/or in step (B3), the temperature of reaction with the reduced thiol groups is 20°C to 30°C or 20°C to 25°C. In some embodiments, in step (B1) and/or in step (B3), the temperature of reaction with the reduced thiol groups is room temperature. In some embodiments, in step (B1) and/or in step (B3), the temperature of reaction with the reduced thiol groups is 4°C, 6°C, 8°C, 10°C, 13°C, 17°C, 20°C, 23°C, 27°C, 30°C, 34°C or 37°C.

In some embodiments, in step (B1) and/or in step (B3), the time of reaction with the reduced thiol groups is 0.5h to 6h, 0.5h to 4h, 0.5h to 2h, 1h to 2h or 0.5h to 1h. In some modifications, in step (B1) and/or in step (B3), the time of reaction with the reduced thiol groups is 0.5h, 1h, 2h, 3h, 4h 5h or 6h.

In some embodiments, the temperature and time of reaction with the reduced thiol groups in step (B1) and/or in step (B3) are independent.

In some embodiments, in step (B1) and/or in step (B3), the temperature of reaction with the reactive groups is 10°C to 37°C, the time of reaction with the reduced thiol groups is 2 h to 12 h.

In some embodiments, in step (B1) and/or in step (B3), the temperature of reaction with the reactive groups is 10°C to 30°C, 15°C to 30°C or 25°C to 30°C. In some embodiments, in step (B1) and/or in step (B3), the temperature of reaction with the reactive groups is 4°C, 6°C, 8°C, 10°C, 13°C, 17°C, 20°C, 23°C, 27°C, 30°C, 34°C, 35°C or 37°C.

In some embodiments, in step (B1) and/or in step (B3), the time of reaction with the reactive groups is 2 h to 10 h, 4 h to 10 h, 8 h to 10 h. In some embodiments, in step (B1) and/or in step (B3), the time of reaction with the reactive groups is 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h, 9 h, 10 h, 11 h, 12 h.

In some embodiments, the temperature and time of reaction with the reactive groups in step (B1) and/or in step (B3) are independent.

In some embodiments, in step (B1), according to the amount of the antibody, the first payload unit is excess.

In some embodiments, the molar ratio of the first payload units and the antibody in step (B1) is 1:1 to 50:1, 1:1 to 20:1, 1:1 to 10:1, 1:1 to 8:1, 1:1 to 6:1, 1:1 to 5:1, 2:1 to 8:1, or 2:1 to 6:1.

In some embodiments, in step (B1), the molar ratio of the first thio-bridging reagent and the antibody is 1:1 to 10:1. In some embodiment, in step (B1), the molar ratio of the first thio-bridging reagent and the antibody is 1:1, 1.05:1, 2:1, 3:1, 3.3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1 or 10:1.

In some embodiments, in step (B1), when the first linker-payload reacts with the reactive groups in the first thio-bridging reagent, the molar ratio of the first linker-payload and the antibody is 1:1 to 10:1. In some embodiments, in the step (B1), when the first linker-payload reacts with the reactive groups in the first thio-bridging reagent, the molar ratio of the first linker-payload and the antibody is 1:1, 1.05:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1 or 10:1.

In some embodiments, in step (B1), when the first linker-payload reacts with the reduced thiol groups, the molar ratio of the first linker-payload and the antibody is 2:1 to 20:1. In some embodiments, in step (B1), when the first linker-payload reacts with the reduced thiol groups, the molar ratio of the first linker-payload and the antibody is 2:1, 3:1, 4:1, 5:1, 6:1, 20:3, 7:1, 8:1, 9:1, 10:1, 15:1 or 20:1.

In some embodiments, in step (B3), according to the amount of the antibody, the second payload unit is excess.

In some embodiments, the molar ratio of the second payload units and the antibody in step (B3) is 1:1 to 30:1, 1:1 to 20:1, 1:1 to 10:1, 1:1 to 8:1, 1:1 to 6:1, 1:1 to 5:1, 2:1 to 8:1, or 2:1 to 6:1.

In some embodiments, in step (B3), the molar ratio of the second thio-bridging reagent and the antibody is 1:1 to 10:1. In some embodiments, in step (b), the molar ratio of the second thio-bridging reagent and the antibody is 1:1, 1.5:1, 2:1, 3:1, 3.8:1, 4:1, 4.8:1 or 5:1.

In some embodiments, in step (B3), when the second linker-payload reacts with the reactive groups in the second thio-bridging reagent, the molar ratio of the second linker-payload and the antibody is 1:1 to 10:1. In some embodiments, in step (B3), when the second linker-payload reacts with the reactive groups in the second thio-bridging reagent, the molar ratio of the second linker-payload and the antibody is 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1 or 10:1.

In some embodiments, in step (B3), when the second linker-payload reacts with the reduced thiol groups, the molar ratio of the second linker-payload and the antibody is 2:1 to 20:1. In some embodiments, in step (B3), when the second linker-payload reacts with the reduced thiol groups, the molar ratio of the second linker-payload and the antibody is 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 12:1, 13:1, 14:1, 15:1, 16:1, 17:1, 18:1, 19:1 or 20:1.

In the present disclosure, the metal chelators are used to trap the excessive transition metal ions, to modify the antibody with site-specificity.

In the present disclosure, there is no specific limitation to the metal chelators, as long as the metal chelators can trap the excessive transition metal ions and do not affect the reduction of the disulfide bonds within the antibody. In some embodiments of the present application, the metal chelators are selected from a group consisting of ethylene diamine tetraacetic acid (EDTA), nitrilotriacetic acid (NTA), diethylenetriaminepentaacetic acid (DTPA), citric Acid (CA), tartaric acid (TA), gluconic acid (GA) or N-(2-hydroxyethyl) ethylenediamine-N, N', N'-triacetic acid (HEDTA).

In some embodiments, the metal chelators are selected from a group consisting of EDTA, NTA and DTPA, or their sodium salt. In some embodiments, the metal chelators in step (B1) and (B3) is Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na).

In some embodiments, the molar ratio of the metal chelators and the antibody in step (B1) is 1:1 to 100:1, 10:1 to 100:1, 20:1 to 100:1, 20:1 to 80:1, 20:1 to 70:1, 30:1 to 60:1, 40:1 to 50:1, 35:1 to 60:1, 40:1 to 55:1.

In some embodiments, the molar ratio of the metal chelators and the antibody in step (B3) is 1:1 to 100:1, 1:1 to 60:1, 1:1 to 50:1, 1:1 to 20:1, 1:1 to 10:1, 1:1 to 8:1, 1:1 to 6:1, 1:1 to 5:1, 2:1 to 8:1, 2:1 to 6:1.

In some embodiments, the second reductant in step (B2) is the same as the first reductant in step (A1). In some embodiments, the second reductant in step (B2) is different with the first reductant in step (A1). In some embodiments, the second reductant in step (B2) independently is tris (2-carboxyethyl) phosphine (TCEP).

In some embodiments, in step (B2), one of the interchain disulfide bond in the product prepared with step (A1) and (B1) is reduced completely without the transition metal ions. In some embodiments, in step (B2), three of the interchain disulfide bonds in the product prepared with step (A1), (A2) and (B1) are reduced completely without the transition metal ions. In some embodiments, one of the interchain disulfide bond or two of the interchain disulfide bonds in the product prepared with step (A1), (A2) and (B1) is(are) reduced with the transition metal ions.

In some embodiments, the molar ratio of the second reductant and the antibody in step (B2) independently is 0.05:1 to 20:1, 3:1 to 20:1, 3:1 to 10:1, 4:1 to 10:1, 5:1 to 9:1, 6:1 to 9:1, 6:1 to 8:1, 1:1 to 5:1, 1:1 to 1:4:1, 1:1 to 3:1, 1:1 to 2:1, 1.5:1 to 3:1.

In some embodiments, with step (A1), step (B1) and step (B2) without the transition metal ions, the molar ratio of the second reductant and the antibody in step (B2) independently is 1:1 to 3:1, 2:1 to 1:1, 1.5:1 to 1:1 or 1.2:1 to 1:1.

In some embodiments, with step (A1), step (A2), step (B1) and step (B2) without the transition metal ions, the molar ratio of the second reductant and the antibody in step (B2) independently is 3:1 to 20:1, 6:1 to 20:1, 4:1 to 10:1. In some embodiments, with step (A1), step (A2), step (B1) and step (B2) without the transition metal ions, the molar ratio of the second reductant and the antibody in step (B2)

independently is 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, 15:1, 16:1, 17:1, 18:1, 19:1 or 20:1.

In some embodiments, in step (B2), the incubation temperature is 0°C to 37°C, the incubation time is 0.2 h to 24 h.

In some embodiments, in step (B2), the incubation temperature is 0°C, 2°C, 4°C, 6°C, 8°C, 10°C, 12°C, 14°C, 16°C, 18°C, 20°C, 22°C, 24°C, 26°C, 28°C, 30°C, 32°C, 34°C or 36°C.

In some embodiments, with step (A1), step (B1) and step (B2) without the transition metal ions, the incubation temperature of the second reductant in step (B2) is 5°C to 37°C, 10°C to 37°C, 15°C to 37°C, 20°C to 37°C or 25°C to 37°C. In some embodiments, with step (A1), step (B1) and step (B2) without the transition metal ions, the incubation temperature in step (B2) is room temperature or 37°C.

As used herein, the term “room temperature” refers to 23°C±2°C, 25°C±5°C or 20°C±5°C.

In some embodiments, with step (A1), step (A2), step (B1) and step (B2), the incubation temperature of the second reductant in step (B2) is 0°C to 30°C, 0°C to 25°C, 0°C to 20°C, 0°C to 15°C or 0°C to 10°C. In some embodiments, with step (A1), step (A2), step (B1) and step (B2), the incubation temperature in step (B2) is 4°C, 6°C, 8°C, 10°C, 12°C or 14°C.

In some embodiments, incubation time in step (B2) independently is 0.5h to 24h, 1h to 10h, 1h to 8h, 1h to 5h, 1h to 3h, 2h to 4h, 1h to 4h, or 2h to 5h. In some embodiments, in step (B2), the incubation temperature is 1h, 2h, 3h, 4h, 5h, 6h, 7h, 8h, 9h, 10h, 12, 14h, 16h, 18h, 20h, 22h or 24h.

In some embodiments, with step (A1), step (B1) and step (B2) without the transition metal ions, the incubation time of the second reductant in step (B2) is 0.5h to 18h, 0.5h to 12h, 1h to 10h, 1h to 8h, 1h to 5h, 1h to 3h, 2h to 4h, 1h to 4h, or 2h to 5h. In some embodiments, with step (A1), step (B1) and step (B2) without the transition metal ions, the incubation time in step (B2) is 1h, 2h, 3h, 4h, 5h, 6h, 7h, 8h, 9h or 10h.

In some embodiments, with step (A1), step (A2), step (B1) and step (B2) without the transition metal ions, the incubation time in step (B2) is 3h, 4h, 5h, 6h, 7h, 8h, 9h, 10h, 12h, 14h, 16h, 18h, 20h, 22h or 24h.

In some embodiments, introducing the transition metal ions, two of the interchain disulfide bonds are selectively reduced. In some embodiments, with step (A1), step (A2), step (B1) and step (B2), in step (B2), the molar ratio of the second reductant and the transition metal ions is 1:0.05 to 1:40, and/or the molar ratio of the second reductant and the antibody is 2.5:1 to 20:1, and/or the incubation time is 1h

to 24h. In some embodiments, with step (A1), step (A2), step (B1) and step (B2), in step (B2), the molar ratio of the second reductant and the transition metal ions is 1:0.05, 1:0.08, 1:0.1, 1:0.2, 1:0.3, 1:0.4, 1:0.5, 1:0.6, 1:0.7, 1:0.8, 1:0.9, 1:1, 1:2, 1:4, 1:6, 1:8, 1:10, 1:12, 1:14, 1:16, 1:18 or 1:20. In some embodiments, with step (A1), step (A2), step (B1) and step (B2), in step (B2), the molar ratio of the second reductant and the antibody is 2.5:1, 3:1, 5:1, 7:1, 9:1, 11:1, 13:1, 15:1, 17:1, 19:1 or 20:1. In some embodiments, with step (A1), step (A2), step (B1) and step (B2), in step (B2), the incubation time is 1h, 2h, 4h, 6h, 8h, 10h, 12h, 14h, 16h, 18h, 20h, 22 or 24h. In some embodiments, with step (A1), step (A2), step (B1) and step (B2), in step (B2), the molar ratio of the second reductant and the transition metal ions is 1:0.05 to 1:40, and/or the molar ratio of the second reductant and the antibody is 3:1 to 15:1, and the incubation time is 1h to 12h. In some embodiments, with step (A1), step (A2), step (B1) and step (B2), in step (B2), the molar ratio of the second reductant and the transition metal ions is 1:0.05 to 1:40, and/or the molar ratio of the second reductant and the antibody is 2.5:1 to 15:1, and the incubation time is 12 to 24h.

In some embodiments, introducing the transition metal ions, one of the interchain disulfide bonds are selectively reduced. In some embodiments, with step (A1), step (A2), step (B1) and step (B2), in step (B2), the molar ratio of the second reductant and the transition metal ions is 1:0.4 to 1:100, and/or the molar ratio of the second reductant and the antibody is 0.8:1 to 2.5:1, and/or the incubation time is 0.5h to 24h. In some embodiments, with step (A1), step (A2), step (B1) and step (B2), in step (B2), the molar ratio of the second reductant and the transition metal ions is 1:0.5, 1:1, 1:4, 1:8, 1:12, 1:24, 1:30, 1:40, 1:50, 1:50, 1:70, 1:80, 1:90, 1:100. In some embodiments, with step (A1), step (A2), step (B1) and step (B2), in step (B2), the molar ratio of the second reductant and the antibody is 0.8:1, 1:1, 1.2:1, 1.4:1, 1.6:1, 1.8:1, 2:1, 2.2:1, 2.4:1, 2.5:1. In some embodiments, with step (A1), step (A2), step (B1) and step (B2), in step (B2), the incubation time is 0.5h, 1h, 2h, 4h, 6h, 8h, 10h, 12h, 14h, 16h, 18h, 20h, 22 or 24h. In some embodiments, with step (A1), step (A2), step (B1) and step (B2), in step (B2), the molar ratio of the second reductant and the transition metal ions is 1:0.4 to 1:100, and/or the molar ratio of the second reductant and the antibody is 0.8:1 to 2:1, and the incubation time is 0.5h to 24h. In some embodiments, with step (A1), step (A2), step (B1) and step (B2), in step (B2), the molar ratio of the second reductant and the transition metal ions is 1:0.54 to 1:100, and/or the molar ratio of the second reductant and the antibody is 2:1 to 2.5:1, and the incubation time is 1h to 9h.

Without to bound by any theory, the reductant selectively reduces disulfide bonds in buffer system. The buffer system of step (A1), (A3) and (B2) independently is MES buffer, Bis-Tris buffer, PIPES buffer, MOPS buffer, BES buffer, HEPES buffer, DIPSO buffer, MOBS buffer, MOPSO buffer, TES

buffer, ACES buffer, TAPSO buffer, PBS, Acetate buffer, ADA buffer BTP buffer, HEPPSO buffer, POPSO buffer, EPPS buffer or Tris buffer.

As used herein, the term “MES buffer” refers to 2-(N-morpholino) ethane sulfonic acid buffer.

As used herein, the term “Bis-Tris buffer” refers to Bis(2-hydroxyethyl) amino-tris(hydroxymethyl)methane buffer.

As used herein, the term “PIPES buffer” refers to piperazine-1,4-bisethanesulfonic acid buffer.

As used herein, the term “MOPS buffer” refers to 3-morpholinopropanesulfonic Acid buffer.

As used herein, the term “BES buffer” refers to N, N-Bis (2-hydroxyethyl)-2-aminoethanesulphonic acid buffer.

As used herein, the term “HEPES buffer” refers to 4-hydroxyethyl piperazine ethane sulfonic acid buffer.

As used herein, the term “DIPSO buffer” refers to 3-[bis(2-hydroxyethyl) amino]-2-hydroxypropanesulphonic acid buffer.

As used herein, the term “MOBS buffer” refers to 3-morpholinopropanesulfonic Acid buffer.

As used herein, the term “MOPSO buffer” refers to 3-(N-morpholino)-2-hydroxy-1-propanesulfonic acid buffer.

As used herein, the term “TES buffer” refers to 2-[tris(hydroxymethyl)methylamino]-1-ethanesulfonic acid buffer.

As used herein, the term “ACES buffer” refers to N-(carbamoylmethyl)taurine buffer.

As used herein, the term “TAPSO buffer” refers to 3-[N-tris-(hydroxymethyl) methylamino]-2-hydroxypropanesulphonic acid buffer.

As used herein, the term “PBS” refers to phosphate buffer saline.

As used herein, the term “ADA buffer” refers to N-(Carbamoylmethyl) iminodiacetic acid buffer.

As used herein, the term “PB buffer” refers to refers to phosphate buffer.

As used herein, the term “BTP buffer” refers to Bis-tris propane buffer.

As used herein, the term “Heppso buffer” refers to N-(Hydroxyethyl) piperazine-N'-2-hydroxypropanesulfonic acid buffer.

As used herein, the term “POPSO buffer” refers to piperazine-N, N'-bis (2-hydroxy-propane sulfonic) acid buffer.

As used herein, the term “EPPS buffer” refers to 4-(2-Hydroxyethyl)-1-piperazinepropanesulfonic acid buffer.

As used herein, the term “Tris buffer” refers to tris(hydroxymethyl)aminomethane buffer.

In some embodiments, the buffer system of step (A1), (A3) and (B2) independently is MES buffer, Bis-Tris buffer, MOPS buffer, BES buffer, HEPES buffer, DIPSO buffer, MOBS buffer, MOPSO buffer, TES buffer, ACES buffer or TAPSO buffer.

In some embodiments, the buffer system of step (A1), (A3) and (B2) independently is Bis-Tris buffer, MOPS buffer, BES buffer, HEPES buffer, DIPSO buffer, MOBS buffer, MOPSO buffer, TES buffer, ACES buffer or TAPSO buffer.

In some embodiments, the buffer system is BES buffer.

In some embodiments, the pH value of the buffer system is 5.8 to 8.0. In some embodiments, the pH value of the buffer system is 6.0 to 7.4. In some embodiments, the pH value of the buffer system is 6.7 to 7.4. In some embodiments, the pH value of the buffer system is 6.0, 6.2, 6.5, 6.8, 7.0, 7.2 or 7.4.

In some embodiments, the buffer system of step (A1) and (A3) is same. In some embodiments, the buffer system of step (A1) and (A3) is different. In some embodiments, the buffer system of step (A1) and (B2) is same. In some embodiments, the buffer system of step (A1) and (B2) is different.

The concentration of the buffer system of step (A1), (A3) and (B2) in the method independently is ranging from 10 mM to 100 mM (mmol/L). optionally, the concentration of the buffer system of step (A1), (A3) or (B2) in the method is 20 mM to 100 mM, 20 mM to 80 mM, 20 mM to 60 mM, 20 mM to 40 mM, 40 mM to 80 mM, 40 mM to 60 mM, 30 mM to 80 mM, 30 mM to 60 mM, 50 mM to 80 mM, or 30 mM to 70 mM.

In some embodiments, the concentration of buffer system of step (A1) and (A3) is same. In some embodiments, the concentration of buffer system of step (A1) and (A3) is different. In some

embodiments, the concentration of buffer system of step (A1) and (B2) is same. In some embodiments, the concentration of buffer system of step (A1) and (B2) is different.

In some embodiments, the method also comprises the following step: introducing a compound which contains at least one thiol group to consume the excessive first linker-payload and the excessive second linker-payload. In some embodiments, the compound is cysteine.

In some embodiments, the method also comprises the following step: purifying and recovering the product from (B1) and/or (B3).

In some embodiments, the resultant antibody-drug conjugates are recovered by any suitable purification method, such as using a de-salting column, size exclusion chromatography, ultrafiltration, dialysis, ultrafiltration (UF) - diafiltration (DF), and the like. If needed, further ADC enrichment (e.g., D2) may be applied in some case using hydrophobic interaction chromatography (HIC).

In some embodiments, in step (B1) and/or (B3), the resultant ADC is purified by a desalting column, size exclusion chromatography, ultrafiltration, dialysis and/or the like. In some embodiments of the present application, in step (B1) and/or (B3), the resultant ADC is purified by a desalting column.

In some embodiments, a linker of the first linker-payload and the second linker payload is selected from any one of which the one terminal can be connected to the reduced thiol group of the antibody or the reactive groups of the thio-bridging reagent, and the other terminal can be connected to a payload of the payload.

As used herein, the term “linker” refers to a substituted molecule which contains at least two substituted groups, one of which can covalently bond a drug molecule and the other of which can covalently couple to an antibody or the reactive groups of the thio-bridging reagent.

In some embodiments, when the first linker-payload and/or the second linker-payload react(s) with the reduced thiol groups, the linker of the first linker-payload and the second linker-payload independently includes a cleavable linker or a noncleavable linker. Cleavable linkers can be chemically labile and enzyme-labile linkers. Due to the high plasma stability and good intracellular cleaving selectivity and efficiency, enzyme-labile linkers are broadly selected as cleavable linker candidates in ADCs. In some embodiments, enzyme-labile linkers may include a peptide unit (-AAs-), -Maleimidocaproyl-(-MC-), -p-aminobenzyl alcohol-(-PAB-), or -MC-peptide unit-PAB-. In some embodiments, the peptide unit is dipeptides, tripeptides, tetrapeptides or pentapeptides.

In some embodiments, without the limitation, the dipeptides can be valine-alanine (VA), valine-citrulline (VC), alanine-asparagine (AD), alanine-phenylalanine (AF), phenylalanine-lysine (FK), alanine-lysine (AK), alanine-valine (AV), valine-lysine (VK), lysine-lysine (KK), phenylalanine-citrulline (FC), leucine-citrulline (LC), isoleucine-citrulline (IC), tryptophan-citrulline (WC) or phenylalanine-alanine (FA). In some embodiments, the dipeptides can be valline-citrulline- (-Val-Cit-), -valline-lysine- (-Val-Lys-), -valline-arginine- (-Val-Arg-), -phenylalanine-citrulline- (-Phe-Cit-), -phenylalanine-lysine-(-Phe-Lys-), and -phenylalanine-arginine- (-Phe-Arg-). Typical enzyme-labile linkers include -Val-Cit- and -Phe-Lys-, which can be recognized by cathepsin B.

In some embodiments, without the limitation, the tripeptides can be alanine-alanine-asparagine (AAD), glycine-valine-citrulline (GVC), glycine-glycine-glycine (GGG), phenylalanine-phenylalanine-lysine (FFK), glutamic acid-valine-citrulline (EVC), or glycine-phenylalanine-lysine (GFK).

In some embodiments, without the limitation, the tetrapeptides can be glycine-glycine-phenylalanine-glycine (GGFG).

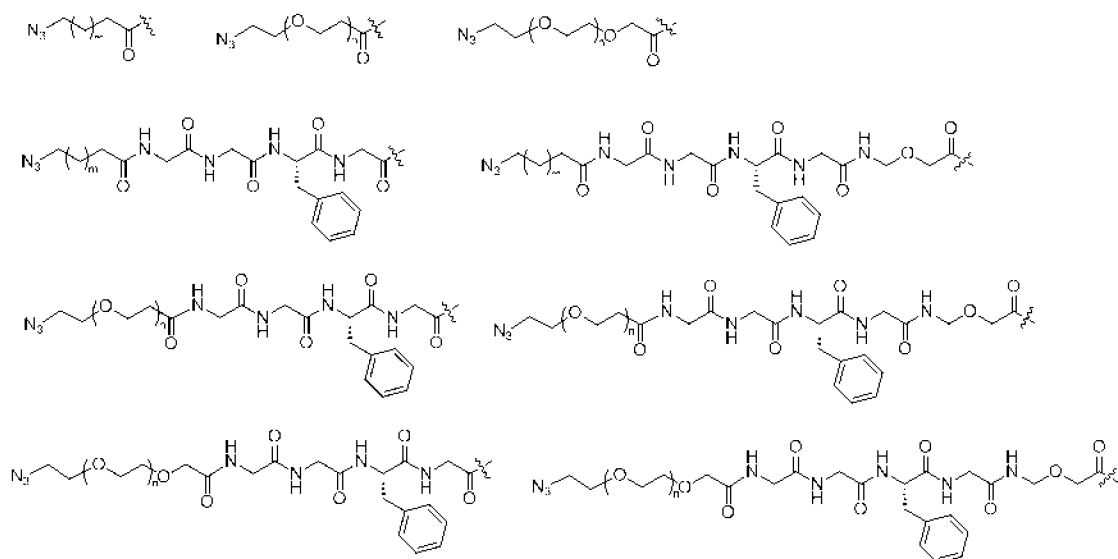
In some embodiments, without the limitation, when the first linker-payload and/or the second linker-payload react(s) with the reduced thiol groups, the linker of the first linker-payload and the second linker-payload can be MC-VA-PAB, MC-VC-PAB, MC-AD-PAB, MC-AF-PAB, MC-FK-PAB, MC-AK-PAB, MC-AV-PAB, MC-VK-PAB, MC-KK-PAB, MC-FC-PAB, MC-LC-PAB, MC-IC-PAB, MC-WC-PAB or MC-FA-PAB independently. In some embodiments, without the limitation, when the first linker-payload and/or the second linker-payload react(s) with the reduced thiol groups, the linker of the first linker-payload and the second linker-payload can be MC-AAD-PAB, MC-GVC-PAB, MC-GGG-PAB, MC-FFK-PAB, MC-EVC-PAB, or MC-GFK-PAB independently. In some embodiments, without the limitation, when the first linker-payload and/or the second linker-payload react(s) with the reduced thiol groups, the linker of the first linker-payload and the second linker-payload can be MC-GGFG.

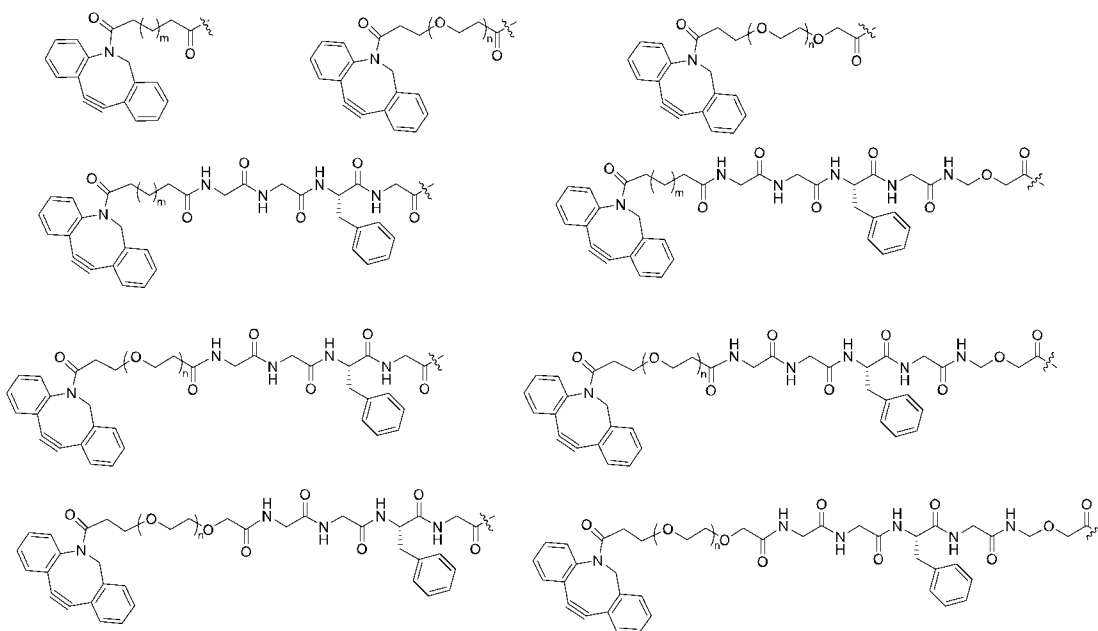
In some embodiments, the linker comprises a maleimide bearing a drug, an organic chloride bearing a drug, an organic bromide bearing a drug, an organic iodide bearing a drug and/or vinylpyrimidine bearing a drug.

In some embodiments, the linker includes a maleimide bearing a drug, an organic chloride bearing a drug, an organic bromide bearing a drug, an organic iodide bearing a drug and/or vinylpyrimidine bearing a drug.

In some embodiments, when the first linker-payload and/or the second linker-payload react(s) with the reactive groups in the thio-bridging reagent, the linker of the first linker-payload and/or the second linker-payload further include(s) azido and/or dibenzocyclooctyne (DBCO). In some embodiments, when the linker of the first linker-payload and/or the second linker-payload contains azido, the reactive groups of the thio-bridging group contain DBCO. In some embodiments, when the linker of the first linker-payload and/or the second linker-payload contains DBCO, the reactive groups of the thio-bridging group contain azido.

In some embodiments, when the linker of the first linker-payload and/or the second linker-payload react(s) with the reactive groups in the thio-bridging reagent, the linker of the first linker-payload and the second linker-payload is independently selected from any one of the groups consisting of





wherein, n of the linker is integer of 0-20, 0-18, 0-15, 0-13, 0-10, 0-7, 0-5 or 0-3, m is integer of 0-20, 0-18, 0-15, 0-13, 0-10, 0-7, 0-5 or 0-3, optionally, n is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10, m is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10.

As used herein, the term “payload” refers to any cytotoxic molecule at least one substituted group or a partial structure allowing connection to a linker structure. The payload may kill cancer cells and/or inhibit growth, proliferation, or metastasis of cancer cells, thereby reducing, alleviating, or eliminating one or more symptoms of a disease or disorder.

In some embodiments, the payload is a cytotoxic drug, a cytokine, a nucleic acid, a radionuclide, a kinase or derivatives thereof. In some embodiments, the payload includes but not limited to topoisomerases inhibitor and tubulin inhibitors.

Exemplary payloads are monomethyl auristatin E (MMAE), monomethyl auristatin D (MMAD), monomethyl auristatin EF(MMAF), calicheamicins (CLM), mertansine (DM1), maytansinoids, duocarmycins, anthracyclines, pyrrolobenzodiazepine dimers, amatoxin, quinolinealkaloid, Dxd, doxorubicin hydrochloride, methotrexate, erlotinib, bortezomib, fulvestrant, sunitib imatinib mesylate, letrozole, finasunate, platins such as oxaliplatin, carboplatin, and cisplatin, finasunate, fluorouracil, rapamycin, leucovorin, lapatinib, lonafamib, sorafenib, gefitinib, capmtothecin, topotecan, bryostatin, adezelesin, anthracyclin, carzelesin, bizelesin, dolastatin, auristatins, duocarmycin, eleutherobin, taxols such as paclitaxel or docetaxel, cyclophosphamide, doxorubicin, vincristine, prednisone or prednisolone, other alkylating agents such as mechlorethamine, chlorambucil, and ifosfamide, antimetabolites such as azathioprine or mercaptopurine, vincristine, vinblastine, vinorelbine, vindesine,

etoposide, teniposide, etoposide phosphate, epipodophyllotoxins, actinomycin, daunorubicin, valrubicin, idarubicin, edrecolomab, epirubicin bleomycin, plicamycin or mitomycin, and salts thereof.

In some embodiments, the payload is deruxtecan (DXd), cyanine 3 (Cy3), MMAE, MMAD or MMAF. In some embodiments of the present application, the payload is MMAE, DXd or Cy3.

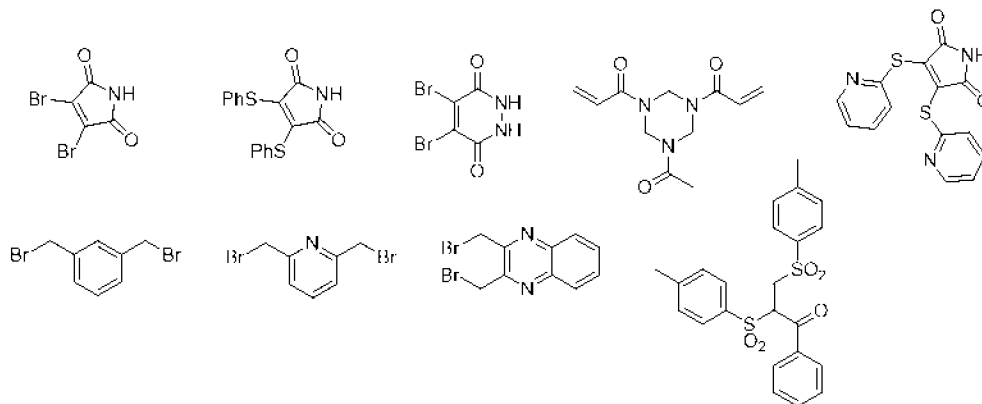
The linker-payload is a chemical moiety, which is synthesized by connecting a linker to a payload. Depending on the desired payload and selected linker, those skilled in the art can select suitable method for coupling them together. For example, some conventional coupling methods, such as amine coupling methods, may be used to form the desired linker-payload which still contains reactive groups for conjugating to the antibodies through covalent linkage. A drug-maleimide complex (i.e., maleimide linking drug) is taken as an example of the payload bearing reactive group in the present disclosure. Most common reactive group capable of bonding to thiol group in ADC preparation is maleimide. Additionally, organic chloride, bromides, iodides also are frequently used.

The linker-payload could be any physical active compound, or any compound used to diagnose, prevent or treat a disease, such as MC-GGFG-DXd, MC-VC-PAB-MMAE, MC-VC-PAB-MMAD, and MC-VC-PAB-MMAF.

In some embodiments, the first linker-payload and the second linker-payload are same. In some embodiments, the first linker-payload and the second linker-payload are different.

The first thio-bridging reagent and the second thio-bridging reagent independently contain at least two substituted groups allowing a re-bridging of the thiol groups.

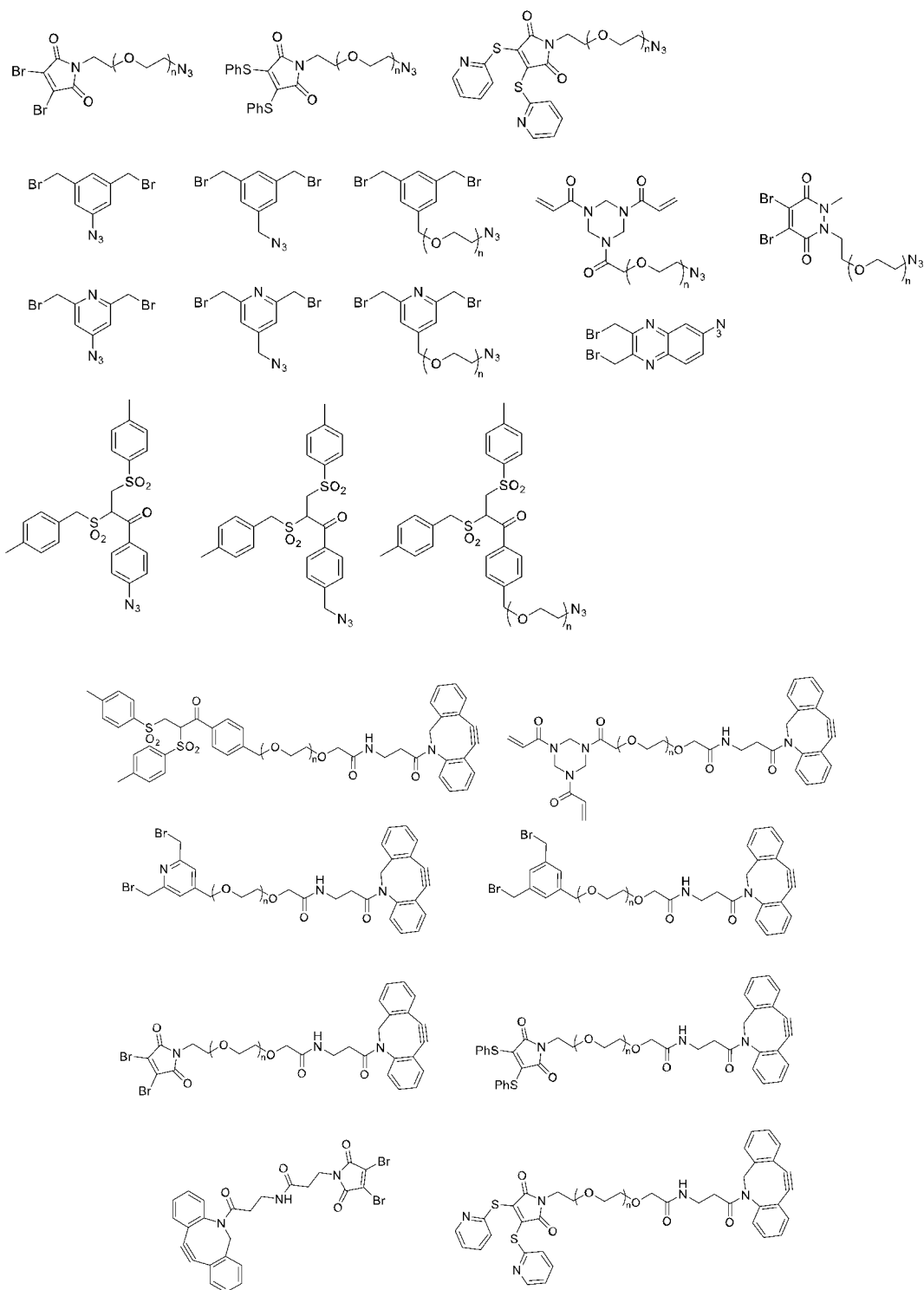
In some embodiments, without the limitation, the first thio-bridging reagent and the second thio-bridging reagent are independently selected from the group consisting of



In some embodiments, the reactive groups contain azido and/or dibenzocyclooctyne (DBCO).

In some embodiments, the thio-bridging reagent and the reactive groups are connected by alkyl group or polyethylene glycol (PEG).

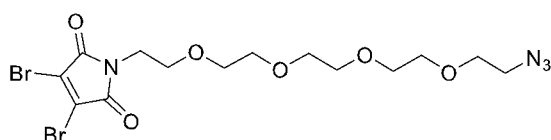
In some embodiments, without the limitation, the first thio-bridging reagent bearing reactive groups and the second thio-bridging reagent bearing reactive groups are independently selected from the groups consisting of



wherein, n of the first thio-bridging reagent bearing reactive groups and the second thio-bridging reagent bearing reactive groups is integer of 0-20, 0-18, 0-15, 0-13, 0-10, 0-7, 0-5 or 0-3.

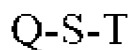
In some embodiments, the first thio-bridging reagent bearing reactive groups could be different from the second thio-bridging reagent bearing reactive groups. In some embodiments, the first thio-bridging reagent bearing reactive groups could be the same as the second thio-bridging reagent bearing reactive groups.

In some embodiments, the first thio-bridging reagent bearing reactive groups and the second thio-bridging reagent bearing reactive groups are dibromomaleimide-PEG4-N₃ having the following formula

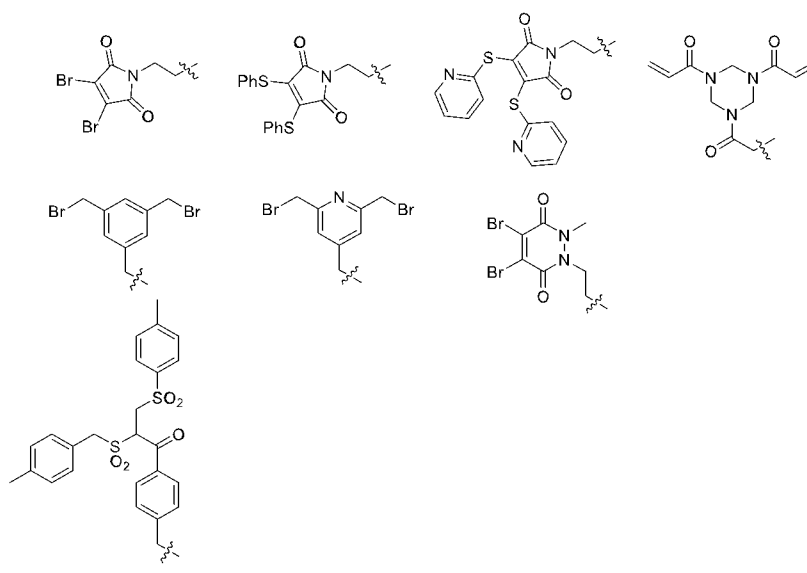


On appropriate conditions, the reactive groups could react with the linker-payloads, and the linker-payload is connected to thio-bridging reagent by covalence. With the change of reactive groups or linker-payloads, the reaction products maybe change. In the disclosure, the products of different reactive groups and linker-payloads are collectively referred to as thio-bridging reagent bearing linker-payload.

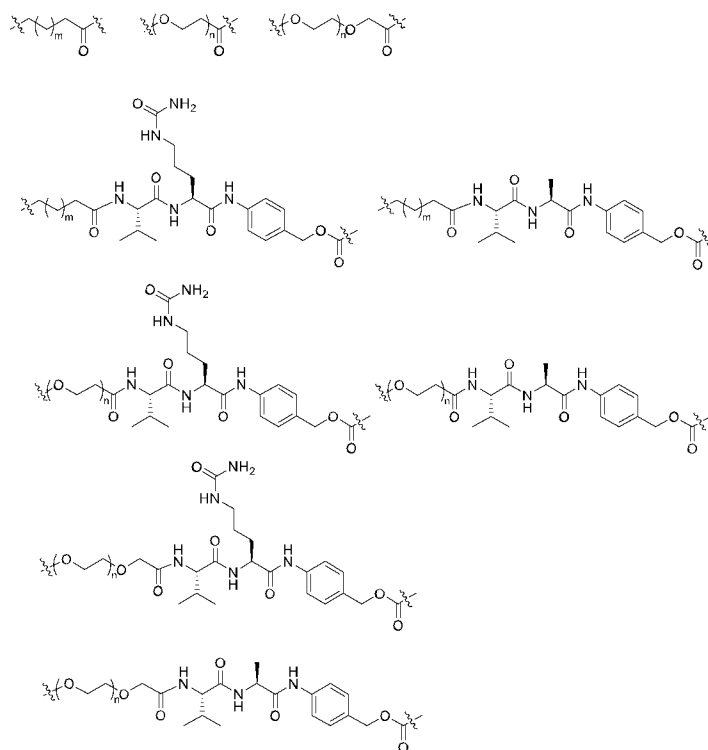
In some embodiments, the first thio-bridging reagent bearing the first linker-payload and the second thio-bridging reagent bearing the second linker-payload have the following formula:

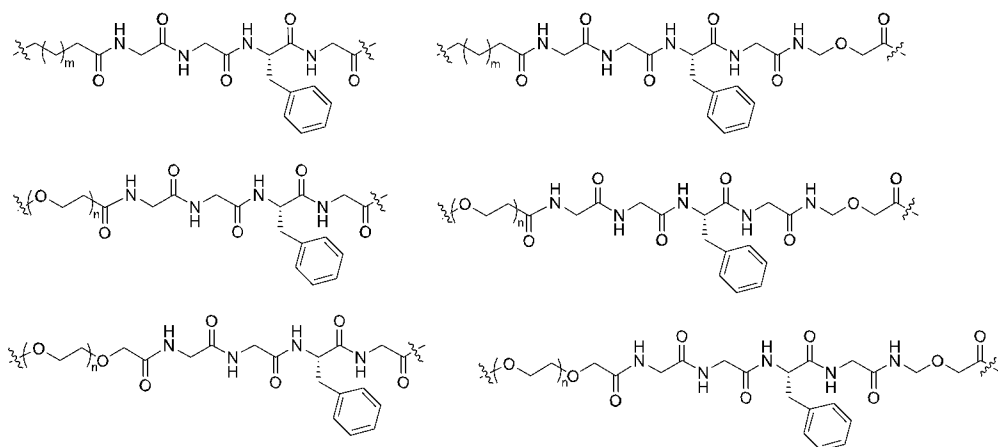


Wherein, Q is selected from the groups consisting of



S is selected from a cleavable linker or a non-cleavable linker, without the limitation, S is selected from the groups consisting of

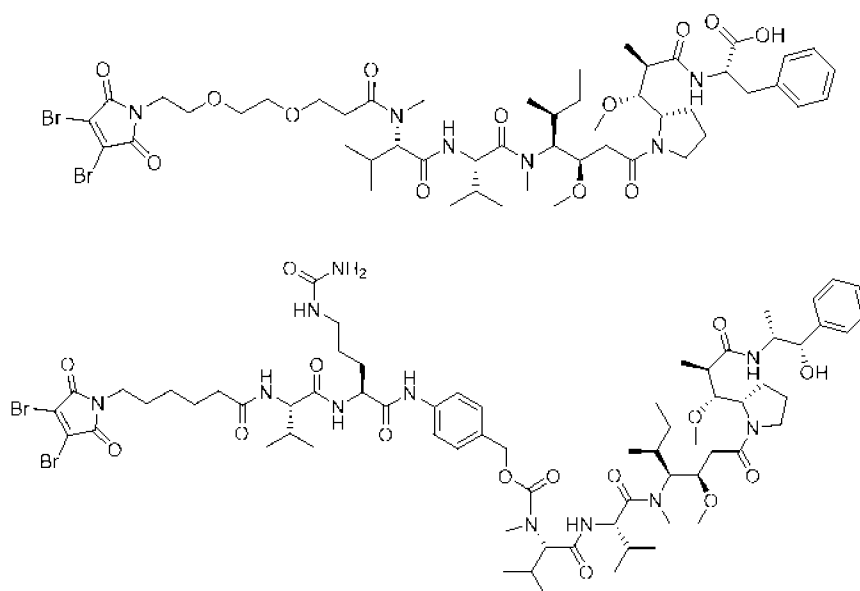


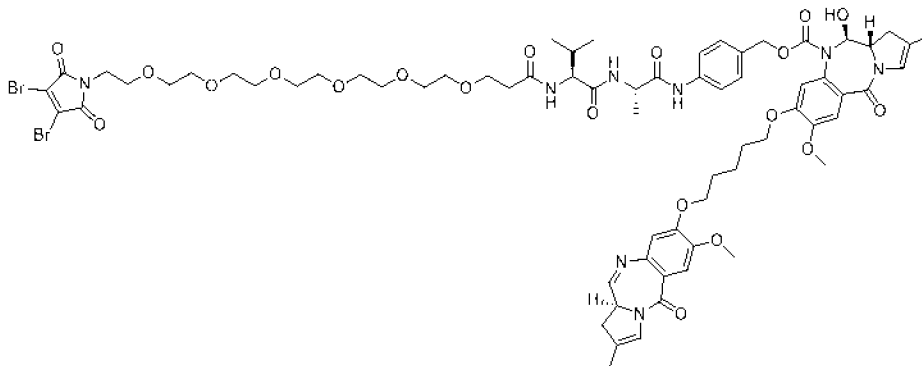


wherein, n is 0-20, m is 0-20, optionally, n is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10, m is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10.

T is payload.

In some embodiments, the first thio-bridging reagent bearing the first linker-payload and the second thio-bridging reagent bearing the second linker-payload, without the limitation, are selected from the groups consisting of





Thus, the first payload units include the first linker-payload, the first thio-bridging reagent bearing reactive groups, the first thio-bridging reagent bearing first linker-payload.

The second payload units include the second linker-payload, the second thio-bridging reagent bearing reactive groups, the second thio-bridging reagent bearing the second linker-payload.

In some embodiments, the first payload units are the first linker-payloads, the first thio-bridging reagent bearing reactive groups or the first thio-bridging reagent bearing first linker-payload.

In some embodiments, the second payload units are the second linker-payloads, the second thio-bridging reagent bearing reactive groups, or the second thio-bridging reagent bearing the second linker-payload.

In some embodiments, the payload of the first thio-bridging reagent bearing the first linker-payload and that of the second thio-bridging reagent bearing the second linker-payload are different. In some embodiments, the linker of the first thio-bridging reagent bearing the first linker-payload and that of the second thio-bridging reagent bearing the second linker-payload could be different. In some embodiments, the linker of the first thio-bridging reagent bearing the first linker-payload and that of the second thio-bridging reagent bearing the second linker-payload could be the same. In some embodiments, the thio-bridging reagent of the first thio-bridging reagent bearing the first linker-payload and that of the second thio-bridging reagent bearing the second linker-payload could be different. In some embodiments, the thio-bridging reagent of the first thio-bridging reagent bearing the first linker-payload and that of the second thio-bridging reagent bearing the second linker-payload could be the same.

As used herein, the term “end capping reagent” refers to a compound which does not bear a drug and contains at least one substituted group which can covalently couple to an antibody.

In some embodiments, the end capping reagent is the cleavable linker or the noncleavable linker. In some embodiments, the end capping reagent is (2-Aminoethyl) maleimide.

In some embodiments, the antibody is a monoclonal antibody, a polyclonal antibody, a mono-specific antibody or a multi-specific antibody.

As used herein, the term “antibody” refers to any immunoglobulin that binds to a specific antigen. A native intact antibody includes two heavy chains and two light chains. Each heavy chain consists of a variable region and a first, second, and third constant region, while each light chain consists of a variable region and a constant region. The heavy chain from any vertebrate species can be assigned to one of five different classes (or isotypes): IgA, IgD, IgE, IgG, and IgM.

As used herein, the term “hinge region” refers to an antibody includes the portion of a heavy chains molecule that joins the CH1 domain to the CH2 domain. This hinge region includes approximately 25 amino acid residues and is flexible, thus allowing the two N-terminus antigen binding regions to move independently.

As used herein, the term “Fab fragments” refers to the region of the antibody structure that can bind to antigen. It consists of a complete light chain (variable and constant regions) and part of the heavy chain structure (variable and a constant region fragment), the light and heavy chains are connected by a disulfide bond. Fab fragments can be obtained by protease digestion of full-length antibodies. Under the action of papain, human immunoglobulin G can be degraded into two Fab fragments and one Fc fragment; under the action of pepsin, IgG can be degraded into an F(ab')₂ fragment and a pFc' fragment. The F(ab')₂ fragment can be further reduced to form two Fab' fragments.

As used herein, the term “Fc region” refers to a monomeric, dimeric or heterodimeric protein having at least an immunoglobulin CH2 and CH3 domain. The CH2 and CH3 domains can form at least a part of the dimeric region of the protein/molecule (e.g., antibody).

In some embodiments, the antibody is a human antibody, a humanized antibody, a chimeric antibody, or an antigen-binding moiety thereof.

As used herein, the term “human antibody” refers to one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human or a human cell or derived from a non-human source that utilizes human antibody repertoires or other human antibody-encoding sequences. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

As used herein, the term “humanized antibody” refers to a chimeric antibody comprising amino acid residues from non-human heavy chain variable regions (HVRs) and amino acid residues from human FRs. In certain embodiments, a humanized antibody will include substantially all or at least one, and typically two, variable domains, in which all or substantially all of the HVRs (e.g., CDRs) correspond to those of a non-human antibody, and all or substantially all of the FRs correspond to those of a human antibody. A humanized antibody optionally may include at least a portion of an antibody constant region derived from a human antibody. A “humanized form” of an antibody, e.g., a non-human antibody, refers to an antibody that has undergone humanization.

As used herein, the term “chimeric antibody” refers to an antibody in which a portion of the heavy and/or light chain is derived from a particular source or species, while the remainder of the heavy and/or light chain is derived from a different source or species.

There is no specific limitation to the antibody. According to the antigens associated with the disease, those skilled in the art can select suitable antibody useful in the bio-conjugation process of the present application.

In some embodiments of the present application, the antibody means an immunoglobulin and is a molecule containing an antigen-binding site immunospecifically binding to an antigen. In some embodiments of the present application, the class of the antibody is IgG, IgE, IgM, IgD, IgA, or IgY. In some embodiments of the present application, the class of the antibody is IgG.

In some embodiments of the present application, the class of the antibody is IgG1, IgG2, IgG3 or IgG4. In some embodiments, the antibody is IgG1 or IgG4.

In some embodiments of the present application, the antibody is wild type. As use herein, the term “wild type” refers to naturally occurring and without mutation.

In some embodiments of the present application, the antibody includes at least one mutation in the Fc region. In some embodiments, the at least one mutation modulates effector function, or attenuates or eliminates Fc-g receptor binding.

In some embodiments, the one or more mutations are to stabilize the antibody and/or to increase half-life. In some instances, the one or more mutations are to modulate Fc receptor interactions, to reduce or eliminate Fc effector functions such as FcγR, antibody- dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). In additional instances, the one or more mutations are to modulate glycosylation.

In some embodiments, the one or more mutations are located in the Fc region. In some instances, the Fc region includes a mutation at residue position L234, L235, or a combination thereof. In some instances, the mutations include L234 and L235. In some instances, the mutations include L234A and L235A. In some cases, the residue positions are in reference to IgG1.

In some embodiments, the Fc region includes a mutation at residue position L234, L235, D265, N21, K46, L52, or P53, or a combination thereof. In some instances, the mutations include L234 and L235 in combination with a mutation at residue position K46, L52, or P53. In some cases, the residue positions are in reference to IgG1.

In some embodiments, the Fc region includes mutations at L234, L235, and K46. In some cases, the Fc region includes mutations at L234, L235, and L52. In some cases, the Fc region includes mutations at L234, L235, and P53. In some cases, the Fc region includes mutations at D265 and N21. In some cases, the residue position is in reference to IgG1.

In some instances, the Fc region includes L234A, L235A, D265A, N21G, K46G, L52R, or P53G, or a combination thereof. In some instances, the Fc region includes L234A and L235A in combination with K46G, L52R, or P53G. In some cases, the Fc region includes L234A, L235A, and K46G. In some cases, the Fc region includes L234A, L235A, and L52R. In some cases, the Fc region includes L234A, L235A, and P53G. In some cases, the Fc region includes D265A and N21G. In some cases, the residue position is in reference to IgG1.

In some embodiments, the Fc region includes a mutation at residue position L233, L234, D264, N20, K45, L51, or P52. In some instances, the Fc region includes mutations at L233 and L234 in combination with a mutation at residue position K45, L51, or P52. In some cases, the Fc region includes mutations at L233, L234, and K45. In some cases, the Fc region includes mutations at L233, L234, and L51. In some cases, the Fc region includes mutations at L233, L234, and K45. In some cases, the Fc region includes mutations at L233, L234, and P52. In some instances, the Fc region includes mutations at D264 and N20. In some cases, equivalent positions to residue L233, L234, D264, N20, K45, L51, or P52 in an IgG1, IgG2, IgG3, or IgG4 framework are contemplated.

In some embodiments, the Fc region includes L233A, L234A, D264A, N20G, K45G, L51R, or P52G. In some instances, the Fc region includes L233A and L234A. In some instances, the Fc region includes L233A and L234A in combination with K45G, L51R, or P52G. In some cases, the Fc region includes L233A, L234A, and K45G. In some cases, the Fc region includes L233A, L234A, and L51R. In some cases, the Fc region includes L233A, L234A, and K45G. In some cases, the Fc region includes

L233A, L234A, and P52G. In some instances, the Fc region includes D264A and N20G. In some cases, the residue position is in reference to IgG1.

In some embodiments, the human IgG constant region is modified to alter antibody- dependent cellular cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC), e.g., with an amino acid modification described in Natsume et al., 2008 *Cancer Res*, 68(10): 3863- 72; Idusogie et al., 2001 *J Immunol*, 166(4): 2571-5; Moore et al., 2010 *mAbs*, 2(2): 181- 189; Lazar et al., 2006 *PNAS*, 103(11): 4005-4010; Shields et al., 2001 *JBC*, 276(9): 6591- 6604; Stavenhagen et al., 2007 *Cancer Res*, 67(18): 8882-8890; Stavenhagen et al., 2008 *Advan. Enzyme Regul.*, 48: 152-164; Alegre et al, 1992 *J Immunol*, 148: 3461-3468; Reviewed in Kaneko and Niwa, 2011 *Biodrugs*, 25(1): 1-11.

In some embodiments, the antibody of IgG1, IgG2, IgG3 or IgG4 is human or humanized antibody. The information of IgG1, IgG2, IgG3 or IgG4 can be obtained on NCBI or UniProt (<https://www.uniprot.org/>).

In some embodiments of the present application, the antibody is bispecific antibodies. In some embodiments of the present application, the antibody is IgG1 like bispecific antibodies.

In some embodiments of the present application, those skilled in the art can select suitable method to prepare the bispecific antibodies. In some embodiments of the present application, the bispecific antibodies can be obtained by Knobs-in-holes technology (Ridgway J B B, Presta L G, Paul C. 'Knobs-into-holes' engineering of antibody CH3 domains for heavy chain heterodimerization[J].*Protein Engineering*(7):617(2023-08-11).), format chain exchange (FORCE) technology, a common light chain format technology (De Nardis C, Hendriks L J A, Poirier E, et al .A new approach for generating bispecific antibodies based on a common light chain format and the stable architecture of human immunoglobulin G1[J].*Journal of Biological Chemistry*, 2017:jbc.M117.793497.), controlled Fab arm exchange technology (Yanakieva De, Pekar L, Evers A, et al. Beyond bispecificity: Controlled Fab arm exchange for the generation of antibodies with multiple specificities[J]. *MABS*, 2022, 14(1), e2018960), CrossMAb technology (Klein C, Schaefer W, Regula J T. The use of CrossMAb technology for the generation of bi- and multispecific antibodies[J]. *MABS*, 2016, 8(6), P1010-P1020.) or their combination.

As used herein, the term “knobs-into-holes” is used in its broadest sense and encompasses various situations, such as the CH1 domain of one heavy chain with the knob mutations and the CH1 domain of the other heavy chain with the hole mutations, the CH2 domain of one heavy chain with the knob mutations and the CH2 domain of the other heavy chain with the hole mutations, and/or the CH3

domain of one heavy chain with the knob mutations and the CH3 domain of the other heavy chain with the hole mutations. For example, and generally, “knobs-into-holes” may refer to an intra-interface modification between two antibody heavy chains in the CH3 domains: i) in the CH3 domain of one heavy chain (first CH3 domain), an amino acid residue is substituted with another amino acid residue bearing a large side chain, thereby creating a protrusion (“knob”) in the interface in the first CH3 domain; ii) in the CH3 domain of the other heavy chain (second CH3 domain), an amino acid residue is substituted with another amino acid residue bearing a smaller side chain, thereby creating a cavity (“hole”) within the interface in the second CH3 domain, in which a protrusion (“knob”) in the first CH3 domain can be placed.

In some embodiments of the present application, the antibody is selected from any one of cytotoxic antibodies, inhibitors of cell proliferation, regulators of cell activation and interaction, regulators of the human immune system, neutralizations of antigens, antibodies that are immunospecific for viral antigens or antibodies that are immunospecific for microbial antigens.

There is no specific limitation to the antibody. According to the antigens associated with the disease, those skilled in the art can select suitable antibody useful in the bio-conjugation process of the present application.

In some embodiments, the antibody is target-specific, which is targeted to, HER2 (Human Epidermal Growth Factor Receptor 2), TROP2 (TACSTD2, tumor associated calcium signal transducer 2), BCMA (TNFRSF17, TNF receptor superfamily member 17).

In some embodiments, the antibody is Trastuzumab, Sacituzumab or Belantamab.

In some embodiments, the antibody can be obtained commercially or produced by any method known to those skilled in the art.

The present application provides a modification prepared by the method described above.

The resultant modified antibody includes ADC. In some embodiments, the ADC comprises the ADC with D2, the ADC with D4, the ADC with D1, the ADC with D6, the ADC with D3, the ADC with D1+D6, the ADC with D1+D2, the ADC with D1+D4, the ADC with D2+D4, the ADC with D6+D2, the ADC with D6+D1, the ADC with D3+D1, the ADC with D3+D2, the ADC with D0+D6, or the ADC with D0+D2.

In some embodiments, the ADC is Trastuzumab-[MC-VC-PAB-MMAE]₆, Sacituzumab-[MC-VC-PAB-MMAE]₆, Belantamab-[MC-VC-PAB-MMAE]₆, Trastuzumab-[MC-VC-PAB-MMAE]₆.

[MC-GGFG-DXd]₂, Trastuzumab-[MC-GGFG-DXd]₆[Maleimide-PEG4-N3-DBCO-Cy3]₁, Trastuzumab-[Maleimide]₆[MC-VC-PAB-MMAE]₂, Trastuzumab-[Maleimide]₆[Maleimide-PEG4-N3-DBCO-Cy3]₁, Trastuzumab-[MC-VC-PAB-MMAE]₂, Trastuzumab-[Maleimide-PEG4-N3-DBCO-Cy3]₁, Trastuzumab-[Maleimide-PEG4-N3-DBCO-Cy3]₁[MC-VC-PAB-MMAE]₆, Trastuzumab-[Maleimide-PEG4-N3-DBCO-Cy3]₁[MC-VC-PAB-MMAE]₂, Trastuzumab-[Maleimide-PEG4-N3-DBCO-Cy3]₁[MC-VC-PAB-MMAE]₄ and Trastuzumab-[MC-GGFG-DXd]₂[MC-VC-PAB-MMAE]₄.

The disclosure also provides the antibody with site-specific modification, of which two interchain disulfide bonds in the Fab region and one interchain disulfide bond in the hinge region of the antibody are reduced, conjugated, or modified.

In some embodiments, the antibody with site-specific modification (ADC with D6) is prepared by the method includes the step (A1) and (B1), wherein the first payload units are the first linker-payloads.

In some embodiments, the antibody with site-specific modification (ADC with D3) is prepared by the method including the step (A1) and (B1), wherein the first payload units are the first thio-bridging reagent bearing reactive groups which reacts with the first linker-payload.

In some embodiments, the antibody with site-specific modification (ADC with D3) is prepared by the method including the step (A1) and (B1), wherein the first payload units are the first thio-bridging reagent bearing the first linker-payloads.

The disclosure also provides the antibody with site-specific modification, of which one interchain disulfide bonds in the hinge region of the antibody are reduced, conjugated, or modified.

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D2) prepared by the method including the step (A1), (A2), and (B1), wherein the first payload units are the first linker-payloads.

In some embodiments, the antibody with site-specific modification (ADC with D1) is prepared by the method including the step (A1), (A2) and (B1), wherein the first payload units are the first thio-bridging reagent bearing reactive groups which reacts with the first linker-payload.

In some embodiments, the antibody with site-specific modification (ADC with D1) is prepared by the method including the step (A1), (A2) and (B1), wherein the first payload units are the first thio-bridging reagent bearing the first linker-payloads.

The disclosure also provides the antibody with site-specific modification, of which two interchain disulfide bonds in the hinge region of the antibody are reduced, conjugated, or modified.

In some embodiments, the antibody with site-specific modification (ADC with D4) is prepared by the method including the step (A1), (A2), (A3), and (B1), wherein the first payload units are the first linker-payloads.

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D1+D2) prepared by the method including the step (A1), (A2), (B1), (B2) and (B3), wherein the first payload units are the first thio-bridging reagent bearing the first linker-payloads, and the second payload units are the second linker-payloads. Meanwhile, the transition metal ions are introduced in step (B2).

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D1+D2) prepared by the method including the step (A1), (A2), (B1), (B2) and (B3), wherein the first payload units are the first thio-bridging reagent bearing reactive groups which reacts with the first linker-payloads, and the second payload units are the second linker-payloads. Meanwhile, the transition metal ions are introduced in step (B2).

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D2+D2) prepared by the method including the step (A1), (A2), (B1), (B2) and (B3), wherein the first payload units are the first linker-payloads, and the second payload units are the second linker-payloads. Meanwhile, the transition metal ions are introduced in step (B2).

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D2+D1) prepared by the method including the step (A1), (A2), (B1), (B2) and (B3), wherein the first payload units are the first linker-payloads, and the second payload units are the second thio-bridging reagent bearing the second linker-payloads. Meanwhile, the transition metal ions are introduced in step (B2).

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D2+D1) prepared by the method including the step (A1), (A2), (B1), (B2) and (B3), wherein the first payload units are the first linker-payloads, and the second payload units are the second thio-bridging reagent bearing reactive groups which reacts with the second linker-payloads. Meanwhile, the transition metal ions are introduced in step (B2).

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D1+D4) prepared by the method including the step (A1), (A2), (B1), (B2) and (B3), wherein the first payload units are the first thio-bridging reagent bearing the first linker-payloads, and the second payload units are the second linker-payloads. Meanwhile, the transition metal ions are introduced in step (B2).

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D1+D4) prepared by the method including the step (A1), (A2), (B1), (B2) and (B3), wherein the first payload units are the first thio-bridging reagent bearing reactive groups which reacts with the first linker-payloads, and the second payload units are the second linker-payloads. Meanwhile, the transition metal ions are introduced in step (B2).

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D2+D4) prepared by the method including the step (A1), (A2), (B1), (B2) and (B3), wherein the first payload units are the first linker-payloads, and the second payload units are the second linker-payloads. Meanwhile, the transition metal ions are introduced in step (B2).

The disclosure also provides the antibody with site-specific modification, of which two interchain disulfide bonds in the Fab region and two interchain disulfide bonds in the hinge region of the antibody are reduced, conjugated, or modified.

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D6+D2) prepared by the method including the step (A1), (B1), (B2) and (B3), wherein the first payload units are the first linker-payloads, and the second payload units are the second linker-payloads.

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D6+D1) prepared by the method including the step (A1), (B1), (B2) and (B3), wherein the first payload units are the first linker-payloads, and the second payload units are the second thio-bridging reagent bearing reactive groups which reacts with the second linker-payloads.

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D6+D1) prepared by the method including the step (A1), (B1), (B2) and (B3), wherein the first payload units are the first linker-payloads, and the second payload units are the second thio-bridging reagent bearing the second linker-payloads.

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D3+D2) prepared by the method including the step (A1), (B1), (B2) and (B3), wherein the first payload

units are the first thio-bridging reagent bearing the first linker-payloads, and the second payload units are the second linker-payloads.

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D3+D2) prepared by the method including the step (A1), (B1), (B2) and (B3), wherein the first payload units are the first thio-bridging reagent bearing reactive groups which reacts with the first linker-payloads, and the second payload units are the second linker-payloads.

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D3+D1) prepared by the method includes the step (A1), (B1), (B2) and (B3), wherein the first payload units are the first thio-bridging reagent bearing the first linker-payloads, and the second payload units are the second thio-bridging reagent bearing the second linker-payloads.

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D3+D1) prepared by the method includes the step (A1), (B1), (B2) and (B3), wherein the first payload units are the first thio-bridging reagent bearing reactive groups which reacts with the first linker-payloads, and the second payload units are the second thio-bridging reagent bearing reactive groups which reacts with the second linker-payloads.

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D0+D2) prepared by the method including the step (A1), (B1), (B2) and (B3), wherein the first payload units are the first thio-bridging reagent, and the second payload units are the second linker-payloads.

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D0+D1) prepared by the method includes the step (A1), (B1), (B2) and (B3), wherein the first payload units are the first thio-bridging reagent, and the second payload units are the second thio-bridging reagent bearing the second linker-payloads.

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D0+D1) prepared by the method includes the step (A1), (B1), (B2) and (B3), wherein the first payload units are the thio-bridging reagent, and the second payload units are the second thio-bridging reagent bearing reactive groups which reacts with the second linker-payloads.

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D2+D6) prepared by the method including the step (A1), (A2), (B1), (B2) and (B3), wherein the first payload units are the first linker-payloads, and the second payload units are the second linker-payloads.

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D1+D6) prepared by the method including the step (A1), (A2), (B1), (B2) and (B3), wherein the first payload units are the first thio-bridging reagent bearing reactive groups which reacts with the first linker-payloads, and the second payload units are the second linker-payloads.

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D1+D6) prepared by the method including the step (A1), (A2), (B1), (B2) and (B3), wherein the first payload units are the first thio-bridging reagent bearing the first linker-payloads, and the second payload units are the second linker-payloads.

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D2+D3) prepared by the method including the step (A1), (A2), (B1), (B2) and (B3), wherein the first payload units are the first linker-payloads, and the second payload units are the second thio-bridging reagent bearing the second linker-payloads.

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D2+D3) prepared by the method including the step (A1), (A2), (B1), (B2) and (B3), wherein the first payload units are the first linker-payloads, and the second payload units are the second thio-bridging reagent bearing reactive groups which reacts with the second linker-payloads.

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D1+D3) prepared by the method includes the step (A1), (A2), (B1), (B2) and (B3), wherein the first payload units are the first thio-bridging reagent bearing the first linker-payloads, and the second payload units are the second thio-bridging reagent bearing the second linker-payloads.

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D1+D3) prepared by the method includes the step (A1), (A2), (B1), (B2) and (B3), wherein the first payload units are the first thio-bridging reagent bearing reactive groups which reacts with the first linker-payloads, and the second payload units are the second thio-bridging reagent bearing reactive groups which reacts with the second linker-payloads.

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D0+D6) prepared by the method including the step (A1), (A2), (B1), (B2) and (B3), wherein the first payload units are the first thio-bridging reagent, and the second payload units are the second linker-payloads.

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D0+D3) prepared by the method includes the step (A1), (A2), (B1), (B2) and (B3), wherein the first payload units are the first thio-bridging reagent, and the second payload units are the second thio-bridging reagent bearing the second linker-payloads.

In some embodiments, provided herein is the antibody with site-specific modification (ADC with D0+D3) prepared by the method includes the step (A1), (A2), (B1), (B2) and (B3), wherein the first payload units are the thio-bridging reagent, and the second payload units are the second thio-bridging reagent bearing reactive groups which reacts with the second linker-payloads.

Various analytical methods can be used to determine the yields and isomeric mixtures of the antibody with site-specific modification. In some embodiments of the present application, the analytical method is HIC-HPLC. HIC-HPLC can separate the antibodies loaded with various numbers of linker-payload. The loading level of payload can be determined based on the ratio of absorbances, e.g., at 250 nm and 280 nm. For example, if a payload (e.g., drug) can absorb at 250 nm while the antibody absorbs at 280nm. The 250/280 ratio therefore increases with drug loading.

The process of generating antibodies with site-specific modification bypasses any need of protein engineering or enzyme catalysis, but is based on native inter-chain disulfide bonds, and only needs novel reductants and transition metal ions. Therefore, the process of the disclosure is less complicate, the homogeneity of the resultant antibodies with site-specific modification (antibody-drug conjugate) is dramatically improved.

pharmaceutical composition

The present application also provides a pharmaceutical composition comprising the antibody with site-specific modification prepared by the method described above and at least a pharmaceutically acceptable ingredient.

Pharmaceutical compositions provided herein may be formulated in any manner known in the art, such as, pharmaceutical compositions provided herein can be formulated for parenteral (e.g., intravenous, intraarterial, intramuscular, intradermal, subcutaneous, or intraperitoneal) administration in dosage unit form (i.e., physically discrete units containing a predetermined quantity of active compound for ease of administration and uniformity of dosage).

Pharmaceutical compositions are formulated to be compatible with their intended route of administration (e.g., intravenous, intraarterial, intramuscular, intradermal, subcutaneous, or intraperitoneal).

Pharmaceutical acceptable carriers for use in the pharmaceutical compositions disclosed herein may include, for example, pharmaceutically acceptable liquid, gel, or solid carriers, aqueous vehicles, nonaqueous vehicles, antimicrobial agents, isotonic agents, buffers, antioxidants, anesthetics, suspending/dispersing agents, sequestering or chelating agents, diluents, adjuvants, excipients, or non-toxic auxiliary substances, other components known in the art, or various combinations thereof.

Suitable components may include, for example, antioxidants, fillers, binders, disintegrants, buffers, preservatives, lubricants, flavorings, thickeners, coloring agents, emulsifiers or stabilizers such as sugars and cyclodextrins. Suitable antioxidants may include, for example, methionine, ascorbic acid, EDTA, sodium thiosulfate, platinum, catalase, citric acid, cysteine, thioglycerol, thioglycolic acid, thiosorbitol, butylated hydroxyanisole, butylated hydroxytoluene, and/or propyl gallate. As disclosed herein, inclusion of one or more antioxidants such as methionine in a composition comprising an antibody or antigen-binding fragment thereof and conjugates provided herein decreases oxidation of the antibody or antigen-binding fragment thereof. This reduction in oxidation prevents or reduces loss of binding affinity, thereby improving antibody stability and maximizing shelf-life. Therefore, in certain embodiments, pharmaceutical compositions are provided that include one or more antibodies or antigen-binding fragments thereof as disclosed herein and one or more antioxidants such as methionine.

In some embodiments, the pharmaceutical compositions can be a liquid solution, suspension, or emulsion. In some embodiments, the pharmaceutical compositions are formulated into an injectable composition. The injectable pharmaceutical compositions may be prepared in any conventional form, such as for example liquid solution, suspension, emulsion, or solid forms suitable for generating liquid solution, suspension, or emulsion. Preparations for injection may include sterile and/or non-pyretic solutions ready for injection, sterile dry soluble products, such as lyophilized powders, ready to be combined with a solvent just prior to use, including hypodermic tablets, sterile suspensions ready for injection, sterile dry insoluble products ready to be combined with a vehicle just prior to use, and sterile and/or non-pyretic emulsions. The solutions may be either aqueous or nonaqueous.

In some embodiments of the present application, the pharmaceutical composition is combined with other therapeutic agents. There is no specific limitation to the other therapeutic agents, as long as the other therapeutic agents can reduce the side effects of the pharmaceutical composition or increase

the efficacy of the pharmaceutical composition. The other therapeutic agents are anti-cancer agents, anti-autoimmune disease agent, anti-emetics, anti-allergic and the like.

In some embodiments of the present application, the anti-cancer agents can include, but not limited to, erlotinib, bortezomib, fulvestrant, sunitib imatinib, mesylate, letrozole, finasunate, platins such as oxaliplatin, carboplatin, and cisplatin, finasunate, fluorouracil, rapamycin, leucovorin, lapatinib, lonafamib, sorafenib, gefitinib, capmtothecin, topotecan, bryostatin, adezelesin, anthracyclin, carzelesin, bizelesin, dolastatin, auristatins, duocarmycin, eleutherobin, taxols such as paclitaxel or docetaxel, cyclophosphamide, doxorubicin, vincristine, prednisone or prednisolone, other alkylating agents such as mechlorethamine, chlorambucil, and ifosfamide, antimetabolites such as azathioprine or mercaptopurine, other microtubule inhibitors (vinca alkaloids like vincristine, vinblastine, vinorelbine and vindesine, as well as taxanes), podophyllotoxins (etoposide, teniposide, etoposide phosphate, and epipodophyllotoxins), topoisomerase inhibitors, other cytotoxins such as actinomycin, daunorubicin, valrubicin, idarubicin, epirubicin, bleomycin, plicamycin, mitomycin and the like.

The disclosure provides the use of the antibody with site-specific modification provided herein in the manufacture of a therapeutic agent for preventing, diagnosing or treating a disease.

As use herein, the term “treat” of any disease refers to alleviating or ameliorating the disease (i.e., slowing or arresting the development of the disease or at least one of the clinical symptoms thereof); or alleviating or ameliorating at least one physical parameter or biomarker associated with the disease, including those which may not be discernible to the patient. For cancer, “treating” may refer to dampen or slow the tumor or malignant cell growth, proliferation, or metastasis, or some combination thereof. For tumors, “treatment” includes removal of all or part of the tumor, inhibiting or slowing tumor growth and metastasis, delaying the development of a tumor, or some combination thereof.

As used herein, the term “prevent” of any disease refers to the prophylactic treatment of the disease; or delaying the onset or progression of the disease.

In some embodiments, the disease is a tumor or cancer. In some embodiments, the disease is an autoimmune disease and the like.

In some embodiments of the present application, the cancer can include, but not limited to, carcinoma, lymphoma, blastema, sarcoma, and leukemia or lymphoid malignancies. More particular examples of the cancer include squamous cell cancer (e.g., epithelial squamous cell cancer), lung cancer including small-cell lung cancer, non-small cell lung cancer (“NSCLC”), adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric

or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, as well as head and neck cancer.

The disclosure provides the method of preventing, diagnosing or treating a disease in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of the antibody with site-specific modification prepared by the method described above.

As use herein, the term “subject” refers to mammals, primates (e.g., humans, male or female), dogs, rabbits, guinea pigs, pigs, rats and mice. In certain embodiments, the subject is a primate. In yet other embodiments, the subject is a human.

As used herein, the term “a therapeutically effective amount” refers to an amount of the ADC of the present application that will elicit the biological or medical response of a subject, for example, ameliorate symptoms, alleviate conditions, slow or delay disease progression, or prevent a disease, etc. The therapeutically effective amount will vary with the type and severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions. In some embodiments of the present application, the therapeutically effective amount is based on a variety of factors, such as the type of disease, the age, weight, sex, medical condition of the patient, the severity, of the condition, the route of administration, and the particular antibody employed. In some embodiments of the present application, the therapeutically effective amount can vary widely, but can be determined routinely using standard methods. In some embodiments of the present application, the therapeutically effective amount can be adjusted based on the pharmacokinetic or pharmacodynamic parameters, which may include clinical effects such as toxic effects and/or laboratory values.

It will be readily apparent to those skilled in the art that other suitable modifications and adaptations of the present application described herein are obvious and may be made using suitable equivalents without departing from the scope of the disclosure or the embodiments disclosed herein. Having now described the disclosure in detail, the same will be more clearly understood by reference to the following examples, which are included for purposes of illustration only and are not intended to be limiting. Further, unless specifically described otherwise, the reagent and the solvent described in the description can be easily obtained from a commercial supplier.

EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

Reagent and Manufacturer

Trastuzumab is commercially available from Roche.

Sacituzumab and Belantamab are commercially available from MedChemExpress.

EDTA is commercially available from Aladdin.

DMA (Dimethylacetamide) is commercially available from Aldrich Sigma.

MC-VC-PAB-MMAE is commercially available from Levena biopharma.

MC-GGFG-DXd is commercially available from Levena biopharma.

Desalting column (type: 40K, 0.5 mL, REF:87766, Lot SJ251704) is commercially available from Thermo Scientific.

DBCO-Cy3 is commercially available from Confluore.

TCEP is commercially available from Bidepharm.

Dibromomaleimide is commercially available from Aladdin.

The reagents used in examples, include but not limited to 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), 1-Hydroxybenzotriazole (HOBt), N,N-Diisopropylethylamine (DIPEA), ethyl acetate (EtOAc), N,N-Dimethylformamide (DMF), Bicyclic amidine (DBU), 2-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDCI), trifluoroacetic acid (TFA), dichloromethane (DCM), tert-butylchlorodiphenylsilane (TBDPSCI) are commercially available.

Synthesis Procedure A of the compound according to the present application

To a solution of TCEP (286.6 mg, 1.0 mmol, 2.0 eq.) in DMF(3mL) was added HATU (190 mg, 0.5mmol, 1 eq) followed by DIPEA (2.0 mmol, 4.0eq) under N₂ atmosphere. The mixture was stirred for 30min and the amine reagent (0.5mmol, depending on the structure of the compound) was added. The reaction was stirred at room temperature for 1h. The reaction mixture was purified by RP-HPLC using a C18 column yielding the desired product.

Synthesis Procedure A-1 of the compound according to the present application

The product prepared by the synthesis procedure A was dissolved in DCM(3mL) and TFA(0.3mL) was added. The mixture was stirred for 1h, LCMS showed reaction was completed. The mixture was concentrated and the residue was taken up by distilled water, washed with EtOAc twice. The aqueous layer was lyophilized to give corresponding product.

Synthesis Procedure B of the compound according to the present application

To a solution of TCEP (286.6 mg, 1.0 mmol, 2.0 eq.) in DMF(3mL) was added HOBt (67.5 mg, 0.5mmol, 1 eq) and EDCI (95.5mg, 0.5mmol, 1.0eq), followed by DIPEA(2.0 mmol, 4.0eq) under N₂ atmosphere. The mixture was stirred for 30 min and the amine reagent (0.5mmo, depending on the structure of the compound) was added. The reaction was stirred at room temperature for 1h. The reaction mixture was purified by RP-HPLC using a C18 column yielding the corresponding product.

Synthesis Procedure B-1 of the compound according to the present application

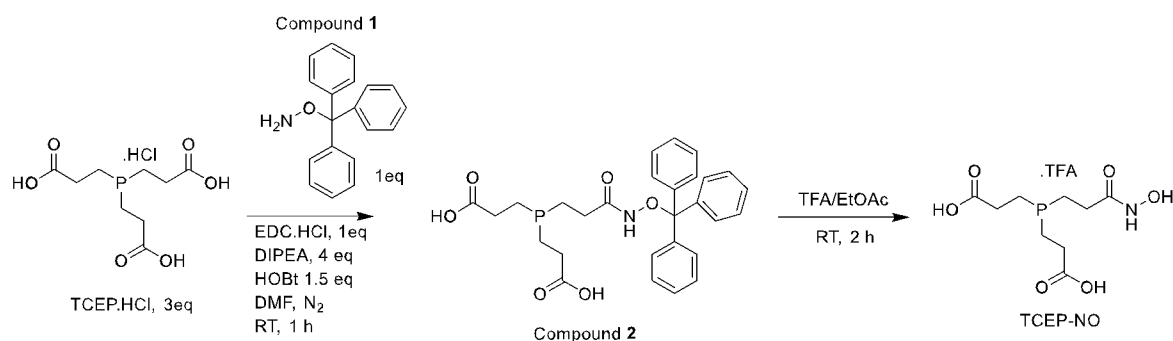
The product prepared by the synthesis procedure B was dissolved in DCM (3mL) and TFA (0.3mL) was added. The mixture was stirred for 1h, LCMS showed reaction was completed. The mixture was concentrated and the residue was taken up by distilled water, washed with EtOAc twice. The aqueous layer was lyophilized to give corresponding product.

Homogeneity Assays

The drug/antibody ratio (DAR) and product distribution were analyzed using HIC-HPLC (Agilent1200) with a TSK gel Butyl-NPR column (2.5μm, 4.6 mm * 3.5cm) (commercially available from Tosoh Biosciences) at a flow rate of 0.5 mL/min at 25 °C. Solvent A was 1.5 M (NH₄)₂SO₄ and 50 mM K₂HPO₄ 3H₂O. Solvent B was 75%v/v 21.3 mM KH₂PO₄, 28.6mM K₂HPO₄ 3H₂O and 25%v/v isopropanol. The washout procedure is as follows:

Time [min]	Solvent A [%]	Solvent B [%]
0.00	100.0	0.0
2.00	100.0	0.0
15.00	0.0	100.0
17.00	0.0	100.0
18.00	100.0	0.0
20.00	100.0	0.0

Example 1: Synthesis of TCEP-NO-Trtyl and TCEP-NO

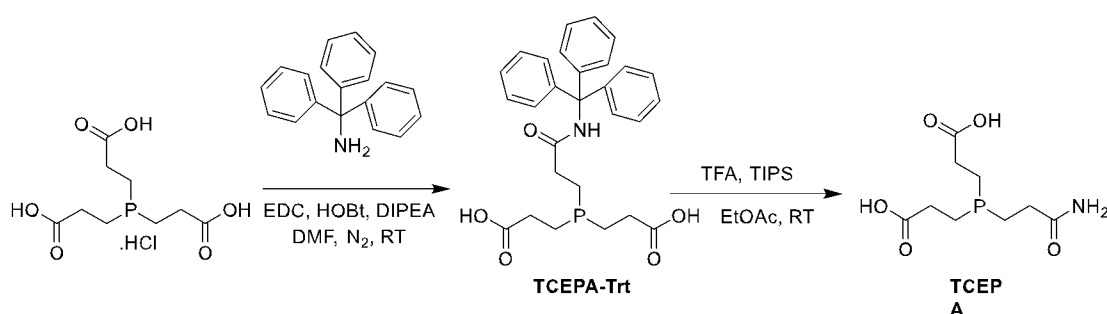


Compound 1 O-trithylhydroxylamine (NH₂-O-Trt, 176 μmol, 48.5 mg, 1eq.), EDC (176 μmol 33.7 mg, 1 eq.) and HOBt (352 μmol, 53.8 mg, 2 eq.) were dissolved in 1.5 mL DMF (degassed) under an inert atmosphere. This solution was added to TCEP (528 μmol, 150 mg, 3 eq.) dissolved in 1.5 mL

degassed DMF containing DIPEA (704 μmol , 123 μL , 4 eq.) under an inert atmosphere. The reaction was stirred at room temperature for 60 min. After that, DMF was removed in vacuo and the residue was added CH_3COOH (0.1 N, 5ml) and EtOAc (2 ml). The resulting mixture was stirred for 5 min and filtered to yield Compound 2 (TCEP-NO-Trtyl) as a white solid (61.2 mg, 12.2%). For Compound 2 (also called TCEP-NO-Trtyl, or TCEP-19-int1), $\text{MS}[\text{M}-\text{H}]^- = 506.2$, Exact mass calc. for $\text{C}_{28}\text{H}_{30}\text{NO}_6\text{P}$ is 507.18. ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 7.40-7.15 (m, 15H), 2.48-2.38 (m, 4H), 2.22 (s, 1H), 2.12-1.52 (m, 7H).

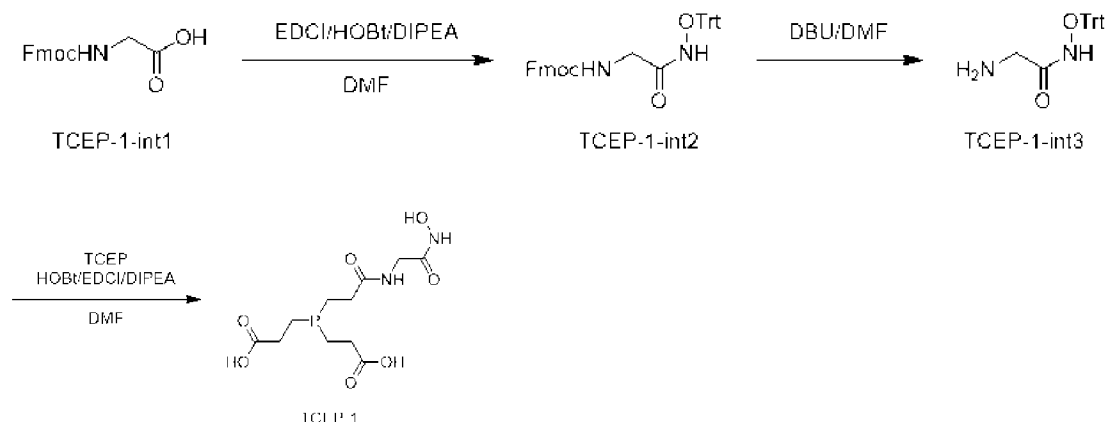
Without further purification, Compound 2 was added EtOAc (2 ml), 5% TFA and 5% TIPS. The reaction was continued for another 2 h. Then H_2O (5 ml) and EtOAc (10 ml) were introduced. The aqueous phase was further washed with EtOAc (10 ml) twice and concentrated to afford TCEP-NO (13 mg). For TCEP-NO, $\text{MS}[\text{M}-\text{H}]^- = 263.94$, exact mass calc. for $\text{C}_9\text{H}_{16}\text{NO}_6\text{P}$ is 265.07. ^1H -NMR (400 MHz, Deuterium Oxide): δ 2.93-2.85 (m, 4H), 2.73-2.56 (m, 8H).

Example 2: Synthesis of TCEPA



Tritylamine (176 μmol , 45.6 mg, 1 eq.), EDC (176 μmol 33.7 mg, 1 eq.) and HOBt (352 μmol , 53.8 mg, 2 eq.) were dissolved in 1.5 mL DMF (degassed) under an inert atmosphere. This solution was added to TCEP (528 μmol , 150 mg, 3 eq.) dissolved in 1.5 mL degassed DMF containing DIPEA (704 μmol , 123 μL , 4 eq.) under an inert atmosphere. The reaction was stirred at room temperature for 60 min. After that, DMF was removed in vacuo and the residue was added CH_3COOH (0.1 N, 5ml) and EtOAc (20 ml). The resulting mixture was washed with H_2O three times and the organic phase was concentrated followed by petroleum ether precipitation to yield TCEPA-Trt as a white solid, without further purification which was added EtOAc (2 ml), 5% TFA and 5% TIPS. The reaction was continued for another 2 h. Then H_2O (5 ml) and EtOAc (10 ml) were introduced. The aqueous phase was further washed with EtOAc (10 ml) twice and concentrated to afford TCEPA (8 mg). $\text{MS}[\text{M}+\text{H}]^+ = 250.18$, exact mass calc. for $\text{C}_9\text{H}_{16}\text{NO}_5\text{P}$ is 249.08. ^1H NMR (400 MHz, Deuterium Oxide) δ 2.85-2.70 (m, 4H), 2.61-2.43 (m, 6H), 2.15-2.06 (m, 2H).

Example 3: Synthesis of TCEP-1



1. TCEP-1-int2

To a solution of TCEP-1-int1 (3.0 g, 10.0 mmol, 1.0 eq, Fmoc-Glycine, Bidepharm) in DMF (30mL) was added HOBt (1.64 g, 12.0mmol, 1.2 eq) and EDCI (2.32g, 12mmol, 1.2eq), followed by DIPEA (4.4mL, 25.2mmol, 2.5eq) under N₂ atmosphere. The mixture was stirred for 30min and the Compound 1 O-Trithylhydroxylamine (2.78g, 10mmol, 1.0eq, Bidepharm) was added. The reaction mixture was stirred at room temperature for 1h and poured into ice-water. The precipitate was collected by filtration and washed with water. The filter cake was dried over vacuum to give the crude product TCEP-1-int2 (4.5g, 80% yield, white solid), which was used in next step directly without further purification.

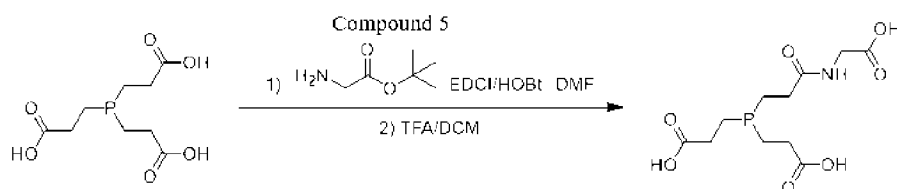
2. TCEP-1-int3

To a solution of TCEP-1-int2 (4.5 g, 8.1mmol, 1.0 eq) in DMF (25mL) was added DBU (5mL). The resulting mixture was stirred for 0.5h at room temperature, LCMS showed reaction was completed. The mixture was poured into ice-water, and extracted with EtOAc, dried over vacuum and purified with flash column (EtOAc/petroleum ether=0~50%) to give product TCEP-1-int3 (2.0 g, 77% yield, white solid).

3. TCEP-1

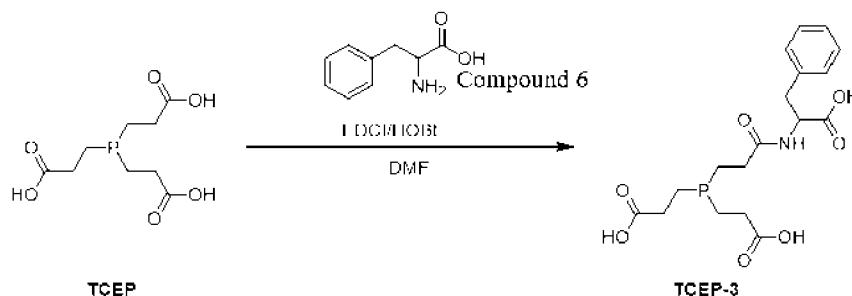
TCEP-1 was synthesized as the synthesis procedure B-1 wherein TCEP-1-int3 was the amine reagent, yielding TCEP-1 (45.1 mg, 28%) as white solid. MS[M-H]⁻ = 321.15, exact mass calc. for C₁₁H₁₉N₂O₇P is 322.25. ¹H-NMR (400 MHz, Deuterium Oxide): δ 3.99 (s, 0.64H), 3.87 (s, 1.34 H), 2.96 – 2.81 (m, 6H), 2.63-2.56 (m, 6H).

Example 4: Synthesis of TCEP-2



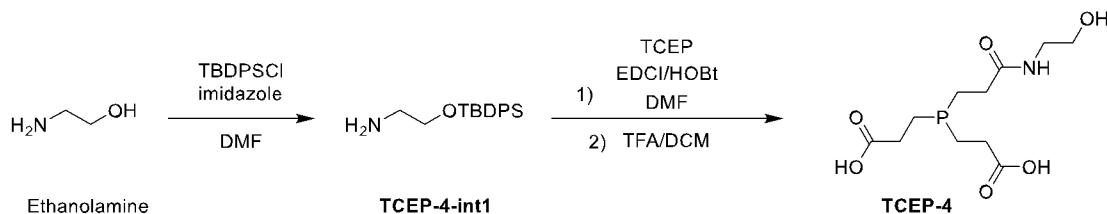
TCEP-2 was synthesized as the synthesis procedure B-1 wherein the Compound 5 (tert-Butyl glycinate, Bidepharm) was the amine reagent, yielding TCEP-2 (52.3 mg, 34%) as white solid. MS[M-H]⁻ = 306.18, exact mass calc. for C₁₁H₁₈NO₇P is 307.24. ¹H NMR (400 MHz, Deuterium Oxide): δ 3.99 (s, 2H), 3.00 – 2.76 (m, 6H), 2.60 (dtd, J = 14.0, 7.0, 3.8 Hz, 6H).

Example 5: Synthesis of TCEP-3



TCEP-3 was synthesized as the procedure B wherein the compound 6 (DL-Phenylalanine, Adamas) is amine reagent, yielding TCEP-3 (65.0 mg, 33%) as white solid. MS[M-H]⁻ = 396.24, exact mass calc. for C₁₈H₂₄NO₇P is 397.13. ¹H NMR (400 MHz, Deuterium Oxide): δ 7.43 - 7.25 (m, 5H), 4.71 (dd, J = 10.3, 4.8 Hz, 1H), 3.31 (dd, J = 13.9, 4.8 Hz, 1H), 2.91 (dd, J = 13.9, 10.3 Hz, 1H), 2.86 - 2.59 (m, 6H), 2.53 - 2.27 (m, 6H).

Example 6: Synthesis of TCEP-4

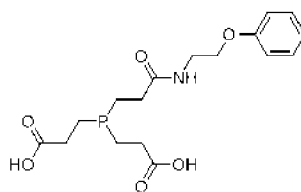


1. TCEP-4-int1

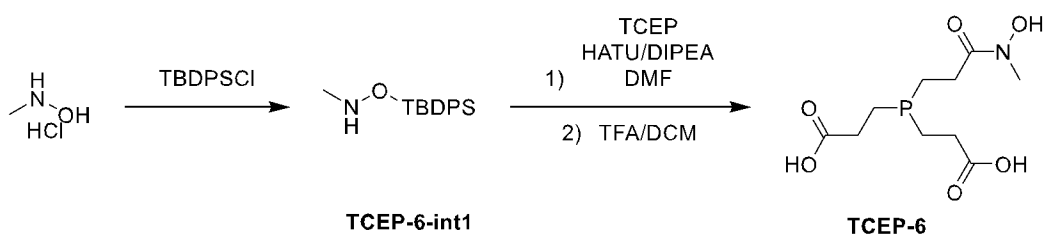
To a solution of Ethanolamine (610 mg, 10mmol, 1.0eq, Adamas) in DMC (20mL) was added imidazole (15mmol, 1.5eq) followed by TBDPSCl (10mmol, 1.0 eq, Adamas) at 0°C. The mixture was stirred for 2h at room temperature. TLC showed reaction was completed, the reaction mixture was washed with water and brine, organic layer was dried over Na₂SO₄ and filtered. The filtrate was concentrated to give the crude product, which was used in next step directly without further purification.

2. TCEP-4

TCEP-4 was synthesized as the synthesis procedure B-1 wherein TCEP-4-int1 was amine reagent, yielding TCEP-4 (60.0 mg, 40.8%) as white solid. MS[M-H]⁻ = 292.25, exact mass calc. for C₁₁H₂₀NO₆P is 293.10. ¹H NMR (400 MHz, Deuterium Oxide): δ 4.23 (t, J = 5.4 Hz, 1H), 3.64 (t, J = 5.4 Hz, 1H), 3.48 (t, J = 5.3 Hz, 1H), 3.32 (t, J = 5.5 Hz, 1H), 2.98 - 2.78 (m, 6H), 2.60 (dp, J = 13.4, 6.8 Hz, 6H).

Example 7: Synthesis of TCEP-5**TCEP-5**

TCEP-5 was synthesized as the procedure B wherein (2-phenoxy-ethylamine, Bidepharm) was amine reagent, yielding TCEP-5 (105.0 mg, 56.8%) as white solid. $MS[M-H]^- = 368.24$, exact mass calc. for $C_{17}H_{24}NO_6P$ is 369.13. ^1H NMR (400 MHz, Deuterium Oxide): δ 7.34 (dd, $J = 8.5, 7.2$ Hz, 2H), 7.00 (dd, $J = 19.2, 7.7$ Hz, 3H), 4.14 (t, $J = 5.1$ Hz, 2H), 3.56 (t, $J = 5.1$ Hz, 2H), 2.78 (ddt, $J = 41.6, 20.1, 6.9$ Hz, 6H), 2.60 - 2.43 (m, 6H).

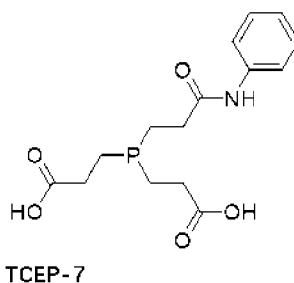
Example 8: Synthesis of TCEP-6**1. TCEP-6-int1**

To a solution of N-Methylhydroxylamine hydrochloride (830 mg, 10mmol, 1.0eq, Bidepharm) in DMC (20mL) was added imidazole (15mmol, 1.5eq) followed by TBDPSCI (10mmol, 1.0 eq, Adamas) at 0°C. The mixture was stirred for 2h at room temperature. TLC showed reaction was completed, the reaction mixture was washed with water and brine, organic layer was dried over Na_2SO_4 and filtered. The filtrate was concentrated to give the crude product, which was used in next step directly without further purification.

2. TCEP-6

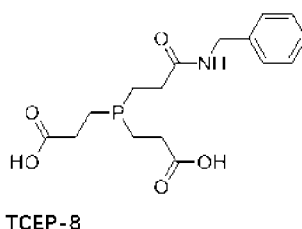
TCEP-6 was synthesized as the procedure A-1 wherein TCEP-6-int1 was amine reagent, yielding TCEP-6 (13.0 mg, 9.3%) as white solid. $MS[M+H]^+ = 280.22$, exact mass calc. for $C_{10}H_{18}NO_6P$ is 279.09. ^1H NMR (400 MHz, Deuterium Oxide): δ 3.15 (s, 3H), 3.01 - 2.80 (m, 4H), 2.64-2.45 (m, 6H), 2.17-2.08 (m, 2H).

Example 9: Synthesis of TCEP-7



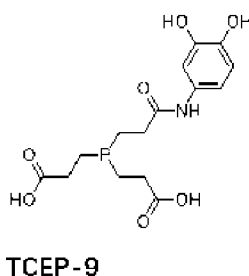
TCEP-7 was synthesized as the synthesis procedure B wherein (Phenylamine, Adamas) was amine reagent, yielding TCEP-7 (73.0 mg, 45.0% yield) as white solid. MS[M-H]⁻ = 324.21, exact mass calc. for C₁₅H₂₀NO₅P is 325.11. ¹H NMR (400 MHz, Deuterium Oxide): δ 7.42 (d, J = 4.3 Hz, 4H), 7.25 (p, J = 4.5 Hz, 1H), 2.96 (ddt, J = 32.1, 18.3, 6.9 Hz, 6H), 2.75 - 2.50 (m, 6H).

Example 10: Synthesis of TCEP-8



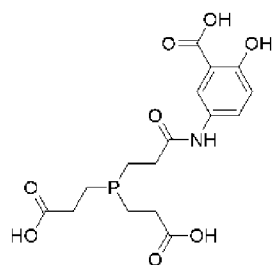
TCEP-8 was synthesized as the synthesis procedure B wherein (Benzylamine, Adamas) was amine reagent, yielding TCEP-8 (85.6 mg, 50.5% yield) as white solid. MS[M-H]⁻ = 338.23, exact mass calc. for C₁₆H₂₂NO₅P is 339.12. ¹H NMR (400 MHz, Deuterium Oxide): δ 7.43 - 7.27 (m, 5H), 4.36 (s, 2H), 2.93 - 2.77 (m, 6H), 2.63 - 2.45 (m, 6H).

Example 11: Synthesis of TCEP-9



TCEP-9 was synthesized as the synthesis procedure A wherein (4-Aminobenzene-1,2-diol, Bidepharm) was amine reagent, yielding TCEP-9 (63.8 mg, 35.7%) as white solid. MS[M-H]⁻ = 356.20, exact mass calc. for C₁₅H₂₀NO₇P is 357.10. ¹H NMR (400 MHz, Deuterium Oxide): δ 6.97 (d, J = 2.4 Hz, 1H), 6.87 (d, J = 8.5 Hz, 1H), 6.78 (dd, J = 8.5, 2.5 Hz, 1H), 2.92 (ddt, J = 17.8, 10.0, 7.0 Hz, 6H), 2.61 (dq, J = 13.9, 6.7 Hz, 6H).

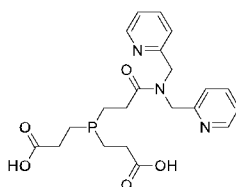
Example 12: Synthesis of TCEP-10



TCEP-10

TCEP-10 was synthesized as the synthesis procedure A wherein (5-Amino-2-hydroxybenzoic acid, Bidepharm) was amine reagent, yielding TCEP-10 (53.7mg, 27.9%) as white solid. MS[M-H]⁻ = 384.20, exact mass calc. for C₁₆H₂₀NO₈P is 385.09. ¹H NMR (400 MHz, Deuterium Oxide) δ 7.76 (d, J = 2.7 Hz, 1H), 7.41 (dd, J = 8.9, 2.7 Hz, 1H), 6.89 (d, J = 8.9 Hz, 1H), 3.00 - 2.83 (m, 6H), 2.61 (dq, J = 13.9, 6.7 Hz, 6H).

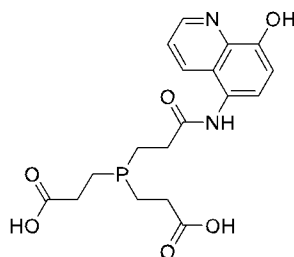
Example 13: Synthesis of TCEP-11



TCEP-11

TCEP-11 was synthesized as the synthesis procedure B wherein (Bis(pyridin-2-ylmethyl)amine, Shanghai Acme Biochemical Co., Ltd) was amine reagent, yielding TCEP-11 (10.5 mg, 4.9%) as brown solid. MS[M+H]⁺ = 432.24, exact mass calc. for C₂₁H₂₆N₃O₅P is 431.16. ¹H NMR (400 MHz, Deuterium Oxide) δ 8.82 - 8.68 (m, 2H), 8.58 - 8.36 (m, 2H), 8.01 - 7.81 (m, 4H), 5.34 (s, 1H), 5.29 (s, 1H), 5.04 (d, J = 2.9 Hz, 2H), 4.48 (s, 1H), 3.16 (dt, J = 19.2, 6.5 Hz, 1H), 2.95 - 2.79 (m, 3H), 2.73 - 2.48 (m, 5H), 2.20 (ddt, J = 36.1, 11.6, 7.5 Hz, 2H).

Example 14: Synthesis of TCEP-12

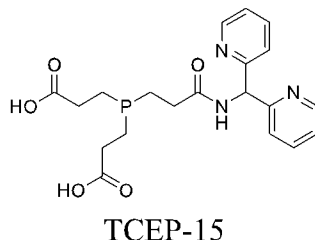


TCEP-12

TCEP-12 was synthesized as the synthesis procedure A wherein (5-Amino-8-hydroxyquinoline, Bidepharm) was amine reagent, yielding TCEP-12 (33.2 mg, 16.9%) as white solid. MS[M-H]⁻ = 391.24, exact mass calc. for C₁₈H₂₁N₂O₆P is 392.11. ¹H NMR (400 MHz, Deuterium Oxide) δ 9.02 -

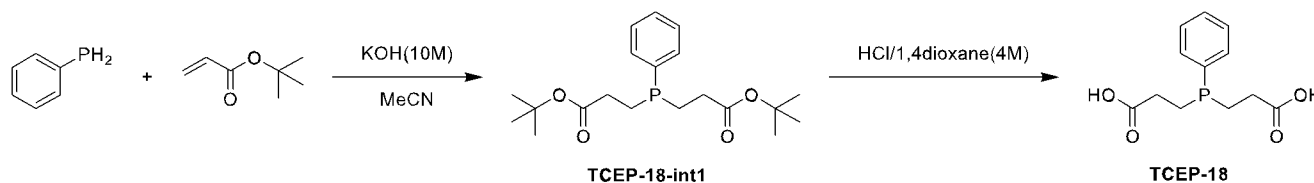
8.96 (m, 2H), 8.05 - 7.98 (m, 1H), 7.84 (s, 1H), 7.64 (d, $J = 8.4$ Hz, 1H), 7.44 (d, $J = 8.4$ Hz, 1H), 2.90 - 2.80 (m, 2H), 2.67 - 2.56 (m, 6H), 2.26 - 2.17 (m, 4H).

Example 15: Synthesis of TCEP-15



TCEP-15 was synthesized as the synthesis procedure B wherein (Bis(pyridin-2-yl) methanamine, Bidepharm) was amine reagent, yielding TCEP-15 (21.7mg, 10.4%) as white solid. $MS[M+H]^+ = 418.26$, exact mass calc. for $C_{20}H_{24}N_3O_5P$ is 417.15. ^1H NMR (400 MHz, Deuterium Oxide) δ 8.69 (td, $J = 6.2, 1.6$ Hz, 2H), 8.37 (dtd, $J = 15.8, 7.9, 1.7$ Hz, 2H), 7.93 - 7.79 (m, 4H), 3.09 - 2.72 (m, 6H), 2.70 - 2.52 (m, 6H).

Example 16: Synthesis of TCEP-18



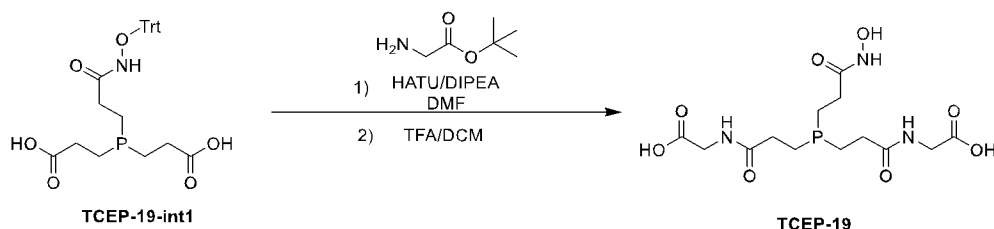
1. TCEP-18-int1

Phenyl phosphine (110 mg, 1.0 mmol, Adamas) was dissolved in acetonitrile (5 ml, degassed) in a flame-dried, round bottom flask under $N_2(g)$. Potassium hydroxide (10N, 10ul) was added to this mixture, and the resulting solution was cooled to $0^\circ C$. Tert-Butyl acrylate (0.44 ml, 3.0 mmol, Adamas) was added. Upon complete addition of Tert-Butyl acrylate, the reaction was heated at $50^\circ C$ and stirred for 8 hours. The reaction mixture was taken up by EtOAc (10mL), then washed with brine (2x5 ml). The organic layer was dried over Na_2SO_4 , filtered, and concentrated in vacuo. The residue was purified by flash column (EtOAc/petroleum ether = 0~20%(v/v)) to give product TCEP-18-int1 as a clear liquid (254 mg, 69.4%).

2. TCEP-18

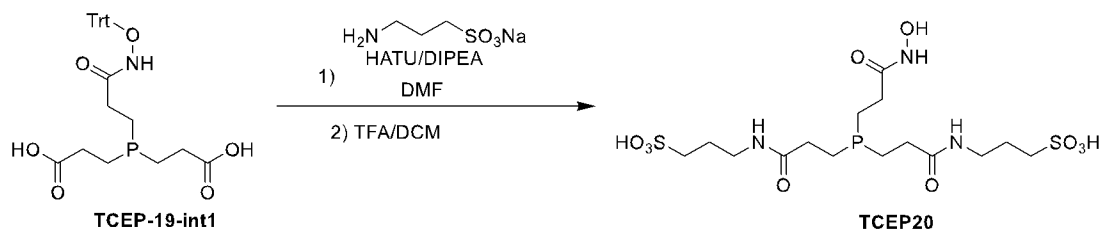
The solution of TCEP-18-int1 (254 mg, 0.69 mmol) in HCl/1,4-dioxane (4M, Adamas) was stirred for 2h at room temperature under N_2 atmosphere. LCMS showed reaction was completed and the mixture was concentrated to remove 1,4-dioxane, the resulting residue was taken up by water and lyophilized to give TCEP-18 (152.7mg, 88.2%) as white solid. $MS[M-H]^- = 253.19$, exact mass calc. for $C_{12}H_{15}O_4P$ is 254.07. ^1H NMR (400 MHz, DMSO- d_6): δ 7.74 (dd, $J = 10.9, 7.3$ Hz, 2H), 7.62 - 7.49 (m, 3H), 2.46-2.35 (m, 2H), 2.33 - 2.00 (m, 6H).

Example 17: Synthesis of TCEP-19



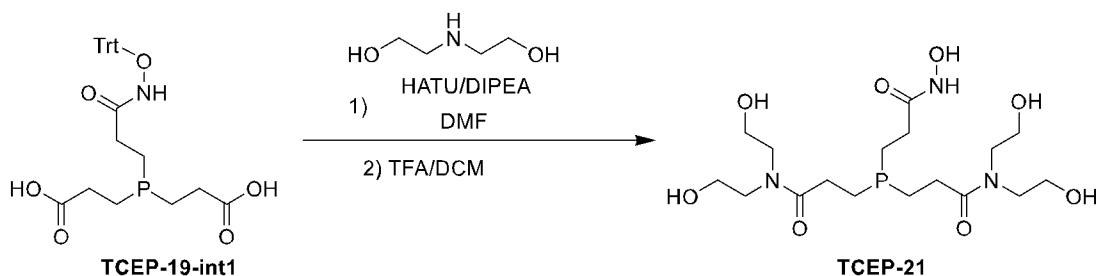
To a solution of TCEP-19-int1 (200 mg, 0.39 mmol, 1.0 eq.) in DMF(3mL) was added HATU (380 mg, 1.0 mmol, 2.5 eq) followed by DIPEA (174 μ L, 1.0 mmol, 2.5eq) at 0°C under N₂ atmosphere. The mixture was stirred for 30min, tert-Butyl glycinate (1mmol, Adamas) was added. The reaction was stirred at room temperature for 1h. The reaction mixture was purified by RP-HPLC using a C18 column yielding the protected product. The product was dissolved in DCM(3mL) and TFA (0.5 mL) was added. The mixture was stirred for 1h, LCMS showed reaction was completed. The mixture was concentrated and the residue was taken up by distilled water (10mL), washed with EtOAc(2*5mL). The aqueous layer was lyophilized to give TCEP-19 (10.2 mg, 6.8%) as brown solid. MS[M+H]⁺ = 380.24, exact mass calc. for C₁₃H₂₂N₃O₈P is 379.31. ¹H-NMR (400 MHz, Deuterium Oxide): δ 3.99 (s, 4H), 2.93-2.84 (m, 4H), 2.76 - 2.53 (m, 8H).

Example 18: Synthesis of TCEP-20



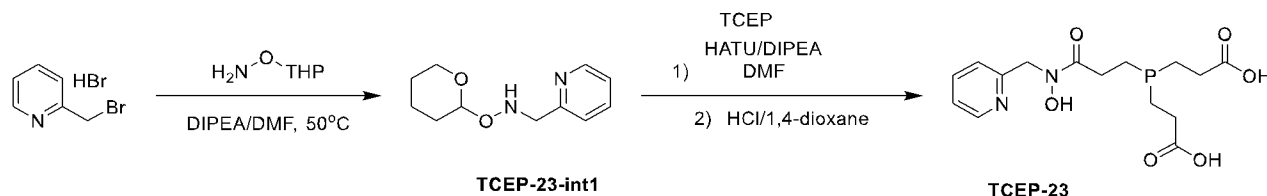
To a solution of TCEP-19-int1 (200 mg, 0.39 mmol, 1.0 eq.) in DMF(3mL) was added HATU (380 mg, 1.0 mmol, 2.5 eq) followed by DIPEA (174 μ L, 1.0 mmol, 2.5eq) at 0°C under N₂ atmosphere. The mixture was stirred for 30min, Sodium 3-Aminopropane-1-Sulfonate (1mmol, Adamas) was added. The reaction was stirred at room temperature for 1h. The reaction mixture was purified by RP-HPLC using a C18 column yielding the protected product. The product was dissolved in DCM(3mL) and TFA (0.5 mL) was added. The mixture was stirred for 1h, LCMS showed reaction was completed. The mixture was concentrated and the residue was taken up by distilled water(10mL), washed with EtOAc(2*5mL). The aqueous layer was lyophilized to give TCEP-20 (23.7 mg, 11.7%) as brown solid. MS[M-H]⁻ = 506.22, exact mass calc. for C₁₅H₃₀N₃O₁₀PS₂ is 507.51. ¹H NMR (400 MHz, Deuterium Oxide): δ 3.27 (t, J = 6.8 Hz, 4H), 2.91 - 2.85 (m, 4H), 2.80-2.71 (m, 4H), 2.69-2.61 (m, 2H), 2.58-2.49 (m, 6H), 1.94 - 1.85 (m, 4H).

Example 19: Synthesis of TCEP-21



To a solution of TCEP-19-int1 (200 mg, 0.39 mmol, 1.0 eq.) in DMF(3mL) was added HATU (380 mg, 1.0 mmol, 2.5 eq) followed by DIPEA (174 μ L, 1.0 mmol, 2.5eq) at 0°C under N₂ atmosphere. The mixture was stirred for 30min, Diethanolamine (1mmol, Bidepharm) was added. The reaction was stirred at room temperature for 1h. The reaction mixture was purified by RP-HPLC using a C18 column yielding the protected product. The product was dissolved in DCM(3mL) and TFA (0.5 mL) was added. The mixture was stirred for 1h, LCMS showed reaction was completed. The mixture was concentrated and the residue was taken up by distilled water(10mL), washed with EtOAc (2*5mL). The aqueous layer was lyophilized to give TCEP-20 (13.8 mg, 7.8 yield) as white solid. MS[M+H]⁺ = 440.27, exact mass calc. for C₁₇H₃₄N₃O₈P is 439.45. ¹H NMR (400 MHz, Deuterium Oxide): δ 4.50 - 4.28 (m, 4H), 3.78 (t, J = 5.2 Hz, 4H), 3.38 (t, J = 5.1 Hz, 4H), 3.17 (q, J = 5.2 Hz, 4H), 2.96-2.80 (m, 4H), 2.68-2.51 (m, 8H).

Example 20: Synthesis of TCEP-23



1. TCEP-23-int1

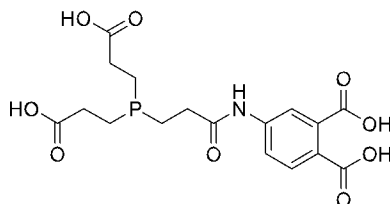
To a solution of 2-(Aminooxy)tetrahydro-2H-pyran (1.17g, 10mmol, 2.0eq, Bidepharm) in DMF(15mL) was added DIPEA(3.5mL, 20mmol, 4.eq) followed by 2-(Bromomethyl)pyridine hydrobromide (1.3g, 5.0mmol, 1.0eq, Adamas). The mixture was stirred for 16h at 50°C. The reaction mixture was poured into water (100mL), extracted with EtOAc (30mL*3). The organic layer was washed with brine (30mL), dried over Na₂SO₄ and filtered. The filtrate was concentrated and purified by flash column, to give TCEP-23-int1 (800mg, 80%), as colorless oil.

2. TCEP-23

To a solution of TCEP (286.6 mg, 1.0 mmol, 2.0 eq.) in DMF(3mL) was added HATU (190 mg, 0.5mmol, 1 eq) followed by DIPEA (2.0 mmol, 4.0eq) under N₂ atmosphere. The mixture was stirred for 30min and TCEP-23-int1 (100mg, 0.5mmol, 1.0 eq) was added. The reaction was stirred at room temperature for 4h. The reaction mixture was purified by RP-HPLC using a C18 column yielding the desired product. The product was dissolved in HCl/1,4-dioxane (3mL). The mixture was stirred for 1h, LCMS showed reaction was completed. The mixture was concentrated and the residue was taken up

by distilled water, lyophilized to give TCEP-23 (17.9 mg, 10.0 %) as white solid. $MS[M+H]^+ = 357.19$, exact mass calc. for $C_{15}H_{21}N_2O_6P$ is 356.31. ^1H -NMR (400 MHz, Deuterium Oxide): δ 8.73 (dd, $J = 6.3, 1.7$ Hz, 1H), 8.59 (td, $J = 7.9, 1.6$ Hz, 1H), 8.02 (dd, $J = 6.4, 3.4$ Hz, 2H), 5.21 (s, 2H), 3.21 - 2.97 (m, 2H), 2.95 - 2.80 (m, 4H), 2.69-2.56 (m, 6H).

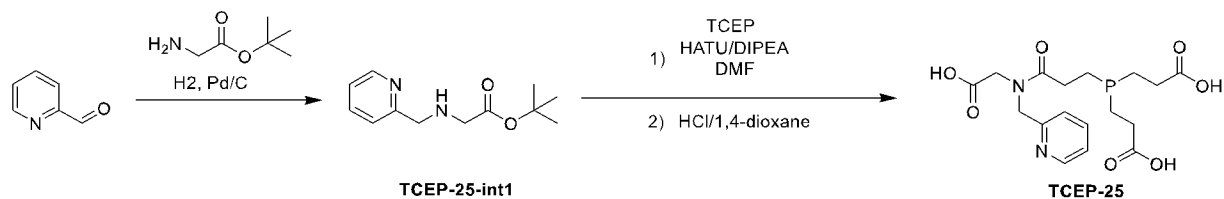
Example 21: Synthesis of TCEP-24



TCEP-24

TCEP-24 was synthesized as the synthesis procedure A wherein (4-Aminophthalic acid, Bidepharm) was amine reagent, yielding TCEP-24 (21.5 mg, 10.4% yield) as white solid. $MS[M-H]^- = 412.22$, exact mass calc. for $C_{17}H_{20}NO_9P$ is 413.09. ^1H NMR (400 MHz, Deuterium Oxide): δ 7.87 (d, $J = 8.5$ Hz, 1H), 7.77 (d, $J = 2.1$ Hz, 1H), 7.71 - 7.64 (m, 1H), 3.08-3.00 (m, 2H), 2.96-2.86 (m, 4H), 2.70-2.59 (m, 6H).

Example 22: Synthesis of TCEP-25



1. TCEP-25-int1

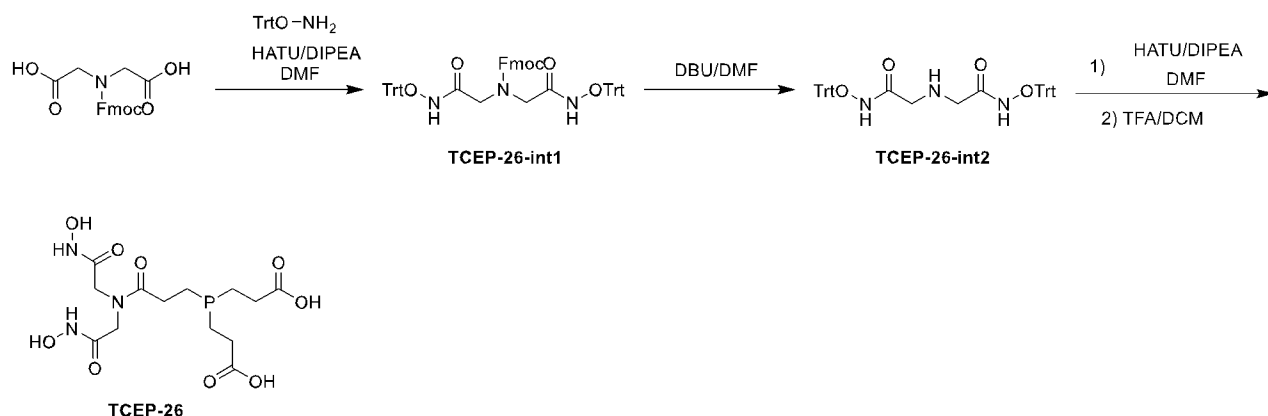
To a solution of 2-Pyridinecarboxaldehyde (1.0g, 10mmol, 1.0 eq. Adamas) and tert-Butyl glycinate (1.3g, 10.0 mmol, 1.0 eq) in MeOH (25mL) was added Pd/C (150mg) and two drops of AcOH. The mixture was degassed 3 times and purged with H_2 , then stirred for 16h at room temperature under H_2 atmosphere. The reaction mixture was filtered through a Celite pad and the filtrate was concentrate then purified by flash column, to give TCEP-25-int1 (1.6g, 72.0%) as yellow oil.

2. TCEP-25

To a solution of TCEP (286.6 mg, 1.0 mmol, 2.0 eq.) in DMF (3mL) was added HATU (190 mg, 0.5mmol, 1 eq) followed by DIPEA (2.0 mmol, 4.0eq) under N_2 atmosphere. The mixture was stirred for 30min and TCEP-25-int1 (111mg, 0.5mmol, 1.0 eq) was added. The reaction was stirred at room temperature for 4h. The reaction mixture was purified by RP-HPLC using a C18 column yielding the desired product. The product was dissolved in HCl/1,4-dioxane (3mL). The mixture was stirred for 1h, LCMS showed reaction was completed. The mixture was concentrated and the residue was taken up by distilled water, lyophilized to give TCEP-25 (51.3 mg, 17.2 % yield) as white solid. $MS[M+H]^+ =$

399.25, exact mass calc. for $C_{17}H_{23}N_2O_7P$ is 398.12. ^1H NMR (400 MHz, Deuterium Oxide): δ 8.60 (dd, $J = 5.9, 1.6$ Hz, 1H), 8.45 (td, $J = 8.0, 1.6$ Hz, 1H), 7.92 (d, $J = 8.3$ Hz, 1H), 7.87 (ddd, $J = 7.5, 5.9, 1.3$ Hz, 1H), 4.88 (s, 2H), 4.39 (s, 2H), 2.84 - 2.68 (m, 6H), 2.51-2.41 (m, 6H).

Example 23: Synthesis of TCEP-26



1. TCEP-26-int1

To a solution of Fmoc-aminodiacetic acid (1.8 g, 5.0 mmol, 1.0 eq, Bidepharm) in DMF (30mL) was added HATU (4.3 g, 11.0 mmol, 2.2 eq) followed by DIPEA (2.0 mmol, 4.0eq) under N_2 atmosphere. The mixture was stirred for 30min and O-Tritylhydroxylamine (3.0 g, 11.0 mmol, 2.2 eq) was added. The reaction was stirred at room temperature for 4h. The reaction mixture was poured into water (200mL). The precipitate was collected by filtration and the filter cake was dried over vacuum to give TCEP-23-int1 (4.0 g, 92.0%), as white solid.

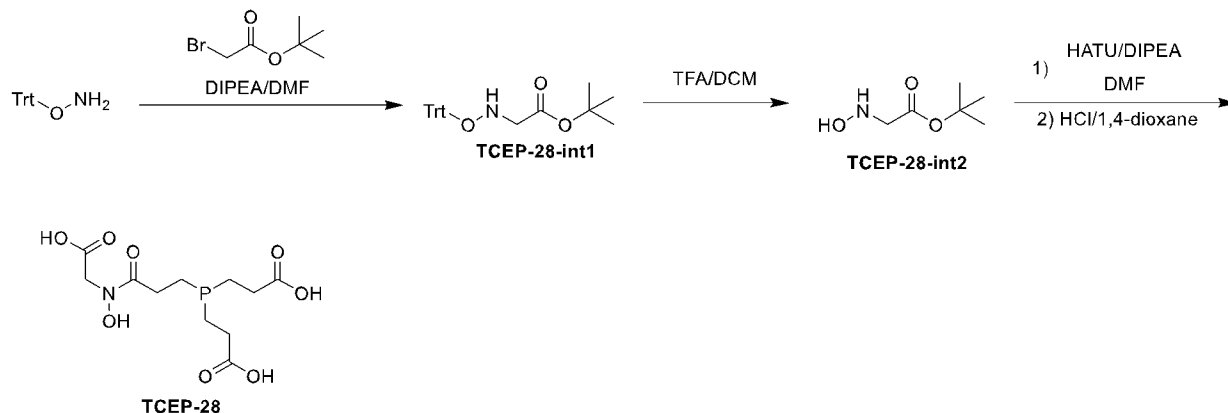
2. TCEP-26-int2

To a solution of TCEP-26-int1 (2.0g, 2.3mmol, 1.0 eq) in DMF (10mL) was added DBU (2mL). The mixture was stirred for 1h at room temperature, then poured into ice-water(100mL), extracted with EtOAc (50mL*3). The combined organic layer was washed with brine, dried over Na_2SO_4 and filtered. The filtrate was concentrated and purified by flash column (EtOAc/petroleum ether = 0~50%, v/v) to give TCEP-26-int2 (1.2 g, 80%).

3. TCEP-26

The compound was synthesized as the synthesis procedure A-1 wherein TCEP-26-int2 was amine reagent, yielding TCEP-26 (31.5 mg, 21.3 % yield) as white solid. $MS[M+H]^+ = 396.17$, exact mass calc. for $C_{13}H_{22}N_3O_9P$ is 395.11. ^1H NMR (400 MHz, Deuterium Oxide) δ 4.13 (s, 2H), 3.97 (s, 2H), 2.85-2.77(m, 4H), 2.54-2.47(m, 6H), 2.18-2.08(m, 2H).

Example 24: Synthesis of TCEP-28



1. TCEP-28-int1

To a solution of O-Tritylhydroxylamine (1.4 g, 5.0 mmol, 1.0 eq) in DMF (15mL) was added DIPEA (1.7mL, 10mmol, 2.eq) followed by tert-Butyl bromoacetate (1.0 g, 5.0mmol, 1.0eq, Adamas). The mixture was stirred for 16h at 50°C. The reaction mixture was poured into water (100mL), extracted with EtOAc (30mL*3). The organic layer was washed with brine (30mL), dried over Na₂SO₄ and filtered. The filtrate was concentrated and purified by flash column, to give TCEP-28-int1 (1.4g, 70%), as white solid.

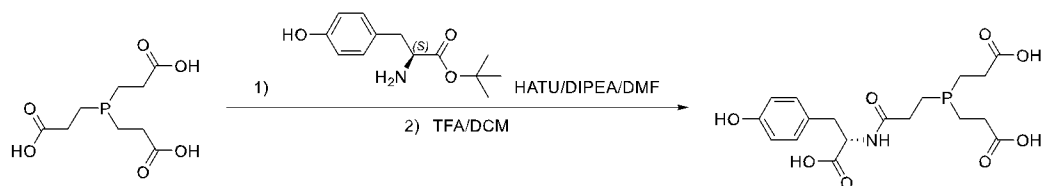
2. TCEP-28-int2

To a solution of TCEP-28-int1 (1.4 g, 3.6 mmol, 1.0 eq) in DCM (15mL) was added TFA (1.5mL). The mixture was stirred for 2h at room temperature. The reaction mixture was concentrated and purified by flash column, to give TCEP-28-int2 (380 mg, 71.8%), as colorless oil.

3. TCEP-28

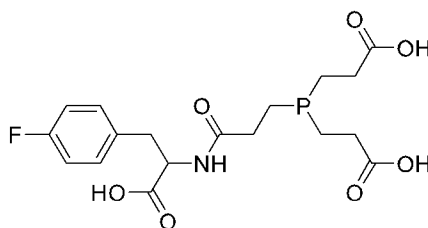
To a solution of TCEP (286.6 mg, 1.0 mmol, 2.0 eq.) in DMF(3mL) was added HATU (190 mg, 0.5mmol, 1 eq) followed by DIPEA (2.0 mmol, 4.0eq) under N₂ atmosphere. The mixture was stirred for 30min and TCEP-28-int2 (73.5mg, 0.5mmol, 1.0 eq) was added. The reaction was stirred at room temperature for 2h. The reaction mixture was purified by RP-HPLC using a C18 column yielding the desired product. The product was dissolved in HCl/1,4-dioxane (3mL, Adamas). The mixture was stirred for 1h, LCMS showed reaction was completed. The mixture was concentrated and the residue was taken up by distilled water, lyophilized to give TCEP-28 (43.6 mg, 27.1 % yield) as white solid. MS[M-H] = 322.16, exact mass calc. for C₁₁H₁₈NO₈P is 323.08. ¹H NMR (400 MHz, Deuterium Oxide): δ 4.36 (s, 2H), 2.86-2.77 (m, 6H), 2.56-2.48 (m, 6H).

Example 25: Synthesis of TCEP-30



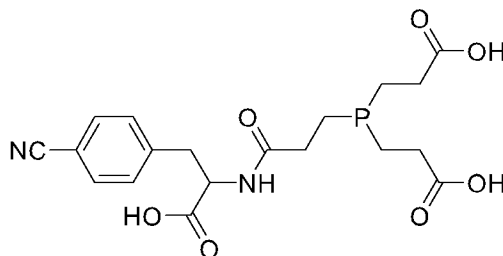
TCEP-30 was synthesized as the synthesis procedure A-1 wherein [tert-Butyl L-tyrosinate, Adamas) was amine reagent, yielding TCEP-30 (25.3 mg, 12.25%) as white solid. MS[M+H]⁺ = 414.23, exact mass calc. for C₁₈H₂₄NO₈P is 413.12. ¹H NMR (400 MHz, Deuterium Oxide): δ 7.24-7.14 (m, 2H), 6.91-6.82 (m, 2H), 4.67 (dd, J = 10.3, 4.7 Hz, 1H), 3.26 (dd, J = 14.0, 4.7 Hz, 1H), 2.92-2.64 (m, 7H), 2.57-2.30 (m, 6H).

Example 26: Synthesis of TCEP-31



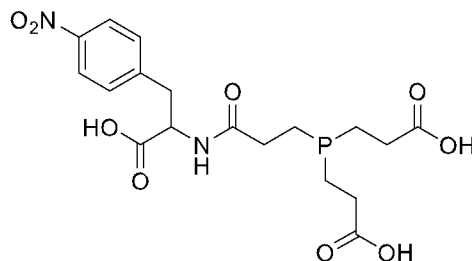
TCEP-31 was synthesized as the synthesis procedure A wherein (DL-3-(4-Fluorophenyl)alanine, Bidepharm) was amine reagent, yielding TCEP-31 (27.1 mg, 13.10%) as white solid. MS[M+H]⁺ = 416.01, exact mass calc. for C₁₈H₂₃NO₇P is 415.12. ¹H NMR (400 MHz, Deuterium Oxide): δ 7.36 - 7.26 (m, 2H), 7.12 (t, J = 8.8 Hz, 2H), 4.70 (dd, J = 10.1, 4.8 Hz, 1H), 3.31 (dd, J = 14.0, 4.9 Hz, 1H), 2.95 (dd, J = 14.0, 10.1 Hz, 1H), 2.90 - 2.65 (m, 6H), 2.56-2.40 (m, 6H).

Example 27: Synthesis of TCEP-32



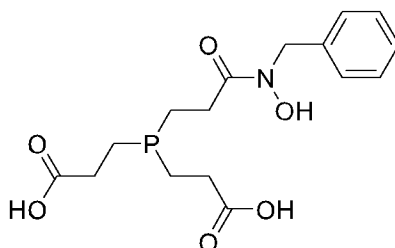
TCEP-32 was synthesized as the synthesis procedure A wherein (DL-4-Cyanophenylalanine, Bidepharm) was amine reagent, yielding TCEP-32 (18.5 mg, 8.76%) as white solid. MS[M+H]⁺ = 423.24, exact mass calc. for C₁₉H₂₃N₂O₇P is 422.12. ¹H NMR (400 MHz, Deuterium Oxide): δ 7.77 (d, J = 8.1 Hz, 2H), 7.49 (d, J = 8.1 Hz, 2H), 4.77 - 4.71 (m, 1H), 3.42 (dd, J = 14.0, 5.0 Hz, 1H), 3.05 (dd, J = 14.0, 10.0 Hz, 1H), 2.91 - 2.70 (m, 6H), 2.68 - 2.39 (m, 6H).

Example 28: Synthesis of TCEP-33



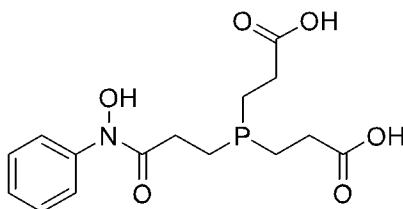
TCEP-33 was synthesized as the synthesis procedure A wherein (DL-4-nitro-phenylalanine, Bidepharm) was amine reagent, yielding TCEP-33 (20.7 mg, 9.37%) as white solid. $MS[M+H]^+ = 443.24$, exact mass calc. for $C_{19}H_{23}N_2O_9P$ is 442.11. ^1H NMR (400 MHz, Deuterium Oxide): δ 8.22 (d, $J = 8.2$ Hz, 2H), 7.54 (d, $J = 8.2$ Hz, 2H), 4.84-4.80 (m, 1H), 3.47 (dd, $J = 14.0, 4.8$ Hz, 1H), 3.11 (dd, $J = 13.9, 10.3$ Hz, 1H), 2.91-2.73 (m, 6H), 2.58-2.38 (m, 6H).

Example 29: Synthesis of TCEP-34



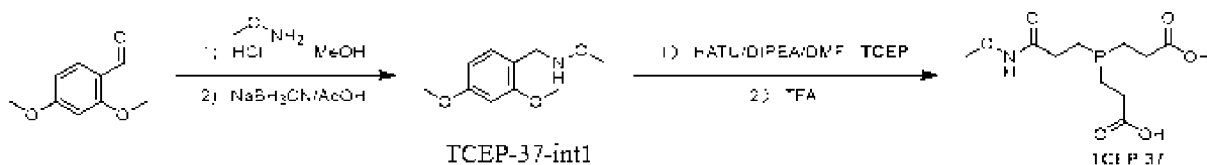
TCEP-34 was synthesized as the synthesis procedure A wherein (N-Benzylhydroxylamine hydrochloride, Bidepharm) was amine reagent, yielding TCEP-34 (15.7 mg, 8.85%) as white solid. $MS[M+H]^+ = 356.05$, exact mass calc. for $C_{16}H_{22}NO_6P$ is 355.12. ^1H NMR (400 MHz, Deuterium Oxide): δ 7.55-7.34 (m, 5H), 4.84 (s, 2H), 3.14 (dt, $J = 19.8, 6.9$ Hz, 2H), 2.92 (dt, $J = 18.2, 7.0$ Hz, 4H), 2.62 (dq, $J = 14.5, 7.3$ Hz, 6H).

Example 30: Synthesis of TCEP-35



TCEP-35 was synthesized as the synthesis procedure A wherein (N-Phenylhydroxylamine, Bidepharm) was amine reagent, yielding TCEP-35 (17.1 mg, 10.0%) as white solid. $MS[M+H]^+ = 341.97$, exact mass calc. for $C_{15}H_{21}NO_6P$ is 341.10. ^1H NMR (400 MHz, Deuterium Oxide): δ 7.57-6.88 (m, 5H), 3.27-3.22 (m, 1H), 2.92-2.78 (m, 3H), 2.65-2.53 (m, 6H), 2.29-1.98 (m, 2H).

Example 31: Synthesis of TCEP-37



1. TCEP-37-int1

To a solution of 2,4-Dimethoxybenzaldehyde (1.66 g, 10.0 mmol, 1.0 eq, Adamas) in MeOH (25mL) was added O-methylhydroxylamine hydrochloride (1.66g, 20.0mmol, 2.0 eq, Bidepharm). The resulting mixture was stirred for 16 h at room temperature, LCMS showed reaction was completed. The mixture was concentrated and the residue was taken up by AcOH(20mL), NaBH₃CN was added and stirred for 5h at room temperature. LCMS showed reaction was completed. The reaction mixture was concentrated and residue was poured into ice-water(200mL) extracted with EtOAc(50mL*3). The combined organic layer was dried over Na₂SO₄ and filtered. The filtrate was concentrated over vacuum and purified with flash column (EtOAc/petroleum ether=0~50%) to give product TCEP-37-int1 (N-(2,4-dimethoxybenzyl)-O-methyl hydroxylamine, 1.5 g, 76.1%, colorless oil).

2. TCEP-37

TCEP-37 was synthesized as the synthesis procedure A-1 wherein TCEP-73-int1 was amine reagent, yielding TCEP-37 (12.8 mg, 9.14 %) as white solid. MS[M+H]⁺ = 280.18, exact mass calc. for C₁₀H₁₈NO₆P is 279.09. ¹H NMR (400 MHz, Deuterium Oxide): δ (s, 3H), 2.89 (dt, J = 18.4, 7.1 Hz, 4H), 2.72 (dd, J = 18.1, 6.6 Hz, 2H), 2.61 (dq, J = 13.9, 6.7 Hz, 6H).

Example 32: Preparation of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate (The ADC with D6)

The ADC was prepared in a one-pot reaction:

(1) ZnCl₂ (0.024 mM) and reductant TCEP-NO-Trtyl prepared by Example 1 (0.048 mM) were added to a solution of a monoclonal antibody Trastuzumab (0.012 mM, in BES buffer, pH7.0, 20 mM) and the reaction mixture was allowed to stay at 4 °C for 18h;

(2) EDTA-2Na (0.6 mM) and MC-VC-PAB-MMAE (0.096 mM) in DMA was introduced and the reaction was continued at room temperature for 1h;

(3) The reaction mixture was subjected to purification using a desalting column.

Example 33: Preparation of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate (The ADC with D6)

The method of Example 33 is similar to Example 32, and the difference was that the reductant was TCEP-NO prepared by Example 1.

Examples 34-57 and the comparative example 5: Preparation of ADCs with D6

The method of examples 34-57 was similar to example 32, and the differences were the kinds of reductant and the antibody, the molar ratio of the reductant and the antibody, and/or the molar ratio of the molar ratio of the Zn^{2+} and the reductant which are shown in as follow table. Meanwhile, and the incubation time in step (1) is 16h in examples 55-57.

No.	Reductant	Reductant (mM)	Reductant/mAb (Molar Ratio)	ZnCl ₂ (mM)	ZnCl ₂ /Reductant (Molar Ratio)	Antibody (mAb)
E34	TCEP-1	0.054	4.5:1	0.024	0.44:1	Trastuzumab
E35	TCEP-2	0.144	12:1	0.024	0.17:1	Trastuzumab
E36	TCEP-3	0.120	10:1	0.024	0.20:1	Trastuzumab
E37	TCEP-4	0.120	10:1	0.024	0.20:1	Trastuzumab
E38	TCEP-5	0.144	12:1	0.024	0.25:1	Trastuzumab
E39	TCEP-6	0.103	8.6:1	0.024	0.23:1	Trastuzumab
E40	TCEP-7	0.036	3:1	0.012	0.34:1	Trastuzumab
E41	TCEP-8	0.048	4:1	0.024	0.50:1	Trastuzumab
E42	TCEP-9	0.048	4:1	0.024	0.50:1	Trastuzumab
E43	TCEP-10	0.048	4:1	0.024	0.50:1	Trastuzumab
E44	TCEP-15	0.048	4:1	0.024	0.50:1	Trastuzumab
E45	TCEP-18	0.054	4.5:1	0.024	0.44:1	Trastuzumab
E46	TCEP-19	0.077	6.4:1	0.024	0.31:1	Trastuzumab
E47	TCEP-20	0.103	8.6:1	0.024	0.23:1	Trastuzumab
E48	TCEP-23	0.077	6.4:1	0.024	0.31:1	Trastuzumab
E49	TCEP-24	0.046	3.8:1	0.024	0.53:1	Trastuzumab
E50	TCEP-25	0.103	8.6:1	0.024	0.23:1	Trastuzumab
E51	TCEP-26	0.096	8:1	0.024	0.25:1	Trastuzumab
E52	TCEP-28	0.103	8.6:1	0.024	0.23:1	Trastuzumab
E53	TCEPA	0.077	6.4:1	0.024	0.31:1	Belantamab
E54	TCEPA	0.042	3.5:1	0.024	0.57:1	Sacituzumab
E55	TCEP-30	0.077	6.4:1	0.024	0.31:1	Trastuzumab
E56	TCEP-31	0.077	6.4:1	0.024	0.31:1	Trastuzumab
E57	TCEP-33	0.077	6.4:1	0.024	0.31:1	Trastuzumab
C5	TCEP	0.048	4:1	0	0:1	Trastuzumab

“E” was short for Example, “C” was short for Comparative Example, and mAb is short for monoclonal antibody.

The homogeneity assays results of examples 32-57 and the comparative example 5 were shown as follows:

Table 1

NO.	Figure	Reductant	D0 (%)	D2 (%)	D4 (%)	D6 (%)	D8 (%)
E32	1	TCEP-NO-Trtyl	0	11	9	66	14
E33	2	TCEP-NO	0	4	16	70	10
E34	3A	TCEP1	0	0	3.75	90.59	5.66
E35	3B	TCEP-2	0	1.19	2.81	93.02	2.98
E36	3C	TCEP-3	0	0	3.09	95.52	1.39
E37	3D	TCEP-4	0	0	2.78	88.40	8.82
E38	3E	TCEP-5	0	0	2.27	88.04	9.69

NO.	Figure	Reductant	D0 (%)	D2 (%)	D4 (%)	D6 (%)	D8 (%)
E39	3F	TCEP-6	0	3.11	5.47	82.56	8.86
E40	3G	TCEP-7	0	1.4	2.502	83.08	13.07
E41	3H	TCEP-8	0	2.79	3.91	89.59	3.71
E42	4A	TCEP-9	0	13.36	5.52	69.33	11.79
E43	4B	TCEP-10	0	0	1.96	90.39	7.65
E44	4C	TCEP-15	0	1.50	6.41	89.06	3.04
E45	4D	TCEP-18	0	3.26	5.68	80.77	10.30
E46	4E	TCEP-19	0	5.13	5.76	79.65	9.47
E47	4F	TCEP-20	0	2.40	3.30	74.78	19.53
E48	4G	TCEP-23	0	2.12	4.86	85.68	7.35
E49	4H	TCEP-24	0	1.91	1.65	81.56	14.87
E50	5A	TCEP-25	0	0.75	4.28	91.37	3.61
E51	5B	TCEP-26	0	0	3.03	90.67	6.30
E52	5C	TCEP-28	0	0	3.20	92.75	4.06
E53	5D	TCEPA	0	6.77	11.44	71.23	10.57
E54	5E	TCEPA	0	0	0	77.51	22.49
E55	5F	TCEP-30	0	1.11	3.38	92.55	2.97
E56	5G	TCEP-31	0	1.54	5.39	93.07	0
E57	5H	TCEP-33	0	2.20	10.16	87.65	0
C5	6B	TCEP	0	0	9	10	81

As shown in the above table, linker-payloads (MC-VC-PAB-MMAE) were successfully linked to the antibody, which indicated ADCs of example 32-57 prepared by the reductant in the present application were successfully synthesized.

According to examples 32-57 and comparative example 5, the reductant in the present application could increase the homogeneity of the ADC with D6 compared with the traditional method using TCEP without Zn^{2+} , which successfully demonstrated that combination of the transition metal ions and the novel reductants is responsible for higher level of D6 in the resultant ADCs. Further, the selective reductant ability of TCEP-3 is best, with a D6 content of up to 95.52%. Meanwhile, the selective reduction ability of TCEP-1, TCEP-2, TCEP-10, TCEP-25, TCEP-26, TCEP-28, TCEP-30, TCEP-31 and TCEP-33 is also wonderful, with a D6 content of up to 90%.

According to the results of examples 53 and 54, the reductants in the present application are suitable for preparing the ADC with different antibody.

Examples 58-78: Preparation of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate

The preparation of examples 58-78 was similar to the preparation of ADC with D6 of Example 33, but it adjusted the reductant, the molar ratio of the reductant and the monoclonal antibody, the molar ratio of the ZnCl_2 and the monoclonal antibody, and/or the reduction time in step (a) which were shown as follows:

No.	Reductant	Reductant/mAb (Molar Ratio)	ZnCl ₂ /mAb (Molar Ratio)	ZnCl ₂ / Reductant (Molar Ratio)	mAb (mM)	Reductant time in step (1) (h)
E58	TCEPA	2.8:1	2:1	0.71:1	0.012	18
E59	TCEPA	3.5:1	2:1	0.57:1	0.012	18
E60	TCEPA	3.8:1	2:1	0.52:1	0.012	18
E61	TCEPA	4:1	2:1	0.5:1	0.012	18
E62	TCEPA	4.2 :1	2:1	0.47:1	0.012	18
E63	TCEPA	4.4:1	2:1	0.45:1	0.012	18
E64	TCEPA	4.6 :1	2:1	0.43:1	0.012	18
E65	TCEPA	3.8:1	1:1	0.26:1	0.012	18
E66	TCEPA	4:1	1:1	0.25:1	0.012	18
E67	TCEPA	4.2:1	1:1	0.23:1	0.012	18
E68	TCEPA	4.4:1	1:1	0.22:1	0.012	18
E69	TCEPA	4.6:1	1:1	0.21:1	0.012	18
E70	TCEP-NO	5:1	1:1	0.20:1	0.012	6
E71	TCEP-NO	6:1	1:1	0.16:1	0.012	6
E72	TCEP-NO	7:1	1:1	0.14:1	0.012	6
E73	TCEP-NO	8:1	1:1	0.125:1	0.012	6
E74	TCEP-NO	9:1	1:1	0.11:1	0.012	6
E75	TCEP-NO	10:1	1:1	0.1:1	0.012	6
E76	TCEP-NO	11:1	1:1	0.09:1	0.012	6
E77	TCEP-NO	12:1	1:1	0.083:1	0.012	6
E78	TCEP-NO	13:1	1:1	0.077:1	0.012	6

The homogeneity assays results were shown as follows:

Table 2

Example	Figure	Reductant/mAb (Molar Ratio)	Reductant time in step (1) (h)	D0 (%)	D2 (%)	D4 (%)	D6 (%)	D8 (%)
E58	7A	2.8:1	18	0	11.11	13.35	75.54	0
E59	7B	3.5:1	18	0	5.35	8.07	84.71	1.87
E60	8	3.8:1	18	0	5	10	81	4
E61	9	4:1	18	0	4	10	81	5
E62	10	4.2 :1	18	0	3	6	83	8
E63	11	4.4:1	18	0	3	6	83	7
E64	12	4.6 :1	18	0	3	7	82	8
E65	13	3.8:1	18	0	5	24	70	1
E66	14	4:1	18	0	4	17	76	3
E67	15	4.2:1	18	0	3	15	78	4
E68	16	4.4:1	18	0	4	17	75	4
E69	17	4.6:1	18	0	3	12	79	6
E70	18A	5:1	6	0	23.69	32.30	44.01	0
E71	18B	6:1	6	0	13.74	21.65	64.60	0
E72	18C	7:1	6	0	11.16	22.20	66.64	0
E73	19A	8:1	6	0	4.69	5.88	81.04	8.40
E74	19B	9:1	6	0	3.81	5.02	82.00	9.17
E75	19C	10:1	6	0	2.79	4.07	83.81	9.33

Example	Figure	Reductant/mAb (Molar Ratio)	Reductant time in step (1) (h)	D0 (%)	D2 (%)	D4 (%)	D6 (%)	D8 (%)
E76	19D	11:1	6	0	1.14	2.74	82.19	13.93
E77	19E	12:1	6	0	1.25	2.70	82.22	13.84
E78	19F	13:1	6	0	1.95	2.79	81.04	14.23

The results of Examples 58-78 were shown in Table 2. As the results shown in table 2, linker-payloads (MC-VC-PAB-MMAE) were successfully linked to Trastuzumab, which indicated ADCs of Examples 58-78 prepared by TCEPA or TCEP-NO were successfully synthesized. TCEPA and TCEP-NO could be used as a reductant in antibody modification and preparation of ADC with D6.

According to the results of table 2, the highest proportion of D6 was 84.71 %, when the molar ratio of the ZnCl_2 and the monoclonal antibody was 2 and the molar ratio of the reductant and the antibody was 2.8:1 to 4.6:1. And D6 was at least 40% when the molar ratio of the ZnCl_2 and the monoclonal antibody was 1 and the molar ratio of the reductant and the antibody was 3.8:1 to 13:1. The resultant ADCs with high level of D6 showed that, the molar ratio of the reductant and the antibody ranging from 2.5 to 13 was benefit for improving the homogeneity of ADCs.

According to examples 70-78, when the molar ratio of the reductant and the antibody is from 5:1 to 13:1, the reduction time in step (1) is shortened to 6h. When the molar ratio of the reductant and the antibody is from 6:1 to 13:1 and the reduction time in step (1) is 6h, the content of D6 is up to 65%, 70%, even to 75% or 80%.

Examples 79-90: Preparation of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate with different molar ratio of the ZnCl_2 and the reductant

The preparation of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate was similar to the preparation of ADC of Example 32, but the reductant was TCEPA or TCEP-NO.

The reductant, the molar ratio of the ZnCl_2 and the reductant and the reductant time in step (1) were shown in follow table.

Comparative examples 1-4: Preparation of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate (the concentration of Zn^{2+} is 0)

The preparation of comparative example 1 was similar to the preparation of ADC of Example 32, but the reductant was TCEPA. The preparation of comparative example 2 was similar to that of Example 73, the preparation of comparative example 3 was similar to that of Example 75, the preparation of comparative example 4 was similar to that of Example 77, the difference is that the concentration of ZnCl_2 in step (1) is 0.

Example	Reductant	ZnCl ₂ / Reductant (Molar Ratio)	mAb (mM)	Reductant/mAb (Molar Ratio)	The reductant time in step (1) (h)
C1	TCEPA	0	0.012	4:1	18
C2	TCEP-NO	0	0.012	8:1	6
C3	TCEP-NO	0	0.012	10:1	6
C4	TCEP-NO	0	0.012	12:1	6
E79	TCEPA	0.25:1	0.012	4	18
E80	TCEPA	0.5:1	0.012	4	18
E81	TCEPA	1:1	0.012	4	18
E82	TCEPA	2:1	0.012	4	18
E83	TCEPA	3:1	0.012	4	18
E84	TCEPA	4:1	0.012	4	18
E85	TCEPA	7:1	0.012	4	18
E86	TCEPA	7.5:1	0.012	4	18
E87	TCEPA	15:1	0.012	3.2:1	18
E88	TCEPA	30:1	0.012	3.2:1	18
E89	TCEP-NO	0.22:1	0.012	9:1	4
E90	TCEP-NO	3.33:1	0.012	9:1	4

The homogeneity assays results of examples 79-90 and comparative examples 1-4 were shown as follows:

Table 3

Example	Figure	ZnCl ₂ / Reductant (Molar Ratio)	D0 (wt%)	D2 (wt%)	D4 (wt%)	D6 (wt%)	D8 (wt%)
C1	20	0	0	2	18	20	60
C2	21A	0	0	0	24.55	30.92	44.54
C3	21B	0	0	0	6.97	17.14	75.89
C4	21C	0	0	0	0	11.56	88.44
E79	22	0.25:1	0	3	12	79	6
E80	23	0.5:1	0	3	6	83	8
E81	24	1:1	0	3	5	82	10
E82	25	2:1	0	3	6	83	8
E83	26	3:1	0	0	5	83	12
E84	27	4:1	0	0	6	81	13
E85	28	7:1	0	0	5	79	16
E86	29A	7.5:1	0	0	5.29	79.31	15.40
E87	29B	15:1	0	2.94	6.64	85.07	5.36
E88	29C	30:1	0	2.08	7.43	78.90	11.59
E89	29D	0.22:1	0	4.12	5.79	77.34	12.75
E90	29E	3.33:1	4.81	2.83	4.49	63.04	24.83

As the results shown in table 3, linker-payloads (MC-VC-PAB-MMAE) were successfully linked to Trastuzumab, which indicated ADCs prepared with TCEPA/TCEP-NO and Zn²⁺ of example 79-90 were successfully synthesized.

According to the results of Table 3, The proportion of D6 in comparative examples 1-4 was only 11%, 17%, 20% or 31%, but the proportion of D6 in Examples 79-90 was at least 60%. It showed that Zn^{2+} was important to improve the homogeneity of ADCs with D6. Further, the molar ratio of Zn^{2+} and the reductant ranging from 0.05 to 30 was benefit for improving the homogeneity of ADCs.

Examples 91-102: Preparation of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate in different buffers

The preparation of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate was similar to the preparation of ADC of Example 82, the molar ratio of the TCEPA and the antibody, and the buffer system was as follows:

Example	The buffer system	TCEPA/mAb (Molar Ratio)	ZnCl ₂ /mAb (Molar Ratio)	pH
E91	MOBS buffer	4:1	2:1	7.4
E92	TAPSO buffer	4:1	2:1	7.4
E93	DIPSO buffer	4:1	2:1	7.4
E94	MOPSO buffer	4:1	2:1	7.4
E95	ACES buffer	4:1	2:1	7.4
E96	Bis-Tris buffer	3.5:1	2:1	6.7
E97	PIPES buffer	3.5:1	2:1	6.7
E98	HEPES buffer	3.5:1	2:1	7.0
E99	MOPS buffer	3.5:1	2:1	7.0
E100	TES buffer	3.5:1	2:1	7.4
E101	PB	3.5:1	2:1	6.7
E102	ADA buffer	3.5:1	2:1	6.7

mAb is short for monoclonal antibody. All the buffers were commercially available from Macklin.

The homogeneity assays results were shown as follows:

Table 4

N0.	Figure	D0 (wt%)	D2 (wt%)	D4 (wt%)	D6 (wt%)	D8 (wt%)
E91	30	0	2	5	85	8
E92	31	0	0	4	87	9
E93	32	0	0	4	88	8
E94	33	0	3	5	85	7
E95	34	0	0	7	86	7
E96	35A	0.96	3.89	4.05	84.65	6.46
E97	35B	1.09	11.27	14.10	71.45	2.09
E98	35C	0	1.22	4.83	86.55	7.40
E99	35D	1.04	4.67	5.54	83.54	5.21
E100	35E	0	3.70	3.90	88.11	4.28
E101	35F	1.41	8.83	20.36	66.55	2.85
E102	35G	0	7.59	17.42	52.10	22.89

The results of examples 91-102 were shown in Table 4. As the results shown in table 4, linker-payloads (MC-VC-PAB-MMAE) were successfully linked to Trastuzumab, which indicated ADCs in different buffers were successfully synthesized.

According to the results of Table 4, The proportion of D6 was more than 50% in examples 91-102. Further, the proportion of D6 was more than 70% in examples 91-100, and the highest proportion of D6 was 88%. It showed that the buffer system in examples 91-102 were benefit for improving the homogeneity of ADCs with D6 and the proportion of D6.

Examples 103-107: Preparation of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate with different pH value

The preparation of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate was similar to the preparation of ADC of Example 82, but it adjusted the buffer pH value and the molar ratio of TCEPA and the antibody as follows:

Example	The buffer system	TCEPA/mAb (Molar Ratio)	pH
E103	BES buffer	3.5:1	6.4
E104	BES buffer	3.5:1	6.7
E105	BES buffer	3.5:1	7.4
E106	MES buffer	3.5:1	5.8
E107	MES buffer	3.5:1	6.1

The homogeneity assays results were shown as follows:

Table 5

No.	Figure	D0 (wt%)	D2 (wt%)	D4 (wt%)	D6 (wt%)	D8 (wt%)
E103	36A	0	6.21	8.05	80.67	5.07
E104	36B	0	5.34	13.90	77.02	3.75
E105	36C	0	2.73	14.61	79.28	3.38
E106	36D	1.43	14.67	25.41	58.49	0
E107	36E	0	13.45	24.28	60.53	1.74

The results of examples 103-107 were shown in Table 5. As the results shown in table 5, linker-payloads (MC-VC-PAB-MMAE) were successfully linked to Trastuzumab, which indicated ADCs with buffers pH value in a range from 5.8 to 6.7 were successfully synthesized.

According to the results of Table 5, The proportion of D6 was more than 58% in examples 103-107. Further, the proportion of D6 was more than 75% in examples 103-105, and the highest proportion of D6 was 80%.

Examples 108-110: Preparation of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate with different concentrations of the buffer system

The preparation of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate was similar to the preparation of ADC of Example 82, but it adjusted the buffer concentration of the buffer system and the molar ratio of TCEPA and the antibody which were shown as follows:

Example	The buffer system	The concentration of the buffer system	TCEPA/mAb (Molar Ratio)
E108	BES buffer	40 mM	3.5:1
E109	BES buffer	60 mM	3.5:1
E110	BES buffer	80 mM	3.5:1

The homogeneity assays results were shown as follows:

Table 6

No.	Figure	D0 (wt%)	D2 (wt%)	D4 (wt%)	D6 (wt%)	D8 (wt%)
E108	37A	0	5.83	6.32	84.06	3.79
E109	37B	1.70	5.14	3.72	85.50	3.95
E110	37C	0	7.10	6.95	83.64	2.31

As show in table 6, the results showed the concentration of the buffer systems of examples 108-110 are useful to increase the content of D6.

Examples 111-119: Preparation of Trastuzumab-[MC-VC-PAB-MMAE]₆ conjugate (the reduction time and/or the reductant temperature in step (1) is different)

The method of examples 111-119 is the same as example 32, and the difference is the reduction time and/or the reductant temperature in step (1), the reductant, the molar ratio of the reductant and the antibody and/or the molar ratio of the ZnCl₂ and the antibody which are shown as follows,

No.	The reduction temperature in step (1) (°C)	The reduction time in step (1) (h)	The reductant	Reductant/mAb (Molar Ratio)	ZnCl ₂ / Reductant (Molar Ratio)
E111	4	4	TCEP-NO	9:1	0.22:1
E112	4	4	TCEP-NO	10:1	0.1:1
E113	4	6	TCEP-NO	10:1	0.1:1
E114	4	8	TCEP-NO	10:1	0.1:1
E115	4	10	TCEP-NO	10:1	0.1:1
E116	4	22	TCEPA	3.5:1	0.57:1
E117	10	16	TCEPA	3.2:1	0.63:1
E118	15	16	TCEPA	3.5:1	0.57:1
E119	25	6	TCEPA	3.9:1	0.51:1

The homogeneity assays results were shown as follows:

Table 7

No.	Figure	The reduction temperature and time in step (1)	D0 (%)	D2 (%)	D4 (%)	D6 (%)	D8 (%)
E111	38A	4°C, 4h	0	4.12	5.79	77.34	12.75
E112	38B	4°C, 4h	0	3.49	9.03	85.31	2.17
E113	38C	4°C, 6h	0	2.00	5.23	88.91	3.86
E114	38D	4°C, 8h	0	0	3.19	88.59	8.22
E115	38E	4°C, 10h	0	0	2.09	85.57	12.34
E116	38F	4°C, 22h	0	4.42	7.84	87.75	0
E117	39A	10°C, 16h	0	2.82	8.48	84.42	4.28
E118	39B	15°C, 16h	0	5.35	8.07	84.71	1.87
E119	39C	25°C, 6h	1.05	2.63	5.76	75.35	15.22

As shown in examples 111-116 in table 7, the results showed the content of D6 is up to 75%, even to 80, 85 or 88% when the molar ratio of the reductant and the antibody is 3.5:1, 9:1 or 10:1 and the reductant time in step (1) is from 4h to 22h, which also indicates that increasing the molar ratio of the reductant and the antibody, the method displayed here is with less reduction time cost.

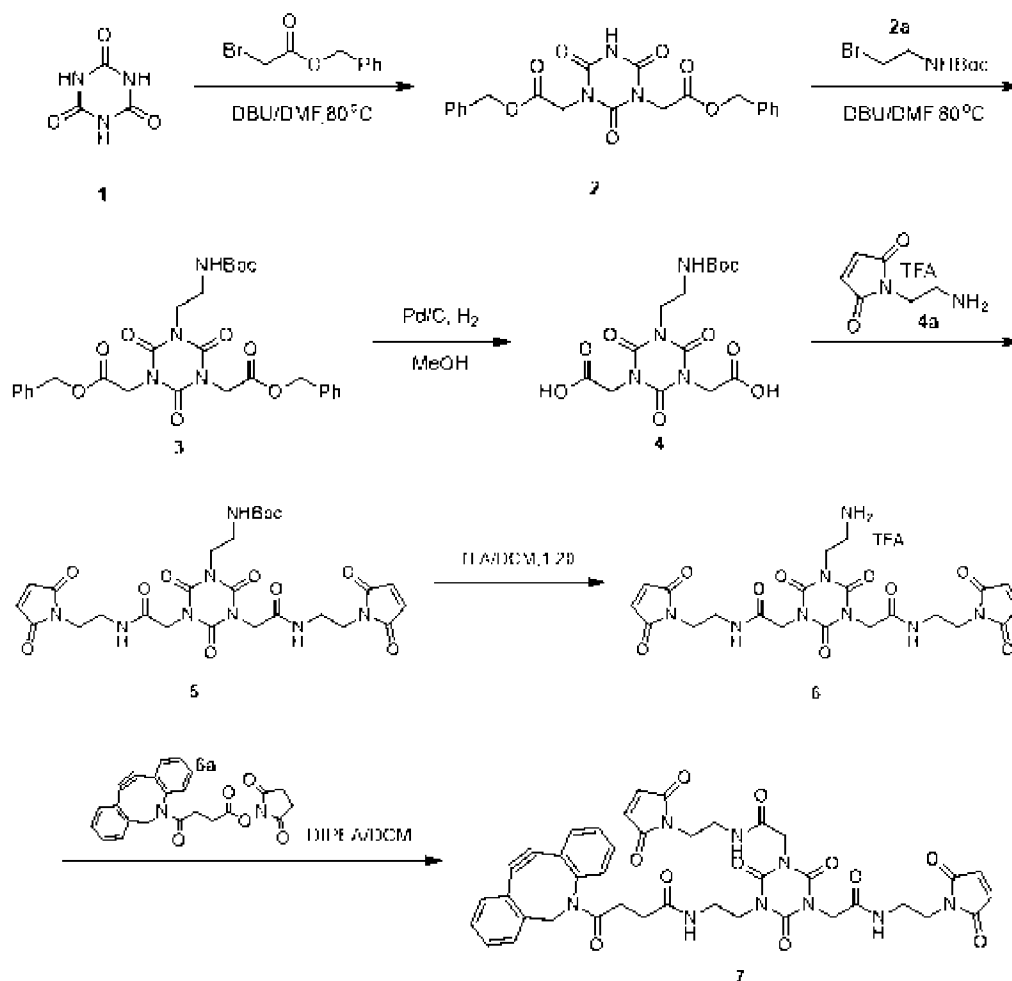
As shown in examples 116-119 in table 7, the results showed the content of D6 is up to 75%, 85%, even to 87% when the reductant temperature in step (1) is from 4°C to 25°C.

Example 120: Preparation of Trastuzumab-[Bismaleimide-DBCO]₃ conjugate

(1) ZnCl₂ (0.024 mM) and reductant TCEPA (0.048 mM) were added to a solution of a monoclonal antibody Trastuzumab (0.012 mM, in BES buffer, pH7.0, 20 mM) and the reaction mixture was allowed to stay at 4 °C for 18h;

(2) EDTA-2Na (0.6 mM) and Bismaleimide-DBCO (0.045mM) in DMA was introduced and the reaction was continued at room temperature for 1h; then recovering Trastuzumab-[Bismaleimide-DBC]₃ using a desalting column.

Wherein, Bismaleimide-DBCO was prepared as follows:



Intermediate 2: To a mixture of 1 (6.5g, 50.0mmol, 1.0 eq) in DMF(100mL) was added DBU(30.7 g, 0.2 mol, 4.0eq). The mixture was stirred for 1h at 80°C, and Benzyl 2-bromoacetate (25.4g, 0.11mol, 2.2 eq). The resulting mixture was stirred for 16h at 80°C. The mixture was poured into ice-water (600mL), extracted with EtOAc (200mL*3). The combined organic layer was washed with brine (200mL), dried over Na₂SO₄ and filtered. The filtrate was concentrated, and purified by flash column (EtOAc/petroleum ether = 0~40%) to give product 2 (4.5g, 21.0%) as white solid.

Intermediate 3: To a mixture of intermediate 2 (4.5g, 10.58 mmol, 1.0 eq) in DMF(50mL) was added DBU(3.22g, 21.16 mmol, 2.0eq). The mixture was stirred for 0.5h at 80°C, and tert-Butyl N-(2-bromoethyl) carbamate (3.56g, 15.87 mmol, 1.5 eq). The resulting mixture was stirred for 6h at 80°C. TLC showed compound 1 was consumed completely. The mixture was poured into ice-water (300mL), extracted with EtOAc (100mL*3). The combined organic layer was washed with brine(100mL), dried over Na₂SO₄ and filtered. The filtrate was concentrated, and purified by flash column (EtOAc/petroleum ether = 0~40%) to give product 3 (4.8 g, 79.8%) as white solid.

Intermediate 4: To a mixture of intermediate 3 (4.8 g, 8.44 mmol, 1.0 eq) in THF (50mL) was added Pd/C (500mg, 10% Pd/C, wetted with ca. 55% Water). The mixture was degassed 3 times and purged with H₂. The resulting mixture was stirred for 2h at room temperature under H₂ atmosphere. TLC showed intermediate 3 was consumed completely. The mixture was filtered through a Celite pad and filtrate was concentrated to give product 4 (3.0 g, 91.5%) as white solid.

Intermediate 5: To a mixture of intermediate 4 (50 mg, 1.00 mmol, 1.0 eq) in DMF(5mL) was added HATU (0.92 g, 2.4 mmol, 2.4 eq) and DIPEA (0.65g, 5.0 mmol, 5.0 eq). The mixture was stirred for 0.5h at room temperature, and 4a (0.51g, 2.0 mmol, 2.0 eq) was added. The resulting mixture was stirred for 2h at room temperature. LCMS showed intermediate 4 was consumed completely. The mixture was quenched by adding HCl (0.5M, 2mL), and purified by RP-column (water/MeCN = 10~70%), and the eluent was lyophilized to give product 5 (0.51 g, 80.3%) as white solid.

Intermediate 6: To a mixture of intermediate 5 (100 mg, 0.16 mmol, 1.0 eq) in DCM (2mL) was added TFA(100uL). The mixture was stirred for 1 hours at room temperature. LCMS showed intermediate 5 was consumed completely. The mixture was concentrated and the residue was taken up by water(10mL), then lyophilized to give product 6 (100 mg, 97.8%) as white solid.

Compound 7 (Bismaleimide-DBCO): To a mixture of intermediate 6 (50 mg, 93.9 umol, 1.0 eq) in DCM (2mL) was added 6a (37.8 mg, 93.9 umol, 1.0 eq) followed by DIPEA(17uL, 93.9 umol, 1.0 eq). The mixture was stirred for 1h at room temperature. LCMS showed intermediate 6 was consumed completely. The mixture was concentrated and the residue was purified by prep-HPLC (water/MeCN = 10~50%), and the eluent was lyophilized to give product 7 (28.8 mg, 3.41%) as white solid.

MS(M+H)⁺ = 820.19, exact mass calc. for C₄₀H₃₇N₉O₁₁ is 819.26. ¹H NMR (400 MHz, DMSO-*d*₆): δ 8.17 (t, J = 6.0 Hz, 2H), 7.85 (d, J = 6.4 Hz, 1H), 7.64 (dd, J = 18.4, 7.3 Hz, 2H), 7.47 (d, J = 10.3 Hz, 3H), 7.38 – 7.24 (m, 3H), 6.96 (s, 4H), 5.02 (d, J = 14.0 Hz, 1H), 4.22 (s, 4H), 3.68 (t, J = 6.6 Hz, 2H), 3.65-3.58 (m, 4H), 3.43 (t, J = 6.2 Hz, 3H), 3.24-3.20 (m, 3H), 3.16-3.10 (m, 5H).

The homogeneity assays results were shown as follows:

NO.	Figure	The type of the ADCs	D3 (%)	D4 (%)
E120	40	D3	82.93	17.07

As shown in the table, the result demonstrated that the content of Trastuzumab-[Bismaleimide-DBCO]₃ was generally up to 82%, which indicated the process of method was benefit for site-specific modifying the antibody with D3 and improving the homogeneity.

Example 121: Preparation of Trastuzumab-[MC-VC-PAB-MMAE]₆[MC-GGFG-DXd]₂ (The ADC with D6+D2)

(1) ZnCl₂ (0.024 mM) and reductant TCEPA (0.048 mM) were added to a solution of a monoclonal antibody Trastuzumab (0.012 mM) in BES buffer (pH7.0, 20 mM) and the reaction mixture was allowed to stay at 4 °C for 18h;

(2) EDTA-2Na (0.6 mM) and MC-VC-PAB-MMAE (0.08 mM) in DMA was introduced and the reaction was continued at room temperature for 1h, then recovering the resulting product using a desalting column;

(3) The resulting product of step (2) and the second reductant TCEP (0.02 mM) were incubating at 37°C for 3h; a second linker-payload MC-GGFG-DXd (0.05 mM) added and the reaction was continued at room temperature for 2h, then recovering the resulting product using a desalting column.

Wherein about six drug molecules MC-VC-PAB-MMAE were coupled to Trastuzumab on average, and about two drug molecules MC-GGFG-DXd were coupled to Trastuzumab on average.

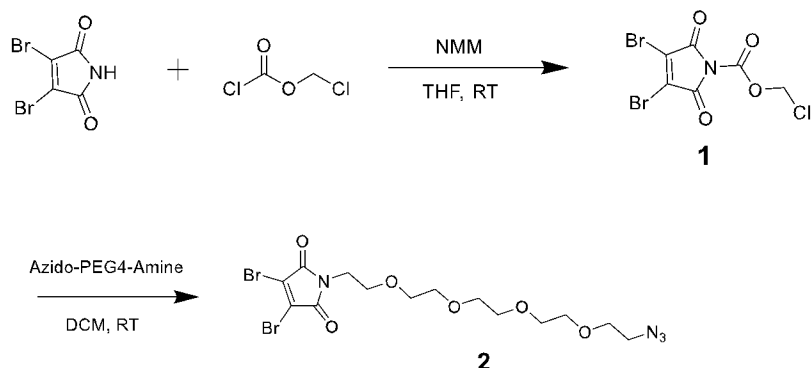
The homogeneity assays results were shown as follows:

NO.	Figure	D2 (%)	D4 (%)	D6 (%)	D8 (%)
E121-step (2)	41A	4.48	4.97	86.49	4.06
NO.	Figure	D2 (%)	D4(%)	D6+D2 (%)	D8 (%)
E121-step (3)	41B	3.67	3.88	84.81	7.65

As shown the above table, the result demonstrated that the content of the ADC with D6+D2 was generally up to 84.81%, which indicated the process of method was benefit for site-specific modifying the antibody with D6+D2 and improving the homogeneity.

Example 122: Preparation of Trastuzumab-[MC-GGFG-DXd]₆[Maleimide-PEG4-N3-DBCO-Cy3]₁ (The ADC with D6+D1)

1. Synthesis of dibromomaleimide-PEG4-N3



To a solution of 3,4-dibromomaleimide (127 mg, 0.5 mmol, 1 eq) and N-methylmorpholine (0.22 mL, 2 mmol, 4 eq) in THF (3.5 mL), chloromethyl chloroformate (0.18 mL, 2 mmol, 4 eq) was added and the mixture was stirred for 20 min at room temperature. Then DCM (10 mL) was added, the organic phase was washed with H₂O, dried over MgSO₄ and the solvent removed in vacuo to yield the title product 1 (139 mg, 0.4 mmol, 80%).

A solution of Azido-PEG4-Amine (105 mg, 0.4 mmol, 1 eq, Xi'an Confluore Biological Technology Co., Ltd) in dichloromethane (2 mL) was added to a stirred solution of product 1 (139 mg, 0.4 mmol, 1 eq) in dichloromethane (2 mL).

After 30 minutes, dichloromethane (6 mL) was added and the solution washed with a 0.68 M acetate buffer pH 5 (10 mL), water (1 mL), and dried with MgSO₄. Concentration in vacuo followed by purification by column chromatography (100% EtOAc as the mobile phase) yielded dibromomaleimide-PEG4-N3 (the title product 2) as a pale yellow oil (150 mg, 0.3 mmol, 75%).

2. Preparation of Trastuzumab-[MC-GGFG-DXd]₆[Maleimide-PEG4-N3-DBCO-Cy3]₁

(1) ZnCl₂ (0.024 mM) and reductant TCEPA (0.048 mM) were added to a solution of a monoclonal antibody Trastuzumab (0.012 mM) in BES buffer (pH7.0, 20 mM) and the reaction mixture was allowed to stay at 4 °C for 18h;

(2) EDTA-2Na (0.6 mM) and MC-GGFG-DXd (0.096 mM) in DMA was introduced and the reaction was continued at room temperature for 1h, then recovering the product using a desalting column;

(3) The resulting product of step (2) and the second reductant TCEP (0.02 mM) were incubating at room temperature for 2h; a thio-bridging reagent [Dibromomaleimide-PEG4-N3] (0.012 mM) added

and the reaction was continued at room temperature for 2 h; then a second linker-payload [DBCO-Cy3] (0.05 mM) added and the reaction was continued at room temperature for 4h.

(4) purifying the resultant product using a desalting column.

Wherein about six drug molecules MC-GGFG-DXd were coupled to Trastuzumab on average, and about one drug molecule Maleimide-PEG4-N3-DBCO-Cy3 was coupled to Trastuzumab on average.

The homogeneity assays results were shown as follows:

NO.	Figure	D4 (%)	D6 (%)	D8 (%)
E122-step (2)	42A	4.58	92.20	3.23
NO.	Figure	D4+D1 (%)	D6+D1 (%)	D8 (%)
E122-step (4)	42B	4.26	92.25	3.50

As shown the above table, the result demonstrated that the content of the ADC with D6+D1 was generally up to 90%%, which indicated the process of method was benefit for site-specific modifying the antibody with D6+D1 and improving the homogeneity.

Example 123: Preparation of Trastuzumab-[Maleimide]₆[MC-VC-PAB-MMAE]₂ (The ADC with D0+D2)

(1) ZnCl₂ (0.024 mM) and reductant TCEPA (0.048 mM) were added to a solution of a monoclonal antibody Trastuzumab (0.012 mM) in BES buffer (pH7.0, 20 mM) and the reaction mixture was allowed to stay at 4 °C for 18h;

(2) introducing EDTA (0.6mM) and (2-Aminoethyl) maleimide (0.1 mM) to react with the reduced thiol groups resulted from step (1) at room temperature for 1h, then recovering the product using a desalting column to afford Trastuzumab-[Maleimide]₆;

(3) Trastuzumab-[Maleimide]₆ and the second reductant TCEP (0.02 mM) were incubating at 37°C for 18h; a second linker-payload MC-VC-PAB-MMAE (0.05 mM) was added and the reaction was continued at room temperature for 2h.

(4) purifying the resultant product using a desalting column.

The homogeneity assays results were shown as follows:

NO.	Figure	D0(%)	/	/
E123-step (2)	43A	100	/	/
NO.	Figure	D0 (%)	D0+D1 (%)	D0+D2 (%)
E123-step (4)	43B	24.30	5.18	70.53

As shown in the above table, the result demonstrated that the content of the ADC with D0+D2 was generally up to 70.53%, which indicated the process of method was benefit for site-specific modifying the antibody with D0+D2 and improving the homogeneity.

Example 124: Preparation of Trastuzumab-[Maleimide]₆[Maleimide-PEG4-N3-DBCO-Cy3]₁ (The ADC with D0+D1)

(1) ZnCl₂ (0.024 mM) and reductant TCEPA (0.048 mM) were added to a solution of a monoclonal antibody Trastuzumab (0.012 mM) in BES buffer (pH7.0, 20 mM) and the reaction mixture was allowed to stay at 4 °C for 18h;

(2) introducing EDTA (0.6mM) and (2-Aminoethyl) maleimide (0.1 mM) to react with reduced thiol groups resulted from step (1) at room temperature for 1h, then recovering the product using a desalting column to afford Trastuzumab-[Maleimide]₆;

(3) Trastuzumab-[Maleimide]₆ and the second reductant TCEP (0.02 mM) were incubating at 37°C for 18h. the second thio-bridging reagent with the reactive group dibromomaleimide-PEG4-N3 (0.013 mM) to react with reduced thiol groups, the reaction temperature is 24°C and the reaction time is 3 h;

(4) incubating resulting product and DBCO-Cy3 (0.02 mM) in MES (20mM, pH6.7), the reaction temperature is 25°C and the reaction time is 8 h, then recovering Trastuzumab-[Maleimide]₆[Maleimide-PEG4-N3-DBCO-Cy3] using a desalting column.

The homogeneity assays results were shown as follows:

No.	Figure	D0 (%)	D0+D1 (%)	D0+D2 (%)
E124	44	17.00	79.40	3.60

As shown in the above table, the result demonstrated that the content of the ADC with D0+D1 was generally up to 79.40%, which indicated the process of method was benefit for site-specific modifying the antibody with D0+D1 and improving the homogeneity.

Example 125: Preparation of Trastuzumab-[MC-VC-PAB-MMAE]₂ conjugate (The ADC with D2)

(1) ZnCl₂ (0.012 mM) and reductant TCEPA prepared (0.048 mM) were added to a solution of a monoclonal antibody Trastuzumab (0.012 mM, in BES buffer, pH7.0, 20 mM) and the reaction mixture was allowed to stay at 4 °C for 18h;

(2) DHAA (0.072 mM) was added to the mixture of step (1) and vortexed for mixing. The mixture was then incubated in darkness at room temperature for 2h;

(3) optionally, purification with a desalting column to remove excess DHAA;

(4) EDTA (0.06 mM) and MC-VC-PAB-MMAE (0.048 mM) in DMA were added and the mixture was then incubated at room temperature for 1h.

(5) The reaction mixture was subjected to purification using a de-salting column.

Examples 126-147: Preparation of Trastuzumab-[MC-VC-PAB-MMAE]₂ conjugate (The ADC with D2)

The method of examples 126-147 is similar to example 125, and the difference is the parameters in step (1) and in step (2), the different parameters are shown as follows,

No.	DHAA/mAb (Molar Ratio)	Antibody (mM)	Oxidation reaction
E126	8:1	0.012	RT, 2h
E127	10:1	0.012	RT, 2h
E128	12:1	0.012	RT, 2h
E129	14:1	0.012	RT, 2h
E130	8:1	0.012	RT, 2h and purification after oxidation
E131	8:1	0.012	4 °C, 5h
E132	10:1	0.012	RT, 2h and purification after oxidation
E133	10:1	0.012	4 °C, 5h

RT is short for room temperature.

No.	Reductant	Reductant/antibody (molar ratio)	DHAA/mAb (Molar Ratio)	Antibody (mM)	The time in step (1) (h)
E134	TCEP-NO	4:1	4:1	0.012	2
E135	TCEP-NO	4:1	4:1	0.012	4
E136	TCEP-NO	4:1	6:1	0.012	4
E137	TCEP-NO	4:1	8:1	0.012	2
E138	TCEP-NO	4:1	8:1	0.012	4
E139	TCEP-NO	4:1	10:1	0.012	4
E140	TCEP-NO	6:1	7:1	0.012	4
E141	TCEP-NO	6:1	10:1	0.012	4
E142	TCEP-NO	6:1	13:1	0.012	4
E143	TCEP-NO	6:1	16:1	0.012	4
E144	TCEP-NO	10:1	10:1	0.012	4
E145	TCEP-NO	10:1	14:1	0.012	4
E146	TCEP-NO	10:1	18:1	0.012	4
E147	TCEP-NO	10:1	22:1	0.012	4

The homogeneity assays results were shown as follows:

Table 8

No.	Figure	D0 (wt%)	D2 (wt%)	D4 (wt%)	D6 (wt%)	D8 (wt%)
E125	45	4	93	3	0	0
E126	46	7	93	0	0	0
E127	47	8	92	0	0	0
E128	48	10	90	0	0	0
E129	49	12	88	0	0	0
E130	50	5	95	0	0	0
E131	51	6	86	5	3	0
E132	52	6	94	0	0	0

No.	Figure	D0 (wt%)	D2 (wt%)	D4 (wt%)	D6 (wt%)	D8 (wt%)
E133	53	7	88	5	0	0
E134	54A	7.24	81.27	11.49	0	0
E135	54B	22.43	71.72	5.85	0	0
E136	54C	26.93	73.07	0	0	0
E137	54D	12.42	85.32	2.26	0	0
E138	54E	32.55	67.45	0	0	0
E139	54F	33.57	66.43	0	0	0
E140	54G	2.34	60.64	33.30	3.72	0
E141	54H	4.50	87.68	7.82	0	0
E142	55A	6.19	92.05	1.77	0	0
E143	55B	8.49	91.51	0	0	0
E144	55C	2.23	71.71	26.06	0	0
E145	55D	4.48	91.94	3.58	0	0
E146	55E	7.36	92.64	0	0	0
E147	55F	8.06	91.94	0	0	0

The results of Examples 125-147 were shown in Table 8. As the results shown in table 8, linker-payloads (MC-VC-PAB-MMAE) were successfully linked to Trastuzumab, which indicated ADCs with D2 were successfully synthesized.

According to the results of Table 8, The proportion of D2 in Example 125-133 was more than 80%. The results showed that the oxidation reaction was benefit for improving the homogeneity of ADCs with D2, and it also showed that oxidation reaction was benefit for antibody site-specific modification, especially benefit for ADCs with site-specific conjugation.

The proportion of D6 was up to 95% when performing purification after oxidation, which indicated that the purification could improve the homogeneity of ADCs with D2 significantly.

As shown in examples 134-147, the content of D2 is up to 60%, even to 70%, 80% and 90% when the molar ratio of the reductant and the antibody is from 4:1 to 10:1 and the reduction time in step (1) is shortened to 2h or 4h, which is with less reduction time cost. As shown in examples 125-147, when the reductant time in step (1) is 2h to 18h, the content of D2 is up to 60%, even to 70%, 80% and 90%.

As shown in example 125-147, the content of D2 is up to 60%, 70%, 80%, 85%, even to 90% or 95% when the molar ratio of DHAA and the antibody is 4:1 to 22:1.

Examples 148-150: Preparation of Trastuzumab-[MC-VC-PAB-MMAE]₂ conjugate (the oxidation temperature and/or time in step (2) is different)

The method of examples 148-150 is similar to example 126, and the difference is the oxidation temperature and/or time in step (2) and the molar ratio of the reductant and the antibody, which are shown in table 9.

Table 9

No.	Figure	Reductant/ antibody (molar ratio)	The oxidation temperature and time in step (2)	D0(%)	D2(%)	D4(%)	D6(%)	D8(%)
E148	56A	3.2:1	37°C, 1h	2.86	80.80	15.20	1.14	0
E149	56B	3.5:1	4°C, 24h	2.69	87.97	9.34	0	0
E150	56C	3.5:1	4°C, 48h	4.18	95.83	0	0	0

As shown in table 9 and in example 126, the results showed the content of D6 is up to 80%, even to 95% when the oxidation temperature in step (2) is from 4°C to 37°C, and the oxidation time in step (2) is from 1h to 48h.

Examples 151-166 and Comparative example 6: Preparation of Trastuzumab-[MC-VC-PAB-MMAE]₂ conjugate by using the different buffer system

The method of examples 151-166 and comparative example 6 is similar to example 126, and the difference is the buffer system which is shown in table 9. Meanwhile, the molar ratio of the reductant and the antibody is 3.5:1 in examples 151-166 and comparative example 6.

The homogeneity assays results were shown as follows:

Table 10

No.	Figure	The buffer system	D0(%)	D2(%)	D4(%)	D6(%)	D8(%)
E151	57A	Bis-Tris, pH 6.7	5.51	87.96	6.53	0	0
E152	57B	PIPES, pH 6.7	4.46	79.81	15.73	0	0
E153	57C	PB, pH 6.7	38.34	61.67	0	0	0
E154	57D	HEPES, pH 7.0	6.06	91.51	2.43	0	0
E155	57E	MOPS, pH 7.0	6.36	87.38	6.26	0	0
E156	57F	DIPSO, pH 7.4	7.19	92.81	0	0	0
E157	57G	MOBS, pH 7.4	6.18	93.83	0	0	0
E158	57H	MOPSO, pH 7.4	5.70	92.18	2.12	0	0
E159	58A	TES, pH 7.4	5.66	91.75	2.59	0	0
E160	58B	ACES, pH 7.4	7.71	92.29	0	0	0
E161	58C	TAPSO, pH 7.4	4.77	92.26	2.98	0	0
E162	58D	MES pH 5.8	3.48	60.48	32.66	3.39	0
E163	58E	MES pH 6.1	4.12	73.51	22.37	0	0
E164	58F	BES, pH 6.4	3.57	76.45	19.98	0	0
E165	58G	BES, pH 6.7	1.79	82.25	14.77	1.20	0
E166	58H	BES, pH 7.4	3.05	94.48	2.48	0	0
C6	59	ADA, pH 6.7	86.47	13.53	0	0	0

As shown in table 10, the results showed the types and the pH value of the buffer system will impact the content of D2 by impacting the reduction kinetics and selectivity. The buffer systems of examples 151-166 are useful to increase the content of D2, and the pH value of the buffer system is from 5.8 to 7.4.

Example 167: preparation of Trastuzumab-[Maleimide-PEG4-N3-DBCO-Cy3]₁ (the ADC with D1)

(1) TCEPA (0.048 mM) and ZnCl₂ (0.012 mM) were added to a solution of Trastuzumab (0.012 mM) in BES buffer (20 mM, pH7.0) and the reaction mixture was vortexed for mixing, then the reaction mixture was incubated at 4°C for 18h;

(2) Adding DHAA (0.096mM) to selectively re-oxidize the reduced thiol groups in Fab region resulted from step (1) at 25°C for 2h;

(3) Introducing EDTA (0.6mM) and a first thio-bridging reagent dibromomaleimide-PEG4-N3 (0.013 mM) to react with reduced thiol groups resulted from step (3), the reaction temperature is 25°C and the reaction time is 1h, then recovering the product using a desalting column to afford Trastuzumab-[Maleimide-PEG4-N3]₁;

(4) incubating Trastuzumab-[Maleimide-PEG4-N3]₁(0.013 mM) and DBCO-Cy3 (0.02 mM) in BES buffer (20 mM, pH7.0), the reaction temperature is 25°C and the reaction time is 8 h.

(5) The reaction mixture was subjected to purification using a de-salting column.

The homogeneity assays results were shown as follows:

No.	Figure	D0(%)	D1(%)	D2(%)
E167	60	13.07	83.81	3.13

As shown in the above table, the result demonstrated that the content of the ADC with D1 was generally up to 83.81%, which indicated the process of method was benefit for site-specific modifying the antibody with D1 and improving the homogeneity.

Example 168: preparation of Trastuzumab-[Maleimide-PEG4-N3-DBCO-Cy3]₁[MC-VC-PAB-MMAE]₆ (the ADC with D1+D6)

(1) introducing Trastuzumab-[Maleimide-PEG4-N3-DBCO-Cy3]₁ prepared from example 167 (0.008 mM) and TCEPA (0.08 mM) in BES buffer (20 mM, pH7.0), the reaction temperature is 24°C and the reaction time is 24 h;

(2) introducing MC-VC-PAB-MMAE (0.14 mM) to solution from step (1), and the reaction mixture was allowed to stay at 24 °C for 1 h, then recovering Trastuzumab-[Maleimide-PEG4-N3-DBCO-Cy3]₁[MC-VC-PAB-MMAE]₆ using a desalting column.

The homogeneity assays results were shown as follows:

No.	Figure	D6(%)	D1+D6 (%)	D2+D6 (%)
E168	61	7.79	88.02	4.20

As shown in the above table, the result demonstrated that the content of the ADC with D1+D6 was generally up to 88.02%, which indicated the process of method was benefit for site-specific modifying the antibody with D1+D6 and improving the homogeneity.

Example 169: preparation of Trastuzumab-[Maleimide-PEG4-N3-DBCO-Cy3]₁[MC-VC-PAB-MMAE]₂ (the ADC with D1+D2)

(1) incubating ZnCl₂ (0.8 mM), the second reductant TCEP-6 (0.0144 mM) and Trastuzumab-[Maleimide-PEG4-N3-DBCO-Cy3]₁ prepared from example 167 (0.008 mM) in BES buffer (pH7.0, 20mM) and the reaction mixture was allowed to stay at 4°C for 4h;

(2) introducing EDTA (3 mM) to trap Zn²⁺, and introducing MC-VC-PAB-MMAE (0.048 mM) to react with the reduced thiol groups resulted from step (2), the reaction temperature is 25°C and the reaction time is 2h;

(3) the reaction mixture was subjected to purification using a desalting column.

The homogeneity assays results were shown as follows:

No.	Figure	D1(%)	D2 (%)	D1+D2 (%)	D4%	D1+D4 (%)
E169	62	3.72	7.92	73.80	4.06	10.51

With step (1), one of the interchain disulfide bonds in the ADC with D1 was reduced. As shown in the above table, the result demonstrated that the content of the ADC with D1+D2 was generally up to 73.8%, which indicated the process of method was benefit for site-specific modifying the antibody with D1+D2 and improving the homogeneity.

Examples 170-171: preparation of Trastuzumab-[Maleimide-PEG4-N3-DBCO-Cy3]₁[MC-VC-PAB-MMAE]₄ (the ADC with D1+D4)

(1) TCEPA (0.048 mM) and ZnCl₂ (0.012 mM) were added to a solution of Trastuzumab (0.012 mM) in BES buffer (20 mM, pH7.0) and the reaction mixture was vortexed for mixing, then the reaction mixture was incubated at 4°C for 14h;

(2) Adding DHAA (0.096mM) to selectively re-oxidize the reduced thiol groups mainly in Fab region resulted from step (1) at 25°C for 2h;

(3) Introducing EDTA (0.6mM) and a first thio-bridging reagent dibromomaleimide-PEG4-N3 (0.013 mM) to react with the reduced thiol groups resulted from step (3), the reaction temperature is 25°C and the reaction time is 1.5h, then recovering the product using a desalting column to afford Trastuzumab-[Maleimide-PEG4-N3]₁;

(4) incubating Trastuzumab-[Maleimide-PEG4-N3]₁ from step (3) and DBCO-Cy3 (0.02 mM) in BES buffer (20 mM, pH7.0), the reaction temperature is 25°C and the reaction time is 8 h;

(5) The reaction mixture was subjected to purification using a de-salting column;

(6) incubating ZnCl₂ (0.8 mM), the second reductant TCEP-3 (0.0384 mM) or TCEP-6 (0.0336 mM) and the product from step (5) in BES buffer (pH7.0, 20mM) and the reaction mixture was allowed to stay at 4°C for 16h;

(7) introducing EDTA (3mM) to trap Zn^{2+} , and introducing MC-VC-PAB-MMAE (0.08 mM) to react with the reduced thiol groups resulted from step (6), the reaction temperature is 25°C and the reaction time is 2h;

(8) the reaction mixture was subjected to purification using a desalting column.

The homogeneity assays results were shown as follows:

No.	Figure	Reductant	D1(%)	D2 (%)	/	/
E170-step (5)	63A	TCEPA	89.43	10.57	/	/
No.	Figure	Reductant	D2(%)	D1+D4 (%)	D6(%)	D1+D6 (%)
E170-step (8)	63B	TCEP-3	2.01	84.48	2.83	10.69
E171-step (8)	63C	TCEP-6	3.74	84.17	2.38	9.71

With step (6), two of the interchain disulfide bonds in the ADC with D1 were reduced. As shown in the above table, the result demonstrated that the content of the ADC with D1+D4 was generally up to 84%, which indicated the process of method was benefit for site-specific modifying the antibody with D1+D4 and improving the homogeneity.

Example 172: Preparation of Trastuzumab-[MC-GGFG-DXd]₂[MC-VC-PAB-MMAE]₄ conjugate (The ADC with D2+D4)

(1) TCEPA (0.048 mM) and $ZnCl_2$ (0.012 mM) were added to a solution of Trastuzumab (0.012 mM) in BES buffer (20 mM, pH7.0) and the reaction mixture was vortexed for mixing, then the reaction mixture was incubated at 4°C for 16h;

(2) Adding DHAA (0.096mM) to selectively re-oxidize the reduced thiol groups in Fab region resulted from step (1) at 25°C for 2h;

(3) Introducing EDTA (0.6mM) and MC-GGFG-DXd (0.048 mM) in DMA to react with remained thiol groups from step (2) and the reaction was incubated at room temperature for 1.5 h;

(4) The reaction mixture was subjected to purification using a de-salting column;

(5) incubating $ZnCl_2$ (0.8 mM), the second reductant TCEP-3 (0.072 mM) and the product from step (4) in BES buffer (pH7.0, 20mM) and the reaction mixture was allowed to stay at 4°C for 16h;

(6) introducing EDTA (3mM) to trap Zn^{2+} , and introducing MC-VC-PAB-MMAE (0.08 mM) to react with the reduced thiol groups resulted from step (5), the reaction temperature is 25°C and the reaction time is 2h;

(7) the reaction mixture was subjected to purification using a desalting column.

Examples 173-174: Preparation of Trastuzumab-[MC-VC-PAB-MMAE]₂[MC-GGFG-DXd]₄ conjugate (The ADC with D2+D4)

(1) TCEPA (0.042 mM) and ZnCl₂ (0.024 mM) were added to a solution of Trastuzumab (0.012 mM) in BES buffer (20 mM, pH7.0) and the reaction mixture was vortexed for mixing, then the reaction mixture was incubated at 4°C for 16h;

(2) Adding DHAA (0.12mM) to selectively re-oxidize the reduced thiol groups in Fab region resulted from step (1) at 25°C for 2h;

(3) Introducing EDTA (0.12mM) and MC-VC-PAB-MMAE (0.048 mM) in DMA to react with remained thiol groups from step (2) and the reaction was incubated at room temperature for 1.5 h;

(4) The reaction mixture was subjected to purification using a de-salting column;

(5) incubating ZnCl₂ (example 173: 0.36 mM; example 174: 0.024 mM), the second reductant TCEP-6 (example 173: 0.042 mM; example 174: 0.054 mM) and the product from step (4) in BES buffer (pH7.0, 20mM) and the reaction mixture was allowed to stay at 3°C for 1h;

(6) introducing EDTA (1.2mM) to trap Zn²⁺, and introducing MC-GGFG-DXd (0.096 mM) to react with the reduced thiol groups resulted from step (5), the reaction temperature is 25°C and the reaction time is 2h;

(7) the reaction mixture was subjected to purification using a desalting column.

The homogeneity assays results were shown as follows:

No.	Figure	Reductant	D1(%)	D2 (%)	D4 (%)	D6 (%)
E172-step (4)	64A	TCEPA	11.30	85.66	2.54	0.51
No.	Figure	Reductant	D0(%)	D2 (%)	D4 (%)	D6 (%)
E173-step (4)	65A	TCEPA	2.60	91.38	6.02	0
E174-step (4)	/	TCEPA	2.59	91.25	6.16	0
No.	Figure	Reductant	D4(Dxd)+D2 (MMAE) (%)	D2(DXd)+D4 (MMAE) (%)	D6 (%)	/
E172-step (7)	64B	TCEP-3	2.58	82.23	15.19	/
No.	Figure	Reductant	D2+D2 (%)	D2+D4 (%)	D2+D6 (%)	D8 (%)
E173-step (7)	65B	TCEP-6	1.5	81.7	14.2	2.6
E174-step (7)	65C	TCEP-6	4.41	62.84	29.88	2.87

As shown in the above table, the result demonstrated that the content of the ADC with D2+D4 was generally up to 60%, which indicated the process of method was benefit for site-specific modifying the antibody with D2+D4 and improving the homogeneity.

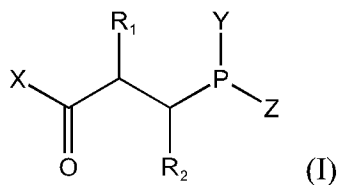
As shown in examples 173-174, the result demonstrated that the selective reduction ability increases in step (5) when the molar ratio of the transition metal ions and the reductant increases in step (5).

While particular embodiments have been described, alternatives, modifications, variations, improvements, and substantial equivalents that are or may be presently unforeseen may arise to applicants or others skilled in the art. Accordingly, the appended claims as filed and as they may be

amended are intended to embrace all such alternatives, modifications, variations, improvements, and substantial equivalents.

C l a i m s

1. A reductant having the following formula (I):



or a salt, solvate, stereoisomer thereof, which characterized in that,

R_1 is H, $-NH_2$, $-C(O)(R_3R_4)$, optionally substituted C_1 - C_5 alkyl group, optionally substituted C_1 - C_5 hydroxyalkyl group, or optionally substituted aryl group;

R_3 is N, NH or O;

R_4 is H, optionally substituted C_1 - C_5 alkyl group, optionally substituted C_1 - C_5 hydroxyalkyl group, or optionally substituted aryl group;

R_2 is H, optionally substituted C_1 - C_5 alkyl group, or optionally substituted C_1 - C_5 hydroxyalkyl group;

X is OH, optionally substituted C_1 - C_5 alkoxy group or $-NR_5R_6$,

R_5 and R_6 independently are H, C_0 - C_5 hydroxyalkyl group, optionally substituted C_1 - C_5 alkyl group, optionally substituted C_2 - C_8 carboxy alkyl group, optionally substituted C_1 - C_5 alkoxy group, optionally substituted heteroaryl alkyl group, optionally substituted aryl alkoxy group, optionally substituted arylalkyl group, optionally substituted aryl group, C_1 - C_5 alkyl sulfonyl group, or $-(CH_2)n^1(OCH_2CH_2O)n^2CH(R_8)CO(R_7)$,

R_7 is C_0 - C_5 hydroxyalkyl group, $-NHOH$,

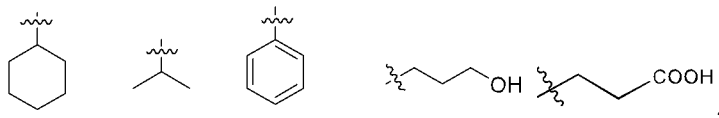
R_8 is H, optionally substituted arylalkyl group,

n^1 and n^2 independently are the number 0, 1, 2, 3, 4,

Y is the same as X, or Y is an ester or amide of X,

Z is the same as X or Y, or

Y and Z independently are selected from the group consisting of



X, Y and Z are not at the same time.

2. The reductant of claim 1, which characterized in that,

R_1 is H, and R_2 is H.

3. The reductant of claim 1, which characterized in that,

X is $-\text{OCH}_3$, $-\text{OCH}_2\text{CH}_3$, or $-\text{O}(\text{CH}_3)_2$.

4. The reduction of claim 1 or 2, which characterized in that,

X is $-\text{NR}_5\text{R}_6$,

R_5 is H, and

R_6 is H, C_0 - C_5 hydroxyalkyl group, C_1 - C_5 alkoxy group, optionally substituted heteroaryl alkyl group, optionally substituted aryl alkoxy group, optionally substituted aryl group, optionally substituted arylalkyl group, C_1 - C_5 alkyl sulfonyl group or $-(\text{CH}_2)_{n^1}(\text{OCH}_2\text{CH}_2\text{O})_{n^2}\text{CH}(\text{R}_8)\text{CO}(\text{R}_7)$,

R_7 is C_0 - C_3 hydroxyalkyl group or $-\text{NHOH}$,

R_8 is H or optionally substituted arylalkyl group,

n^1 and n^2 independently are the number 0.

5. The reductant of claim 4, which characterized in that,

R_6 is H, C_0 - C_2 hydroxyalkyl group, C_1 - C_3 alkoxy group, C_1 - C_3 alkyl sulfonyl group, bipyridyl group, benzyl group, aryl alkoxy group, phenyl group which is optionally substituted with OH, carboxy or pyridyl group, or $-\text{CH}(\text{R}_8)\text{CO}(\text{R}_7)$,

R_7 is OH or $-\text{NHOH}$,

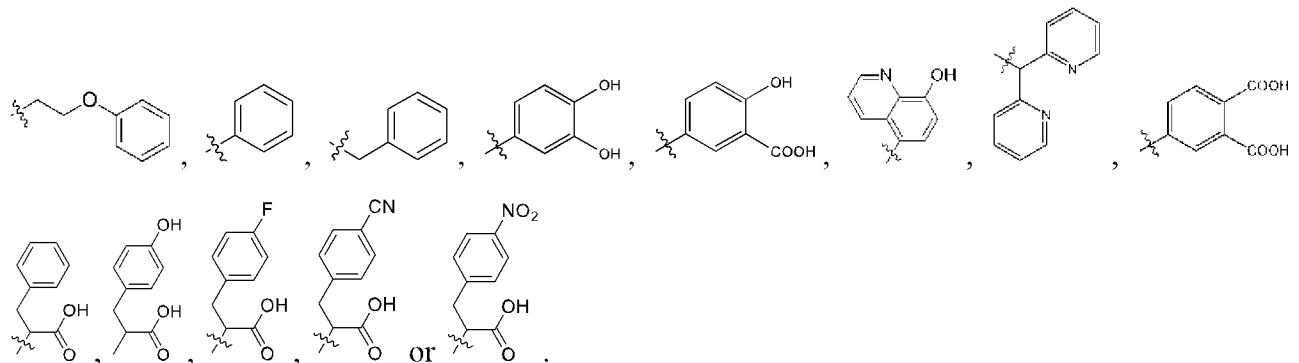
R_8 is H or benzyl group which is optionally substituted with $-\text{OH}$, halogen, cyano group or nitro group.

6. The reductant of claim 1 or 2, which characterized in that,

X is $-\text{NR}_5\text{R}_6$,

R_5 is H, and

R_6 is H, OH, $-\text{CH}_2\text{OH}$, $-(\text{CH}_2)_2\text{OH}$, $-\text{CH}_3$, $-\text{CH}_2\text{CH}_3$, $-\text{CH}_2\text{COOH}$, $-(\text{CH}_2)_2\text{COOH}$, $-(\text{CH}_2)_3\text{COOH}$, $-(\text{CH}_2)_4\text{COOH}$, $-(\text{CH}_2)_5\text{COOH}$, $-\text{OCH}_3$, $-\text{OCH}_2\text{CH}_3$, $-\text{CH}_2\text{CONHOH}$, $-\text{OC}(\text{C}_6\text{H}_5)_3$, $-(\text{CH}_2)_3\text{S}(\text{O})_2\text{OH}$,



7. The reductant of claim 1 or 2, which characterized in that,

X is $-\text{NR}_5\text{R}_6$,

R_5 is OH,

R_6 is $\text{C}_1\text{-C}_5$ alkyl group, optionally substituted heteroaryl alkyl group, optionally substituted arylalkyl group, optionally substituted aryl group, or $-(\text{CH}_2)^{n^1}(\text{OCH}_2\text{CH}_2\text{O})^{n^2}\text{CH}(\text{R}_8)\text{CO}(\text{R}_7)$,

R_7 is $\text{C}_0\text{-C}_5$ hydroxyalkyl group,

R_8 is H,

n^1 and n^2 independently are the number 0, 1, 2, 3, 4.

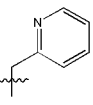
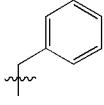
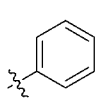
8. The reductant of claim 7, which characterized in that,

R_6 is $\text{C}_1\text{-C}_3$ alkyl group, heteroaryl alkyl group which comprises a heteroatom N, optionally substituted benzyl group, optionally substituted phenyl group, or $-\text{CH}(\text{R}_8)\text{CO}(\text{R}_7)$,

R_7 is $\text{C}_0\text{-C}_3$ hydroxyalkyl group,

R_8 is H.

9. The reductant of claim 7, which characterized in that,

R_6 is $-\text{CH}_3$, $-\text{CH}_2\text{COOH}$, , , or .

10. The reductant of claim 1 or 2, which characterized in that,

X is $-\text{NR}_5\text{R}_6$,

R_5 and R_6 independently are $\text{C}_1\text{-C}_5$ alkyl group, $\text{C}_0\text{-C}_5$ hydroxyalkyl group, optionally substituted heteroaryl alkyl group or $-(\text{CH}_2)^{n^1}(\text{OCH}_2\text{CH}_2\text{O})^{n^2}\text{CH}(\text{R}_8)\text{CO}(\text{R}_7)$,

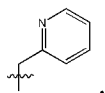
R_7 is $\text{C}_0\text{-C}_5$ hydroxyalkyl group or $-\text{NHOH}$,

R_8 is H,

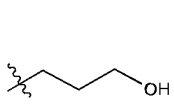
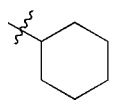
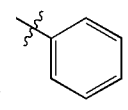
n^1 and n^2 independently are the number 0, 1, 2, 3, 4.

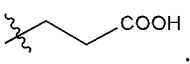
11. The reductant of claim 10, which characterized in that,

R_5 and R_6 independently are methyl, ethyl group, $-(\text{CH}_2)_2\text{OH}$, $-\text{CH}_2\text{COOH}$, $-\text{CH}_2\text{CONHOH}$ or

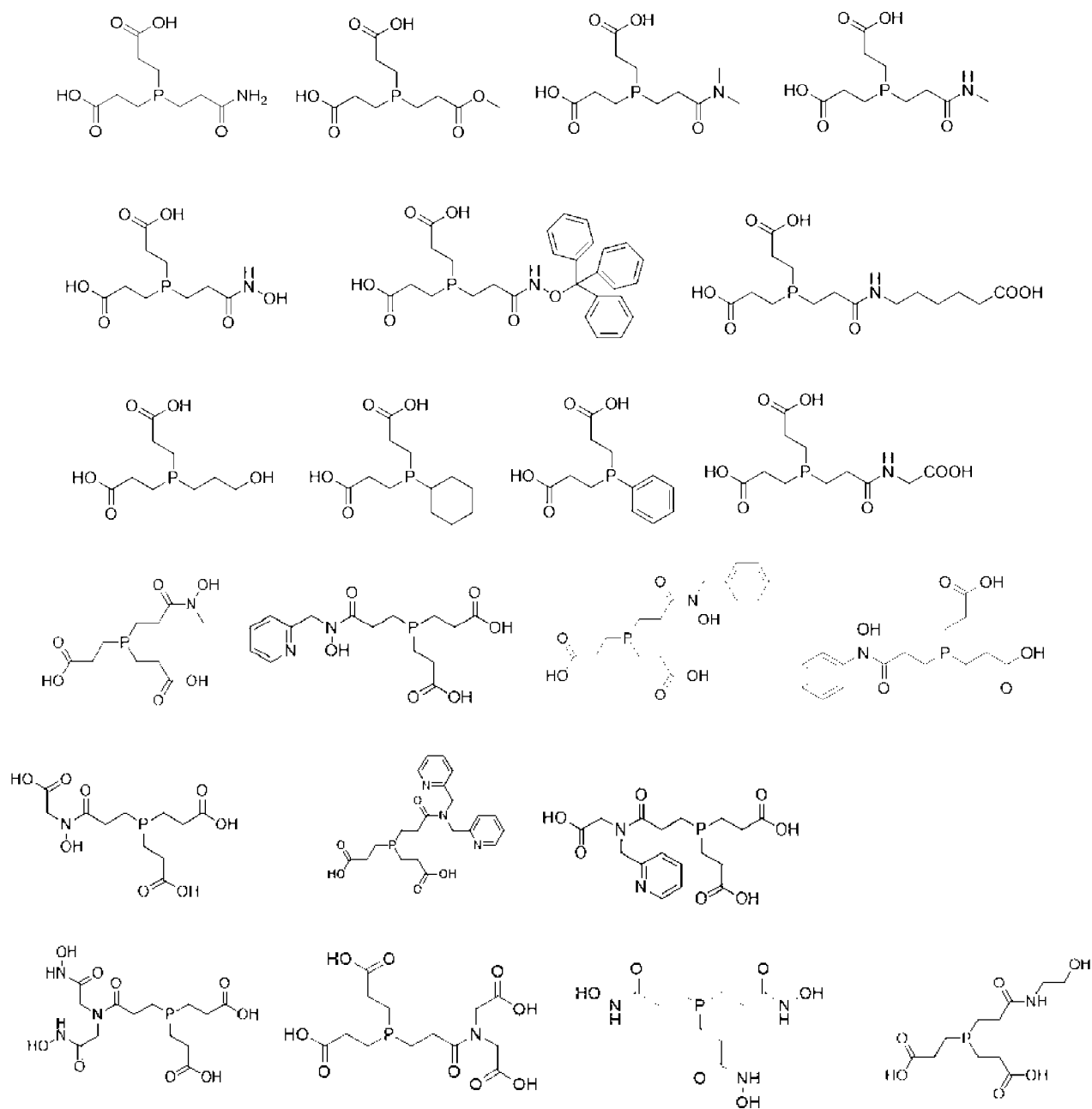


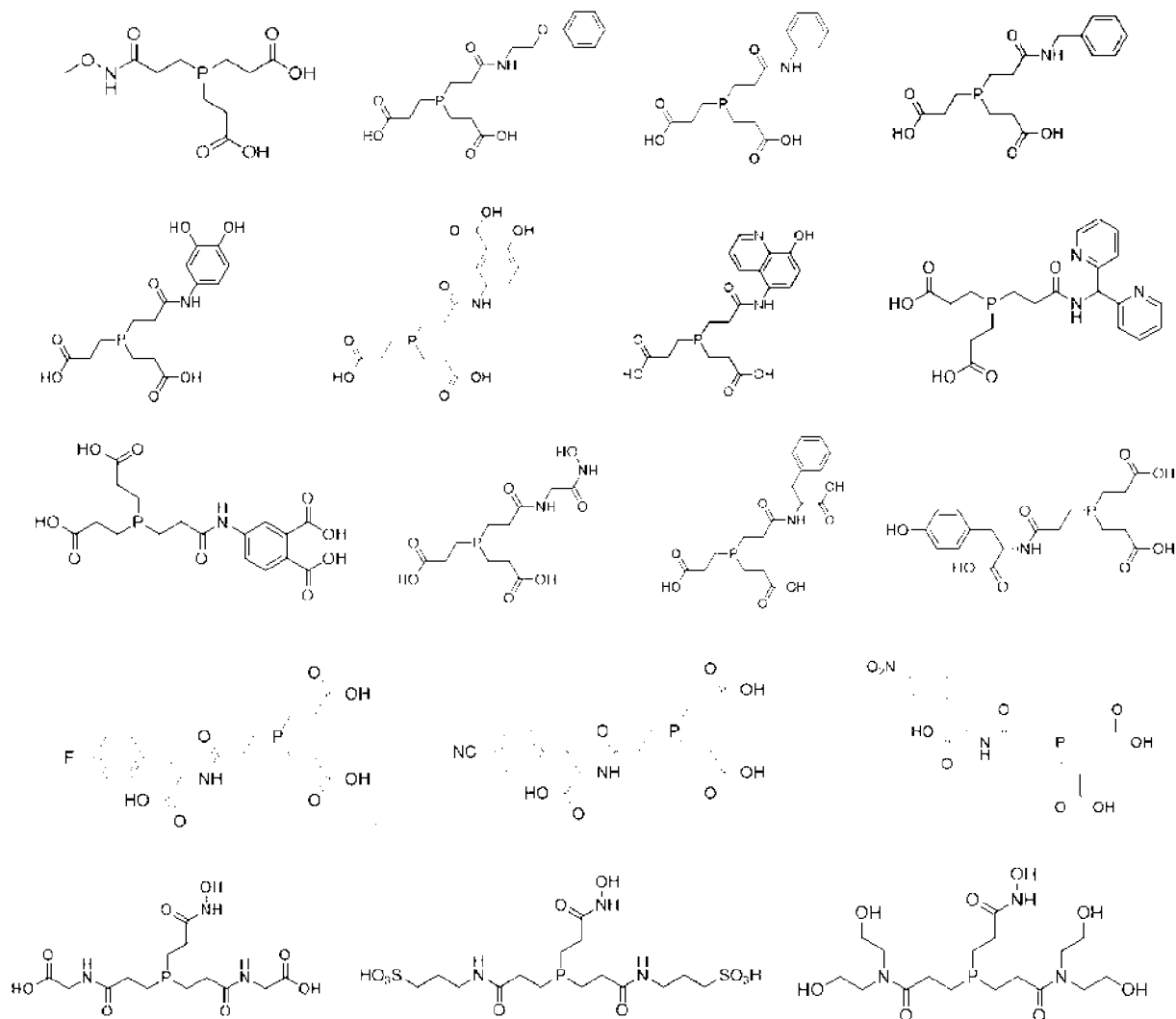
12. The reductant of claim 1 or 2, which characterized in that,

Y is ,  or  ;

Z is  .

13. The reductant of any one of claims 1-12, which characterized in that, the reductant is selected from the group consisting of



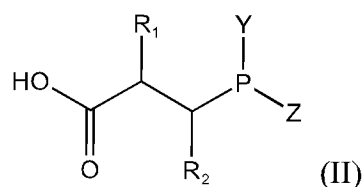


14. A composition comprising a reductant of any one of claims 1-13 and transition metal ions.

15. The composition of claim 14, which characterized in that, the transition metal ions are Zn^{2+} , Cd^{2+} , Hg^{2+} , Ni^{2+} , Co^{2+} or the combination thereof, optionally, the transition metal ions are Zn^{2+} .

16. The composition of claim 14 or 15, which characterized in that, the molar ratio of the transition metal ions and the first reductant is 0.05:1 to 40:1, optionally, the molar ratio of the transition metal ions and the first reductant is 0.25:1 to 30:1, more optionally, the molar ratio of the transition metal ions and the first reductant is 0.25:1 to 15:1.

17. A method of preparing the reductant of any one of claims 1-13, which characterized in that, at least one X' is connected to a compound of formula II by introducing a condensation reagent under an inert atmosphere,



R₁ is H, -NH₂, -C(O)(R₃R₄), optionally substituted C₁-C₅ alkyl group, optionally substituted C₁-C₅ hydroxyalkyl group, or optionally substituted aryl group;

R₃ is N, NH or O;

R₄ is H, optionally substituted C₁-C₅ alkyl group, optionally substituted C₁-C₅ hydroxyalkyl group, or optionally substituted aryl group;

R₂ is H, optionally substituted C₁-C₅ alkyl group, or optionally substituted C₁-C₅ hydroxyalkyl group;

X' is optionally substituted C₁-C₅ alkyl alcohol or NR₅R₆,

R₅ and R₆ independently are H, C₀-C₅ hydroxyalkyl group, optionally substituted C₁-C₅ alkyl group, optionally substituted C₂-C₈ carboxy alkyl group, optionally substituted C₁-C₅ alkoxy group, optionally substituted heteroaryl alkyl group, optionally substituted aryl alkoxy group, optionally substituted arylalkyl group, optionally substituted aryl group, C₁-C₅ alkyl sulfonyl group, or - (CH₂)^{n¹}(OCH₂CH₂O)^{n²}CH(R₈)CO(R₇),

R₇ is C₀-C₅ hydroxyalkyl group, -NHOH,

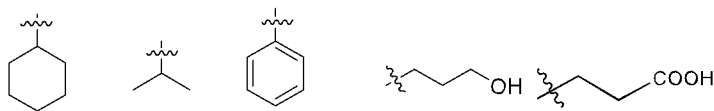
R₈ is H, optionally substituted arylalkyl group,

n¹ and n² independently are the number 0, 1, 2, 3, 4,

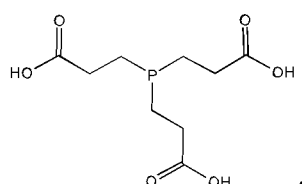
Y is the same as X, or Y is an ester or amide of X,

Z is the same as X or Y, or

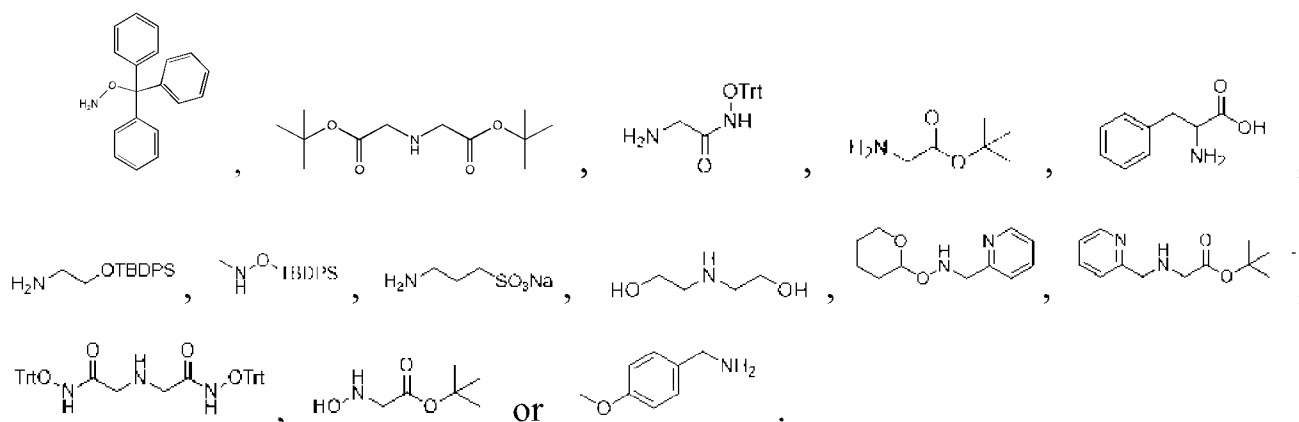
Y and Z independently are selected from the group consisting of



18. The method of claim 17, which characterized in that, the compound of formula II is



19. The method of claim 17, which characterized in that, the X' is 2-phenoxy-ethylamine, Phenylamine, Benzylamine, 4-Aminobenzene-1,2-diol, 5-Amino-2-hydroxybenzoic acid, Bis(pyridin-2-ylmethyl)amine, 5-Amino-8-hydroxyquinoline, Bis(pyridin-2-yl) methanamine, 4-Aminophthalic acid, tert-Butyl L-tyrosinate, DL-3-(4-Fluorophenyl)alanine, DL-4-Cyanophenylalanine, DL-4-nitrophenylalanine, N-Benzylhydroxylamine hydrochloride, N-Phenylhydroxylamine,



20. Use of the reductant of any one of claims 1-13 or the composition of any one of claims 14-16 in reducing the interchain disulfide bonds of an antibody.

21. The use of claim 20, which characterized in that, in the preparation of an antibody with site-specific modifications, optionally, the antibody with site-specific modifications is an antibody drug conjugate (ADC), more optionally, the ADC is the ADC with D2, the ADC with D4, the ADC with D1, the ADC with D6, the ADC with D3, the ADC with D1+D6, the ADC with D1+D2, the ADC with D1+D4, the ADC with D2+D4, the ADC with D6+D2, the ADC with D6+D1, the ADC with D3+D1, the ADC with D3+D2, the ADC with D0+D6, or the ADC with D0+D2.

22. A method of preparing an antibody with site-specific modification, comprising steps of

(A1) incubating a reductant of any one of claims 1-13 a salt, solvate, stereoisomer thereof as a first reductant and the transition metal ions in the presence of an antibody in a buffer system to selectively the reduce interchain disulfide bonds within the antibody to afford the antibody bearing reduced thiol groups.

23. The method of claim 22, which characterized in that, two interchain disulfide bonds in Fab region of the antibody and one interchain disulfide bonds in hinge region of the antibody are reduced.

24. The method of claim 22, which characterized in that, the method further comprising step of (A2) introducing oxidant to selectively re-oxidize the reduced thiol groups resulted from step (A1), optionally re-oxidize the reduced thiol groups in Fab region of the antibody.

25. The method of claim 24, which characterized in that, the method further comprising steps of (A3) incubating a second reductant in a buffer system to selectively reduce the interchain disulfide bonds resulted from step (A2), optionally reduce the interchain disulfide bonds in the hinge region of the antibody.

26. The method of any one of claims 22-24, which characterized in that, the method further comprising the following steps,

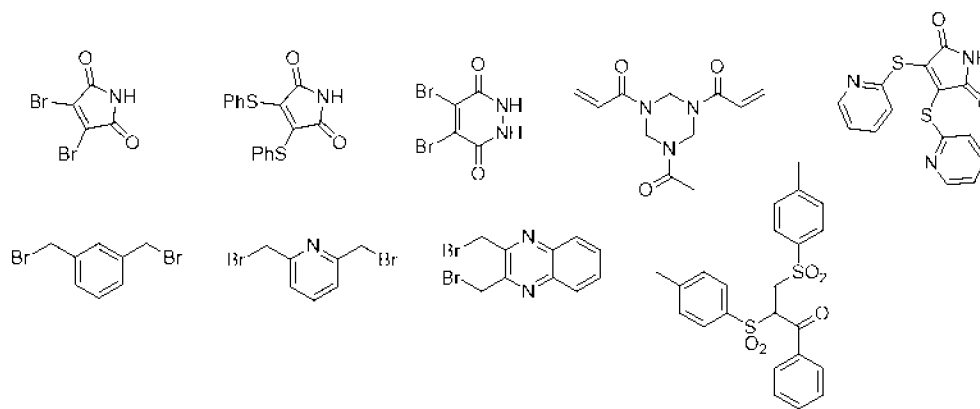
(B1) introducing metal chelators and first payload units to react with the reduced thiol groups resulted from step (A1), step (A2) or step (A3), wherein, the first payload unit is an end capping reagent, a first linker-payload or a first thio-bridging reagent, optionally, the first thio-bridging reagent bears the first linker-payload or reactive groups.

27. The method of claim 26, which characterized in that, the method further comprising step of (B2) incubating a second reductant in a buffer system to reduce interchain disulfide bonds resulted from step (B1), optionally, introducing the transition metal ions; and

(B3) introducing second payload units to react with the reduced thiol groups resulted from step (B2), optionally, introducing the metal chelators, wherein, the second payload unit is a second linker-payload or a second thio-bridging reagent, optionally, the second thio-bridging reagent bears the second linker-payload or reactive groups.

28. The method of claim 26 or 27, which characterized in that, the first thio-bridging reagent and the second thio-bridging independently contain at least two substituted groups allowing a re-bridging of the thiol groups.

29. The method of claim 28, which characterized in that, the first thio-bridging reagent and the second thio-bridging are independently selected from the group consisting of



30. The method of any one of claims 22, 25 and 27, which characterized in that, the molar ratio of the reductant and the antibody in step (A1), (A3) and (B2) independently is 1:1 to 20, 1:1 to 5:1, 1:1 to 3:1, 1:1 to 2:1 or 3:1 to 5:1.

31. The method of claim 30, which characterized in that, the molar ratio of the first reductant and the antibody in step (A1) is 2.8:1 to 13:1, optionally, the molar ratio of the first reductant and the antibody is 3.5:1 to 5:1, 4:1 to 10:1 or 5:1 to 13:1.

32. The method of any one of claims 22, 25 and 27, which characterized in that, the incubation temperature in step (A1), (A3) and (B2) independently is 0°C to 37°C, 0°C to 25°C, 0°C to 15°C, 0°C to 10°C, or 0°C to 5°C.

33. The method of any one of claims 22, 25 and 27, which characterized in that, the incubation time in step (A1) is 2h to 24h, 14h to 24h, 16h to 20h, or 16h to 18h;

incubation time in step (A3) and (B2) independently is 0.5h to 24h, 0.5h to 12h, 1h to 10h, 1h to 8h, or 1h to 5h.

34. The method of claim 33, which characterized in that, in step (A1), the molar ratio of the first reductant and the antibody is 4:1 to 10:1, the incubation time is 1h to 16h.

35. The method of claim 33, which characterized in that, in step (A1), the molar ratio of the first reductant and the antibody is 6:1 to 13:1, the incubation time is 4h to 16h.

36. The method of claim 33, which characterized in that, in step (A1), the molar ratio of the first reductant and the antibody is 2.8:1 to 3:1, the incubation time is 10-24h.

37. The method of claim 22, which characterized in that, the molar ratio of the transition metal ions and the first reductant in step (A1) is 0.05:1 to 40:1, 0.08:1 to 30:1, 0.1:1 to 20:1, 0.2:1 to 8:1, or 0.25:1 to 7.5:1.

38. The method of claim 27, which characterized in that, in step (B2), the molar ratio of the second reductant and the transition metal ions is 1:0.05 to 1:40, and/or the molar ratio of the second reductant and the antibody is 2.5:1 to 20:1, and/or the incubation time is 1h to 24h.

39. The method of claim 27, which characterized in that, in step (B2), the molar ratio of the second reductant and the transition metal ions is 1:0.4 to 1:100, and/or the molar ratio of the second reductant and the antibody is 0.8:1 to 2.5:1, and/or the incubation time is 0.5h to 24h.

40. The method of any one of claim 22 or 27, which characterized in that, the transition metal ions are selected from the group consisting of Zn^{2+} , Cd^{2+} , Hg^{2+} , Ni^{2+} , Co^{2+} or the combination thereof, optionally, the transition metal ions are Zn^{2+} .

41. The method of claim 25 or 27, which characterized in that, the second reductant in step (A3) and (B2) is the same as the first reductant in step (A1).

42. The method of claim 25 or 27, which characterized in that, the second reductant in step (A3) and the second reductant in step (B2) independently are tris (2-carboxyethyl) phosphine (TCEP).

43. The method of claim 24, which characterized in that, the molar ratio of the oxidant and the antibody in step (A2) is 2:1 to 25:1, optionally, the molar ratio of the oxidant and the antibody in step (A2) is 4:1 to 22:1 or 3:1 to 15:1.

44. The method of claim 24, which characterized in that, the oxidant is Dehydroacetic acid (DHAA).

45. The method of claim 24, which characterized in that, in step (A2), the oxidation temperature is 0°C to 37°C, and/or the oxidation time is 1h to 48h, optionally, the oxidation temperature is 0°C to 30°C, and/or the oxidation time is 1h to 5h.

46. The method of any one of claims 22, 25 and 27, which characterized in that, the buffer system of step (A1), (A3) and (B2) independently is selected from a group consisting of MES buffer, Bis-Tris buffer, PIPES buffer, MOPS buffer, BES buffer, HEPES buffer, DIPSO buffer, MOBS buffer, MOPSO buffer, TES buffer, ACES buffer, TAPSO buffer, PBS, Acetate buffer, ADA buffer, BTP buffer, HEPPSO buffer, POPSO buffer, EPPS buffer or Tris buffer,

optionally, the buffer system of step (A1), (A3) and (B2) independently are Bis-Tris buffer, MOPS buffer, BES buffer, HEPES buffer, DIPSO buffer, MOBS buffer, MOPSO buffer, TES buffer, ACES buffer or TAPSO buffer.

47. The method of claim 45, which characterized in that, the concentration of the buffer system is 10 mM to 100 mM, 20 mM to 80 mM, 20 mM to 40 mM, 20 mM to 60 mM, 40 mM to 80 mM or 40 mM to 60 mM.

48. The method of claim 45, which characterized in that, the pH value of the buffer system is 5.5 to 8, preferably, the pH value of the buffer system is 6.7 to 7.4.

49. The method of any one of claim 26 or 27, which characterized in that, the metal chelators in step (B1) and (B3) is Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na).

50. The method of claim 26, which characterized in that, when the first payload units are the first thio-bridging reagent bearing reactive groups, the step (B1) further comprising step of

incubating the metal chelators and the first linker-payloads in the buffer system to react with the reactive groups of the first thio-bridging reagent bearing reactive groups.

51. The method of claim 27, which characterized in that, when the second payload units are the second thio-bridging reagent bearing reactive groups, the step (B3) further comprising step of

incubating the second linker-payloads in the buffer system to react with the reactive groups of the second thio-bridging reagent bearing reactive groups, optionally, introducing the metal chelators.

52. The method of any one of claim 22, which characterized in that, the antibody is a monoclonal antibody, a polyclonal antibody, a mono-specific antibody or a multi-specific antibody, optionally, the antibody is IgG1 or IgG4.

53. The method of any one of claim 26 or 27, which characterized in that, a linker of the first linker-payload and the second linker payload is selected from any one of which the one terminal can be connected to the reduced thiol group of the antibody or the reactive groups of the thio-bridging reagent, and the other terminal can be connected to the payload.

54. The method of any one of claim 26 or 27, which characterized in that, the payload is selected from any one of which contains at least one substituted group allowing a connection from the payload to the linker.

55. A modified antibody prepared by the method of any one of claims 22-54.

56. The modified antibody of claim 55, which characterized in that, the modified antibody is the antibody with site-specific modification, optionally, the modified antibody comprises the ADC with D2, the ADC with D4, the ADC with D1, the ADC with D6, the ADC with D3, the ADC with D1+D6, the ADC with D1+D2, the ADC with D1+D4, the ADC with D2+D4, the ADC with D6+D2, the ADC with D6+D1, the ADC with D3+D1, the ADC with D3+D2, the ADC with D0+D6, or the ADC with D0+D2.

57. A pharmaceutical composition comprising an antibody with site-specific modification prepared by the method of any one of claims 22-54, and at least one pharmaceutically acceptable ingredient.

58. Use of the antibody with site-specific modification prepared by the method of any one of claims 22-54 or the pharmaceutical composition of claim 57 in the manufacture of a therapeutic agent for preventing, diagnosing or treating a disease.

59. A method of preventing or treating a disease in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of the antibody with site-specific modification prepared by the method of any one of claims 22-54.

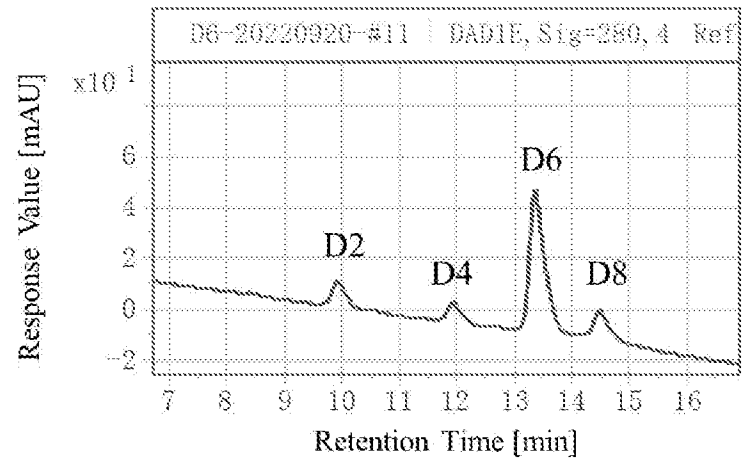


Figure 1

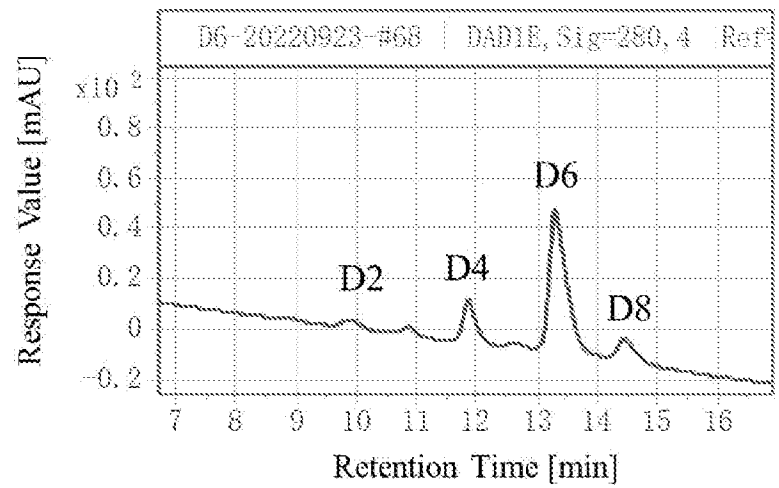


Figure 2

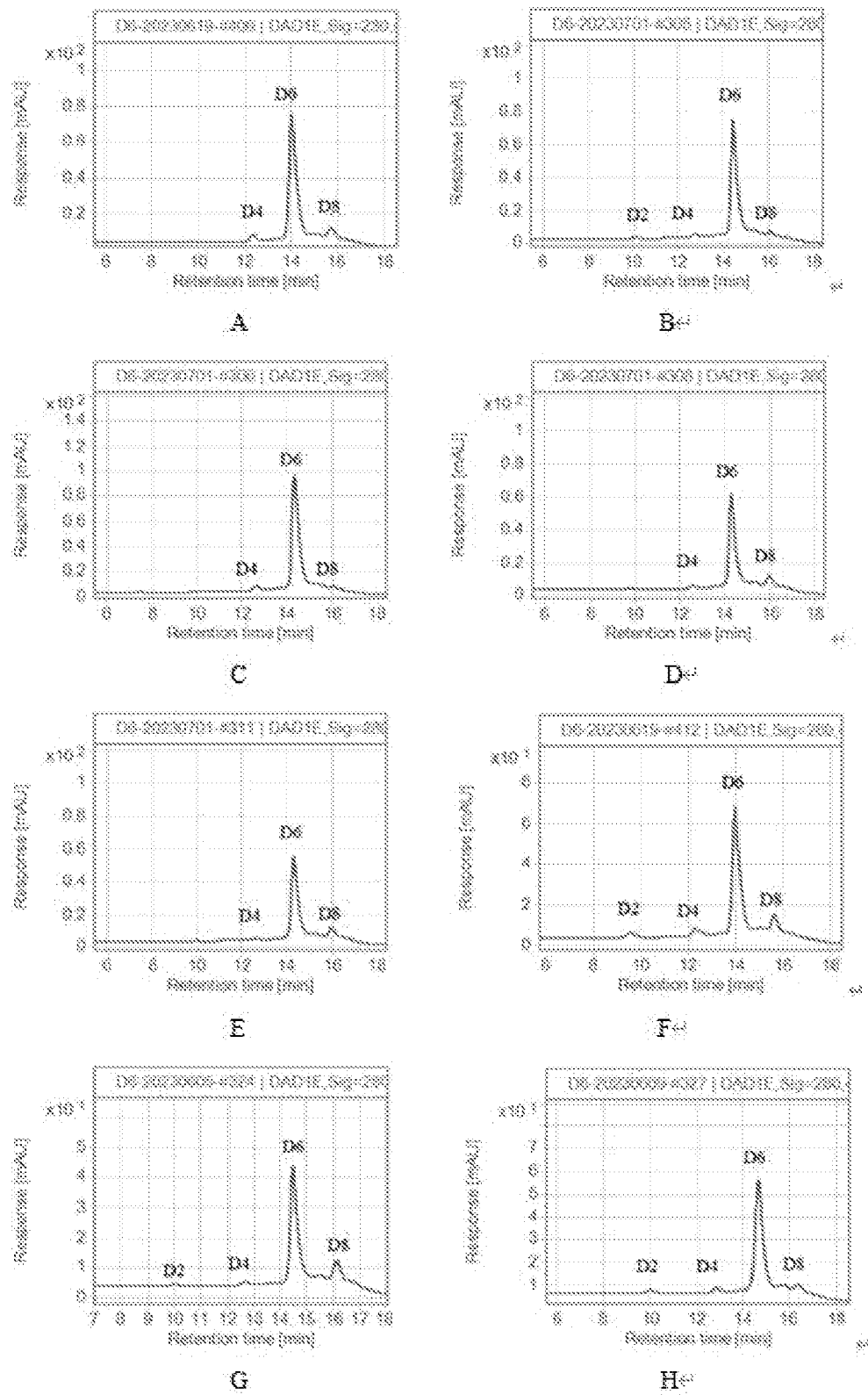


Figure 3

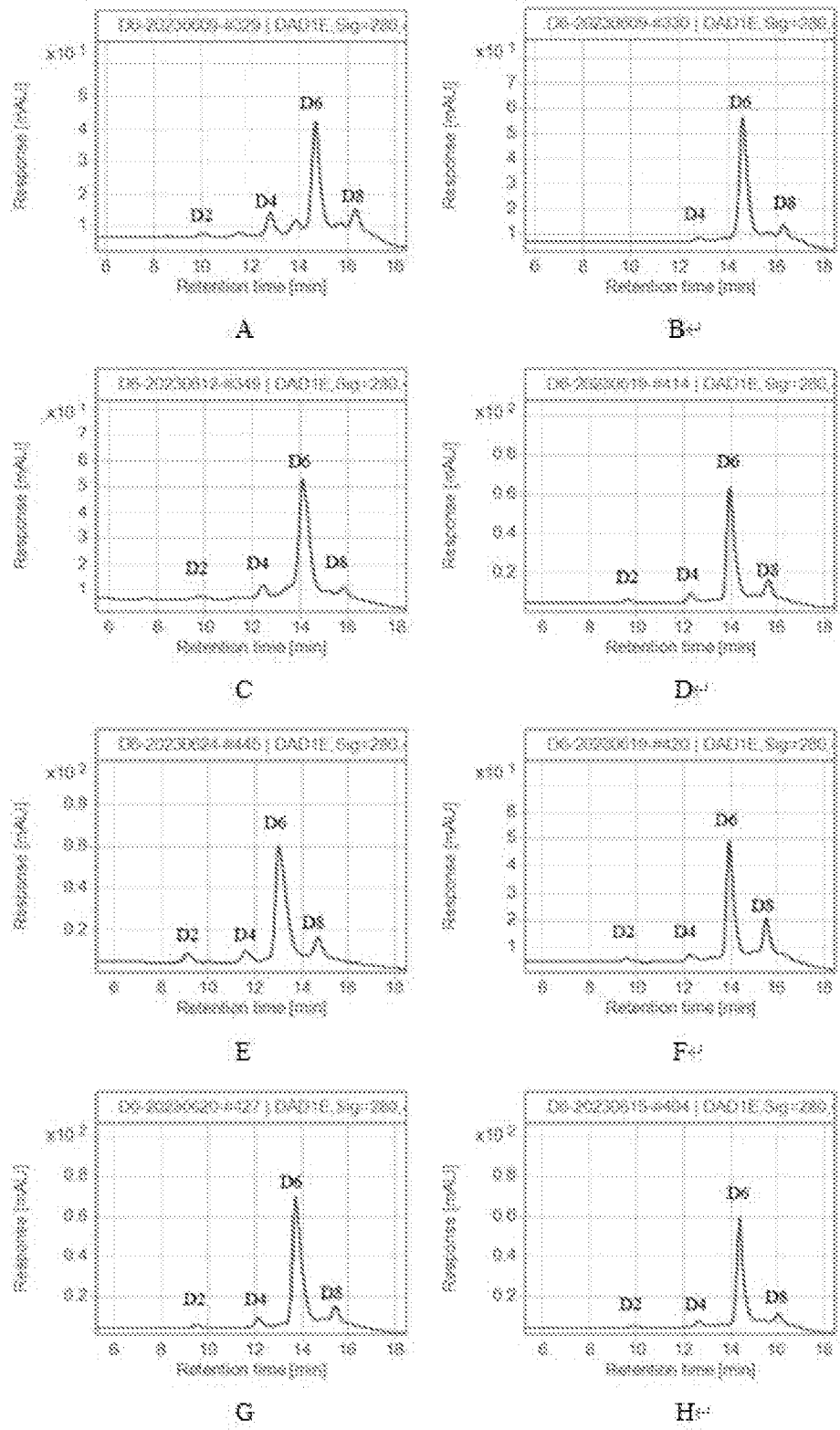


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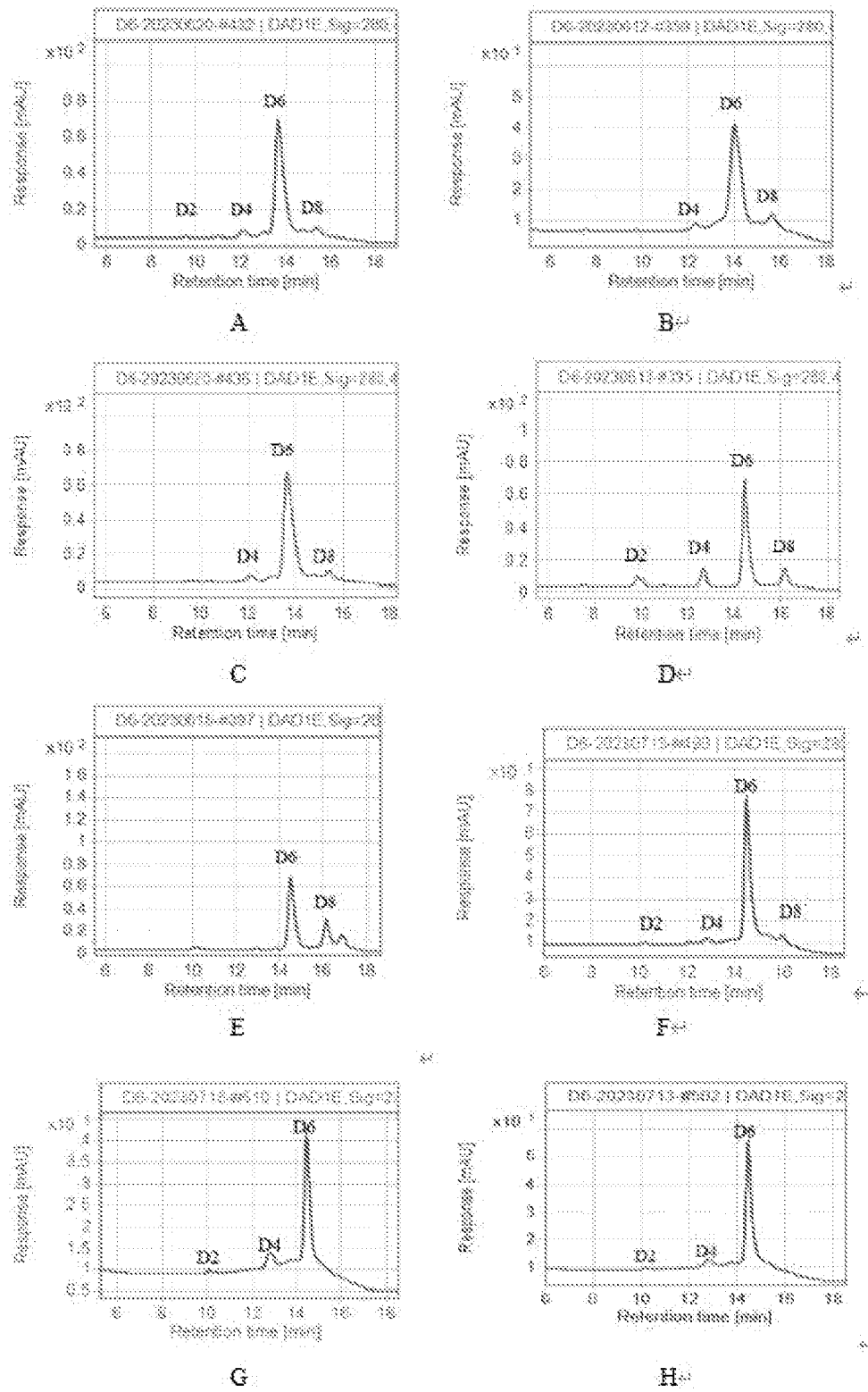


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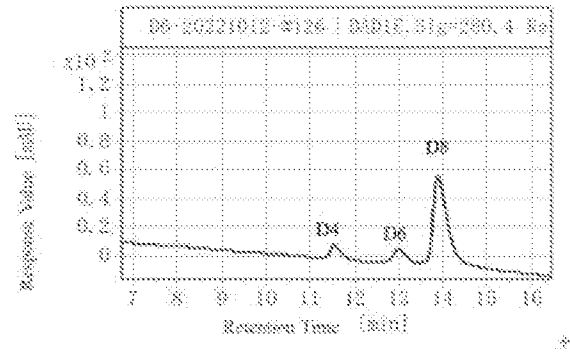


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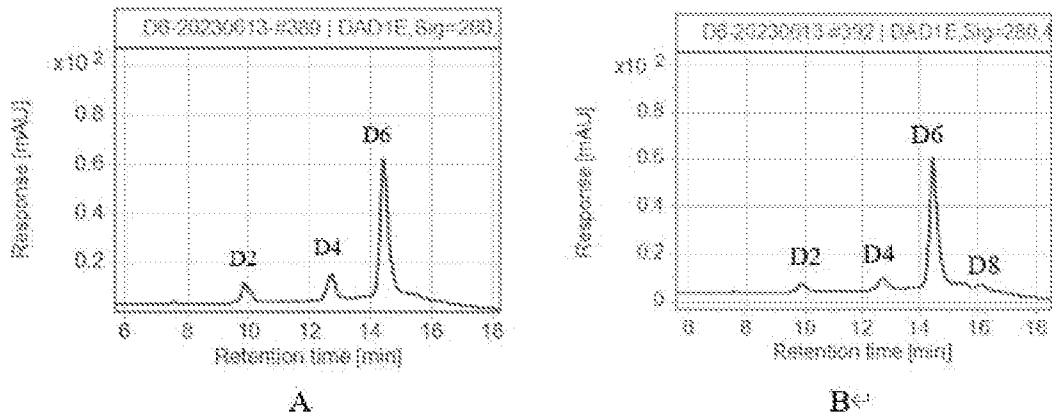


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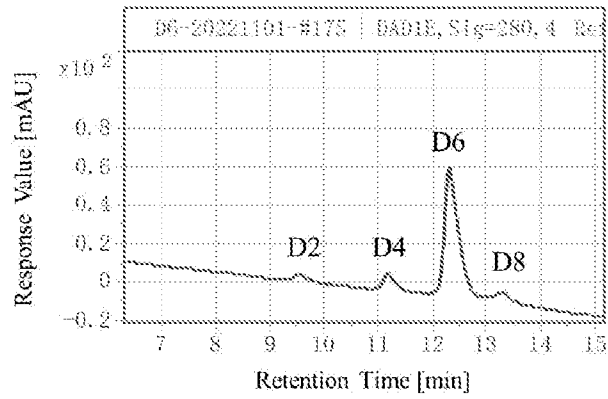


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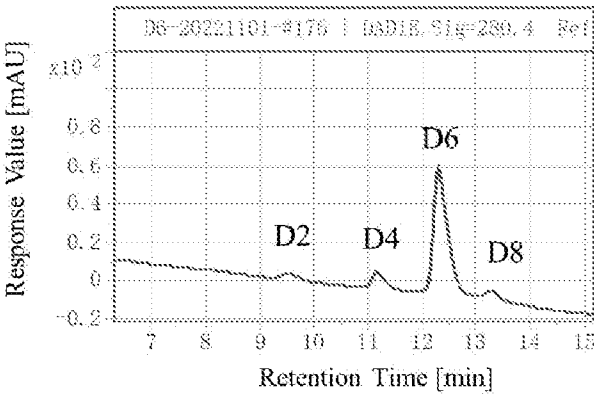


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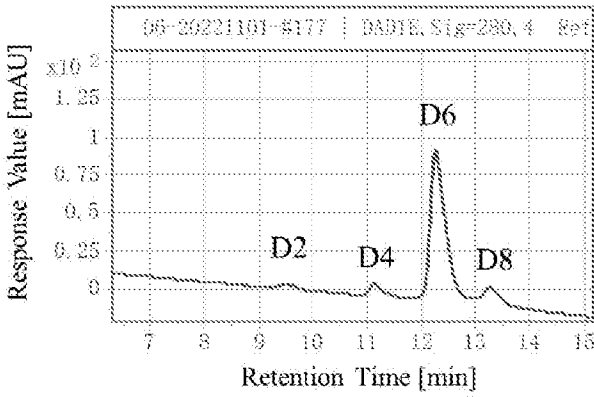


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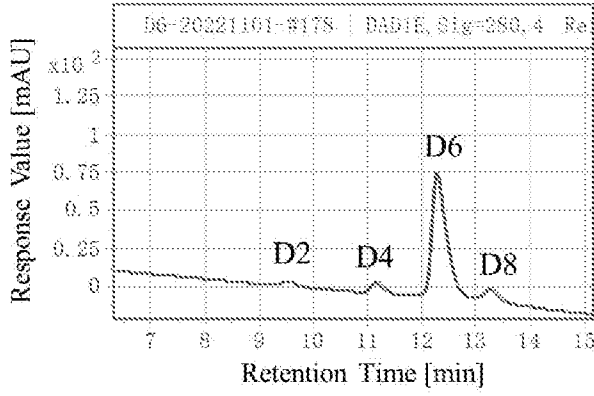


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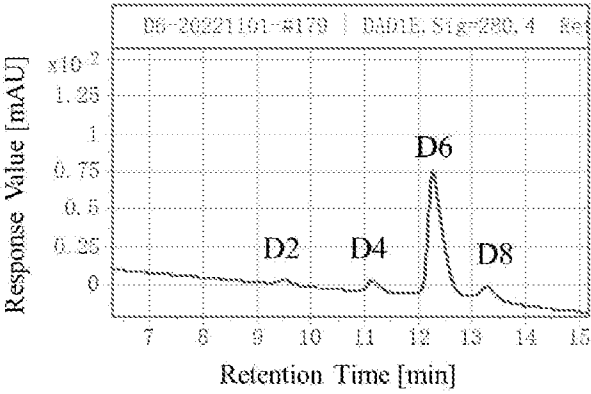


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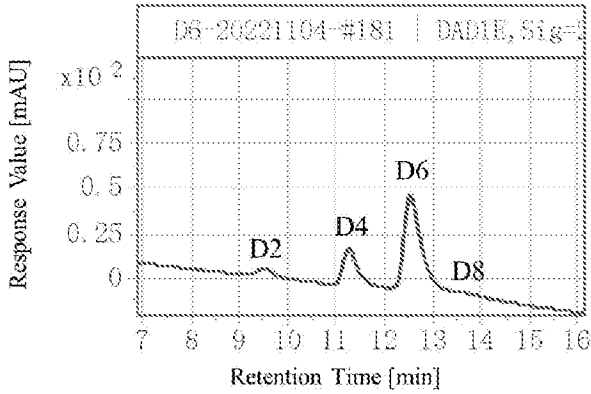


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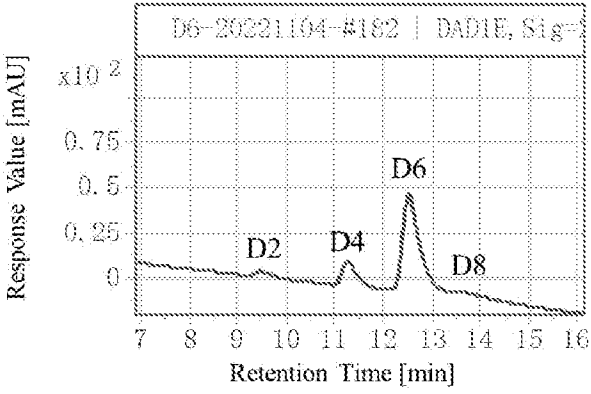


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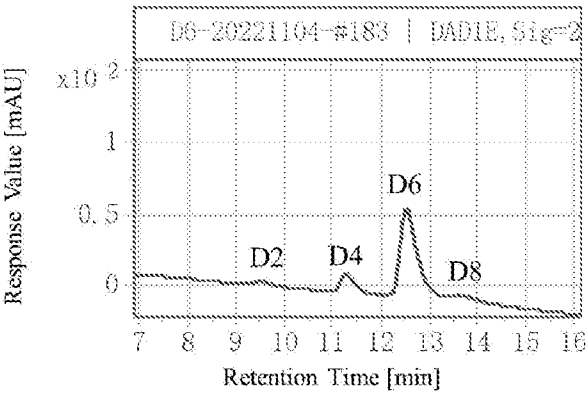


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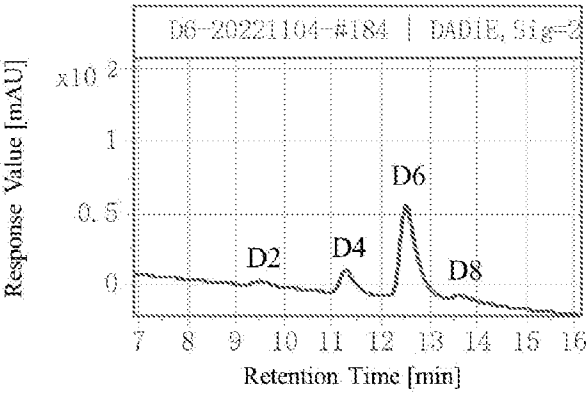


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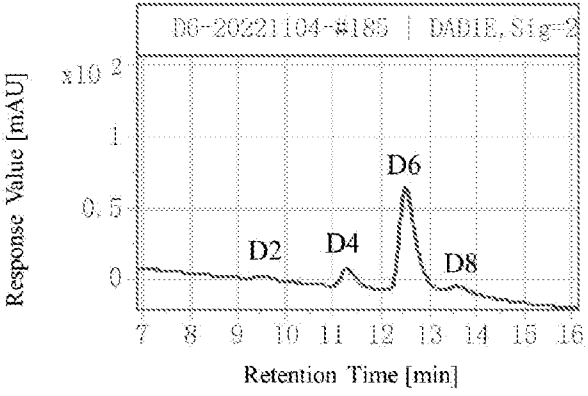


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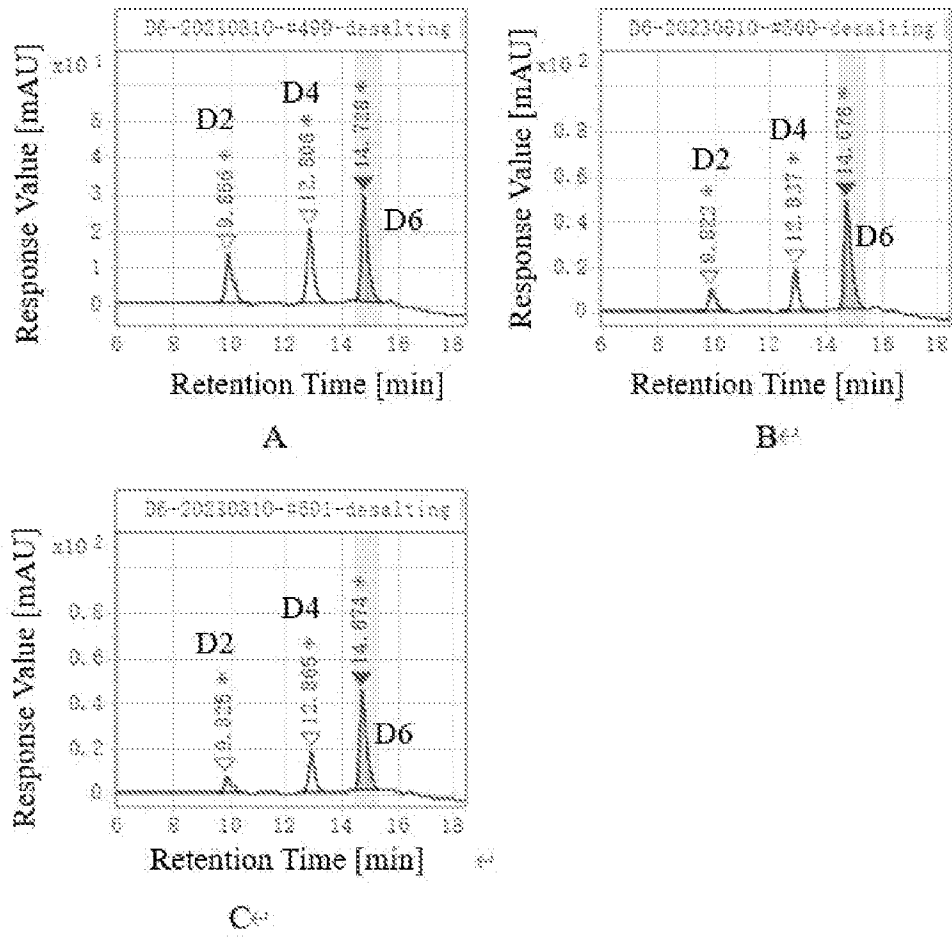


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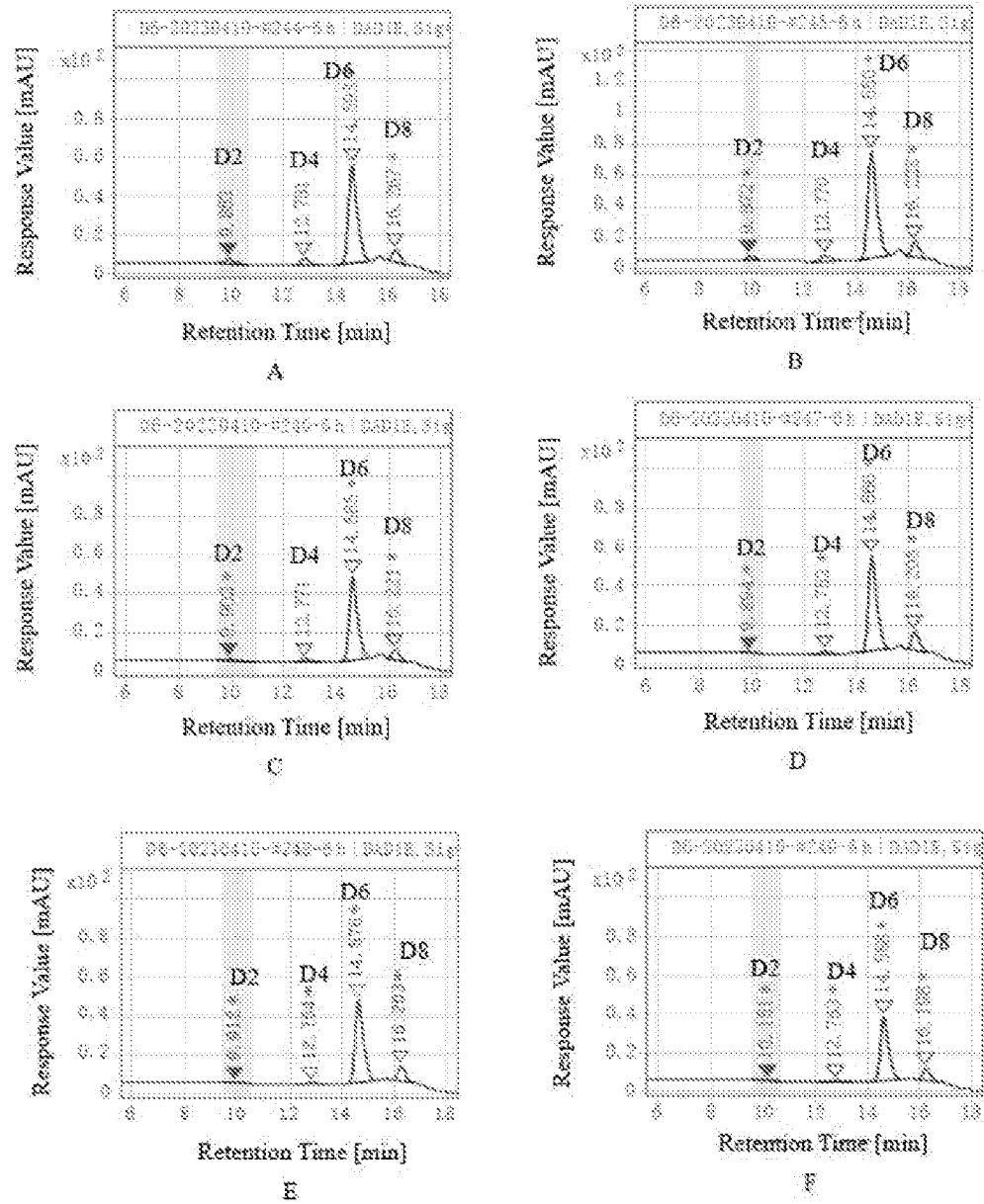


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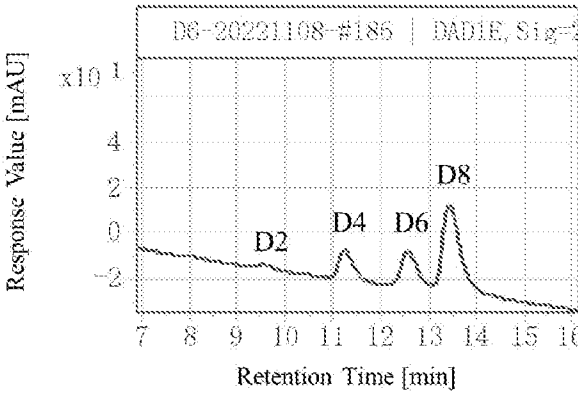


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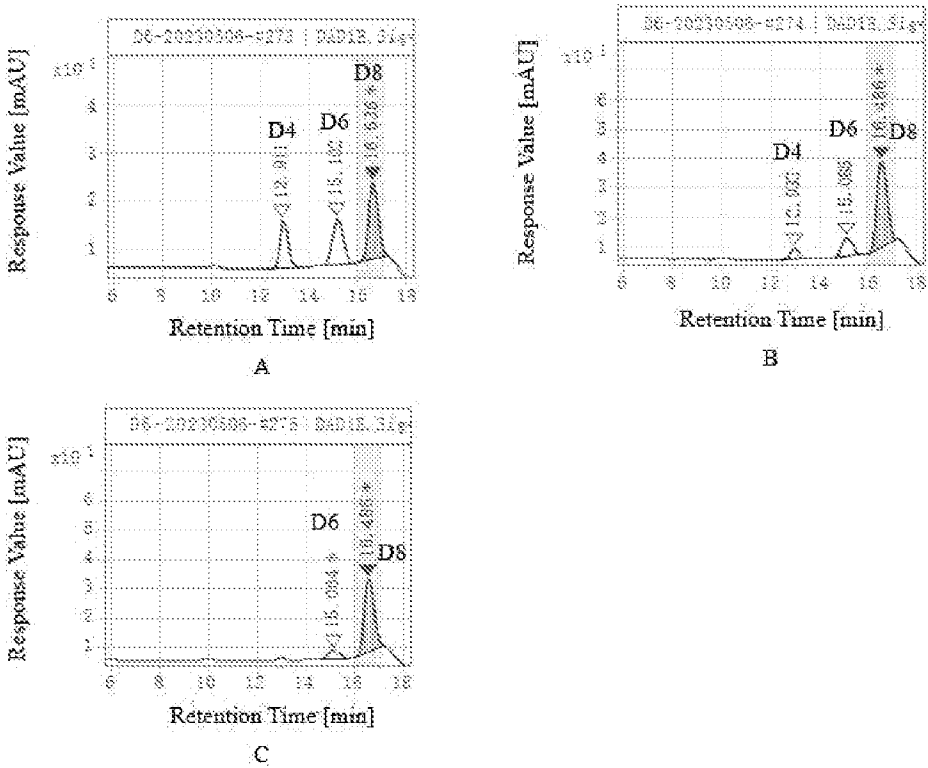


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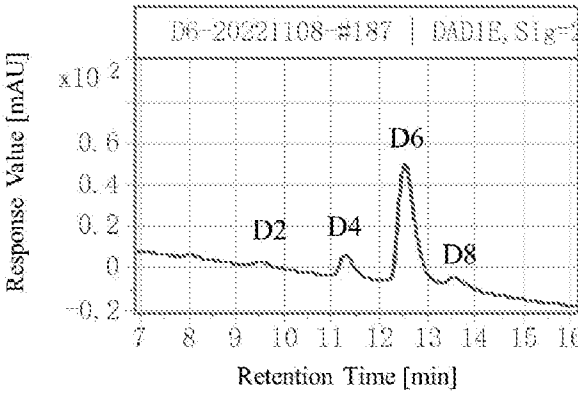


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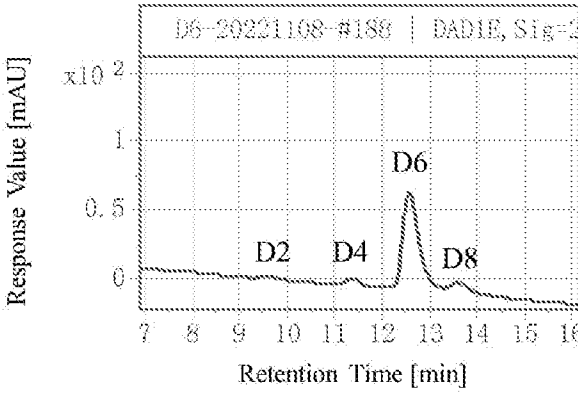


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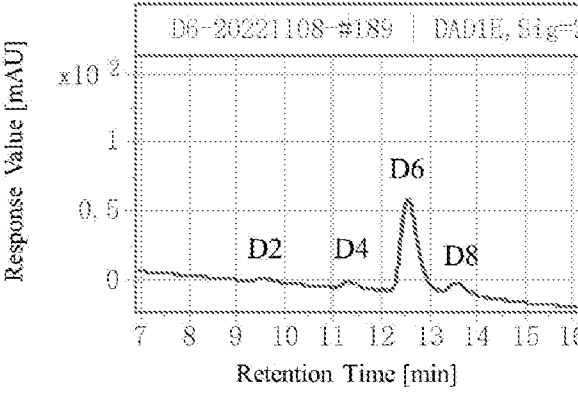


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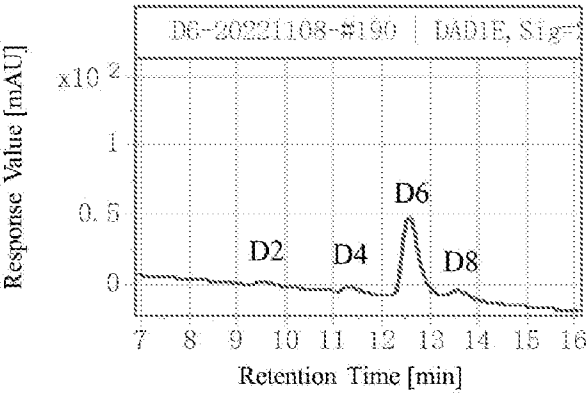


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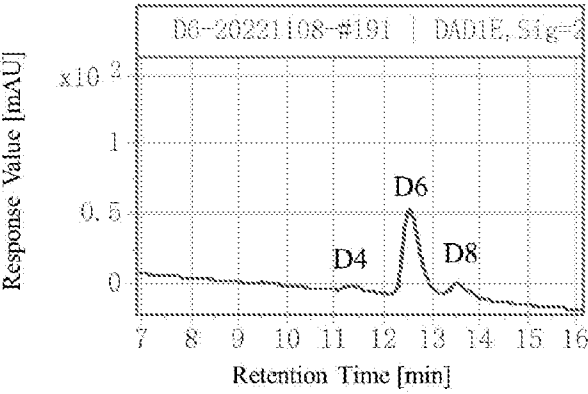


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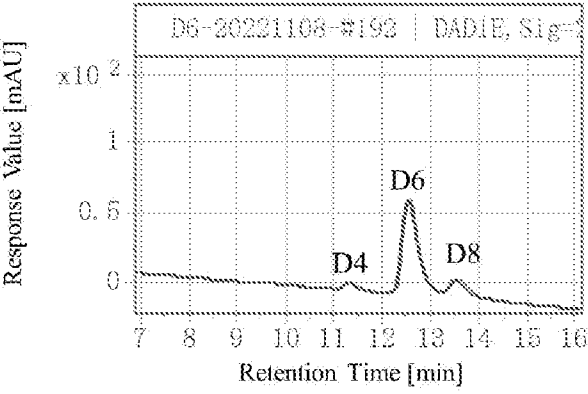


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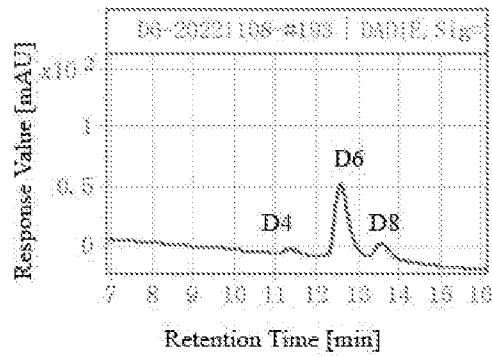


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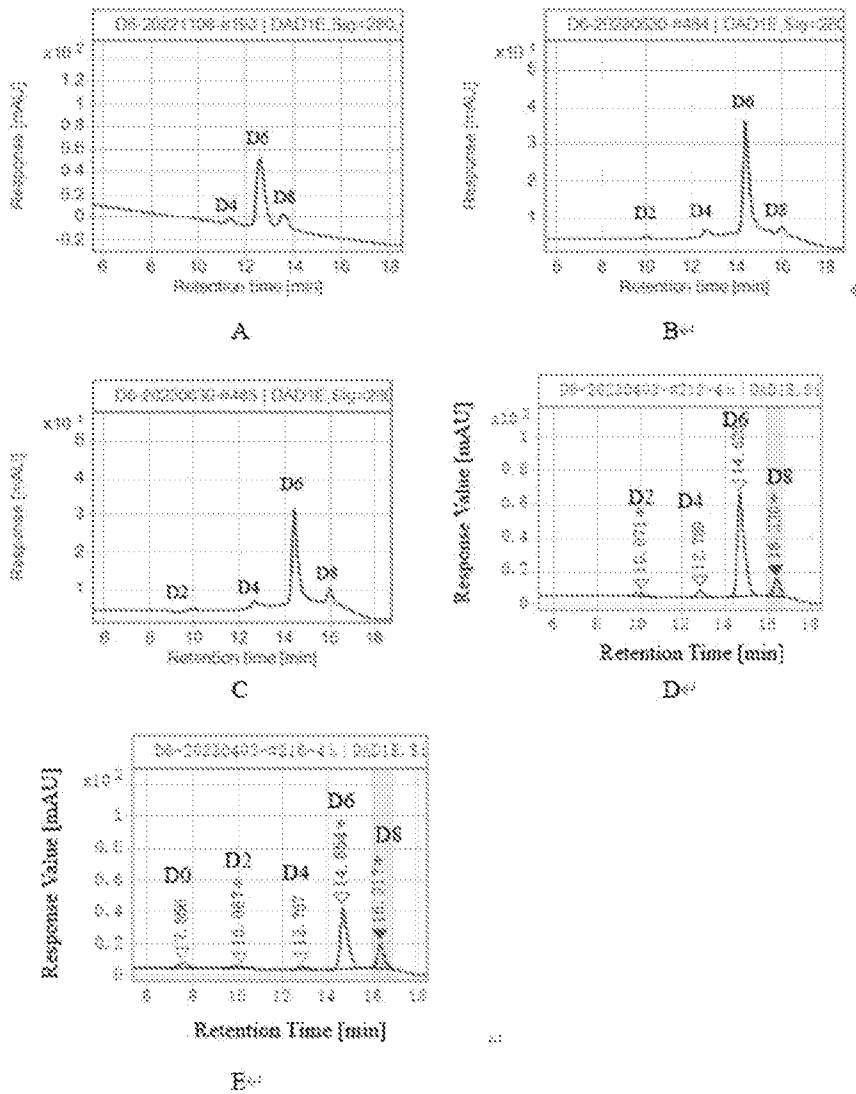


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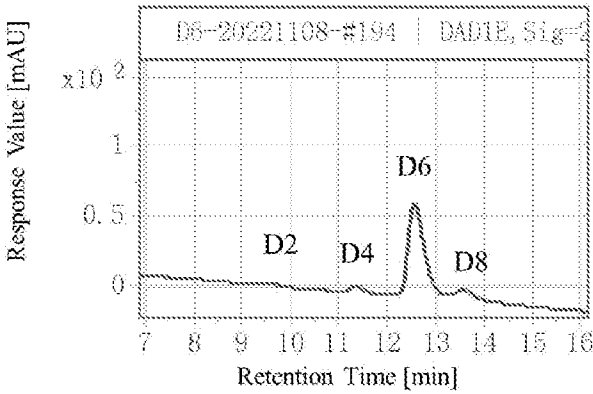


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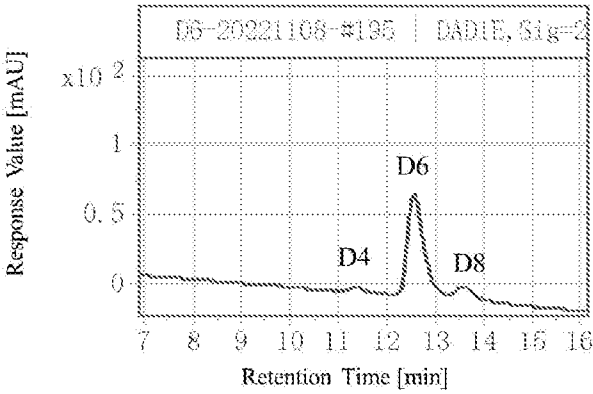


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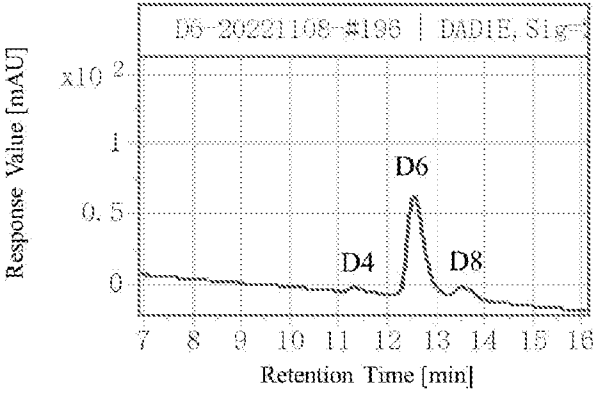


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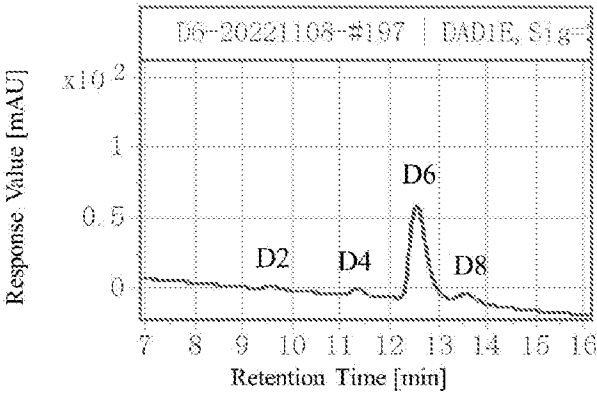


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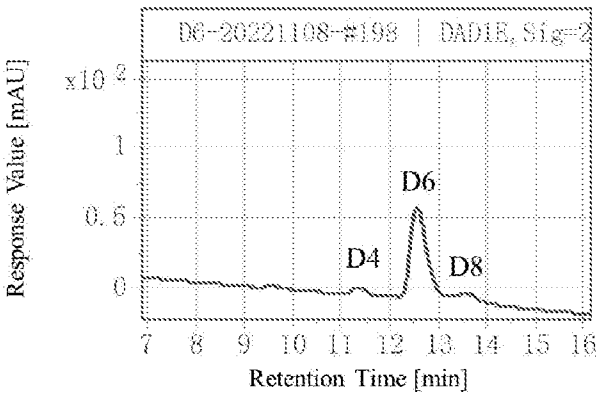


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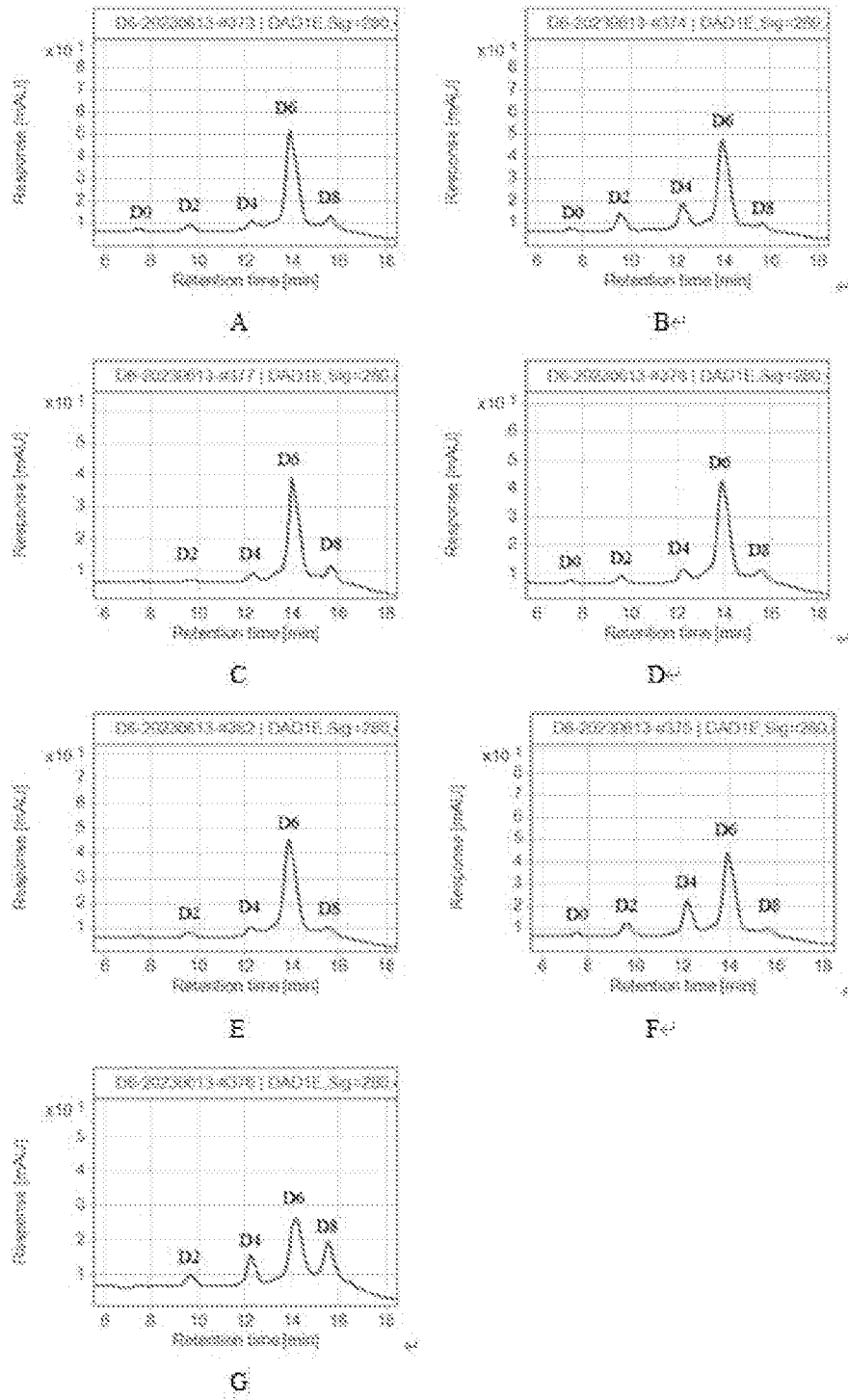


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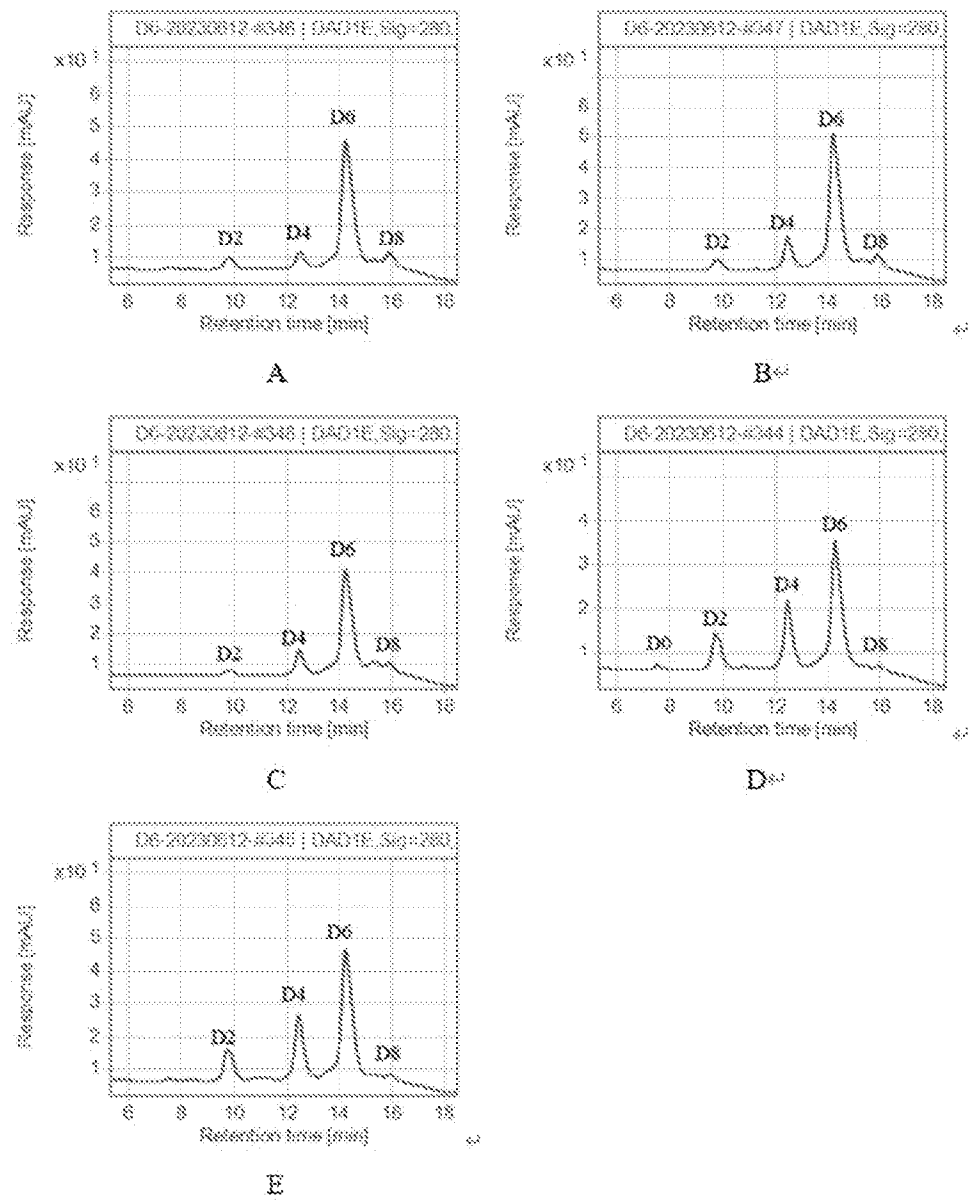


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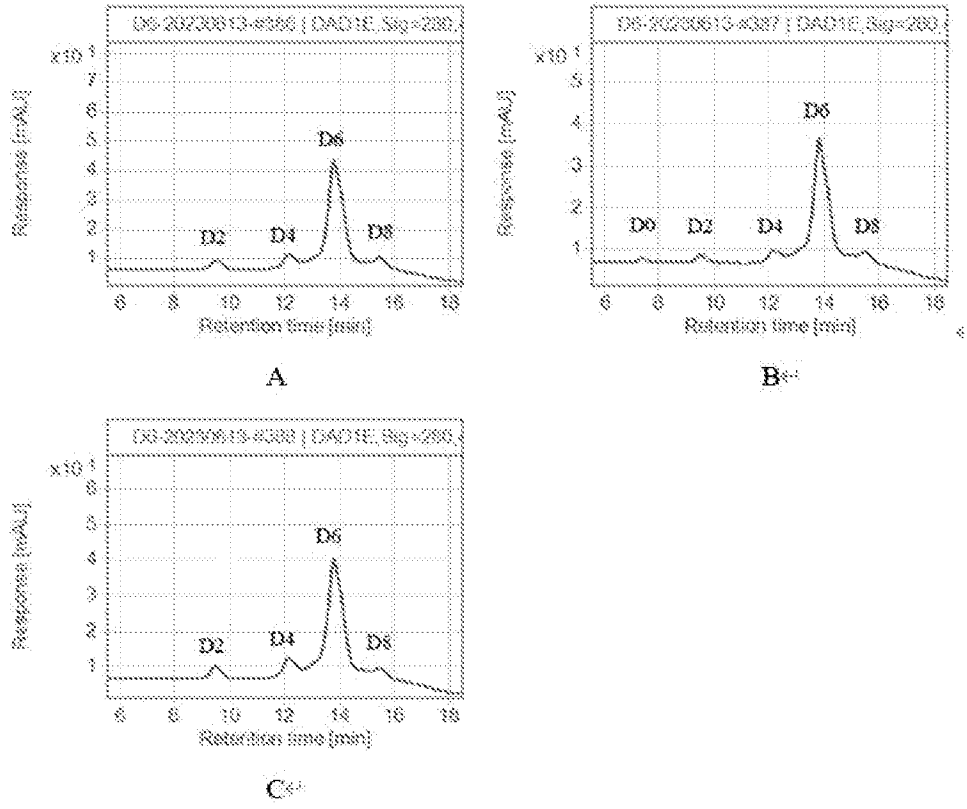


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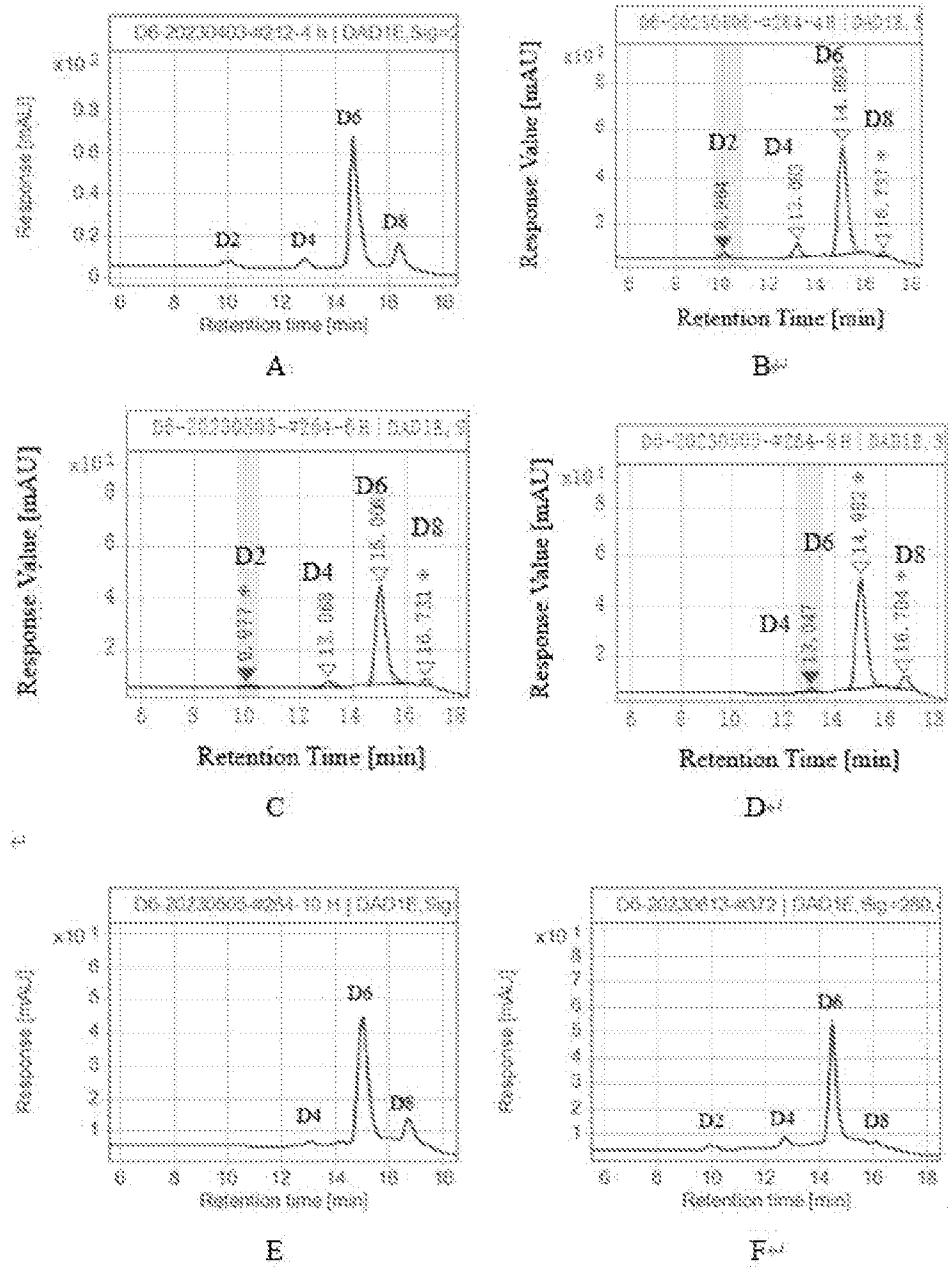


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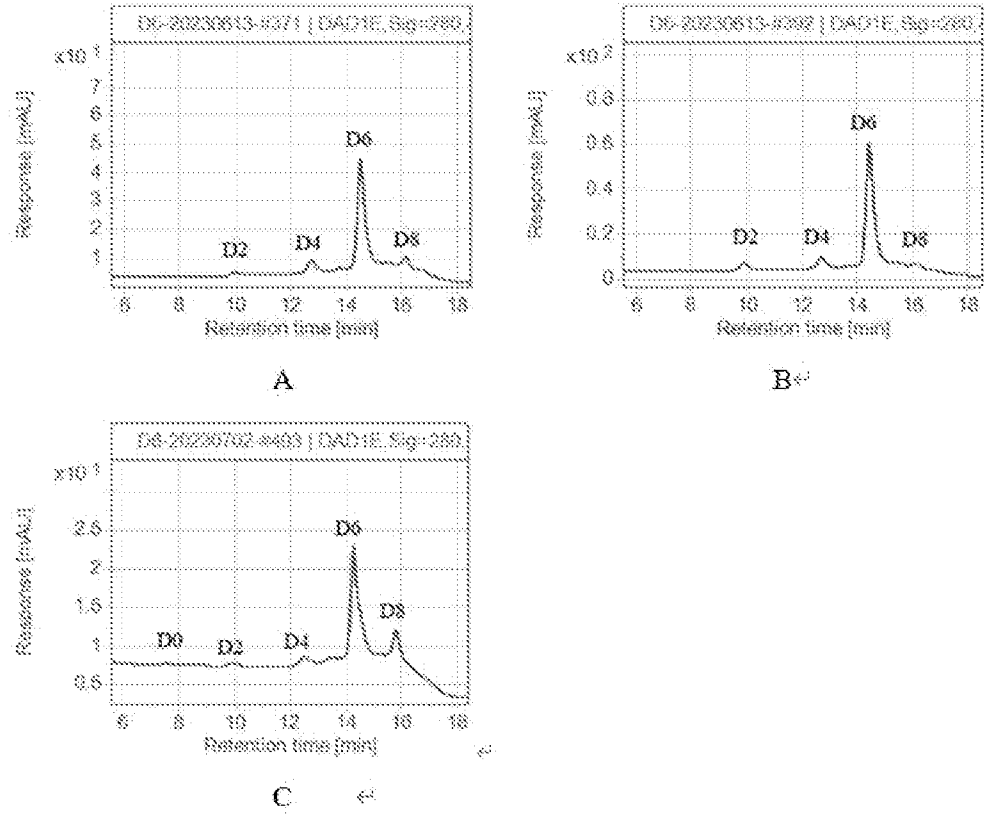


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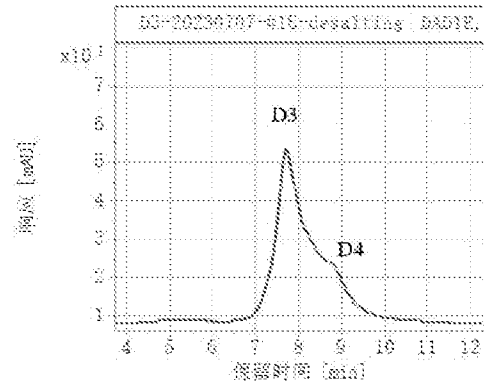


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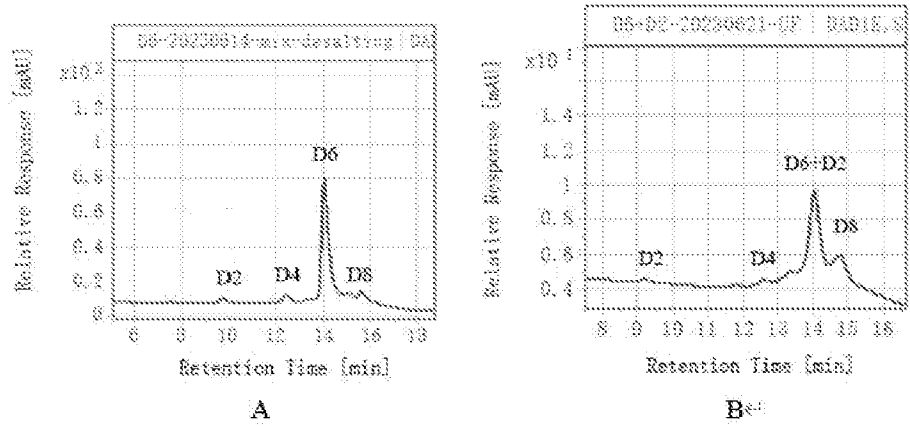


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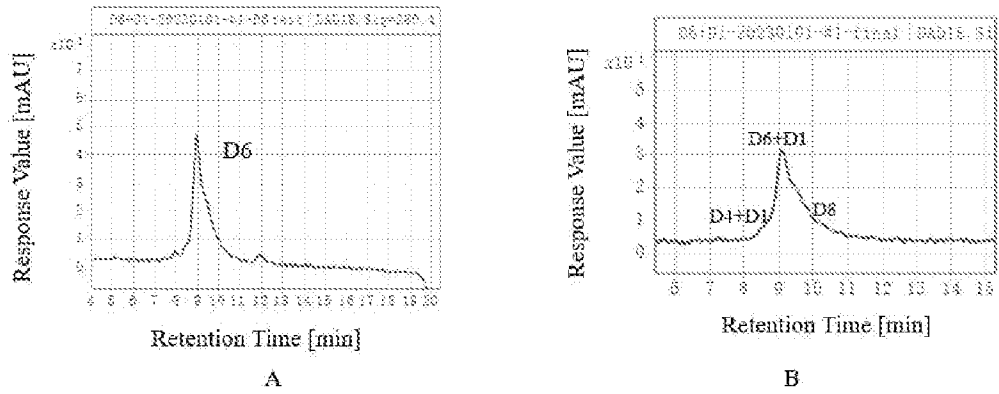


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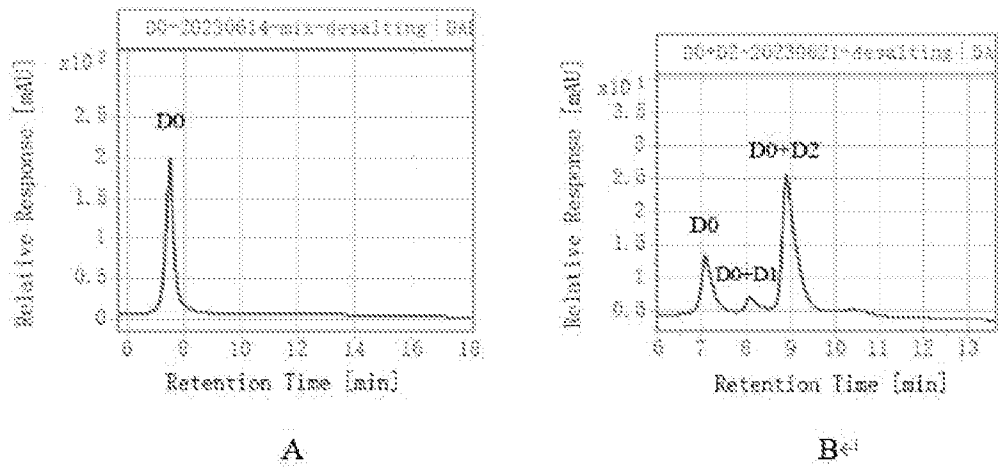


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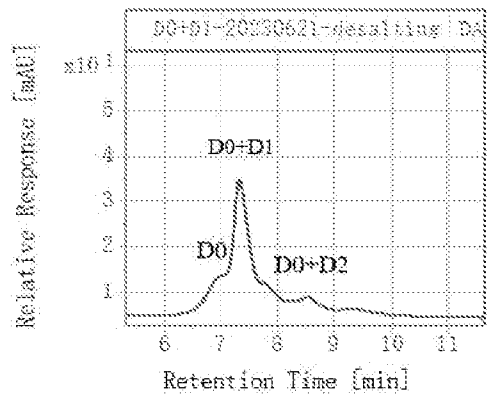


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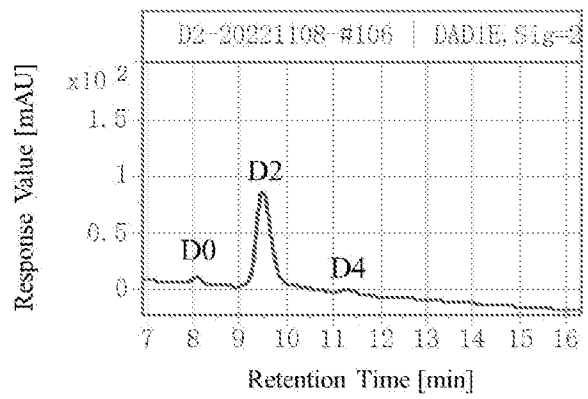


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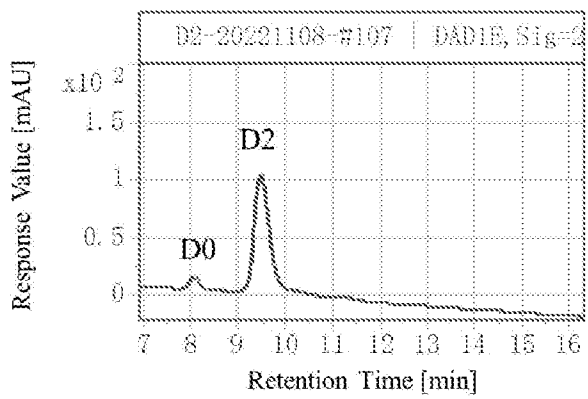


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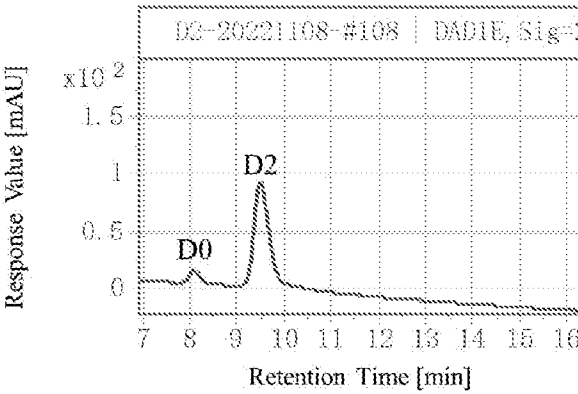


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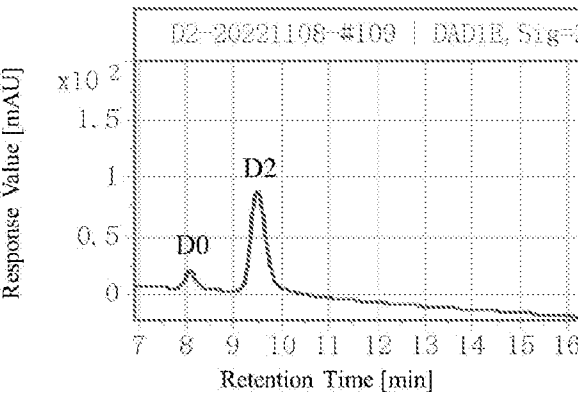


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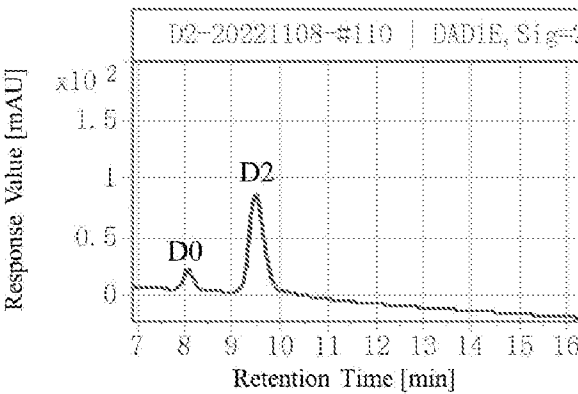


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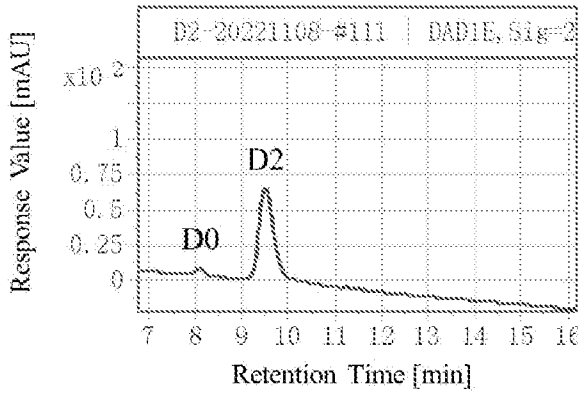


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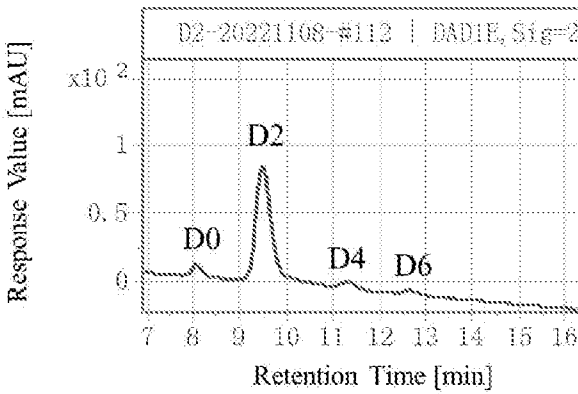


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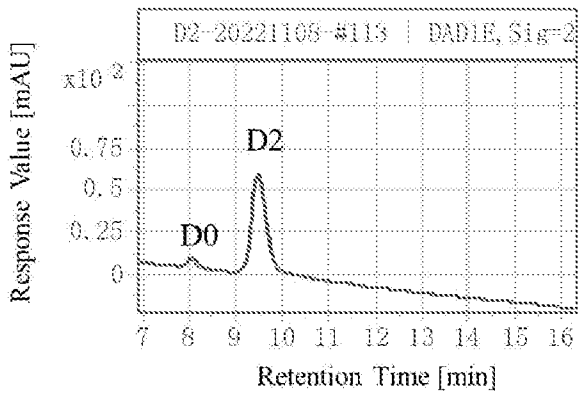


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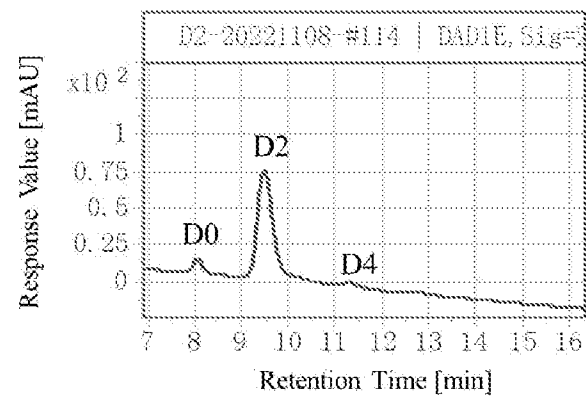


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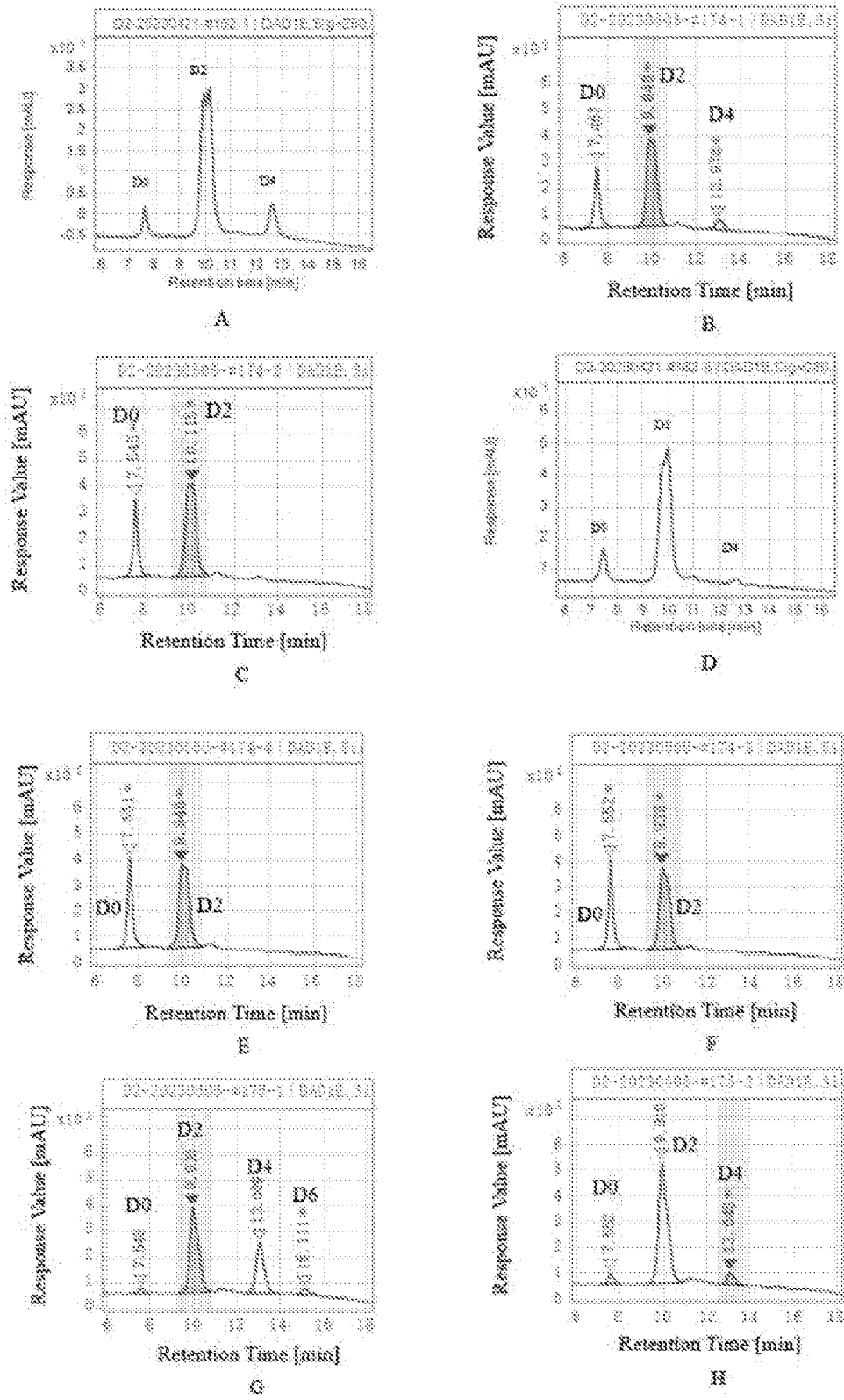


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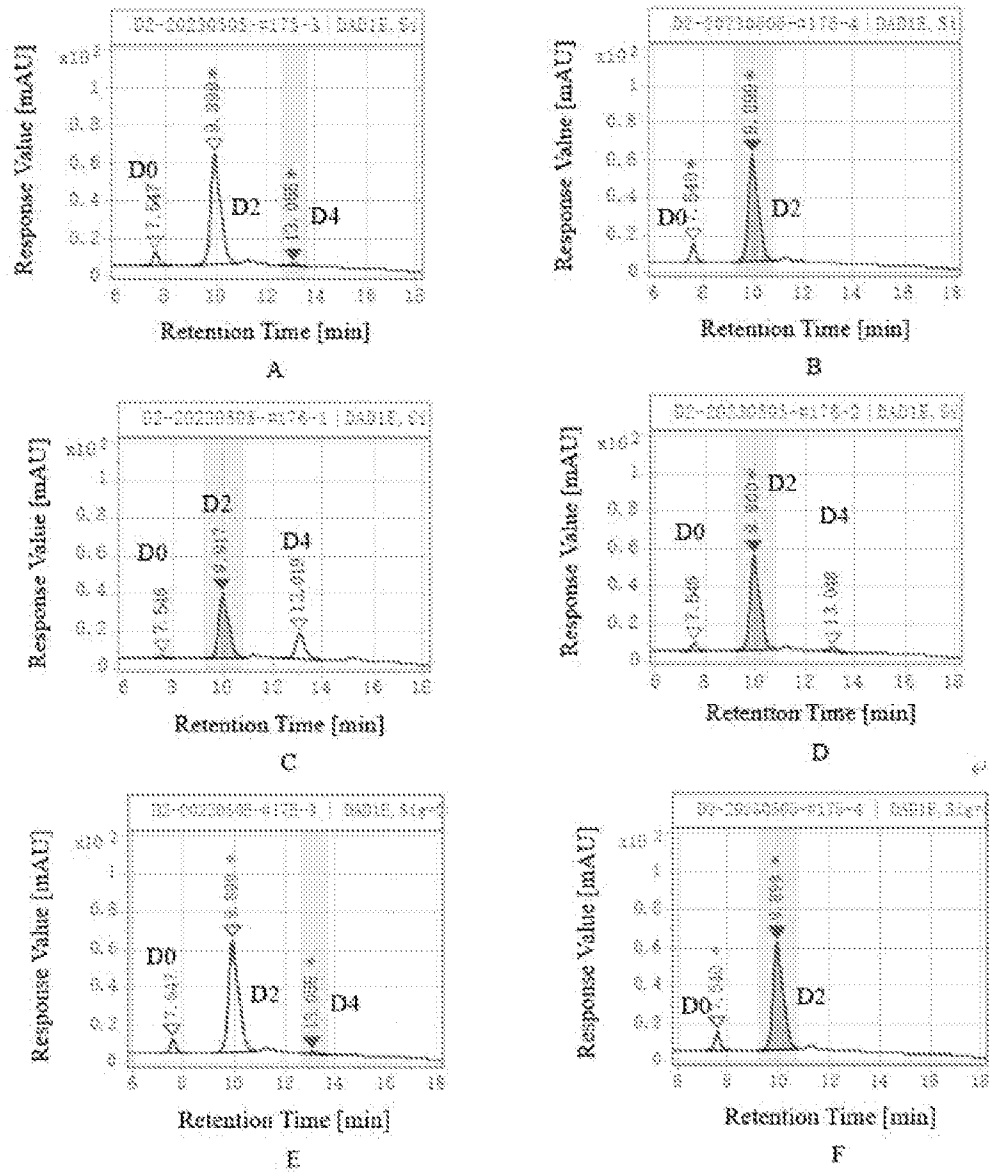


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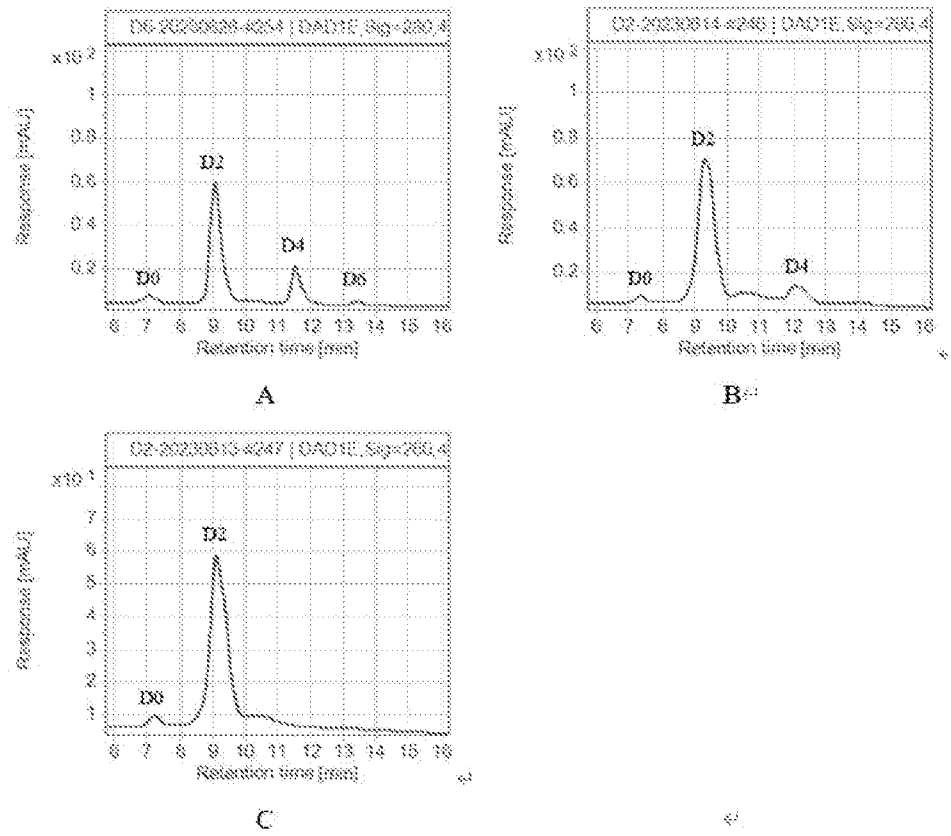


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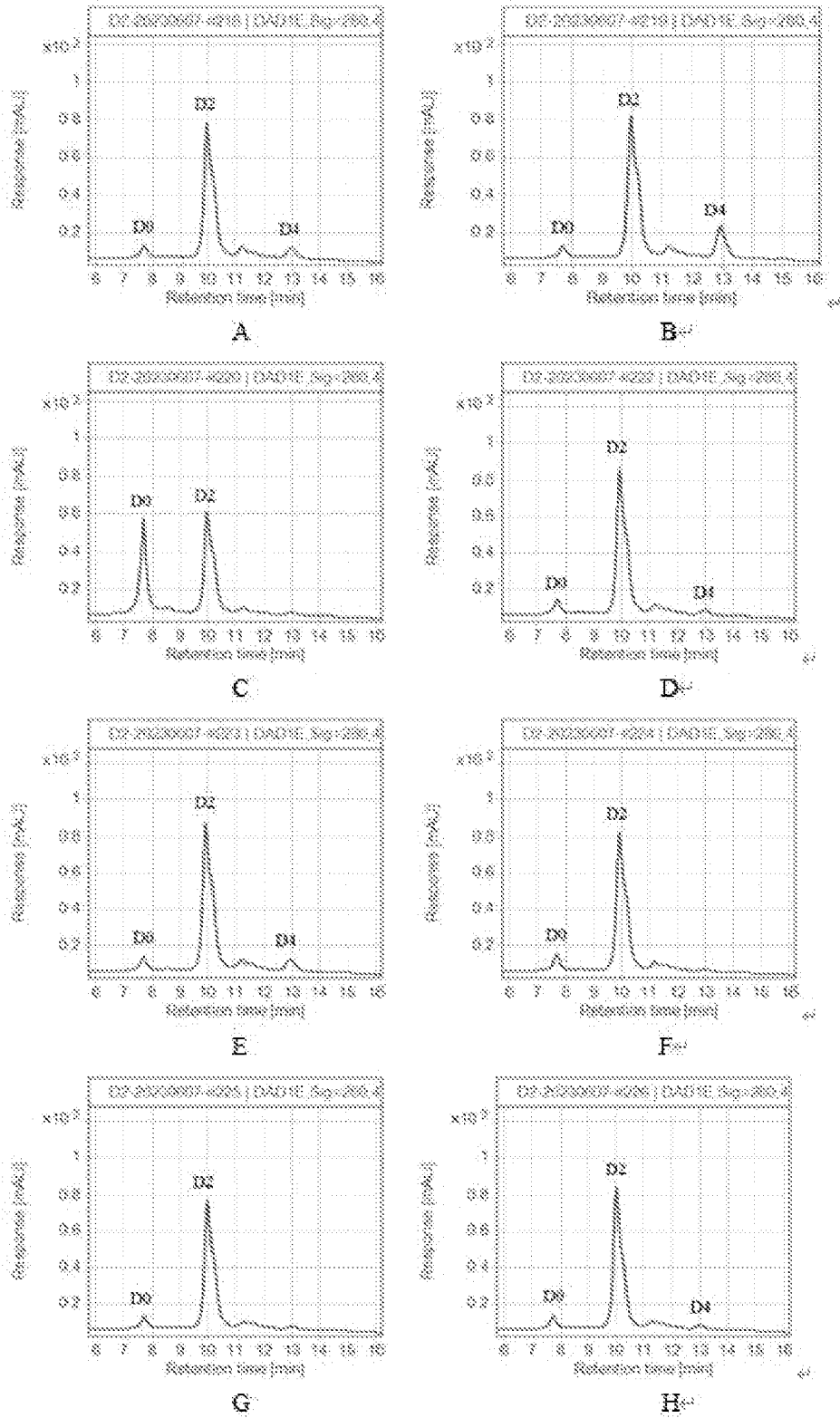


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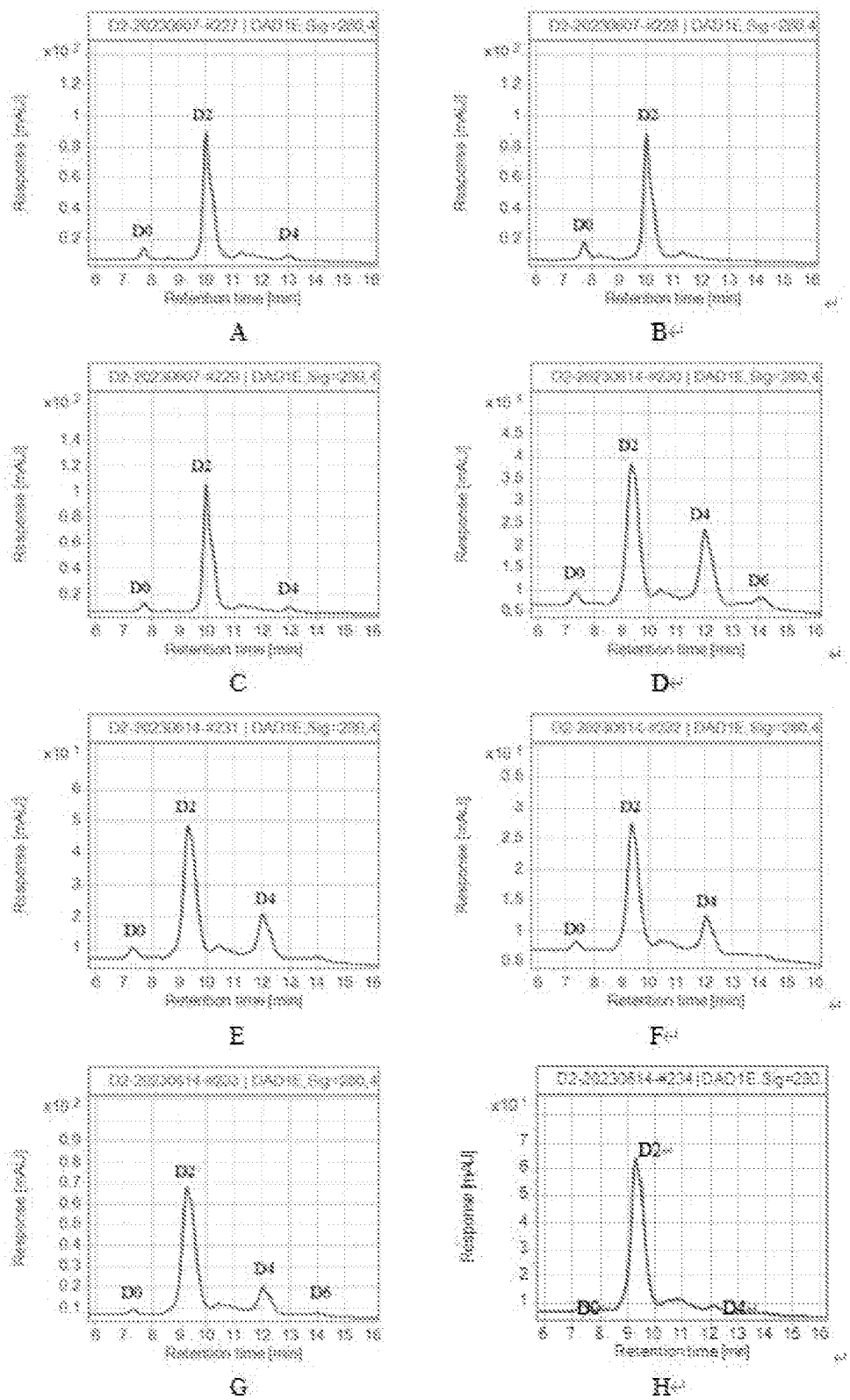


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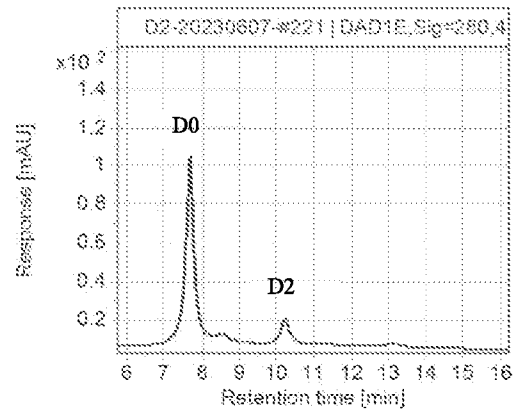


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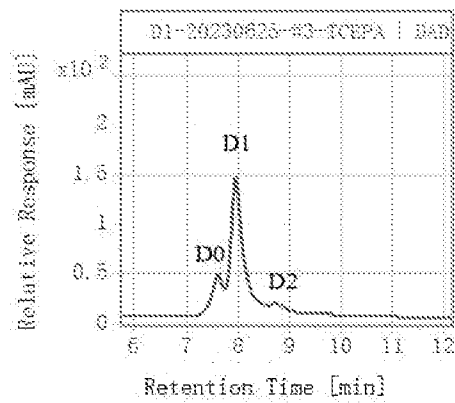


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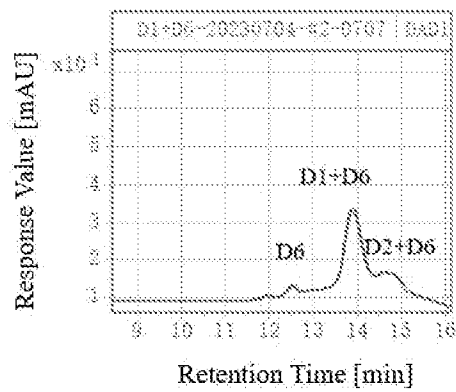


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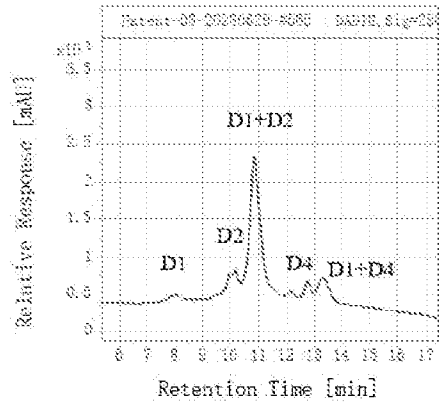


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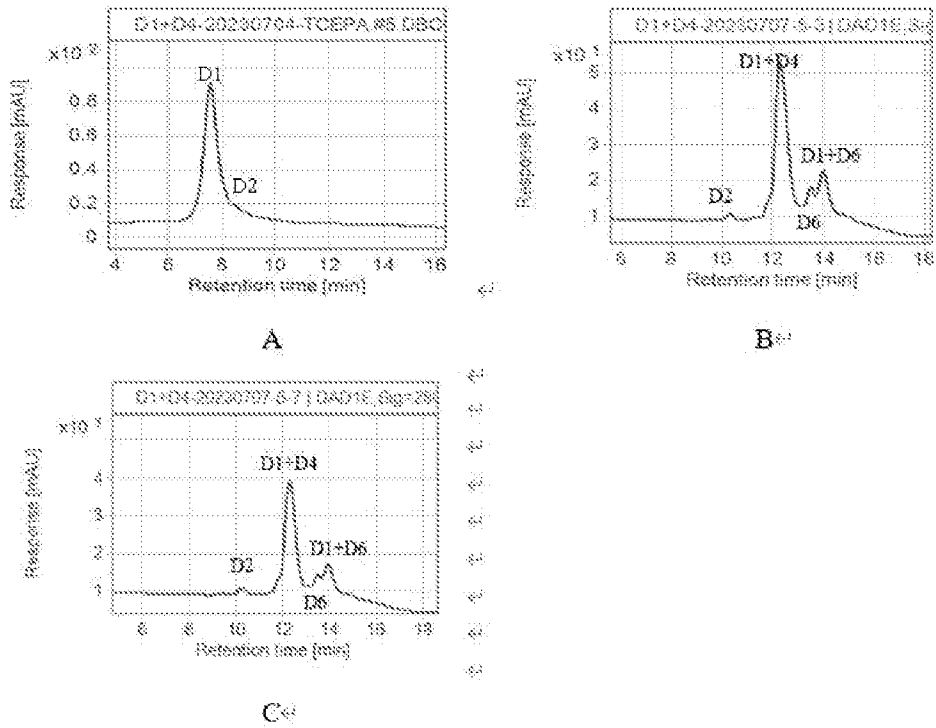


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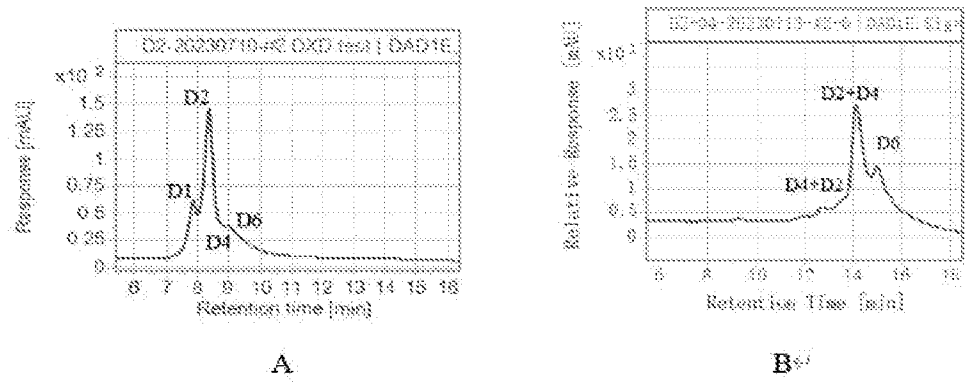


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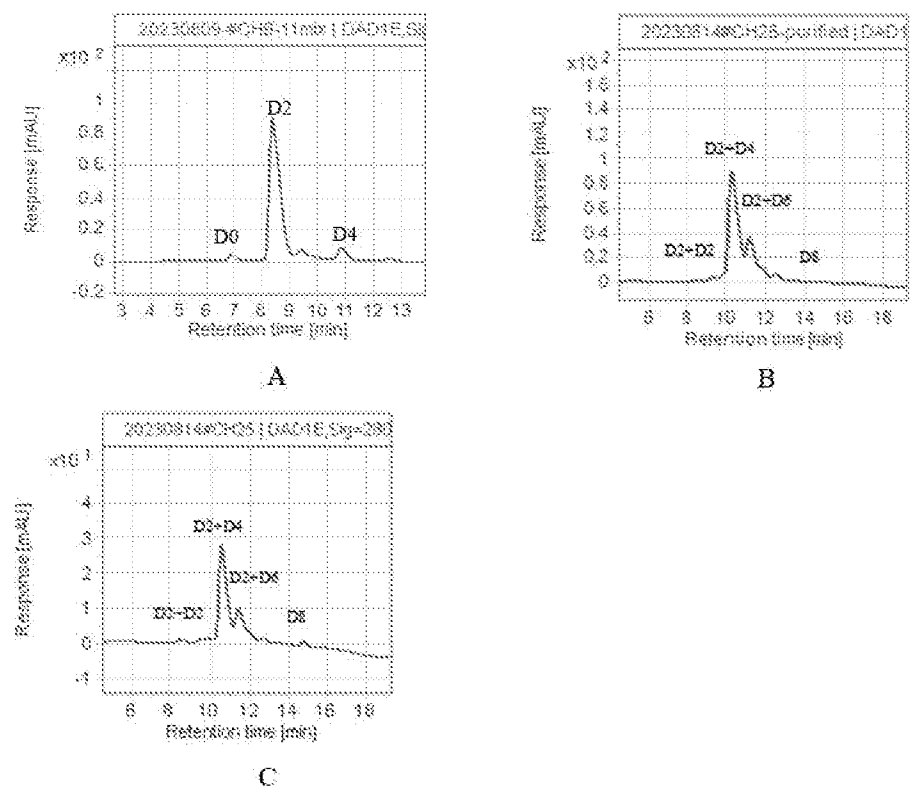


Figure 65

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2023/114320

A. CLASSIFICATION OF SUBJECT MATTER C07F 9/50(2006.01)i; C07K16/00(2006.01)i; C07K16/28(2006.01)i; C07K16/32(2006.01)i; A61K31/662(2006.01)i; A61K31/664(2006.01)i; A61P35/00(2006.01)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) IPC: C07F9/-; C07K16/-; A61K31/-; A61P35/- 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) CNKI,CNTXT,DWPI,ENTXT,STN(CAPLUS,REGISTRY); phosphine, TCEP, antibody, drug, conjugate, ADC, reductant, reduce, reduction, disulfide bond, transition metal, Zn, zinc, Cd, cadmium, Hg, mercury, Ni, nickel, Co, cobalt, carboxyethyl																	
C. DOCUMENTS CONSIDERED TO BE RELEVANT <table border="1"> <thead> <tr> <th>Category*</th> <th>Citation of document, with indication, where appropriate, of the relevant passages</th> <th>Relevant to claim No.</th> </tr> </thead> <tbody> <tr> <td>X</td> <td>CLINE, Daniel J. et al. "New water-solublephosphines as reductants of peptide and protein disulfide bonds: reactivity and membrane permeability" <i>Biochemistry</i>, Vol. 43, 12 November 2004 (2004-11-12), pages 15195-15203, especially page 15196 left column, paragraph 5 and right column, paragraph 2; figure 3 on page 15200; discussion section on page 15201</td> <td>1-13, 17-21</td> </tr> <tr> <td>X</td> <td>US 4315867 A (CHEMISCHE WERKE HUELS AKTIENGESELLSCHAFT) 16 February 1982 (1982-02-16) the whole document, especially column 10, lines 32-33 and 59; column 12, lines 28-29; column 13, table 1; examples 5 and 8-11</td> <td>1-16</td> </tr> <tr> <td>X</td> <td>CN 107922477 A (DAIICHI SANKYO CO LTD) 17 April 2018 (2018-04-17) the whole document, especially examples 5-7, paragraph 244 of the description</td> <td>55-58</td> </tr> <tr> <td>X</td> <td>HE, Pei Yang et al. "Late-stage peptide and protein modifications through phospho-Michael addition reaction" <i>Chem. Commun.</i>, Vol. 56, 14 September 2020 (2020-09-14), pages 12632-12635, especially support material, Scheme S10 on page S13, Scheme S4 on page S11, Scheme S3 on page S10</td> <td>1-13, 17-19</td> </tr> </tbody> </table>			Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	X	CLINE, Daniel J. et al. "New water-solublephosphines as reductants of peptide and protein disulfide bonds: reactivity and membrane permeability" <i>Biochemistry</i> , Vol. 43, 12 November 2004 (2004-11-12), pages 15195-15203, especially page 15196 left column, paragraph 5 and right column, paragraph 2; figure 3 on page 15200; discussion section on page 15201	1-13, 17-21	X	US 4315867 A (CHEMISCHE WERKE HUELS AKTIENGESELLSCHAFT) 16 February 1982 (1982-02-16) the whole document, especially column 10, lines 32-33 and 59; column 12, lines 28-29; column 13, table 1; examples 5 and 8-11	1-16	X	CN 107922477 A (DAIICHI SANKYO CO LTD) 17 April 2018 (2018-04-17) the whole document, especially examples 5-7, paragraph 244 of the description	55-58	X	HE, Pei Yang et al. "Late-stage peptide and protein modifications through phospho-Michael addition reaction" <i>Chem. Commun.</i> , Vol. 56, 14 September 2020 (2020-09-14), pages 12632-12635, especially support material, Scheme S10 on page S13, Scheme S4 on page S11, Scheme S3 on page S10	1-13, 17-19
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☒ 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 "D" document cited by the applicant in the international application "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 05 December 2023		Date of mailing of the international search report 08 December 2023															
Name and mailing address of the ISA/CN CHINA NATIONAL INTELLECTUAL PROPERTY ADMINISTRATION 6, Xitucheng Rd., Jimen Bridge, Haidian District, Beijing 100088, China		Authorized officer MA,Jin Telephone No. (+86) 010-62084479															

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2023/114320

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2022078524 A2 (HANGZHOU DAC BIOTECH CO LTD) 21 April 2022 (2022-04-21) the whole document	1-58

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2023/114320

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.: **59**
because they relate to subject matter not required to be searched by this Authority, namely:

Claim 59 relates to the prevention or treatment of diseases, and does not warrant an international search according to the criteria set out in PCT Rule 39.1(iv).
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/CN2023/114320

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
US	4315867	A	16 February 1982	DE	2902202	A1	11 September 1980
				DE	2902202	C2	21 January 1982
				EP	0014747	A1	03 September 1980
CN	107922477	A	17 April 2018	BR	112017027690	A2	09 October 2018
				IL	256558	A	28 February 2018
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				TWI	725037	B	21 April 2021
				IL	290959	A	01 April 2022
				IL	290959	B	01 December 2022
				IL	290959	B2	01 April 2023
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				EP	3315512	A4	13 February 2019
				EP	3315512	B1	23 November 2022
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				AU	2016286898	B2	08 December 2022
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				JP	6787890	B2	18 November 2020
				WO	2017002776	A1	05 January 2017
WO	2022078524	A2	21 April 2022	WO	2022078524	A3	25 August 2022
				WO	2022078524	A4	08 December 2022
				WO	2023078021	A1	11 May 2023
				WO	2023078273	A1	11 May 2023