

CROSS-REFERENCE TO RELATED APPLICATIONS

10 FIELD OF THE INVENTION

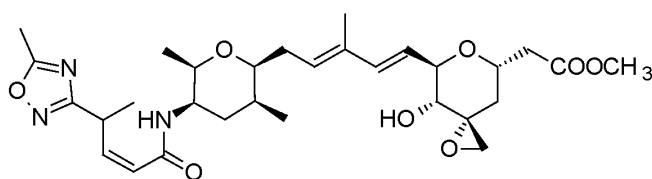
BACKGROUND OF THE INVENTION

Antibody-drug conjugates (ADCs) have demonstrated considerable utility as anti-cancer agents. In an ADC, a therapeutic agent (also referred to as the cytotoxic drug, payload, or toxin) is covalently linked to an antibody whose antigen is expressed by a cancer or other proliferative cell (tumor associated antigen); for example, an antigen expressed in the tumor, either directly on cancer cells, or on cells in the tumor microenvironment (tumor associated antigen). The antibody, by binding to the antigen, delivers the ADC to the cancer site. There, cleavage of the covalent link or degradation of the antibody leads to the release of the therapeutic agent. On the other hand, while the ADC is circulating in the blood system, the therapeutic agent is inactive because of its linkage to the antibody. Thus, the

therapeutic agent used in an ADC can be much more potent than ordinary chemotherapeutic agents because of its highly localized release.

While a number of different drug classes have been tried for delivery via antibodies, only a few drug classes have proved efficacious as antibody-drug conjugates, while having a suitable toxicity profile. One drug class includes analogs of FR901464, such as spliceostatin C and thailanstatin A, which are extremely potent inhibitors of eukaryotic RNA splicing. These compounds bind tightly to the SF3b subunit of the U2 snRNA subcomplex, an essential component of the spliceosome (Puthenveetil, S., *et al.*, *Bioconjugate Chem.*, **2016**, 27:1880-1888). International Patent Application Publication Number WO 20019/060398 discloses certain thailanstatin-based compounds that are reported to be useful as payloads in ADCs and payload-linker compounds useful in connection with ADCs. These ADCs are reported to be useful for treating diseases such as cancer.

International Patent Application Publication Number WO 2019/060398 also discloses synthetic methods as well as intermediates to prepare the compounds disclosed therein. One specific compound is a compound of the following formula III:

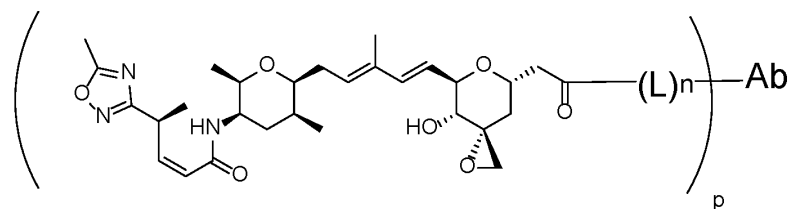


III

There is an ongoing need for novel antibody-drug conjugates to treat diseases such as cancer. There is also a need for novel drug moieties and antibodies that are useful to prepare antibody-drug conjugates. There is currently a need for synthetic methods and intermediates that can be used to prepare the compounds of formula 8* and formula I (or a diastereomer thereof), and salts thereof as well as other compounds and salts thereof described herein. There is also a need for improved methods for preparing intermediate compounds that can be used to prepare the compounds of formula 8* and formula I and salts thereof. The improved methods and intermediates may reduce the number of synthetic steps, cost, time, impurities, purification steps and/or the amount of waste associated with the existing methods for preparing one or more of the compounds described above.

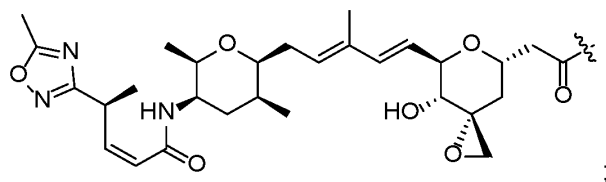
SUMMARY

One embodiment provides an antibody-drug conjugate compound comprising an antibody covalently attached to a drug moiety directly (i.e., without a linker) or through a linker of Formula I:



I

or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein the drug moiety is:



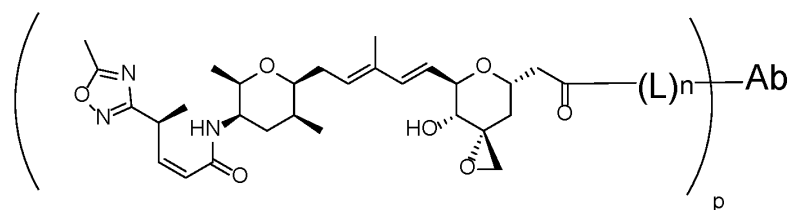
L is a linker;

n is 0 or 1;

p is an integer from 1 to 20 (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20); and

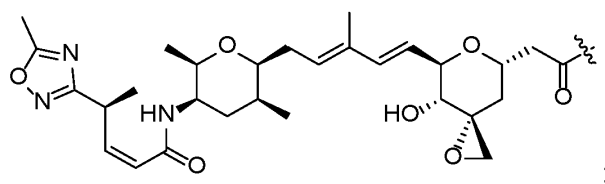
Ab is an antibody.

One embodiment provides an antibody-drug conjugate compound comprising an antibody covalently attached to a drug moiety directly (i.e., without a linker) or through a linker of Formula I:



I

or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein the drug moiety is:



L is a linker;

n is 0 or 1;

p is 1 or more (e.g., 1 to 10 e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10); and

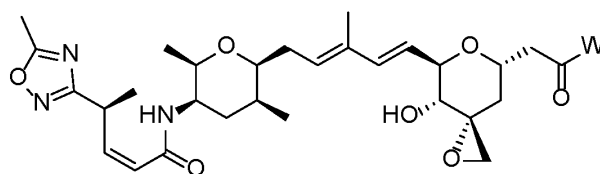
5 Ab is an antibody that binds to Trop2.

One embodiment provides an antibody or fragment thereof comprising an immunoglobulin heavy chain variable region polypeptide and an immunoglobulin light chain variable region polypeptide, wherein:

the immunoglobulin heavy chain variable region polypeptide comprises a complementarity determining region 1 (HCDR1) comprising an amino acid sequence that is at least 90% identical to NYGMN (SEQ ID NO: 14), a complementarity determining region 2 (HCDR2) comprising an amino acid sequence that is at least 90% identical to WINTKTGEPTYAQEFTG (SEQ ID NO: 21), and a complementarity determining region 3 (HCDR3) comprising an amino acid sequence that is at least 90% identical to GYGSSYWYFDV (SEQ ID NO: 18); and/or

the immunoglobulin light chain variable region polypeptide comprises a complementarity determining region 1 (LCDR1) comprising an amino acid sequence that is at least 90% identical to KASQDVSIABA (SEQ ID NO: 27), a complementarity determining region 2 (LCDR2) comprising an amino acid sequence that is at least 90% identical to SASYRYT (SEQ ID NO: 29), and a complementarity determining region 3 (LCDR3) comprising an amino acid sequence that is at least 90% identical to QQHYITPLT (SEQ ID NO: 31).

One embodiment provides a compound of formula II



II

or a salt thereof,

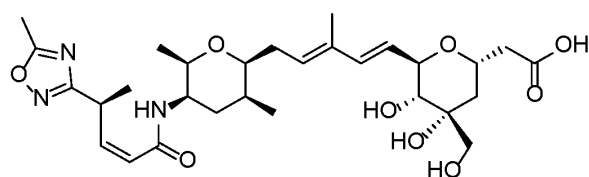
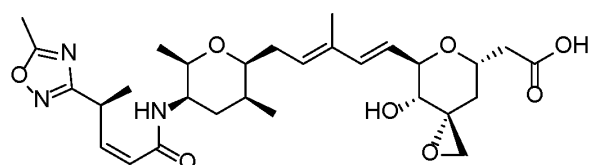
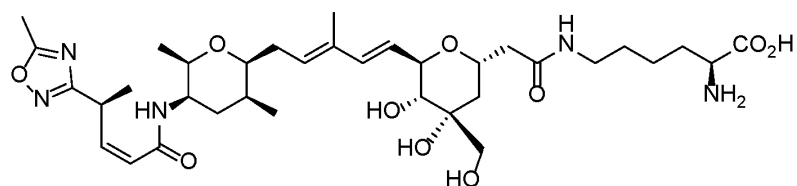
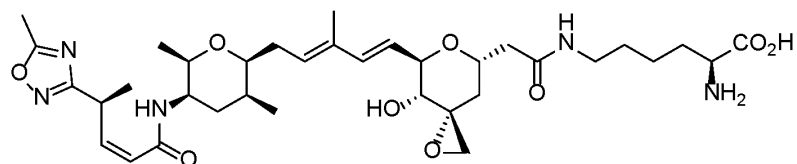
wherein

W is a leaving group or -L-X;

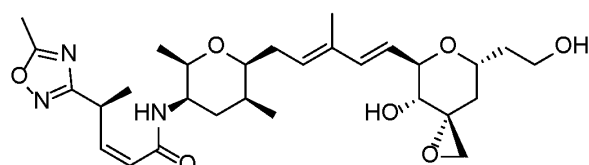
L is a linker; and

X is a reactive group.

One embodiment provides a compound of formula



, or



or a salt thereof (e.g., a pharmaceutically acceptable salt thereof).

One embodiment provides a pharmaceutical composition comprising an antibody-drug conjugate compound of Formula I, and a pharmaceutically acceptable diluent, carrier or excipient.

One embodiment provides method for treating cancer in a patient (e.g., a mammalian patient such as a human patient) in need thereof, comprising administering a therapeutically effective amount of the antibody drug conjugate compound of formula I as described herein or a pharmaceutically acceptable salt thereof, to the patient.

One embodiment provides method for treating cancer in a patient (e.g., a mammalian patient such as a human patient), comprising administering a therapeutically effective amount

of the antibody drug conjugate compound of formula I as described herein or a pharmaceutically acceptable salt thereof, to the patient.

One embodiment provides method for treating cancer in a mammal (e.g., a human) in need thereof, comprising administering a therapeutically effective amount of the antibody drug conjugate compound of formula I as described herein or a pharmaceutically acceptable salt thereof, to the mammal.

One embodiment provides method for treating cancer in a mammal (e.g., a human), comprising administering a therapeutically effective amount of the antibody drug conjugate compound of formula I as described herein or a pharmaceutically acceptable salt thereof, to the mammal.

One embodiment provides an antibody-drug conjugate compound of Formula I or a pharmaceutically acceptable salt as described herein for medical therapy.

One embodiment provides an antibody drug conjugate compound of formula I as described herein or a pharmaceutically acceptable salt thereof, for use in the therapeutic treatment of cancer.

One embodiment provides the use of an antibody-drug conjugate compound of Formula I or a pharmaceutically acceptable salt as described herein for the manufacture of a medicament for the treatment of cancer in a mammal (e.g., a human).

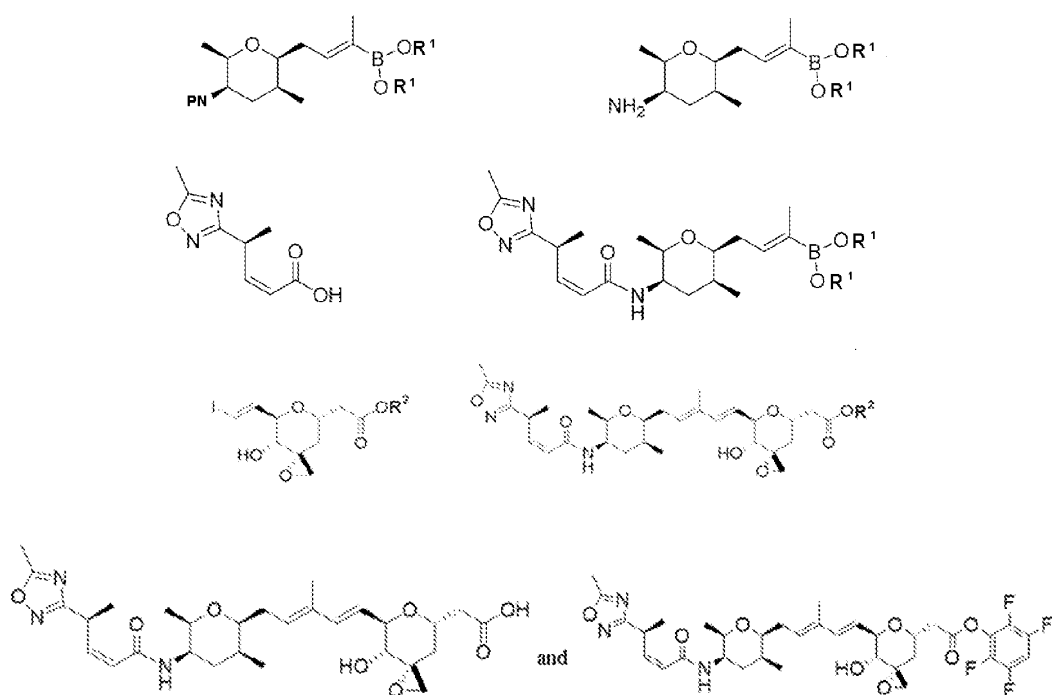
One embodiment provides an intermediate for making an antibody-drug conjugate compound of Formula I as described herein.

One embodiment provides a method for making an antibody-drug conjugate compound of Formula I or a compound of Formula II as described herein.

One embodiment provides an article of manufacture comprising a pharmaceutical composition comprising an antibody-drug conjugate compound of Formula I, a container, and a package insert or label indicating that the pharmaceutical composition can be used to treat cancer.

The present invention also provides new synthetic processes and synthetic intermediates that are useful for preparing the compound of formula 8* and I or salts thereof. The present invention also provides new synthetic processes and synthetic intermediates that are useful for preparing additional compounds as described here.

Accordingly, one embodiment provides a compound selected from the group consisting of:



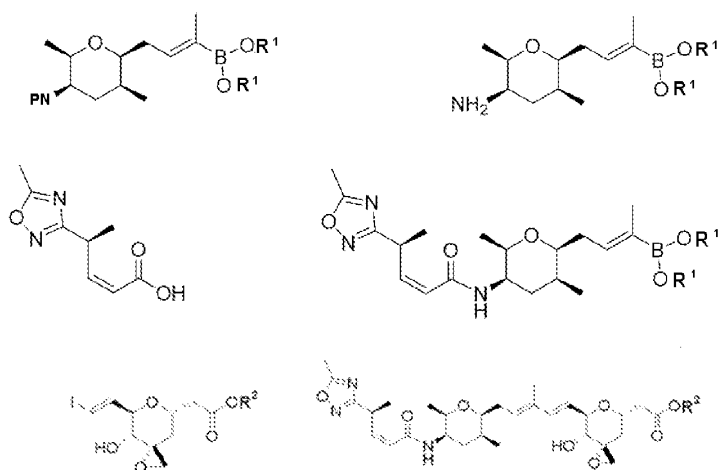
5 wherein:

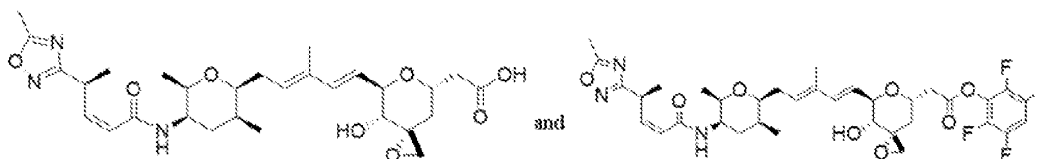
P is a nitrogen protecting group;

each R¹ is independently a (C₁-C₆)alkyl or two R¹ groups together with the atoms to which they are attached form a heterocyclyl optionally substituted with one or more (C₁-C₆)alkyl; and

10 R² is a (C₁-C₆)alkyl;
or a salt thereof.

One embodiment provides a compound selected from the group consisting of:





wherein:

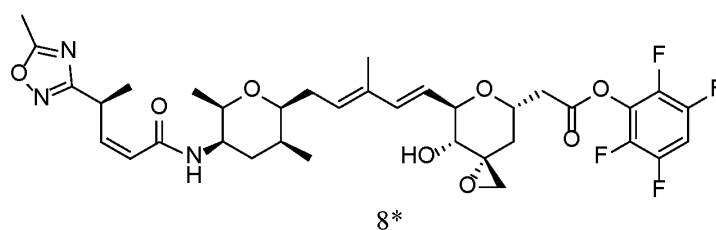
P is an amine nitrogen protecting group;

each R^1 is independently a (C₁-C₆)alkyl, or two R^1 groups together with the atoms to which they are attached form a heterocyclyl optionally substituted with one or more (C₁-C₆)alkyl; and

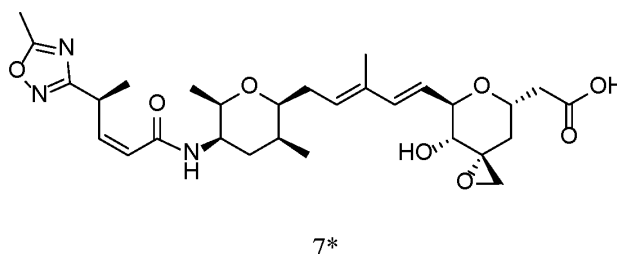
R^2 is a (C₁-C₆)alkyl or phenyl, wherein the phenyl is optionally substituted with one or more halo or nitro;

or a salt thereof.

One embodiment provides a method for preparing a compound of formula 8*:

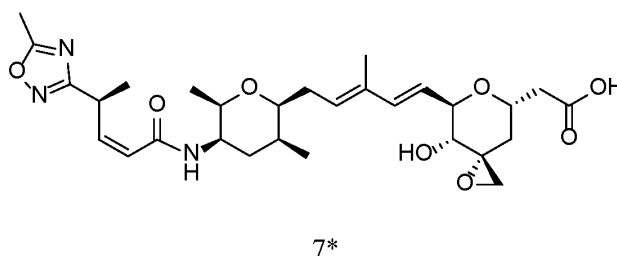


or a salt thereof, comprising converting a corresponding compound of formula 7*:

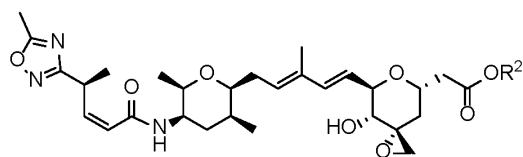


- 5 or a salt thereof, to the compound of formula 8* or a salt thereof.

One embodiment provides a method for preparing a compound of formula 7*:



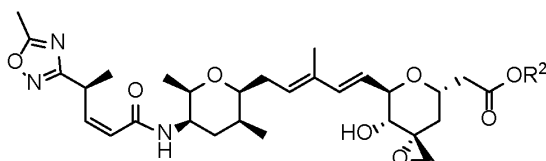
or a salt thereof, comprising converting a corresponding compound of formula 6a*:



6a*

or a salt thereof, to the compound of formula 7 or a salt thereof, wherein R² is a (C₁-C₆)alkyl.

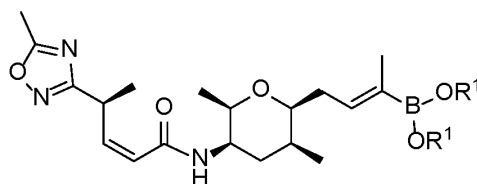
One embodiment provides a method for preparing a compound of formula 6a*:



6a*

5

or a salt thereof, comprising converting a corresponding compound of formula 5a*:



5a*

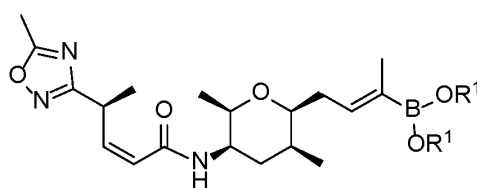
or a salt thereof, to the compound of formula 6a* or a salt thereof,

wherein:

10 each R¹ is independently a (C₁-C₆)alkyl or two R¹ groups together with the atoms to which they are attached form a heterocyclyl optionally substituted with one or more (C₁-C₆)alkyl; and

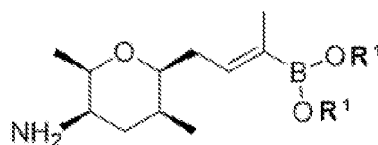
R² is a (C₁-C₆)alkyl.

One embodiment provides a method for preparing a compound of formula 5a*:



5a*

or a salt thereof, comprising converting a corresponding compound of formula 4a*:



4a*

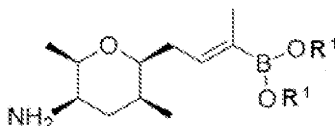
5 or a salt thereof, to the compound of formula 5a or a salt thereof,

wherein:

each R¹ is independently a (C₁-C₆)alkyl or two R¹ groups together with the atoms to which they are attached form a heterocyclyl optionally substituted with one or more (C₁-C₆)alkyl; and

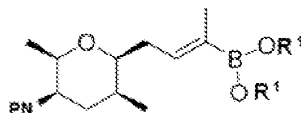
10 R² is a (C₁-C₆)alkyl.

One embodiment provides a method for preparing a compound of formula 4a*:



4a*

or a salt thereof, comprising converting a corresponding compound of formula 3a*:



3a*

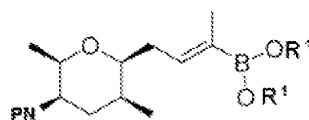
or a salt thereof, to the compound of formula 4a* or a salt thereof,

wherein:

P is an amine protecting group; and

each R¹ is independently a (C₁-C₆)alkyl or two R¹ groups together with the atoms to which they are attached form a heterocyclyl optionally substituted with one or more (C₁-C₆)alkyl.

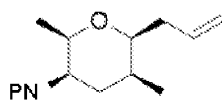
One embodiment provides a method for preparing a compound of formula 3a*:



5

3a*

or a salt thereof, comprising converting a corresponding compound of formula 2a*:



2a*

10 or a salt thereof, to the compound of formula 3a* or a salt thereof,

wherein:

P is an amine protecting group; and

each R¹ is independently a (C₁-C₆)alkyl or two R¹ groups together with the atoms to which they are attached form a heterocyclyl optionally substituted with one or more (C₁-C₆)alkyl.

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One embodiment provides novel synthetic intermediates described herein as well as methods for preparing such intermediates.

BRIEF DESCRIPTION OF THE DRAWINGS

20 Figures 1A-1B. Trop2 ADC linker screen. Fig. 1A shows that the lysine conjugates with non-cleavable linker have highest potency. The PH-1 family of linker-toxins (L-Ts) were conjugated to Trop2-specific monoclonal antibody (hRS7) and the resulting ADCs were tested for therapeutic efficacy against pre-implanted Trop2-expressing NCI-N87 gastric carcinoma tumors in mice. Non-cleavable Lysine linked PH-1 Linker-Toxins were associated

25 with maximal Tumor Growth Inhibition (TGI) *in vivo*. L2 (Lys Non-cleavable) DAR8 showed about 88% TGI. L2 (Lys Non-cleavable) DAR4 showed about 77% TGI. L22 (Lys Non-cleavable) showed about 82% TGI. L92 (Lys cleavable) showed about 68% TGI. L18

(Cys Non-cleavable) showed about 65% TGI. Fig.1B shows the experimental drug antibody ratios (DARs) for the ADCs.

Figures 2A-2E. Antibody binding profile and binding affinity for Trop2. Fig.2A shows the binding profile of the Daiichi mAb (TINA) having K_d (M): 2.46×10^{-8} . Fig.2B shows the binding profile of the Immunomedics mAb (hRS7) having K_d (M): 2.50×10^{-9} . Fig.2C shows the binding profile of M2.1 having K_d (M): 1.17×10^{-8} . Fig.2D shows the binding profile of M2.2 having K_d (M): 1.32×10^{-8} . Fig.2E shows the binding profile of M2.8 having K_d (M): 8.97×10^{-9} .

Figures 3A-3D. M2.1 and M2.8 mAbs recognize human and cynomolgus monkey Trop2. Antibodies were incubated with Chinese Hamster Ovary (CHO) cells that were transiently transfected with species-specific Trop2 cDNAs to test antibodies' binding profile to human, cynomolgus monkey, or mouse Trop2 proteins. Fig.3A shows M2.1 mAb binding profile. Fig.3B shows hRS7 (IMMU-132) mAb binding profile. Fig.3C shows REA916 Ab (positive control) binding profile. Fig.3D shows TINA (DS-1062) mAb binding profile.

Figure 4 shows the assessment for ability of fluorescently labeled Peak Trop2 antibody in binding gastric cancer cells that were pre-bound with Daiichi, or Immunomedics Trop2 antibodies. Moderate binding by Peak Bio mAb even after Trop2 was saturated with Daiichi mAb (TINA) suggests slightly different epitope or partial overlap of epitopes. No binding or steep drop-off by Peak Bio mAb (M2.8) after Trop2 was pre-bound with Immunomedics Trop2 antibody (hRS7) suggests potentially competitive binding for the same epitope. M2.8 mAb epitope is likely to be same as that of hRS7, although further confirmatory study is needed.

Figure 5 shows a representative gel electrophoresis characterization of Trop2 antibody and ADCs. M2.1 antibody (Lane 1) was conjugated at a DAR of 2 (Lane 2) or 4 (Lane 3) with Compound of formula 8*. Non-cleavable M2.1-formula 8* conjugates were compared with Cleavable ADCs using alternative linkers. Thailanstatin cysteine (Lane 4) or a lysine conjugate (Lane 5) were in turn compared with pharmaceutical grade Trodelvy (Lane 6) and Enhertu (Lane 7) that release the payload by a similar mechanism. The Lysine conjugated ADCs were more stable under non-reduced conditions.

Figures 6A-6F show mass spectra demonstrating efficient conjugation of linker toxin (compound 8* termed PH1) to Trop2 mAb and characterization of DAR2 and DAR4 ADCs. Figs. 6A-6C show Trop2 PH1 ADC (DAR2) with an actual DAR of 1.73 and conjugation efficiency of 67%. In particular, Fig.6A shows hydrophobic interaction chromatography (HIC) graph of Trop2 PH1 ADC (DAR2). Fig.6B shows size-exclusion chromatography

(SEC) graph of Trop2 PH1 ADC (DAR2). Fig.6C shows DAR population distribution as determined by mass spectrometry for Trop2 PH1 ADC (DAR2). Figs. 6D-6F show Trop2 PH1 ADC (DAR4) with an actual DAR of 3.62 and conjugation efficiency of 67%. In particular, Fig.6D shows hydrophobic interaction chromatography (HIC) graph of Trop2 PH1 ADC (DAR4). Fig.6E shows size-exclusion chromatography (SEC) graph of Trop2 PH1 ADC (DAR4). Fig.6F shows DAR population distribution as determined by mass spectrometry for Trop2 PH1 ADC (DAR4).

Figure 7 shows internalization of Trop2 mABs following engagement with target receptors in NCI-N87 cells. Internalization assay was performed with Trop2 mAbs: hRS7, M2.1, M2.3, T6-16, TINA. Control Ab used was HuLys 11 targeting chicken lysozyme. T6-16 and TINA appear to internalize more rapidly, but T6-16 shows the highest internalization percentage.

Figure 8 shows internalization of TROP2 ADCs that specifically bind and target human Trop2. As cell-surface Trop2 in human cancer cell lines can be proteolytically cleaved and certain antibody-binding epitopes may be lost, internalization of anti-hTrop2 ADCs were tested on a mouse cell line, CT26, stably overexpressing full-length human Trop2. Percent internalization was calculated using formula: $[V(tx)/M(tx)] - [V(t0)/M(t0)] * 100$ where V is the GMFI of quenched samples (at given time point), and M is the max GMFI/signal when there is 0 quenching (at given time point). Internalization and accumulation of M2.1 and M2.8 ADCs were comparable in CT26 cells overexpressing hTrop2.

Figure 9 shows the payload superiority of PH1 (when conjugated on IMMU-132 antibody hRS7) over SN-38 on IMMU-132 antibody (spider plots, n=10 per arm). PH1 was conjugated on an anti-RSV antibody as a control ADC.

Figure 10 shows the superiority of Trop-2 PH1 ADC over IMMU-132 (spider plots, n=10 per arm). PH1 was conjugated on an anti-RSV antibody as a control ADC.

Figure 11 shows that all tested Trop2 PH1 ADCs regress individual tumors at 3 mg/kg. PH1 was conjugated on an anti-RSV antibody as a control ADC.

Figure 12 shows tested Trop2 ADCs. M2.1 PH1 showed higher TGI than M2.3 PH1. For M2.1 PH1 and M2.3 PH1, DAR4 showed higher TGI than DAR2.

Figure 13 shows that Trop2 PH1 ADC (M2.8 PH1) demonstrated nanomolar potency *in vitro* against various cancer cell lines in multiple indications.

Figures 14A-14F show ADCs targeting Trop2 have differentiated on-target and off-target activities. Figs. 14A-14C show differentiated ADC Targeting Trop2 on-target activity.

In particular, Fig. 14A shows cytotoxicity data using NCI-N87 Gastric cell line (Trop2^{high}). Fig. 14B shows cytotoxicity data using BxPC3 Pancreatic cell line (Trop2^{high}). Fig. 14C shows cytotoxicity data using RT112/84 bladder cell line (Trop2^{heterogenous}). Figs. 14D-14F shows differentiated ADC Targeting Trop2 off-target activity. In particular, Fig. 14D shows cytotoxicity data using 786-0 cell line (Trop2^{negative}). Fig. 14E shows cytotoxicity data using BJ normal human fibroblast cells. Fig. 14F shows cytotoxicity data using HS27 normal human fibroblast cells.

Figure 15 shows Trop2 PH1 ADC at 3mg/kg QWx2 Dosing Regimen was associated with highest TGI. In a Trop2 positive tumor model, mice were administered with ADC at different doses (e.g., 0.5, 1, or 3mg/kg) and regimens (e.g., SD, QW x 2, or QW x 3). Tumor growth inhibition (TGI) were monitored. In this study, DAR4 ADC treatments had higher TGI as compared to DAR2 ADC treatments. There was also dose dependent and/or regimen dependent TGI observed (3mg/kg > 1mg/kg > 0.5mg/kg; and QW x 2 > SD).

Figure 16 shows Trop2 PH1 ADC could achieve durable tumor regression at lower drug-antibody ratio (DAR). In a Trop2 positive tumor model (nude mice bearing human NCI-N87 gastric tumors with expression level IHC3+), mice were dosed at dosing regimen of QW for 2 weeks (QWx2). Horizontal dotted line indicates mean tumor volume of 200 mm³ size at which treatment was initiated. Tumor shrinkage below this line was considered regression. Tumor sizes were monitored. Long term tumor regression (TR) was observed in 50% of treated mice over a period of about 5 months in the group treated with Trop2 PH1 (DAR4) ADC at 3mg/kg. Stable disease was observed in 50% of treated mice over a period of about 5 months in the group treated with Trop2 PH1 (DAR2) ADC at 3mg/kg. Both treatment groups of Trop2 PH1 outperform the treatment group of IMMU-132* at lower DAR (2 or 4 vs 7.6) and/or at lower dose (3mg/kg vs 10 mg/kg). IMMU-132* represents clinical grade IMMU-132 or Sacituzumab govitecan.

Figure 17 shows the cell viability activity (HCT116 cell line) of compound 2A (also referred to as ThA13D2L2) versus compound 2B (also referred to as ThA13D1L2) and the diastomeric mixture (also referred to a ThA13L2).

Figure 18 shows certain exemplary structures of linker-toxins.

Figure 19 shows a linker screening study, antibody binding affinity and antibody internalization assays.

Figure 20 shows comparison of TGI efficacies of ADCs, and antibody cross-species binding profile.

Figures 21A-21B show differentiated NHP safety profile for Trop2 PH1 ADCs.

Figure 22 shows low off-target activity, and *in vitro* potency in various indications.

Figure 23 shows *in vivo* potency of M2.8 PH1 ADC.

Figures 24A-24B show the NMR spectra for compound 3*. Figure 24A shows the proton NMR spectrum of compound 3* and figure 24B shows the carbon NMR spectrum of compound 3*.

Figures 25A-25B show the NMR spectra for compound 4*. Figure 25A shows the proton NMR spectrum of compound 4* and figure 25B shows the carbon NMR spectrum of compound 4*.

Figures 26A-26B show the NMR spectra for compound 5*. Figure 26A shows the proton NMR spectrum of compound 5* and figure 26B shows the carbon NMR spectrum of compound 5*.

Figures 27A-27B show the NMR spectra for compound 6*. Figure 27A shows the proton NMR spectrum of compound 6* and figure 27B shows the carbon NMR spectrum of compound 6*.

Figure 28 shows the proton NMR spectrum of compound 7*.

Figures 29A-29B show the NMR spectra for compound 8*. Figure 29A shows the proton NMR spectrum of compound 8* and figure 29B shows the carbon NMR spectrum of compound 8*.

Figures 30A – 30E shows MDR1-low MES-SA uterine sarcoma cells and isogenic MDR1-high MES-SA/ MX2 cells were grown in the presence or absence of different ADC payloads or their active payload species. Figure 30A shows ThA13 d2 methyl ester, which is a membrane-permeable representative for the lysine conjugates. The lysine adduct is impermeable. Figure 30B shows ThA13 d2 301 (alcohol) species that is released by the cysteine linkers. Figure 30C shows MMAE (vedotin ADCs). Figure 30D shows SN38 (e.g. Trodelvy). Figure 30E shows DXd (e.g. DS-1062). Chemical structures are provided in inset along with their corresponding graphs. In addition, MES-SA/ MX2 cells were also grown in the presence or absence of MDR1-specific inhibitors Elacridar and Tariquidar and MDR1-plus CYP 3A4- inhibitor Valspodar that block the p-Glycoproteins from pumping out the ADC payloads in the cell line expressing high levels of MDR1.

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DETAILED DESCRIPTION

Reference will now be made in detail to certain embodiments of the invention, examples of which are illustrated in the accompanying structures and formulas. While the invention will be described in conjunction with the illustrated embodiments, it will be

understood that they are not intended to limit the invention to those embodiments. On the contrary, the invention is intended to cover all alternatives, modifications, and equivalents, which may be included within the scope of the present invention as defined by the claims.

One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. The present invention is in no way limited to the methods and materials described.

Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs, and are consistent with: Singleton et al (1994) Dictionary of Microbiology and Molecular Biology, 2nd Ed., J. Wiley & Sons, New York, NY; and Janeway, C., Travers, P., Walport, M., Shlomchik (2001) Immunobiology, 5th Ed., Garland Publishing, New York.

DEFINITIONS

Unless stated otherwise, the following terms and phrases as used herein are intended to have the following meanings:

When trade names are used herein, applicants intend to independently include the trade name product formulation, the generic drug, and the active pharmaceutical ingredient(s) of the trade name product.

An “acceptor human framework” for the purposes herein is a framework comprising the amino acid sequence of a light chain variable domain (VL) framework or a heavy chain variable domain (VH) framework derived from a human immunoglobulin framework or a human consensus framework, as defined below. An acceptor human framework “derived from” a human immunoglobulin framework or a human consensus framework may comprise the same amino acid sequence thereof, or it may contain amino acid sequence changes. In some embodiments, the number of amino acid changes are 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, or 2 or less. In some embodiments, the VL acceptor human framework is identical in sequence to the VL human immunoglobulin framework sequence or human consensus framework sequence.

“Affinity” refers to the strength of the sum total of noncovalent interactions between a single binding site of a molecule (*e.g.*, an antibody) and its binding partner (*e.g.*, an antigen). Unless indicated otherwise, as used herein, “binding affinity” refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (*e.g.*, antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (K_d). Affinity can be measured by common methods known in the

art, including those described herein. Specific illustrative and exemplary embodiments for measuring binding affinity are described in the following.

In certain embodiments, an antibody as described herein has dissociation constant (K_d) of $\leq 1\mu\text{M}$, $\leq 100\text{ nM}$, $\leq 10\text{ nM}$, $\leq 5\text{ nM}$, $\leq 4\text{ nM}$, $\leq 3\text{ nM}$, $\leq 2\text{ nM}$, $\leq 1\text{ nM}$, $\leq 0.1\text{ nM}$, $\leq 0.01\text{ nM}$, or $\leq 0.001\text{ nM}$ (*e.g.*, 10^{-8} M or less, *e.g.*, from 10^{-8} M to 10^{-13} M , *e.g.*, from 10^{-9} M to 10^{-13} M).

An “affinity matured” antibody refers to an antibody with one or more alterations in one or more hypervariable regions (HVRs), compared to a parent antibody which does not possess such alterations, such alterations resulting in an improvement in the affinity of the antibody for antigen.

The term “antibody” is used herein in the broadest sense and encompasses various antibody structures, including but not limited to monoclonal antibodies, polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), and antibody fragments so long as they exhibit the desired antigen-binding activity.

An “antibody fragment” refers to a molecule other than an intact antibody that comprises a portion of an intact antibody and that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to F_v, Fab, Fab', Fab'-SH, F(ab')₂; diabodies; linear antibodies; single-chain antibody molecules (*e.g.*, scFv); and multispecific antibodies formed from antibody fragments.

The terms “cancer” and “cancerous” refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth/proliferation. A “tumor” comprises one or more cancerous cells. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia or lymphoid malignancies. More particular examples of such cancers 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 term “early stage breast cancer (EBC)” or “early breast cancer” is used herein to refer to breast cancer that has not spread beyond the breast or the axillary lymph nodes. This

includes ductal carcinoma in situ and stage I, stage IIA, stage IIB, and stage IIIA breast cancers.

Reference to a tumor or cancer as a “Stage 0,” “Stage I,” “Stage II,” “Stage III,” or “Stage IV”, and various sub-stages within this classification, indicates classification of the tumor or cancer using the Overall Stage Grouping or Roman Numeral Staging methods known in the art. Although the actual stage of the cancer is dependent on the type of cancer, in general, a Stage 0 cancer is an in situ lesion, a Stage I cancer is small localized tumor, a Stage II and III cancer is a local advanced tumor which exhibits involvement of the local lymph nodes, and a Stage IV cancer represents metastatic cancer. The specific stages for each type of tumor are known to the skilled clinician.

The term “metastatic breast cancer” means the state of breast cancer where the cancer cells are transmitted from the original site to one or more sites elsewhere in the body, by the blood vessels or lymphatics, to form one or more secondary tumors in one or more organs besides the breast.

An “advanced” cancer is one which has spread outside the site or organ of origin, either by local invasion or metastasis. Accordingly, the term “advanced” cancer includes both locally advanced and metastatic disease.

A “recurrent” cancer is one which has regrown, either at the initial site or at a distant site, after a response to initial therapy, such as surgery.

A “locally recurrent” cancer is cancer that returns after treatment in the same place as a previously treated cancer.

An “operable” or “resectable” cancer is cancer which is confined to the primary organ and suitable for surgery (resection).

A “non-resectable” or “unresectable” cancer is not able to be removed (resected) by surgery.

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.

The “class” of an antibody refers to the type of constant domain or constant region possessed by its heavy chain. There are five major classes of antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.*, IgG₁, IgG₂, IgG₃, IgG₄, IgA₁, and IgA₂. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α , δ , ϵ , γ , and μ , respectively.

The term “cytotoxic agent” as used herein refers to a substance that inhibits or prevents a cellular function and/or causes cell death or destruction. Cytotoxic agents include, but are not limited to, radioactive isotopes (*e.g.*, At²¹¹, I¹³¹, I¹²⁵, Y⁹⁰, Re¹⁸⁶, Re¹⁸⁸, Sm¹⁵³, Bi²¹², P³², Pb²¹² and radioactive isotopes of Lu); chemotherapeutic agents or drugs (*e.g.*,
5 methotrexate, adriamycin, vinca alkaloids (vincristine, vinblastine, etoposide), doxorubicin, melphalan, mitomycin C, chlorambucil, daunorubicin or other intercalating agents); growth inhibitory agents; enzymes and fragments thereof such as nucleolytic enzymes; antibiotics; toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof; and the various antitumor or
10 anticancer agents disclosed below.

“Effector functions” refer to those biological activities attributable to the Fc region of an antibody, which vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of
15 cell surface receptors (*e.g.*, B cell receptor); and B cell activation.

An “effective amount” of an agent, *e.g.*, a pharmaceutical formulation, refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result. The effective amount of the drug for treating cancer may reduce the number of cancer cells; reduce the tumor size; inhibit (*i.e.*, slow to some extent
20 and preferably stop) cancer cell infiltration into peripheral organs; inhibit (*i.e.*, slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the cancer. To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. The effective amount may extend progression free survival (*e.g.*, as measured by
25 Response Evaluation Criteria for Solid Tumors, RECIST, or CA-125 changes), result in an objective response (including a partial response, PR, or complete response, CR), increase overall survival time, and/or improve one or more symptoms of cancer (*e.g.* as assessed by FOSI).

The term “epitope” refers to the particular site on an antigen molecule to which an
30 antibody binds.

The term “Fc region” herein is used to define a C-terminal region of an immunoglobulin heavy chain that contains at least a portion of the constant region. The term includes native sequence Fc regions and variant Fc regions. In one embodiment, a human IgG heavy chain Fc region extends from Cys226, or from Pro230, to the carboxyl-terminus of the

heavy chain. However, the C-terminal lysine (Lys447) of the Fc region may or may not be present. Unless otherwise specified herein, numbering of amino acid residues in the Fc region or constant region is according to the EU numbering system, also called the EU index, as described in Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD, 1991.

“Framework” or “FR” refers to variable domain residues other than hypervariable region (HVR) residues. The FR of a variable domain generally consists of four FR domains: FR1, FR2, FR3, and FR4. Accordingly, the HVR and FR sequences generally appear in the following sequence in VH (or VL): FR1-H1(L1)-FR2-H2(L2)-FR3-H3(L3)-FR4.

The terms “full length antibody,” “intact antibody,” and “whole antibody” are used herein interchangeably to refer to an antibody having a structure substantially similar to a native antibody structure or having heavy chains that contain an Fc region as defined herein.

The terms “host cell,” “host cell line,” and “host cell culture” are used interchangeably and refer to cells into which exogenous nucleic acid has been introduced, including the progeny of such cells. Host cells include “transformants” and “transformed cells,” which include the primary transformed cell and progeny derived therefrom without regard to the number of passages. Progeny may not be completely identical in nucleic acid content to a parent cell, but may contain mutations. Mutant progeny that have the same function or biological activity as screened or selected for in the originally transformed cell are included herein.

A “human antibody” is 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.

A “human consensus framework” is a framework which represents the most commonly occurring amino acid residues in a selection of human immunoglobulin VL or VH framework sequences. Generally, the selection of human immunoglobulin VL or VH sequences is from a subgroup of variable domain sequences. Generally, the subgroup of sequences is a subgroup as in Kabat et al., *Sequences of Proteins of Immunological Interest*, Fifth Edition, NIH Publication 91-3242, Bethesda MD (1991), vols. 1-3. In one embodiment, for the VL, the subgroup is subgroup kappa I as in Kabat et al., *supra*. In one embodiment, for the VH, the subgroup is subgroup III as in Kabat et al., *supra*.

A “humanized” antibody refers to a chimeric antibody comprising amino acid residues from non-human HVRs and amino acid residues from human FRs. In certain embodiments, a humanized antibody will comprise substantially all of 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 comprise 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.

The term “hypervariable region” or “HVR,” as used herein, refers to each of the regions of an antibody variable domain which are hypervariable in sequence and/or form structurally defined loops (“hypervariable loops”). Generally, native four-chain antibodies comprise six HVRs; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). HVRs generally comprise amino acid residues from the hypervariable loops and/or from the “complementarity determining regions” (CDRs), the latter being of highest sequence variability and/or involved in antigen recognition.

An “immunoconjugate” is an antibody conjugated to one or more heterologous molecule(s), including but not limited to a cytotoxic agent.

A “patient” or “individual” or “subject” is a mammal. Mammals include, but are not limited to, domesticated animals (*e.g.*, cows, sheep, cats, dogs, and horses), primates (*e.g.*, humans and non-human primates such as monkeys), rabbits, and rodents (*e.g.*, mice and rats). In certain embodiments, the patient, individual, or subject is a human. In some embodiments, the patient may be a “cancer patient,” *i.e.* one who is suffering or at risk for suffering from one or more symptoms of cancer, in particular gastric or breast cancer.

A “patient population” refers to a group of cancer patients. Such populations can be used to demonstrate statistically significant efficacy and/or safety of a drug.

A “relapsed” patient is one who has signs or symptoms of cancer after remission. Optionally, the patient has relapsed after adjuvant or neoadjuvant therapy.

A cancer or biological sample which “displays HER expression, amplification, or activation” is one which, in a diagnostic test, expresses (including overexpresses) a HER receptor, has amplified HER gene, and/or otherwise demonstrates activation or phosphorylation of a HER receptor.

“Neoadjuvant therapy” or “preoperative therapy” herein refers to therapy given prior to surgery. The goal of neoadjuvant therapy is to provide immediate systemic treatment,

potentially eradicating micrometastases that would otherwise proliferate if the standard sequence of surgery followed by systemic therapy were followed. Neoadjuvant therapy may also help to reduce tumor size thereby allowing complete resection of initially unresectable tumors or preserving portions of the organ and its functions. Furthermore, neoadjuvant
5 therapy permits an in vivo assessment of drug efficacy, which may guide the choice of subsequent treatments.

“Adjuvant therapy” herein refers to therapy given after definitive surgery, where no evidence of residual disease can be detected, so as to reduce the risk of disease recurrence. The goal of adjuvant therapy is to prevent recurrence of the cancer, and therefore to reduce
10 the chance of cancer-related death. Adjuvant therapy herein specifically excludes neoadjuvant therapy.

“Definitive surgery” is used as that term is used within the medical community. Definitive surgery includes, for example, procedures, surgical or otherwise, that result in removal or resection of the tumor, including those that result in the removal or resection of all
15 grossly visible tumor. Definitive surgery includes, for example, complete or curative resection or complete gross resection of the tumor. Definitive surgery includes procedures that occur in one or more stages, and includes, for example, multi-stage surgical procedures where one or more surgical or other procedures are performed prior to resection of the tumor. Definitive surgery includes procedures to remove or resect the tumor including involved
20 organs, parts of organs and tissues, as well as surrounding organs, such as lymph nodes, parts of organs, or tissues. Removal may be incomplete such that tumor cells might remain even though undetected.

“Survival” refers to the patient remaining alive, and includes disease free survival (DFS), progression free survival (PFS) and overall survival (OS). Survival can be estimated
25 by the Kaplan-Meier method, and any differences in survival are computed using the stratified log-rank test.

“Progression-Free Survival” (PFS) is the time from the first day of treatment to documented disease progression (including isolated CNS progression) or death from any cause on study, whichever occurs first.

30 “Disease free survival (DFS)” refers to the patient remaining alive, without return of the cancer, for a defined period of time such as about 1 year, about 2 years, about 3 years, about 4 years, about 5 years, about 10 years, etc., from initiation of treatment or from initial diagnosis. In one aspect of the invention, DFS is analyzed according to the intent-to-treat principle, i.e., patients are evaluated on the basis of their assigned therapy. The events used in

the analysis of DFS can include local, regional and distant recurrence of cancer, occurrence of secondary cancer, and death from any cause in patients without a prior event (e.g, breast cancer recurrence or second primary cancer).

“Overall survival” refers to the patient remaining alive for a defined period of time, such as about 1 year, about 2 years, about 3 years, about 4 years, about 5 years, about 10 years, etc., from initiation of treatment or from initial diagnosis. In the studies underlying the invention the event used for survival analysis was death from any cause.

By “extending survival” is meant increasing DFS and/or OS in a treated patient relative to an untreated patient, or relative to a control treatment protocol. Survival is monitored for at least about six months, or at least about 1 year, or at least about 2 years, or at least about 3 years, or at least about 4 years, or at least about 5 years, or at least about 10 years, etc., following the initiation of treatment or following the initial diagnosis.

By “monotherapy” is meant a therapeutic regimen that includes only a single therapeutic agent for the treatment of the cancer or tumor during the course of the treatment period.

By “maintenance therapy” is meant a therapeutic regimen that is given to reduce the likelihood of disease recurrence or progression. Maintenance therapy can be provided for any length of time, including extended time periods up to the life-span of the subject. Maintenance therapy can be provided after initial therapy or in conjunction with initial or additional therapies. Dosages used for maintenance therapy can vary and can include diminished dosages as compared to dosages used for other types of therapy.

An “isolated antibody” is one which has been separated from a component of its natural environment. In some embodiments, an antibody is purified to greater than 95% or 99% purity as determined by, for example, electrophoretic (e.g., SDS-PAGE, isoelectric focusing (IEF), capillary electrophoresis) or chromatographic (e.g., ion exchange or reverse phase HPLC). For review of methods for assessment of antibody purity, *see, e.g.,* Flatman et al., *J. Chromatogr. B* 848:79-87 (2007).

An “isolated nucleic acid” refers to a nucleic acid molecule that has been separated from a component of its natural environment. An isolated nucleic acid includes a nucleic acid molecule contained in cells that ordinarily contain the nucleic acid molecule, but the nucleic acid molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location.

“Isolated nucleic acid encoding an antibody” refers to one or more nucleic acid molecules encoding antibody heavy and light chains (or fragments thereof), including such

nucleic acid molecule(s) in a single vector or separate vectors, and such nucleic acid molecule(s) present at one or more locations in a host cell.

The term “monoclonal antibody” as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical and/or bind the same epitope, except for possible variant antibodies, *e.g.*, containing naturally occurring mutations or arising during production of a monoclonal antibody preparation, such variants generally being present in minor amounts. In contrast to polyclonal antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. Thus, the modifier “monoclonal” indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including but not limited to the hybridoma method, recombinant DNA methods, phage-display methods, and methods utilizing transgenic animals containing all or part of the human immunoglobulin loci, such methods and other exemplary methods for making monoclonal antibodies being described herein.

A “naked antibody” refers to an antibody that is not conjugated to a heterologous moiety (*e.g.*, a cytotoxic moiety) or radiolabel. The naked antibody may be present in a pharmaceutical formulation.

“Native antibodies” refer to naturally occurring immunoglobulin molecules with varying structures. For example, native IgG antibodies are heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light chains and two identical heavy chains that are disulfide-bonded. From N- to C-terminus, each heavy chain has a variable region (VH), also called a variable heavy domain or a heavy chain variable domain, followed by three constant domains (CH1, CH2, and CH3). Similarly, from N- to C-terminus, each light chain has a variable region (VL), also called a variable light domain or a light chain variable domain, followed by a constant light (CL) domain. The light chain of an antibody may be assigned to one of two types, called kappa (κ) and lambda (λ), based on the amino acid sequence of its constant domain.

A “vial” is a container suitable for holding a liquid or lyophilized preparation. In one embodiment, the vial is a single-use vial, *e.g.* a 20-cc single-use vial with a stopper.

The term “package insert” is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, combination therapy, contraindications and/or warnings concerning the use of such therapeutic products.

- 5 “Percent (%) amino acid sequence identity” with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity.
- 10 Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, Clustal X, Clustal W, ALIGN or Megalign (DNASTAR) software.

- 15 The term “pharmaceutical formulation” refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered.

- A “pharmaceutically acceptable carrier” refers to an ingredient in a pharmaceutical
20 formulation, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative.

- As used herein, “treatment” (and grammatical variations thereof such as “treat” or “treating”) refers to clinical intervention in an attempt to alter the natural course of the
25 individual being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include, but are not limited to, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or
30 improved prognosis. In some embodiments, antibodies of the invention are used to delay development of a disease or to slow the progression of a disease.

 By “co-administering” is meant intravenously administering two (or more) drugs during the same administration, rather than sequential infusions of the two or more drugs.

Generally, this will involve combining the two (or more) drugs into the same IV bag prior to co-administration thereof.

A drug that is administered “concurrently” with one or more other drugs is administered during the same treatment cycle, on the same day of treatment as the one or more other drugs, and, optionally, at the same time as the one or more other drugs. For instance, for cancer therapies given every 3 weeks, the concurrently administered drugs are each administered on day-1 of a 3-week cycle.

A "chemotherapeutic agent" refers to a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclophosphamide (CYTOXAN®); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylmelamine; acetogenins (especially bullatacin and bullatacinone); delta-9-tetrahydrocannabinol (dronabinol, MARINOL®); beta-lapachone; lapachol; colchicines; betulinic acid; a camptothecin (including the synthetic analogue topotecan (HYCAMTIN®), CPT-11 (irinotecan, CAMPTOSAR®), acetylcamptothecin, scoplectin, and 9-aminocamptothecin); bryostatin; calystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); podophyllotoxin; podophyllinic acid; teniposide; cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, chlorophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosoureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics such as the enediyne antibiotics (*e.g.*, calicheamicin, especially calicheamicin gammaII and calicheamicin omegaII (*see, e.g.*, Nicolaou et al., *Angew. Chem Intl. Ed. Engl.*, 33: 183-186 (1994))); CDP323, an oral alpha-4 integrin inhibitor; dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabycin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including ADRIAMYCIN®, morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin, doxorubicin HCl liposome injection (DOXIL®),

liposomal doxorubicin TLC D-99 (MYOCET®), pegylated liposomal doxorubicin (CAELYX®), and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, porfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, 5 tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate, gemcitabine (GEMZAR®), tegafur (UFTORAL®), capecitabine (XELODA®), an epothilone, and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, 10 cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitio stanol, mepitio stanane, testolactone; anti-adrenals such as aminogluthethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diazi quone; 15 elfornithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, OR); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triazi quone; 20 2,2',2'-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine (ELDISINE®, FILDESIN®); dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); thiotepa; taxoid, *e.g.*, paclitaxel (TAXOL®), albumin-engineered nanoparticle formulation of paclitaxel (ABRAXANETM), and docetaxel (TAXOTERE®); chloranbucil; 6-thioguanine; 25 mercaptopurine; methotrexate; platinum agents such as cisplatin, oxaliplatin (*e.g.*, ELOXATIN®), and carboplatin; vincas, which prevent tubulin polymerization from forming microtubules, including vinblastine (VELBAN®), vincristine (ONCOVIN®), vindesine (ELDISINE®, FILDESIN®), and vinorelbine (NAVELBINE®); etoposide (VP-16); ifosfamide; mitoxantrone; leucovorin; novantrone; edatrexate; daunomycin; aminopterin; 30 ibandronate; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid, including bexarotene (TARGRETIN®); bisphosphonates such as clodronate (for example, BONEFOS® or OSTAC®), etidronate (DIDROCAL®), NE-58095, zoledronic acid/zoledronate (ZOMETA®), alendronate (FOSAMAX®), pamidronate (AREDIA®), tiludronate (SKELID®), or risedronate (ACTONEL®); troxacitabine (a 1,3-

dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those that inhibit expression of genes in signaling pathways implicated in aberrant cell proliferation, such as, for example, PKC-alpha, Raf, H-Ras, and epidermal growth factor receptor (EGF-R); vaccines such as THERATOPE® vaccine and gene therapy vaccines, for example, 5 ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine; topoisomerase I inhibitor (*e.g.*, LURTOTECAN®); rmRH (*e.g.*, ABARELIX®); BAY439006 (sorafenib; Bayer); SU-11248 (sunitinib, SUTENT®, Pfizer); perifosine, COX-2 inhibitor (*e.g.*, celecoxib or etoricoxib), proteasome inhibitor (*e.g.*, PS341); bortezomib (VELCADE®); CCI-779; tipifarnib (R11577); orafenib, ABT510; Bcl-2 inhibitor such as oblimersen sodium 10 (GENASENSE®); pixantrone; EGFR inhibitors (*see* definition below); tyrosine kinase inhibitors; serine-threonine kinase inhibitors such as rapamycin (sirolimus, RAPAMUNE®); farnesyltransferase inhibitors such as lonafarnib (SCH 6636, SARASARTM); and pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or more of the above such as CHOP, an abbreviation for a combined 15 therapy of cyclophosphamide, doxorubicin, vincristine, and prednisolone; and FOLFOX, an abbreviation for a treatment regimen with oxaliplatin (ELOXATINTM) combined with 5-FU and leucovorin.

Chemotherapeutic agents as defined herein include “anti-hormonal agents” or “endocrine therapeutics” which act to regulate, reduce, block, or inhibit the effects of 20 hormones that can promote the growth of cancer. They may be hormones themselves, including, but not limited to: anti-estrogens with mixed agonist/antagonist profile, including, tamoxifen (NOLVADEX®), 4-hydroxytamoxifen, toremifene (FARESTON®), idoxifene, droloxifene, raloxifene (EVISTA®), trioxifene, keoxifene, and selective estrogen receptor modulators (SERMs) such as SERM3; pure anti-estrogens without agonist properties, such as 25 fulvestrant (FASLODEX®), and EM800 (such agents may block estrogen receptor (ER) dimerization, inhibit DNA binding, increase ER turnover, and/or suppress ER levels); aromatase inhibitors, including steroidal aromatase inhibitors such as formestane and exemestane (AROMASIN®), and nonsteroidal aromatase inhibitors such as anastrozole (ARIMIDEX®), letrozole (FEMARA®) and aminoglutethimide, and other aromatase 30 inhibitors include vorozole (RIVISOR®), megestrol acetate (MEGASE®), fadrozole, and 4(5)-imidazoles; lutenizing hormone-releasing hormone agonists, including leuprolide (LUPRON® and ELIGARD®), goserelin, buserelin, and triptorelin; sex steroids, including progestins such as megestrol acetate and medroxyprogesterone acetate, estrogens such as diethylstilbestrol and premarin, and androgens/retinoids such as fluoxymesterone, all

transretinoic acid and fenretinide; onapristone; anti-progesterones; estrogen receptor down-regulators (ERDs); anti-androgens such as flutamide, nilutamide and bicalutamide; and pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or more of the above.

5 A “fixed ” or “flat” dose of a therapeutic agent herein refers to a dose that is administered to a human patient without regard for the weight (WT) or body surface area (BSA) of the patient. The fixed or flat dose is therefore not provided as a mg/kg dose or a mg/m² dose, but rather as an absolute amount of the therapeutic agent.

10 A “loading” dose herein generally comprises an initial dose of a therapeutic agent administered to a patient, and is followed by one or more maintenance dose(s) thereof. Generally, a single loading dose is administered, but multiple loading doses are contemplated herein. Usually, the amount of loading dose(s) administered exceeds the amount of the maintenance dose(s) administered and/or the loading dose(s) are administered more frequently than the maintenance dose(s), so as to achieve the desired steady-state
15 concentration of the therapeutic agent earlier than can be achieved with the maintenance dose(s).

 A “maintenance” dose herein refers to one or more doses of a therapeutic agent administered to the patient over a treatment period. Usually, the maintenance doses are administered at spaced treatment intervals, such as approximately every week, approximately
20 every 2 weeks, approximately every 3 weeks, or approximately every 4 weeks, preferably every 3 weeks.

 “Infusion” or “infusing” refers to the introduction of a drug-containing solution into the body through a vein for therapeutic purposes. Generally, this is achieved via an intravenous (IV) bag.

25 An “intravenous bag” or “IV bag” is a bag that can hold a solution which can be administered via the vein of a patient. In one embodiment, the solution is a saline solution (e.g. about 0.9% or about 0.45% NaCl). Optionally, the IV bag is formed from polyolefin or polyvinyl chloride.

 The term “variable region” or “variable domain” refers to the domain of an antibody
30 heavy or light chain that is involved in binding the antibody to antigen. The variable domains of the heavy chain and light chain (VH and VL, respectively) of a native antibody generally have similar structures, with each domain comprising four conserved framework regions (FRs) and three hypervariable regions (HVRs). (*See, e.g., Kindt et al. Kuby Immunology, 6th ed., W.H. Freeman and Co., page 91 (2007).*) A single VH or VL domain may be sufficient to

confer antigen-binding specificity. Furthermore, antibodies that bind a particular antigen may be isolated using a VH or VL domain from an antibody that binds the antigen to screen a library of complementary VL or VH domains, respectively. *See, e.g.,* Portolano et al., *J. Immunol.* 150:880-887 (1993); Clarkson et al., *Nature* 352:624-628 (1991).

5 The term “vector,” as used herein, refers to a nucleic acid molecule capable of propagating another nucleic acid to which it is linked. The term includes the vector as a self-replicating nucleic acid structure as well as the vector incorporated into the genome of a host cell into which it has been introduced. Certain vectors are capable of directing the expression of nucleic acids to which they are operatively linked. Such vectors are referred to herein as
10 “expression vectors.”

A “free cysteine amino acid” refers to a cysteine amino acid residue which has been engineered into a parent antibody, has a thiol functional group (-SH), and is not paired as an intramolecular or intermolecular disulfide bridge.

 “Linker”, “Linker Unit”, or “link” means a chemical moiety comprising a chain of
15 atoms that covalently attaches an antibody to a drug moiety.

 When indicating the number of substituents, the term “one or more” refers to the range from one substituent to the highest possible number of substitution, i.e. replacement of one hydrogen up to replacement of all hydrogens by substituents. The term “substituent” denotes an atom or a group of atoms replacing a hydrogen atom on the parent molecule. The
20 term “substituted” denotes that a specified group bears one or more substituents. Where any group may carry multiple substituents and a variety of possible substituents is provided, the substituents are independently selected and need not to be the same. The term “unsubstituted” means that the specified group bears no substituents. The term “optionally substituted” means that the specified group is unsubstituted or substituted by one or more substituents,
25 independently chosen from the group of possible substituents. When indicating the number of substituents, the term “one or more” means from one substituent to the highest possible number of substitution, i.e. replacement of one hydrogen up to replacement of all hydrogens by substituents.

 The term “alkyl” as used herein refers to a saturated linear or branched-chain
30 monovalent hydrocarbon radical of any length from one to twelve carbon atoms (C₁–C₁₂), wherein the alkyl radical may be optionally substituted independently with one or more substituents described below. In another embodiment, an alkyl radical is one to eight carbon atoms (C₁–C₈), or one to six carbon atoms (C₁–C₆). Examples of alkyl groups include, but

are not limited to, methyl (Me, -CH₃), ethyl (Et, -CH₂CH₃), 1-propyl (n-Pr, n-propyl, -CH₂CH₂CH₃), 2-propyl (i-Pr, i-propyl, -CH(CH₃)₂), 1-butyl (n-Bu, n-butyl, -CH₂CH₂CH₂CH₃), 2-methyl-1-propyl (i-Bu, i-butyl, -CH₂CH(CH₃)₂), 2-butyl (s-Bu, s-butyl, -CH(CH₃)CH₂CH₃), 2-methyl-2-propyl (t-Bu, t-butyl, -C(CH₃)₃), 1-pentyl (n-pentyl, -CH₂CH₂CH₂CH₂CH₃), 2-pentyl (-CH(CH₃)CH₂CH₂CH₃), 3-pentyl (-CH(CH₂CH₃)₂), 2-methyl-2-butyl (-C(CH₃)₂CH₂CH₃), 3-methyl-2-butyl (-CH(CH₃)CH(CH₃)₂), 3-methyl-1-butyl (-CH₂CH₂CH(CH₃)₂), 2-methyl-1-butyl (-CH₂CH(CH₃)CH₂CH₃), 1-hexyl (-CH₂CH₂CH₂CH₂CH₂CH₃), 2-hexyl (-CH(CH₃)CH₂CH₂CH₂CH₃), 3-hexyl (-CH(CH₂CH₃)(CH₂CH₂CH₃)), 2-methyl-2-pentyl (-C(CH₃)₂CH₂CH₂CH₃), 3-methyl-2-pentyl (-CH(CH₃)CH(CH₃)CH₂CH₃), 4-methyl-2-pentyl (-CH(CH₃)CH₂CH(CH₃)₂), 3-methyl-3-pentyl (-C(CH₃)(CH₂CH₃)₂), 2-methyl-3-pentyl (-CH(CH₂CH₃)CH(CH₃)₂), 2,3-dimethyl-2-butyl (-C(CH₃)₂CH(CH₃)₂), 3,3-dimethyl-2-butyl (-CH(CH₃)C(CH₃)₃), 1-heptyl, 1-octyl, and the like.

The term “alkylene” as used herein refers to a saturated linear or branched-chain divalent hydrocarbon radical of any length from one to twelve carbon atoms (C₁–C₁₂), wherein the alkylene radical may be optionally substituted independently with one or more substituents described below. In another embodiment, an alkylene radical is one to eight carbon atoms (C₁–C₈), or one to six carbon atoms (C₁–C₆). Examples of alkylene groups include, but are not limited to, methylene (-CH₂-), ethylene (-CH₂CH₂-), propylene (-CH₂CH₂CH₂-), and the like.

The term “alkenyl” refers to linear or branched-chain monovalent hydrocarbon radical of any length from two to eight carbon atoms (C₂–C₈) with at least one site of unsaturation, i.e., a carbon-carbon, sp² double bond, wherein the alkenyl radical may be optionally substituted independently with one or more substituents described herein, and includes radicals having “cis” and “trans” orientations, or alternatively, “E” and “Z” orientations. Examples include, but are not limited to, ethylenyl or vinyl (-CH=CH₂), allyl (-CH₂CH=CH₂), and the like.

The term “alkenylene” refers to linear or branched-chain divalent hydrocarbon radical of any length from two to eight carbon atoms (C₂–C₈) with at least one site of unsaturation, i.e., a carbon-carbon, sp² double bond, wherein the alkenylene radical may be optionally substituted independently with one or more substituents described herein, and includes radicals having “cis” and “trans” orientations, or alternatively, “E” and “Z” orientations.

Examples include, but are not limited to, ethylenylene or vinylene ($-\text{CH}=\text{CH}-$), allyl ($-\text{CH}_2\text{CH}=\text{CH}-$), and the like.

The term “alkynyl” refers to a linear or branched monovalent hydrocarbon radical of any length from two to eight carbon atoms (C_2-C_8) with at least one site of unsaturation, i.e.,
5 a carbon-carbon, sp triple bond, wherein the alkynyl radical may be optionally substituted independently with one or more substituents described herein. Examples include, but are not limited to, ethynyl ($-\text{C}\equiv\text{CH}$), propynyl (propargyl, $-\text{CH}_2\text{C}\equiv\text{CH}$), and the like.

The term “alkynylene” refers to a linear or branched divalent hydrocarbon radical of any length from two to eight carbon atoms (C_2-C_8) with at least one site of unsaturation, i.e.,
10 a carbon-carbon, sp triple bond, wherein the alkynylene radical may be optionally substituted independently with one or more substituents described herein. Examples include, but are not limited to, ethynylene ($-\text{C}\equiv\text{C}-$), propynylene (propargylene, $-\text{CH}_2\text{C}\equiv\text{C}-$), and the like.

The terms “carbocycle”, “carbocyclyl”, “carbocyclic ring” and “cycloalkyl” refer to a monovalent non-aromatic, saturated or partially unsaturated ring having 3 to 12 carbon atoms
15 (C_3-C_{12}) as a monocyclic ring or 7 to 12 carbon atoms as a bicyclic ring. Bicyclic carbocycles having 7 to 12 atoms can be arranged, for example, as a bicyclo [4,5], [5,5], [5,6] or [6,6] system, and bicyclic carbocycles having 9 or 10 ring atoms can be arranged as a bicyclo [5,6] or [6,6] system, or as bridged systems such as bicyclo[2.2.1]heptane, bicyclo[2.2.2]octane and bicyclo[3.2.2]nonane. Spiro moieties are also included within the
20 scope of this definition. Examples of monocyclic carbocycles include, but are not limited to, cyclopropyl, cyclobutyl, cyclopentyl, 1-cyclopent-1-enyl, 1-cyclopent-2-enyl, 1-cyclopent-3-enyl, cyclohexyl, 1-cyclohex-1-enyl, 1-cyclohex-2-enyl, 1-cyclohex-3-enyl, cyclohexadienyl, cycloheptyl, cyclooctyl, cyclononyl, cyclodecyl, cycloundecyl, cyclododecyl, and the like. Carbocyclyl groups are optionally substituted independently with one or more substituents
25 described herein.

“Aryl” means a monovalent aromatic hydrocarbon radical of 6-20 carbon atoms (C_6-C_{20}) derived by the removal of one hydrogen atom from a single carbon atom of a parent aromatic ring system. Some aryl groups are represented in the exemplary structures as “Ar”. Aryl includes bicyclic radicals comprising an aromatic ring fused to a saturated, partially
30 unsaturated ring, or aromatic carbocyclic ring. Typical aryl groups include, but are not limited to, radicals derived from benzene (phenyl), substituted benzenes, naphthalene, anthracene, biphenyl, indenyl, indanyl, 1,2-dihydronaphthalene, 1,2,3,4-tetrahydronaphthyl,

and the like. Aryl groups are optionally substituted independently with one or more substituents described herein.

“Arylene” means a divalent aromatic hydrocarbon radical of 6-20 carbon atoms (C₆–C₂₀) derived by the removal of two hydrogen atom from a two carbon atoms of a parent aromatic ring system. Some arylene groups are represented in the exemplary structures as “Ar”. Arylene includes bicyclic radicals comprising an aromatic ring fused to a saturated, partially unsaturated ring, or aromatic carbocyclic ring. Typical arylene groups include, but are not limited to, radicals derived from benzene (phenylene), substituted benzenes, naphthalene, anthracene, biphenylene, indenylene, indanylene, 1,2-dihydronaphthalene, 1,2,3,4-tetrahydronaphthyl, and the like. Arylene groups are optionally substituted with one or more substituents described herein.

The terms “heterocycle,” “heterocyclyl” and “heterocyclic ring” are used interchangeably herein and refer to a saturated or a partially unsaturated (i.e., having one or more double and/or triple bonds within the ring) carbocyclic radical of 3 to about 20 ring atoms in which at least one ring atom is a heteroatom selected from nitrogen, oxygen, phosphorus and sulfur, the remaining ring atoms being C, where one or more ring atoms is optionally substituted independently with one or more substituents described below. A heterocycle may be a monocycle having 3 to 7 ring members (2 to 6 carbon atoms and 1 to 4 heteroatoms selected from N, O, P, and S) or a bicycle having 7 to 10 ring members (4 to 9 carbon atoms and 1 to 6 heteroatoms selected from N, O, P, and S), for example: a bicyclo [4,5], [5,5], [5,6], or [6,6] system. Heterocycles are described in Paquette, Leo A.; “Principles of Modern Heterocyclic Chemistry” (W.A. Benjamin, New York, 1968), particularly Chapters 1, 3, 4, 6, 7, and 9; “The Chemistry of Heterocyclic Compounds, A series of Monographs” (John Wiley & Sons, New York, 1950 to present), in particular Volumes 13, 14, 16, 19, and 28; and J. Am. Chem. Soc. (1960) 82:5566. “Heterocyclyl” also includes radicals where heterocycle radicals are fused with a saturated, partially unsaturated ring, or aromatic carbocyclic or heterocyclic ring. Examples of heterocyclic rings include, but are not limited to, morpholin-4-yl, piperidin-1-yl, piperazinyl, piperazin-4-yl-2-one, piperazin-4-yl-3-one, pyrrolidin-1-yl, thiomorpholin-4-yl, S-dioxothiomorpholin-4-yl, azocan-1-yl, azetidin-1-yl, octahydropyrido[1,2-a]pyrazin-2-yl, [1,4]diazepan-1-yl, pyrrolidinyl, tetrahydrofuranyl, dihydrofuranyl, tetrahydrothienyl, tetrahydropyranyl, dihydropyranyl, tetrahydrothiopyranyl, piperidino, morpholino, thiomorpholino, thioxanyl, piperazinyl, homopiperazinyl, azetidiny, oxetanyl, thietanyl, homopiperidinyl, oxepanyl,

thiepanyl, oxazepinyl, diazepinyl, thiazepinyl, 2-pyrrolinyl, 3-pyrrolinyl, indolinyl, 2H-pyranyl, 4H-pyranyl, dioxanyl, 1,3-dioxolanyl, pyrazolinyl, dithianyl, dithiolanyl, dihydropyranyl, dihydrothienyl, dihydrofuranyl, pyrazolidinylimidazolinyl, imidazolidinyl, 3-azabicyclo[3.1.0]hexanyl, 3-azabicyclo[4.1.0]heptanyl, azabicyclo[2.2.2]hexanyl, 3H-indolyl
5 quinolizinyl and N-pyridyl ureas. Spiro moieties are also included within the scope of this definition. Examples of a heterocyclic group wherein 2 ring atoms are substituted with oxo (=O) moieties are pyrimidinonyl and 1,1-dioxo-thiomorpholinyl. The heterocycle groups herein are optionally substituted independently with one or more substituents described herein.

10 The term “heteroaryl” refers to a monovalent aromatic radical of 5-, 6-, or 7-membered rings, and includes fused ring systems (at least one of which is aromatic) of 5-20 atoms, containing one or more heteroatoms independently selected from nitrogen, oxygen, and sulfur. Examples of heteroaryl groups are pyridinyl (including, for example, 2-hydroxypyridinyl), imidazolyl, imidazopyridinyl, 1-methyl-1H-benzo[d]imidazole,
15 [1,2,4]triazolo[1,5-a]pyridine, pyrimidinyl (including, for example, 4-hydroxypyrimidinyl), pyrazolyl, triazolyl, pyrazinyl, tetrazolyl, furyl, thienyl, isoxazolyl, thiazolyl, oxadiazolyl, oxazolyl, isothiazolyl, pyrrolyl, quinolinyl, isoquinolinyl, tetrahydroisoquinolinyl, indolyl, benzimidazolyl, benzofuranyl, cinnolinyl, indazolyl, indolizinyl, phthalazinyl, pyridazinyl, triazinyl, isoindolyl, pteridinyl, purinyl, oxadiazolyl, thiadiazolyl, thiadiazolyl, furazanyl,
20 benzofurazanyl, benzothiophenyl, benzothiazolyl, benzoxazolyl, quinazolinyl, quinoxalinyl, naphthyridinyl, and furopyridinyl. Heteroaryl groups are optionally substituted independently with one or more substituents described herein.

The heterocycle or heteroaryl groups may be carbon (carbon-linked), or nitrogen (nitrogen-linked) bonded where such is possible. By way of example and not limitation,
25 carbon bonded heterocycles or heteroaryls are bonded at position 2, 3, 4, 5, or 6 of a pyridine, position 3, 4, 5, or 6 of a pyridazine, position 2, 4, 5, or 6 of a pyrimidine, position 2, 3, 5, or 6 of a pyrazine, position 2, 3, 4, or 5 of a furan, tetrahydrofuran, thiofuran, thiophene, pyrrole or tetrahydropyrrole, position 2, 4, or 5 of an oxazole, imidazole or thiazole, position 3, 4, or 5 of an isoxazole, pyrazole, or isothiazole, position 2 or 3 of an aziridine, position 2, 3, or 4
30 of an azetidine, position 2, 3, 4, 5, 6, 7, or 8 of a quinoline or position 1, 3, 4, 5, 6, 7, or 8 of an isoquinoline.

By way of example and not limitation, nitrogen bonded heterocycles or heteroaryls are bonded at position 1 of an aziridine, azetidine, pyrrole, pyrrolidine, 2-pyrroline, 3-pyrroline, imidazole, imidazolidine, 2-imidazoline, 3-imidazoline, pyrazole, pyrazoline, 2-

pyrazoline, 3-pyrazoline, piperidine, piperazine, indole, indoline, 1H-indazole, position 2 of a isoindole, or isoindoline, position 4 of a morpholine, and position 9 of a carbazole, or β -carboline.

The term “chiral” refers to molecules which have the property of non-
5 superimposability of the mirror image partner, while the term “achiral” refers to molecules which are superimposable on their mirror image partner.

The term “stereoisomers” refers to compounds which have identical chemical constitution, but differ with regard to the arrangement of the atoms or groups in space.

“Diastereomer” refers to a stereoisomer with two or more centers of chirality and
10 whose molecules are not mirror images of one another. Diastereomers have different physical properties, e.g. melting points, boiling points, spectral properties, and reactivities. Mixtures of diastereomers may separate under high resolution analytical procedures such as electrophoresis and chromatography.

“Enantiomers” refer to two stereoisomers of a compound which are non-
15 superimposable mirror images of one another.

The following definitions are used, unless otherwise described: halo or halogen is fluoro, chloro, bromo, or iodo. Alkyl and alkoxy, etc. denote both straight and branched groups but reference to an individual radical such as propyl embraces only the straight chain radical (a branched chain isomer such as isopropyl being specifically referred to).

20 As used herein, the term “(C_a-C_b)alkyl” wherein a and b are integers refers to a straight or branched chain alkyl radical having from a to b carbon atoms. Thus when a is 1 and b is 6, for example, the term includes methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, t-butyl, n-pentyl and n-hexyl.

The term “amino nitrogen protecting group” (P) refers to any protecting group (e.g., a
25 BOC group) that can protect the amine groups during synthetic processes.

Specific values listed below for radicals, substituents, and ranges, are for illustration only; they do not exclude other defined values or other values within defined ranges for the radicals and substituents.

Specifically, (C₁-C₆)alkyl can be methyl, ethyl, propyl, isopropyl, butyl, iso-butyl,
30 sec-butyl, pentyl, 3-pentyl, hexyl.

Stereochemical definitions and conventions used herein generally follow S. P. Parker, Ed., *McGraw-Hill Dictionary of Chemical Terms* (1984) McGraw-Hill Book Company, New York; and Eliel, E. and Wilen, S., *Stereochemistry of Organic Compounds* (1994) John Wiley & Sons, Inc., New York. Many organic compounds exist in optically active forms, i.e., they

have the ability to rotate the plane of plane-polarized light. In describing an optically active compound, the prefixes D and L, or *R* and *S*, are used to denote the absolute configuration of the molecule about its chiral center(s). The prefixes d and l or (+) and (-) are employed to designate the sign of rotation of plane-polarized light by the compound, with (-) or l meaning
5 that the compound is levorotatory. A compound prefixed with (+) or d is dextrorotatory. For a given chemical structure, these stereoisomers are identical except that they are mirror images of one another. A specific stereoisomer may also be referred to as an enantiomer, and a mixture of such isomers is often called an enantiomeric mixture. A 50:50 mixture of enantiomers is referred to as a racemic mixture or a racemate, which may occur where there
10 has been no stereoselection or stereospecificity in a chemical reaction or process. The terms “racemic mixture” and “racemate” refer to an equimolar mixture of two enantiomeric species, devoid of optical activity.

The phrase “pharmaceutically acceptable salt,” as used herein, refers to pharmaceutically acceptable organic or inorganic salts of an antibody-drug conjugate (ADC).
15 Exemplary salts include, but are not limited, to sulfate, citrate, acetate, oxalate, chloride, bromide, iodide, nitrate, bisulfate, phosphate, acid phosphate, isonicotinate, lactate, salicylate, acid citrate, tartrate, oleate, tannate, pantothenate, bitartrate, ascorbate, succinate, maleate, gentisinate, fumarate, gluconate, glucuronate, saccharate, formate, benzoate, glutamate, methanesulfonate, ethanesulfonate, benzenesulfonate, *p*-toluenesulfonate, and pamoate (*i.e.*,
20 1,1'-methylene-bis-(2-hydroxy-3-naphthoate)) salts. A pharmaceutically acceptable salt may involve the inclusion of another molecule such as an acetate ion, a succinate ion or other counterion. The counterion may be any organic or inorganic moiety that stabilizes the charge on the parent compound. Furthermore, a pharmaceutically acceptable salt may have more than one charged atom in its structure. Instances where multiple charged atoms are part of
25 the pharmaceutically acceptable salt can have multiple counter ions. Hence, a pharmaceutically acceptable salt can have one or more charged atoms and/or one or more counterion.

The following abbreviations are used herein and have the indicated definitions: BME is beta-mercaptoethanol, Boc is *N*-(*t*-butoxycarbonyl), cit is citrulline (2-amino-5-ureido
30 pentanoic acid), DCC is 1,3-dicyclohexylcarbodiimide, DCM is dichloromethane, DEA is diethylamine, DEAD is diethylazodicarboxylate, DEPC is diethylphosphorylcyanoate, DIAD is diisopropylazodicarboxylate, DIEA is *N,N*-diisopropylethylamine, DMA is dimethylacetamide, DMAP is 4-dimethylaminopyridine, DME is ethyleneglycol dimethyl ether (or 1,2-dimethoxyethane), DMF is *N,N*-dimethylformamide, DMSO is

dimethylsulfoxide, DTT is dithiothreitol, EDCI is 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, EEDQ is 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline, ES-MS is electrospray mass spectrometry, EtOAc is ethyl acetate, Fmoc is *N*-(9-fluorenylmethoxycarbonyl), gly is glycine, HATU is O-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate, HOBt is 1-hydroxybenzotriazole, HPLC is high pressure liquid chromatography, ile is isoleucine, lys is lysine, MeCN (CH₃CN) is acetonitrile, MeOH is methanol, Mtr is 4-anisylidiphenylmethyl (or 4-methoxytrityl), NHS is *N*-hydroxysuccinimide, PBS is phosphate-buffered saline (pH 7), PEG is polyethylene glycol or a unit of ethylene glycol (-OCH₂CH₂-), Ph is phenyl, Pnp is *p*-nitrophenyl, MC is 6-maleimidocaproyl, phe is L-phenylalanine, PyBrop is bromo *tris*-pyrrolidino phosphonium hexafluorophosphate, SEC is size-exclusion chromatography, Su is succinimide, TFA is trifluoroacetic acid, TLC is thin layer chromatography, UV is ultraviolet, and val is valine.

15 Antibody Unit (Ab or AB)

As noted above, the term “antibody” (or “Ab” or “AB”) herein is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, monospecific antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments that exhibit the desired biological activity. Accordingly, the term “antibody” includes antibody fragment thereof and thus is used interchangeably with the term “antibody or fragment thereof”. In addition, while certain aspects of the invention described herein refer to antibody-drug conjugates, it is further envisioned that the antibody portion of the conjugate might be replaced with anything that specifically binds or reactively associates or complexes with a receptor, antigen or other receptive moiety associated with a given target-cell population. For example, instead of containing an antibody a conjugate of the invention could contain a targeting molecule (also referred to as a binding agent) that binds to, complexes with, or reacts with a receptor, antigen or other receptive moiety of a cell population sought to be therapeutically or otherwise biologically modified. Example of such molecules include smaller molecular weight proteins, polypeptide or peptides, lectins, glycoproteins, non-peptides, vitamins, nutrient-transport molecules (such as, but not limited to, transferrin), or any other cell binding molecule or substances. In certain aspects, the antibody or other such targeting molecule acts to deliver a drug to the particular target cell population with which the antibody or other targeting molecule interacts.

- In another aspect, the present invention relates to an antibody-drug conjugate compound wherein the antibody AB is selected from: gemtuzumab, trastuzumab (Herceptin®), pertuzumab (Perjeta®), iratumumab, inotuzumab, pinatuzumab, epratuzumab, polatuzumab, coltuximab, lovotuzumab, sacituzumab, anetumab, aprutumab, aratumumab, atezolizumab, avelumab, azintuxizumab, bevacizumab (Avastin®), bivatuzumab, brentuximab, camidanlumab, cantuzumab, cetuximab (Erbix®), cofetuzumab, denintuzumab, durvalumab, elotuzumab, enfortumab, glembatumumab, ibritumomab, iladatuzumab, indatuximab, industuzumab, labetuzumab, ladiratuzumab, laprituximab, lifastuzumab, loncastuximab, lorvotuzumab, lupartumab, milatuzumab, mirvetuximab, naratuximab, natalizumab, necitumumab, obinutuzumab, ocrelizumab, ofatumumab, olaratumab, panitumumab, pertuzumab, rituximab (Rituxan®), rovalpituzumab, sirtratumab, sofituzumab, telisotuzumab, tositumomab, trastuzumab, and vadastuximab. In certain embodiments, the antibody AB is selected from the group consisting of: trastuzumab, pertuzumab, gemtuzumab, and vadastuximab.
- In another aspect, the present invention relates to an antibody-drug conjugate compound wherein the antibody AB is selected from: gemtuzumab, trastuzumab (Herceptin®), pertuzumab (Perjeta®), iratumumab, inotuzumab, pinatuzumab, enapotamab, epratuzumab, polatuzumab, coltuximab, lovotuzumab, sacituzumab, anetumab, aprutumab, aratumumab, atezolizumab, avelumab, azintuxizumab, bevacizumab (Avastin®), belantamab, bivatuzumab, brentuximab, camidanlumab, cantuzumab, cetuximab (Erbix®), cofetuzumab, datopotamab, depatuxizumab, denintuzumab, disatamab, durvalumab, elotuzumab, enfortumab, farletuzumab, gemtuzumab, glembatumumab, ibritumomab, ifinatamab, iladatuzumab, indatuximab, industuzumab, indusatumab, labetuzumab, ladiratuzumab, laprituximab, lifastuzumab, loncastuximab, lorvotuzumab, lupartumab, luveltamab, milatuzumab, mirvetuximab, mecbotamab, moxetumomab, naratuximab, natalizumab, necitumumab, obinutuzumab, ocrelizumab, ofatumumab, olaratumab, opelkibart, ozuriftamab, panitumumab, patritumab, praluzatamab, rituximab (Rituxan®), Raludotatug, rovalpituzumab, sirtratumab, sigvotatugum, sofituzumab, telisotuzumab, tisotumab, tositumomab, trastuzumab, tusamitamab, vadastuximab, vobramitamab, vorsetuzumab, zilovertamab and zalontamab. In certain embodiments, the antibody AB is selected from the group consisting of: trastuzumab, pertuzumab, gemtuzumab, and vadastuximab.

Heteroatoms that may be present on an antibody unit include sulfur (in one embodiment, from a sulfhydryl group of an antibody), oxygen (in one embodiment, from a

carbonyl, carboxyl or hydroxyl group of an antibody) and nitrogen (in one embodiment, from a primary or secondary amino group of an antibody). These hetero atoms can be present on the antibody in the antibody's natural state, or can be introduced into the antibody via chemical modification.

5 In one embodiment, an antibody unit has a sulfhydryl group and the antibody unit bonds via the sulfhydryl group's sulfur atom.

 In another embodiment, the antibody has lysine residues that can react with activated esters (such esters include, but are not limited to, N-hydroxysuccinimide, pentafluorophenyl, and p-nitrophenyl esters) and thus form an amide bond consisting of the nitrogen atom of the
10 antibody unit and a carbonyl. In another embodiment the activated esters include N-hydroxysuccinimide and phenyl esters ($-C(=O)OPh$) wherein the phenyl is optionally substituted with one or more fluoro, chloro, bromo, iodo, or nitro.

 In yet another aspect, the antibody unit has one or more lysine residues that can be chemically modified to introduce one or more sulfhydryl groups. The reagents that can be
15 used to modify lysines include, but are not limited to, N-hydroxysuccinimide acetate ester (NHS ester), N-succinimidyl S-acetylthioacetate (SATA), and 2-Iminothiolane hydrochloride (Traut's Reagent).

 In another embodiment, the antibody unit can have one or more carbohydrate groups that can be chemically modified to have one or more sulfhydryl groups.

20 In yet another embodiment, the antibody unit can have one or more carbohydrate groups that can be oxidized to provide an aldehyde group. The corresponding aldehyde can form a bond with a reactive site such as, for example, hydrazine and hydroxylamine. Other protocols for the modification of proteins for the attachment or association of drugs are described in Coligan *et al.*, *Current Protocols in Protein Science*, Vol. 2, John Wiley & Sons
25 (2002).

 Useful polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of immunized animals. Useful monoclonal antibodies are homogeneous populations of antibodies to a particular antigenic determinant (e.g., a cancer cell antigen, a viral antigen, a microbial antigen, a protein, a peptide, a carbohydrate, a
30 chemical, nucleic acid, or fragments thereof). A monoclonal antibody (mAb) to an antigen-of-interest can be prepared by using any technique known in the art which provides for the production of antibody molecules by continuous cell lines in culture.

 Useful monoclonal antibodies include, but are not limited to, human monoclonal antibodies, humanized monoclonal antibodies, antibody fragments, or chimeric monoclonal

antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art.

The antibody can also be a bispecific antibody. Methods for making bispecific antibodies are known in the art.

- 5 The antibody can be a functionally active fragment, derivative or analog of an antibody that immunospecifically binds to target cells (e.g., tumor-associated antigens, cancer cell antigens, viral antigens, or microbial antigens) or other antibodies that bind to tumor cells or matrix. The “functionally active” fragment, derivative or analog is capable of binding the same antigen / epitope as the intact antibody. In certain embodiments, “functionally active”
- 10 fragment, derivative or analog is able to elicit anti-anti-idiotypic antibodies that recognize the same antigen that the antibody from which the fragment, derivative or analog is derived recognized. Specifically, in an exemplary embodiment the antigenicity of the idiotype of the immunoglobulin molecule can be enhanced by deletion of framework and CDR sequences that are C-terminal to the CDR sequence that specifically recognizes the antigen. To
- 15 determine which CDR sequences bind the antigen, synthetic peptides containing the CDR sequences can be used in binding assays with the antigen by any binding assay method known in the art (e.g., the BIA core assay).

- Other useful antibodies include fragments of antibodies such as, but not limited to, F(ab')₂ fragments, Fab fragments, single chain antibodies, diabodies, triabodies, tetrabodies,
- 20 scFv, scFv-Fc, or any other molecule with the same specificity as the antibody.

- Additionally, recombinant antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are useful antibodies. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as for
- 25 example, those having a variable region derived from a murine monoclonal and human immunoglobulin constant regions. Humanized antibodies are antibody molecules from non-human species having one or more complementarity determining regions (CDRs) from the non-human species and a framework region from a human immunoglobulin molecule. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA
- 30 techniques known in the art.

Completely human antibodies are particularly desirable and can be produced using transgenic mice that are incapable of expressing endogenous immunoglobulin heavy and light chains genes, but which can express human heavy and light chain genes. The transgenic mice are immunized in the normal fashion with a selected antigen, e.g., to produce all or a portion

of a polypeptide of the invention. Monoclonal antibodies directed against the antigen can be obtained using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to
5 produce therapeutically useful IgG, IgA, IgM and IgE antibodies using methods known to one skilled in the art. Other human antibodies can be obtained commercially from numerous companies.

Completely human antibodies that recognize a selected epitope can be generated using a technique referred to as “guided selection.” In this approach a selected non-human
10 monoclonal antibody, e.g., a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope. Human antibodies can also be produced using various techniques known in the art, including phage display libraries.

In other embodiments, the antibody is a fusion protein of an antibody, or a functionally active fragment thereof, for example in which the antibody is fused via a
15 covalent bond (e.g., a peptide bond), at either the N-terminus or the C-terminus to an amino acid sequence of another protein (or portion thereof, preferably at least 10, 20 or 50 amino acid portion of the protein) that is not from an antibody.

Antibodies include analogs and derivatives that are either modified, i.e., by the covalent attachment of any type of molecule as long as such covalent attachment permits the
20 antibody to retain its antigen binding immunospecificity. For example, but not by way of limitation, derivatives and analogs of the antibodies include those that have been further modified, e.g., by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular antibody unit or other protein, etc. Any of numerous chemical modifications can be
25 carried out by known techniques including, but not limited to, specific chemical cleavage, acetylation, formylation, metabolic synthesis in the presence of tunicamycin, etc. Additionally, the analog or derivative can contain one or more unnatural amino acids.

Antibodies can have modifications (e.g., substitutions, deletions or additions) in amino acid residues that interact with Fc receptors. In particular, antibodies can have
30 modifications in amino acid residues identified as involved in the interaction with the FcRn receptor.

Antibodies immunospecific for a cancer cell antigen can be obtained commercially or produced by any method known to one of skill in the art such as, e.g., chemical synthesis or recombinant expression techniques. The nucleotide sequence encoding antibodies

immunospecific for a cancer cell antigen can be obtained, e.g., from the GenBank database or a database like it, literature publications, or by routine cloning and sequencing.

In a specific embodiment, known antibodies for the treatment of cancer can be used. Antibodies immunospecific for a cancer cell antigen can be obtained commercially or
 5 produced by any method known to one of skill in the art such as, e.g., recombinant expression techniques. The nucleotide sequence encoding antibodies immunospecific for a cancer cell antigen (tumor-associated antigens) can be obtained, e.g., from the GenBank database or a database like it, the literature publications, or by routine cloning and sequencing.

Examples of tumor-associated antigens (TAA) include, but are not limited to, TAA
 10 (1)-(100) listed herein. For convenience, in most cases, information relating to these antigens, all of which are known in the art, is listed herein and includes, in most cases, names, alternative names, GenBank accession numbers and primary reference(s), following nucleic acid and protein sequence identification conventions of the National Center for Biotechnology Information (NCBI). Nucleic acid and protein sequences corresponding to
 15 TAA (1)-(100) are available in public databases such as GenBank. Tumor-associated antigens targeted by antibodies include all amino acid sequence variants and isoforms possessing at least about 70%, 80%, 85%, 90%, or 95% sequence identity relative to the sequences identified in the cited references, or which exhibit substantially the same biological properties or characteristics as a TAA having a sequence found in the cited references. For
 20 example, a TAA having a variant sequence generally is able to bind specifically to an antibody that binds specifically to the TAA with the corresponding sequence listed. The sequences and disclosure in the reference specifically recited herein are expressly incorporated by reference.

Specific non-limiting examples of TAA include:

25 (1) BMPR1B (bone morphogenetic protein receptor-type IB, GenBank accession no. NM_001203); ten Dijke, P., *et al.*, *Science*, 1994, **264** (5155):101-104; *Oncogene*, 1997, **14**(11):1377-1382; WO 2004/063362 (Claim 2); WO 2003/042661 (Claim 12); US 2003/134790 (Page 38-39); WO 2002/102235 (Claim 13; Page 296); WO 2003/055443 (Page 91-92); WO 2002/99122 (Example 2; Page 528-530); WO 2003/029421
 30 (Claim 6); WO 2003/024392 (Claim 2; Fig 112); WO 2002/98358 (Claim 1; Page 183); WO 2002/54940 (Page 100-101); WO 2002/59377 (Page 349-350); WO 2002/30268 (Claim 27; Page 376); WO 2001/48204 (Example; Fig 4) NP_001194 bone morphogenetic protein receptor, type IB/pid=NP_001194.1 - Cross-references: MIM:603248; NP_001194.1; AY065994.

(2) E16 (LAT1, SLC7A5, GenBank accession no. NM_003486); *Biochem. Biophys. Res. Commun.*, 1999, **255**(2), 283-288; *Nature*, 1998, **395**(6699):288-291; Gaugitsch, H.W., *et al.*, *J. Biol. Chem.*, 1992, **267**(16):11267-11273; WO 2004/048938 (Example 2); WO 2004/032842 (Example IV); WO 2003/042661 (Claim 12); WO 2003/016475 (Claim 1); WO 2002/78524 (Example 2); WO 2002/99074 (Claim 19; Page 127-129); WO 2002/86443 (Claim 27; Pages 222, 393); WO 2003/003906 (Claim 10; Page 293); WO 2002/64798 (Claim 33; Page 93-95); WO 2000/14228 (Claim 5; Page 133-136); US 2003/224454 (Fig 3); WO 2003/025138 (Claim 12; Page 150); NP_003477 solute carrier family 7 (cationic amino acid transporter, y⁺ system), member 5 /pid=NP_003477.3 - Homo sapiens Cross-references: MIM:600182; NP_003477.3; NM_015923; NM_003486_1.

(3) STEAP1 (six transmembrane epithelial antigen of prostate, GenBank accession no. NM_012449); *Cancer Res.*, 2001, **61**(15), 5857-5860; Hubert, R.S., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, **96** (25):14523-14528; WO 2004/065577 (Claim 6); WO 2004/027049 (Fig 1L); EP 1394274 (Example 11); WO 2004/016225 (Claim 2); WO 2003/042661 (Claim 12); US 2003/157089 (Example 5); US 2003/185830 (Example 5); US 2003/064397 (Fig 2); WO 2002/89747 (Example 5; Page 618-619); WO 2003/022995 (Example 9; Fig 13A, Example 53; Page 173, Example 2; Fig 2A); NP_036581 six transmembrane epithelial antigen of the prostate Cross-references: MIM:604415; NP_036581.1; NM_012449_1.

(4) 0772P (CA125, MUC16, GenBank accession no. AF361486); *J. Biol. Chem.*, 2001, **276**(29):27371-27375; WO 2004/045553 (Claim 14); WO 2002/92836 (Claim 6; Fig 12); WO 2002/83866 (Claim 15; Page 116-121); US 2003/124140 (Example 16). Cross-references: GI:34501467; AAK74120.3; AF361486_1.

(5) MPF (MPF, MSLN, SMR, megakaryocyte potentiating factor, mesothelin, GenBank accession no. NM_005823); Yamaguchi, N., *et al.*, *Biol. Chem.*, 1994, **269**(2), 805-808; *Proc. Natl. Acad. Sci. U.S.A.*, 1999, **96**(20):11531-11536; *Proc. Natl. Acad. Sci. U.S.A.*, 1996, **93**(1):136-140; *J. Biol. Chem.*, 1995, **270**(37):21984-21990; WO 2003/101283 (Claim 14); (WO 2002/102235 (Claim 13; Page 287-288); WO 2002/101075 (Claim 4; Page 308-309); WO 2002/71928 (Page 320-321); WO 9410312 (Page 52-57); Cross-references: MIM:601051; NP_005814.2; NM_005823_1.

(6) Napi3b (NAPI-3B, NPTIb, SLC34A2, solute carrier family 34 (sodium phosphate), member 2, type II sodium-dependent phosphate transporter 3b, GenBank accession no. NM_006424); *J. Biol. Chem.*, 2002, **277**(22):19665-19672; *Genomics*, 1999, **62**(2):281-284; Feild, J.A., *et al.*, *Biochem. Biophys. Res. Commun.*, 1999, **258**(3):578-582);

WO 2004/022778 (Claim 2); EP 1394274 (Example 11); WO 2002/102235 (Claim 13; Page 326); EP 875569 (Claim 1; Page 17-19); WO 2001/57188 (Claim 20; Page 329); WO 2004/032842 (Example IV); WO200175177 (Claim 24; Page 139-140); Cross-references: MIM:604217; NP_006415.1; NM_006424_1.

- 5 (7) Sema 5b (FLJ10372, KIAA1445, Mm.42015, SEMA5B, SEMAG, Semaphorin 5b Hlog, sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5B, GenBank accession no. AB040878); Nagase T., *et al.*, *DNA Res.*, 2000, **7**(2):143-150); WO 2004/000997 (Claim 1); WO 2003/003984 (Claim 1); WO 2002/06339 (Claim 1; Page 50);
- 10 WO 2001/88133 (Claim 1; Page 41-43, 48-58); WO 2003/054152 (Claim 20); WO 20031/01400 (Claim 11); Accession: Q9P283; EMBL; AB040878; BAA95969.1. Genew; HGNC:10737.
- (8) PSCA hlg (2700050C12Rik, C530008O16Rik, RIKEN cDNA 2700050C12, RIKEN cDNA 2700050C12 gene, GenBank accession no. AY358628); Ross *et al.*, *Cancer Res.*, 2002, **62**:2546-2553; US 2003/129192 (Claim 2); US 2004/044180 (Claim 12); US 2004/044179 (Claim 11); US 2003/096961 (Claim 11); US 2003/232056 (Example 5); WO 2003/105758 (Claim 12); US 2003/206918 (Example 5); EP 1347046 (Claim 1); WO 2003/025148 (Claim 20); Cross-references: GI:37182378; AAQ88991.1; AY358628_1.
- (9) ETBR (Endothelin type B receptor, GenBank accession no. AY275463);
- 20 Nakamuta M., *et al.*, *Biochem. Biophys. Res. Commun.*, 1991, **177**:34-39; Ogawa Y., *et al.*, *Biochem. Biophys. Res. Commun.*, 1991, **178**, 248-255; Arai, H., *et al.*, *Jpn. Circ. J.*, 1992, **56**:1303-1307; Arai, H., *et al.*, *J. Biol. Chem.*, 1993, **268**:3463-3470; Sakamoto A., Yanagisawa M., *et al.*, *Biochem. Biophys. Res. Commun.*, 1991, **178**:656-663; Elshourbagy N.A., *et al.*, *J. Biol. Chem.*, 1993, **268**:3873-3879; Haendler B., *et al.*, *J. Cardiovasc.*
- 25 *Pharmacol.*, 1992, **20**:s1-S4; Tsutsumi M., *et al.*, *Gene*, 1999, **228**:43-49; Strausberg, R.L., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 2002, **99**:16899-16903; Bourgeois C., *et al.*, *J. Clin. Endocrinol. Metab.*, 1997, **82**, 3116-3123; Okamoto Y., *et al.*, *Biol. Chem.*, 1997, **272**:21589-21596; Verheij, J.B., *Am. J. Med. Genet.*, 2002, **108**:223-225; Hofstra, R.M.W., *et al.*, *Eur. J. Hum. Genet.*, 1997, **5**:180-185; Puffenberger, E.G., *et al.*, *Cell*, 1994, **79**:1257-1266; Attie T.,
- 30 *et al.*, *Hum. Mol. Genet.*, 1995, **4**:2407-2409; Auricchio A., *et al.*, *Hum. Mol. Genet.*, 1996, **5**:351-354; Amiel J., *et al.* *Hum. Mol. Genet.* **5**, 355-357, 1996; Hofstra R.M.W., *et al.* *Nat. Genet.*, 1996, **12**:445-447; Svensson, P.J., *et al.*, *Hum. Genet.*, 1998, **103**:145-148; Fuchs, S., *et al.*, *Mol. Med.*, 2001, **7**:115-124; Pingault V., *et al.*, *Hum. Genet.*, 2002, 111:198-206; WO 2004/045516 (Claim 1); WO 2004/048938 (Example 2); WO 2004/040000 (Claim 151); WO

2003/087768 (Claim 1); WO 2003/016475 (Claim 1); WO 2003/016475 (Claim 1); WO
 2002/61087 (Fig 1); WO 2003/016494 (Fig 6); WO 2003/025138 (Claim 12; Page 144); WO
 2001/98351 (Claim 1; Page 124-125); EP 522868 (Claim 8; Fig 2); WO 2001/77172 (Claim
 1; Page 297-299); US 2003/109676; US 6,518,404 (Fig 3); US 5,773,223 (Claim 1a; Col 31-
 5 34); WO 2004/001004.

(10) MSG783 (RNF124, hypothetical protein FLJ20315, GenBank accession
 no. NM_017763); WO 2003/104275 (Claim 1); WO 2004/046342 (Example 2); WO
 2003/042661 (Claim 12); WO 2003/083074 (Claim 14; Page 61); WO 2003/018621 (Claim
 1); WO 2003/024392 (Claim 2; Fig 93); WO 2001/66689 (Example 6); Cross-references:
 10 LocusID:54894; NP_060233.2; NM_017763_1.

(11) STEAP2 (HGNC_8639, IPCA-1, PCANAP1, STAMP1, STEAP2,
 STMP, prostate cancer associated gene 1, prostate cancer associated protein 1, six
 transmembrane epithelial antigen of prostate 2, six transmembrane prostate protein, GenBank
 accession no. AF455138) Lab. Invest. 82 (11):1573-1582 (2002)); WO 2003/087306; US
 15 2003/064397 (Claim 1; Fig 1); WO 2002/72596 (Claim 13; Page 54-55); WO 2001/72962
 (Claim 1; Fig 4B); WO 2003/104270 (Claim 11); WO 2003/104270 (Claim 16); US
 2004/005598 (Claim 22); WO 2003/042661 (Claim 12); US 2003/060612 (Claim 12; Fig 10);
 WO 2002/26822 (Claim 23; Fig 2); WO 2002/16429 (Claim 12; Fig 10); Cross-references:
 GI:22655488; AAN04080.1; AF455138_1.

(12) TrpM4 (BR22450, FLJ20041, TRPM4, TRPM4B, transient receptor
 potential cation channel, subfamily M, member 4, GenBank accession no. NM_017636) Xu,
 X.Z., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 2001, **98**(19):10692-10697; *Cell*, 2002, **109**(3):397-
 407; *J. Biol. Chem.*, 2003, **278**(33):30813-30820; US 2003/143557 (Claim 4); WO
 2000/40614 (Claim 14; Page 100-103); WO 2002/10382 (Claim 1; Fig 9A); WO
 25 2003/042661 (Claim 12); WO 2002/30268 (Claim 27; Page 391); US 2003/219806 (Claim
 4); WO 2001/62794 (Claim 14; Fig 1A-D); Cross-references: MIM:606936; NP_060106.2;
 NM_017636_1.

(13) CRIPTO (CR, CR1, CRGF, CRIPTO, TDGF1, teratocarcinoma-derived
 growth factor, GenBank accession no. NP_003203 or NM_003212); Ciccodicola, A., *et al.*,
 30 *EMBO J.*, 1989, **8**(7):1987-1991; *Am. J. Hum. Genet.*, 1991, **49**(3):555-565; US 2003/224411
 (Claim 1); WO 2003/083041 (Example 1); WO 2003/034984 (Claim 12); WO 2002/88170
 (Claim 2; Page 52-53); WO 2003/024392 (Claim 2; Fig 58); WO 2002/16413 (Claim 1; Page
 94-95, 105); WO 2002/22808 (Claim 2; Fig 1); US 5,854,399 (Example 2; Col 17-18); US
 5,792,616 (Fig 2); Cross-references: MIM:187395; NP_003203.1; NM_003212_1.

- (14) CD21 (CR2 (Complement receptor 2) or C3DR (C3d/Epstein Barr virus receptor) or Hs.73792 GenBank accession no. M26004); Fujisaku, *et al.*, *J. Biol. Chem.*, 1989, **264**(4):2118-2125; Weis J.J., *et al.*, *J. Exp. Med.*, 1988, **167**:1047-1066; Moore M., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 1987, **84**:9194-9198; Barel M., *et al.*, *Mol. Immunol.*, 1998, **35**:1025-1031; Weis J.J., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 1986, **83**:5639-5643; Sinha S.K., *et al.*, *J. Immunol.*, 1993, **150**:5311-5320; WO 2004/045520 (Example 4); US 2004/005538 (Example 1); WO 2003/062401 (Claim 9); WO 2004/045520 (Example 4); WO 9102536 (Fig 9.1-9.9); WO 2004/020595 (Claim 1); Accession: P20023; Q13866; Q14212; EMBL; M26004; AAA35786.1.
- (15) CD79b (CD79B, CD79 β , Igb (immunoglobulin-associated beta), B29, GenBank accession no. NM_000626 or 11038674); *Proc. Natl. Acad. Sci. U.S.A.*, 2003, **100**(7):4126-4131; *Blood*, 2002, **100**(9):3068-3076; Muller, *et al.*, *Eur. J. Immunol.*, 1992, **22**(6):1621-1625; WO 2004/016225 (claim 2, Fig 140); WO 2003/087768, US 2004/101874 (claim 1, page 102); WO 2003/062401 (claim 9); WO 2002/78524 (Example 2); US 2002/150573 (claim 5, page 15); US 5,644,033; WO 2003/048202 (claim 1, pages 306 and 309); WO 99/558658, US 6,534,482 (claim 13, Fig 17A/B); WO 2000/55351 (claim 11, pages 1145-1146); Cross-references: MIM:147245; NP_000617.1; NM_000626_1.
- (16) FcRH2 (IFGP4, IRTA4, SPAP1A (SH2 domain containing phosphatase anchor protein 1a), SPAP1B, SPAP1C, GenBank accession no. NM_030764, AY358130); *Genome Res.*, 2003, **13**(10):2265-2270; *Immunogenetics*, 2002, **54**(2):87-95; *Blood*, 2002, **99**(8):2662-2669; *Proc. Natl. Acad. Sci. U.S.A.*, 2001, **98**(17):9772-9777; Xu, M.J., *et al.*, *Biochem. Biophys. Res. Commun.*, 2001, **280**(3):768-775; WO 2004/016225 (Claim 2); WO 2003/077836; WO 2001/38490 (Claim 5; Fig 18D-1-18D-2); WO 2003/097803 (Claim 12); WO 2003/089624 (Claim 25); Cross-references: MIM:606509; NP_110391.2; NM_030764_1.
- (17) HER2 (ErbB2, GenBank accession no. M11730); Coussens, L., *et al.*, *Science*, 1985, **230**(4730):1132-1139; Yamamoto, T., *et al.*, *Nature*, 1986, **319**:230-234; Semba, K., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 1985, **82**:6497-6501; Swiercz, J.M., *et al.*, *J. Cell Biol.*, 2004, **165**:869-880; Kuhns, J.J., *et al.*, *J. Biol. Chem.*, 1999, **274**:36422-36427; Cho H.-S., *et al.*, *Nature*, 1993, **421**:756-760; Ehsani, A., *et al.*, *Genomics*, 1993, **15**:426-429; WO 2004/048938 (Example 2); WO 2004/027049 (Fig 1I); WO 2004/009622; WO 2003/081210; WO 2003/089904 (Claim 9); WO 2003/016475 (Claim 1); US 2003/118592; WO 2003/008537 (Claim 1); WO 2003/055439 (Claim 29; Fig 1A-B); WO 2003/025228

(Claim 37; Fig 5C); WO 2002/22636 (Example 13; Page 95-107); WO 2002/12341 (Claim 68; Fig 7); WO 2002/13847 (Page 71-74); WO 2002/14503 (Page 114-117); WO 2001/53463 (Claim 2; Page 41-46); WO 2001/41787 (Page 15); WO 2000/44899 (Claim 52; Fig 7); WO 2000/20579 (Claim 3; Fig 2); US 5,869,445 (Claim 3; Col 31-38); WO 9630514 (Claim 2; Page 56-61); EP 1439393 (Claim 7); WO 2004/043361 (Claim 7); WO 2004/022709; WO 2001/00244 (Example 3; Fig 4); Accession: P04626; EMBL; M11767; AAA35808.1. EMBL; M11761; AAA35808.1.

(18) NCA (CEACAM6, GenBank accession no. M18728); Barnett T., *et al.*, *Genomics*, 1988, 3:59-66; Tawaragi, Y., *et al.*, *Biochem. Biophys. Res. Commun.*, 1988, **150**:89-96; Strausberg, R.L., *et al.* *Proc. Natl. Acad. Sci. U.S.A.*, 2002, **99**:16899-16903; WO 2004/063709; EP 1439393 (Claim 7); WO 2004/044178 (Example 4); WO 2004/031238; WO 2003/042661 (Claim 12); WO 2002/78524 (Example 2); WO 2002/86443 (Claim 27; Page 427); WO 2002/60317 (Claim 2); Accession: P40199; Q14920; EMBL; M29541; AAA59915.1. EMBL; M18728.

(19) MDP (DPEP1, GenBank accession no. BC017023); *Proc. Natl. Acad. Sci. U.S.A.*, 2002, **99**(26):16899-16903; WO 2003/016475 (Claim 1); WO 2002/64798 (Claim 33; Page 85-87); JP 05003790 (Fig 6-8); WO 9946284 (Fig 9); Cross-references: MIM:179780; AAH17023.1; BC017023_1.

(20) IL20R α (IL20Ra, ZCYTOR7, GenBank accession no. AF184971); Clark H.F., *et al.*, *Genome Res.*, 2003, **13**:2265-2270; Mungall A.J., *et al.*, *Nature*, 2003, **425**:805-811; Blumberg H., *et al.*, *Cell*, 2001, **104**:9-19; Dumoutier L., *et al.*, *J. Immunol.*, 2001, **167**:3545-3549; Parrish-Novak J., *et al.*, *J. Biol. Chem.*, 2002, **277**:47517-47523; Pletnev, S., *et al.*, *Biochemistry*, 2003, **42**:12617-12624; Sheikh F., *et al.*, *J. Immunol.*, 2004, **172**:2006-2010; EP 1394274 (Example 11); US 2004/005320 (Example 5); WO 2003/029262 (Page 74-75); WO 2003/002717 (Claim 2; Page 63); WO 2002/22153 (Page 45-47); US 2002/042366 (Page 20-21); WO 2001/46261 (Page 57-59); WO 2001/46232 (Page 63-65); WO 9837193 (Claim 1; Page 55-59); Accession: Q9UHF4; Q6UWA9; Q96SH8; EMBL; AF184971; AAF01320.1.

(21) Brevican (BCAN, BEHAB, GenBank accession no. AF229053); Gary S.C., *et al.*, *Gene*, 2000, **256**:39-147; Clark H.F., *et al.*, *Genome Res.*, 2003, **13**:2265-2270; Strausberg, R.L., *et al.* *Proc. Natl. Acad. Sci. U.S.A.*, 2002, **99**:16899-16903; US 2003/186372 (Claim 11); US 2003/186373 (Claim 11); US 2003/119131 (Claim 1; Fig 52); US 2003/119122 (Claim 1; Fig 52); US 2003/119126 (Claim 1); US 2003/119121 (Claim 1);

Fig 52); US 2003/119129 (Claim 1); US 2003/119130 (Claim 1); US 2003/119128 (Claim 1; Fig 52); US 2003/119125 (Claim 1); WO 2003/016475 (Claim 1); WO 2002/02634 (Claim 1).

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(23) ASLG659 (B7h, GenBank accession no. AX092328) US20040101899 (Claim 2); WO 2003/104399 (Claim 11); WO 2004/000221 (Fig 3); US 2003/165504 (Claim 1); US 2003/124140 (Example 2); US 2003/065143 (Fig 60); WO 2002/102235 (Claim 13; Page 299); US 2003/091580 (Example 2); WO 2002/10187 (Claim 6; Fig 10); WO 2001/94641 (Claim 12; Fig 7b); WO 2002/02624 (Claim 13; Fig 1A-1B); US 2002/034749 (Claim 54; Page 45-46); WO 2002/06317 (Example 2; Page 320-321, Claim 34; Page 321-322); WO 2002/71928 (Page 468-469); WO 2002/02587 (Example 1; Fig 1); WO 2001/40269 (Example 3; Pages 190-192); WO 2000/36107 (Example 2; Page 205-207); WO 2004/053079 (Claim 12); WO 2003/004989 (Claim 1); WO 2002/71928 (Page 233-234, 452-453); WO 0116318.

(24) PSCA (Prostate stem cell antigen precursor, GenBank accession no. AJ297436) Reiter R.E., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 1998, **95**:1735-1740; Gu Z., *et al.*, *Oncogene*, 2000, **19**:1288-1296; *Biochem. Biophys. Res. Commun.*, 2000, **275**(3):783-788; WO 2004/022709; EP 1394274 (Example 11); US 2004/018553 (Claim 17); WO 2003/008537 (Claim 1); WO 2002/81646 (Claim 1; Page 164); WO 2003/003906 (Claim 10; Page 288); WO 2001/40309 (Example 1; Fig 17); US 2001/055751 (Example 1; Fig 1b); WO 2000/32752 (Claim 18; Fig 1); WO 1998/51805 (Claim 17; Page 97); WO 1998/51824 (Claim 10; Page 94); WO 1998/40403 (Claim 2; Fig 1B); Accession: O43653; EMBL; AF043498; AAC39607.1.

(25) GEDA (GenBank accession No. AY260763); AAP14954 lipoma HMGIC fusion-partner-like protein /pid=AAP14954.1 - Homo sapiens Species: Homo sapiens (human) WO 2003/054152 (Claim 20); WO 2003/000842 (Claim 1); WO 2003/023013 (Example 3, Claim 20); US 2003/194704 (Claim 45); Cross-references: GI:30102449; AAP14954.1; AY260763_1.

(26) BAFF-R (B cell -activating factor receptor, BLyS receptor 3, BR3, GenBank accession No. AF116456); BAFF receptor /pid=NP_443177.1 - Homo sapiens Thompson, J.S., *et al.*, *Science*, 2001, **293**(5537):2108-2111; WO 2004/058309; WO 2004/011611; WO 2003/045422 (Example; Page 32-33); WO 2003/014294 (Claim 35; Fig 5 6B); WO 2003/035846 (Claim 70; Page 615-616); WO 2002/94852 (Col 136-137); WO 2002/38766 (Claim 3; Page 133); WO 2002/24909 (Example 3; Fig 3); Cross-references: MIM:606269; NP_443177.1; NM_052945_1; AF132600.

(27) CD22 (B-cell receptor CD22-B isoform, BL-CAM, Lyb-8, Lyb8, SIGLEC-2, FLJ22814, GenBank accession No. AK026467); Wilson, *et al.*, *J. Exp. Med.*, 10 1991, **173**:137-146; WO 2003/072036 (Claim 1; Fig 1); Cross-references: MIM:107266; NP_001762.1; NM_001771_1.

(28) CD79a (CD79A, CD79 α , immunoglobulin-associated alpha, a B cell-specific protein that covalently interacts with Ig beta (CD79B) and forms a complex on the surface with Ig M molecules, transduces a signal involved in B-cell differentiation), pI: 4.84, 15 MW: 25028 TM: 2 [P] Gene Chromosome: 19q13.2, GenBank accession No. NP_001774.10) WO 2003/088808, US 2003/0228319; WO 2003/062401 (claim 9); US 2002/150573 (claim 4, pages 13-14); WO 9958658 (claim 13, Fig 16); WO 9207574 (Fig 1); US 5,644,033; Ha, *et al.*, *J. Immunol.*, 1992, **148**(5):1526-1531; Mueller, *et al.*, *Eur. J. Biochem.*, 1992, **22**:1621-1625; Hashimoto, *et al.*, *Immunogenetics*, 1994, **40**(4):287-295; 20 Preud'homme, *et al.*, *Clin. Exp. Immunol.*, 1992, **90**(1):141-146; Yu, *et al.*, *J. Immunol.*, 1992, **148**(2) 633-637; Sakaguchi, *et al.*, *EMBO J.*, 1988, **7**(11):3457-3464.

(29) CXCR5 (Burkitt's lymphoma receptor 1, a G protein-coupled receptor that is activated by the CXCL13 chemokine, functions in lymphocyte migration and humoral defense, plays a role in HIV-2 infection and perhaps development of AIDS, lymphoma, 25 myeloma, and leukemia); 372 aa, pI: 8.54 MW: 41959 TM: 7 [P] Gene Chromosome: 11q23.3, GenBank accession No. NP_001707.1) WO 2004/040000; WO 2004/015426; US 2003/105292 (Example 2); US 6,555,339 (Example 2); WO 2002/61087 (Fig 1); WO 2001/57188 (Claim 20, page 269); WO 2001/72830 (pages 12-13); WO 2000/22129 (Example 1, pages 152-153, Example 2, pages 254-256); WO 1999/28468 (claim 1, page 38); 30 US 5,440,021 (Example 2, col 49-52); WO 9428931 (pages 56-58); WO 1992/17497 (claim 7, Fig 5); Dobner, *et al.*, *Eur. J. Immunol.*, 1992, **22**:2795-2799; Barella, *et al.*, *Biochem. J.*, 1995, **309**:773-779.

(30) HLA-DOB (Beta subunit of MHC class II molecule (Ia antigen) that binds peptides and presents them to CD4⁺ T lymphocytes); 273 aa, pI: 6.56 MW: 30820 TM: 1 [P] Gene Chromosome: 6p21.3, GenBank accession No. NP_002111.1); Tonnelle, *et al.*, *EMBO J.*, 1985, **4**(11):2839-2847; Jonsson, *et al.*, *Immunogenetics*, 1989, **29**(6):411-413; Beck, *et al.*, *J. Mol. Biol.*, 1992, **228**:433-441; Strausberg, *et al.*, *Proc. Natl. Acad. Sci USA*, 2002, **99**:16899-16903; Servenius, *et al.*, *J. Biol. Chem.*, 1987, **262**:8759-8766; Beck, *et al.*, *J. Mol. Biol.*, 1996, **255**:1-13; Naruse, *et al.*, *Tissue Antigens*, 2002, **59**:512-519; WO 9958658 (claim 13, Fig 15); US 6,153,408 (Col 35-38); US 5,976,551 (col 168-170); US 6,011,146 (col 145-146); Kasahara, *et al.*, *Immunogenetics*, 1989, **30**(1):66-68; Larhammar, *et al.*, *J. Biol. Chem.*, 1985, **260**(26):14111-14119.

(31) P2X5 (Purinergic receptor P2X ligand-gated ion channel 5, an ion channel gated by extracellular ATP, may be involved in synaptic transmission and neurogenesis, deficiency may contribute to the pathophysiology of idiopathic detrusor instability); 422 aa, pI: 7.63, MW: 47206 TM: 1 [P] Gene Chromosome: 17p13.3, GenBank accession No. NP_002552.2); Le, *et al.*, *FEBS Lett.*, 1997, **418**(1-2):195-199; WO 2004/047749; WO 2003/072035 (claim 10); Touchman, *et al.*, *Genome Res.*, 2000, **10**:165-173; WO 2002/22660 (claim 20); WO 2003/093444 (claim 1); WO 2003/087768 (claim 1); WO 2003/029277 (page 82).

(32) CD72 (B-cell differentiation antigen CD72, Lyb-2), pI: 8.66, MW: 40225 TM: 1 [P] Gene Chromosome: 9p13.3, GenBank accession No. NP_001773.1) WO2004042346 (claim 65); WO 2003/026493 (pages 51-52, 57-58); WO 2000/75655 (pages 105-106); Von Hoegen, *et al.*, *J. Immunol.*, 1990, **144**(12):4870-4877; Strausberg, *et al.*, *Proc. Natl. Acad. Sci USA*, 2002, **99**:16899-16903.

(33) LY64 (Lymphocyte antigen 64 (RP105), type I membrane protein of the leucine rich repeat (LRR) family, regulates B-cell activation and apoptosis, loss of function is associated with increased disease activity in patients with systemic lupus erythematosus); 661 aa, pI: 6.20, MW: 74147 TM: 1 [P] Gene Chromosome: 5q12, GenBank accession No. NP_005573.1) US 2002/193567; WO 9707198 (claim 11, pages 39-42); Miura, *et al.*, *Genomics*, 1996, **38**(3):299-304; Miura, *et al.*, *Blood*, 1998, **92**:2815-2822; WO 2003/083047; WO 9744452 (claim 8, pages 57-61); WO 2000/12130 (pages 24-26).

(34) FcRH1 (Fc receptor-like protein 1, a putative receptor for the immunoglobulin Fc domain that contains C2 type Ig-like and ITAM domains, may have a role in B-lymphocyte differentiation); 429 aa, pI: 5.28, MW: 46925 TM: 1 [P] Gene Chromosome: 1q21-1q22, GenBank accession No. NP_443170.1) WO 2003/077836; WO

2001/38490 (claim 6, Fig 18E-1-18-E-2); Davis, *et al.*, *Proc. Natl. Acad. Sci USA*, 2001, **98**(17):9772-9777; WO 2003/089624 (claim 8); EP 1347046 (claim 1); WO 2003/089624 (claim 7).

(35) IRTA2 (Immunoglobulin superfamily receptor translocation associated 2, a putative immunoreceptor with possible roles in B cell development and lymphomagenesis; deregulation of the gene by translocation occurs in some B cell malignancies); 977 aa, pI: 6.88 MW: 106468 TM: 1 [P] Gene Chromosome: 1q21, GenBank accession No. Human:AF343662, AF343663, AF343664, AF343665, AF369794, AF397453, AK090423, AK090475, AL834187, AY358085; Mouse:AK089756, AY158090, AY506558; NP_112571.1. WO 2003/024392 (claim 2, Fig 97); Nakayama, *et al.*, *Biochem. Biophys. Res. Commun.*, 2000, **277**(1):124-127; WO 2003/077836; WO 2001/38490 (claim 3, Fig 18B-1-18B-2).

(36) TENB2 (TMEFF2, tomoregulin, TPEF, HPP1, TR, putative transmembrane proteoglycan, related to the EGF/hereregulin family of growth factors and follistatin); 374 aa, NCBI Accession: AAD55776, AAF91397, AAG49451, NCBI RefSeq: NP_057276; NCBI Gene: 23671; OMIM: 605734; SwissProt Q9UIK5; GenBank accession No. AF179274; AY358907, CAF85723, CQ782436 WO 2004/074320; JP 2004113151; WO 2003/042661; WO 2003/009814; EP 1295944 (pages 69-70); WO 2002/30268 (page 329); WO 2001/90304; US 2004/249130; US 2004/022727; WO 2004/063355; US 2004/197325; US 2003/232350; US 2004/005563; US 2003/124579; Horie, *et al.*, *Genomics*, 2000, **67**:146-152; Uchida, *et al.*, *Biochem. Biophys. Res. Commun.*, 1999, **266**:593-602; Liang, *et al.*, *Cancer Res.*, 2000, **60**:4907-12; Glynne-Jones, *et al.*, *Int. J. Cancer*, 2001, **94**(2):178-84.

(37) PMEL17 (silver homolog; SILV; D12S53E; PMEL17; SI; SIL); ME20; gp100) BC001414; BT007202; M32295; M77348; NM_006928; McGlinchey, R.P., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 2009, **106**(33):13731-13736; Kummer, M.P., *et al.*, *J. Biol. Chem.*, 2009, **284** (4):2296-2306.

(38) TMEFF1 (transmembrane protein with EGF-like and two follistatin-like domains 1; Tomoregulin-1); H7365; C9orf2; C9ORF2; U19878; X83961; NM_080655; NM_003692; Harms, P.W., *Genes Dev.*, 2003, **17**(21):2624-2629; Gery, S., *et al.*, *Oncogene*, 2003, **22**(18):2723-2727.

(39) GDNF-Ra1 (GDNF family receptor alpha 1; GFRA1; GDNFR; GDNFRA; RETL1; TRNR1; RET1L; GDNFR-alpha1; GFR-ALPHA-1); U95847; BC014962; NM_145793 NM_005264; Kim, M.H., *et al.*, *Mol. Cell. Biol.*, 2009, **29**(8):2264-2277; Treanor, J.J., *et al.*, *Nature*, 1996, **382**(6586):80-83.

- (40) Ly6E (lymphocyte antigen 6 complex, locus E; Ly67, RIG-E, SCA-2, TSA-1); NP_002337.1; NM_002346.2; de Nooij-van Dalen, A.G., *et al.*, *Int. J. Cancer*, 2003, **103**(6): 768-774; Zammit, D.J., *et al.*, *Mol. Cell. Biol.*, 2002, **22**(3):946-952.
- (41) TMEM46 (shisa homolog 2 (*Xenopus laevis*); SHISA2);
 5 NP_001007539.1; NM_001007538.1; Furushima, K., *et al.*, *Dev. Biol.*, 2007, **306**(2):480-492; Clark, H.F., *et al.*, *Genome Res.*, 2003, **13**(10):2265-2270.
- (42) Ly6G6D (lymphocyte antigen 6 complex, locus G6D; Ly6-D, MEGT1); NP_067079.2; NM_021246.2; Mallya, M., *et al.*, *Genomics*, 2002, **80**(1):113-123; Ribas, G., *et al.*, *J. Immunol.*, 1999, **163**(1):278-287.
- 10 (43) LGR5 (leucine-rich repeat-containing G protein-coupled receptor 5; GPR49, GPR67); NP_003658.1; NM_003667.2; Salanti, G., *et al.*, *Am. J. Epidemiol.*, 2009, **170**(5):537-545; Yamamoto, Y., *et al.*, *Hepatology*, 2003, **37**(3):528-533.
- (44) RET (ret proto-oncogene; MEN2A; HSCR1; MEN2B; MTC1; PTC; CDHF12; Hs.168114; RET51; RET-ELE1); NP_066124.1; NM_020975.4; Tsukamoto, H., *et al.*, *Cancer Sci.*, 2009 **100**(10):1895-1901; Narita, N., *et al.*, *Oncogene*, 2009, **28**(34):3058-3068.
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- (45) LY6K (lymphocyte antigen 6 complex, locus K; LY6K; HsJ001348; FLJ35226); NP_059997.3; NM_017527.3; Ishikawa, N. *et al.*, *Cancer Res.*, 2007, **67**(24):11601-11611; de Nooij-van Dalen, A.G., *et al.*, *Int. J. Cancer*, 2003, **103**(6):768-774.
- 20 (46) GPR19 (G protein-coupled receptor 19; Mm.4787); NP_006134.1; NM_006143.2; Montpetit, A. and Sinnett, D., *Hum. Genet.*, 1999, **105**(1-2):162-164; O'Dowd, B.F., *et al.*, *FEBS Lett.*, 1996, **394**(3):325-329.
- (47) GPR54 (KISS1 receptor; KISS1R; GPR54; HOT7T175; AXOR12); NP_115940.2; NM_032551.4; Navenot, J.M., *et al.*, *Mol. Pharmacol.*, 2009, **75**(6):1300-1306; Hata, K., *et al.*, *Anticancer Res.* 2009, **29**(2):617-623.
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- (48) ASPHD1 (aspartate beta-hydroxylase domain containing 1; LOC253982); NP_859069.2; NM_181718.3; Gerhard, D.S., *et al.*, *Genome Res.*, 2004, **14**(10B):2121-2127.
- (49) Tyrosinase (TYR; OCA1A; OCA1A; tyrosinase; SHEP3); NP_000363.1; NM_000372.4; Bishop, D.T., *et al.*, *Nat. Genet.*, 2009, **41**(8):920-925; Nan, H., *et al.*, *Int. J. Cancer*, 2009, **125**(4):909-917.
 30
- (50) TMEM118 (ring finger protein, transmembrane 2; RNFT2; FLJ14627); NP_001103373.1; NM_001109903.1; Clark, H.F., *et al.*, *Genome Res.*, 2003, **13**(10):2265-2270; Scherer, S.E., *et al.*, *Nature*, 2006, **440**(7082):346-351.

(51) GPR172A (G protein-coupled receptor 172A; GPCR41; FLJ11856; D15Ert747e); NP_078807.1; NM_024531.3; Ericsson, T.A., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 2003, **100**(11):6759-6764; Takeda, S., *et al.*, *FEBS Lett.*, 2002, **520**(1-3):97-101.

(52) CD33, a member of the sialic acid binding, immunoglobulin-like lectin family, is a 67-kDa glycosylated transmembrane protein. CD33 is expressed on most myeloid and monocytic leukemia cells in addition to committed myelomonocytic and erythroid progenitor cells. It is not seen on the earliest pluripotent stem cells, mature granulocytes, lymphoid cells, or nonhematopoietic cells (Sabbath, *et al.*, *J. Clin. Invest.*, 1985, **75**:756-56; Andrews, *et al.*, *Blood*, 1986, **68**:1030-5). CD33 contains two tyrosine residues on its cytoplasmic tail, each of which is followed by hydrophobic residues similar to the immunoreceptor tyrosine-based inhibitory motif (ITIM) seen in many inhibitory receptors.

(53) CLL-1 (CLEC12A, MICL, and DCAL2), encodes a member of the C-type lectin/C-type lectin-like domain (CTL/CTLD) superfamily. Members of this family share a common protein fold and have diverse functions, such as cell adhesion, cell-cell signaling, glycoprotein turnover, and roles in inflammation and immune response. The protein encoded by this gene is a negative regulator of granulocyte and monocyte function. Several alternatively spliced transcript variants of this gene have been described, but the full-length nature of some of these variants has not been determined. This gene is closely linked to other CTL/CTLD superfamily members in the natural killer gene complex region on chromosome 12p13 (Drickamer, K., *Curr. Opin. Struct. Biol.*, 1999, **9**(5):585-90; van Rhenen, A., *et al.*, *Blood*, 2007, **110**(7):2659-66; Chen, C.H., *et al.*, *Blood*, 2006, **107**(4):1459-67; Marshall, A.S., *et al.*, *Eur. J. Immunol.*, 2006, **36**(8):2159-69; Bakker, A.B., *et al.*, *Cancer Res.*, 2005, **64**(22):8443-50; Marshall, A.S., *et al.*, *J. Biol. Chem.*, 2004, **279**(15):14792-802). CLL-1 has been shown to be a type II transmembrane receptor comprising a single C-type lectin-like domain (which is not predicted to bind either calcium or sugar), a stalk region, a transmembrane domain and a short cytoplasmic tail containing an ITIM motif.

(54) CD70 (CD27 ligand; CD27-L); cytokine that binds to CD27) plays a role in T-cell activation and induces the proliferation of co-stimulated T-cells and enhances the generation of cytolytic T-cells. CD70 protein is expressed on highly activated lymphocytes such as T- and B-cell lymphomas (Israel, B.F., *et al.*, *Mol. Cancer Ther.*, 2005, **4**(12):2037-44; O'Neill, R.E., *et al.*, *J. Immunol.*, 2017, **199**(10):3700-3710; Leigh, N. D., *et al.*, *J. Immunol.*, 2017, **199**(1):336-347; Itani, H. A., *et al.*, *Circ. Res.*, 2016, **118**(8):1233-43;

Burchill, M.A., *et al.*, *Eur. J. Immunol.*, 2015, **45**(11):3140-9; Allam, A., *et al.*, *J. Immunol.*, 2014, **193**(2):871-8).

(55) CLDN18.2 (Claudin-18 Splice Variant 2); CLDN18 encodes the human gene, Claudin 18. It may also be known as: Claudin-18; SFTA5; and SFTPJ. The encoded protein has an amino acid length of 261 and a mass of 27.9 kDa. CLDN18 is a member of the Claudin family (WO 2013/167295; WO 2016/166122). The tight junction molecule claudin 18 isotype 2 (CLDN 18.2) is a cancer-associated splice variant of Claudin 18. CLDN 18.2 is a 27.8 kDa transmembrane protein comprising four membrane spanning domains with two small extracellular loops (loop1 embraced by hydrophobic region 1 and hydrophobic region 2; loop2 embraced by hydrophobic regions 3 and 4). CLDN 18.2 is a highly selective gastric lineage antigen, exclusively expressed on short-lived differentiated gastric epithelial cells and not detectable in any other normal human tissue. The antigen is ectopically expressed at significant levels in a diversity of human cancers including gastroesophageal and pancreatic cancer (Sahin, U., *et al.*, *Clin. Cancer Res.*, 2008, **14**(23):7624-34). The CLDN18.2 protein is also frequently detected in lymph node metastases of gastric cancer and in distant metastases. CLDN 18.2 seems to be involved in proliferation of CLDN 18.2 positive tumor cells, since down regulation of the target by siRNA technology results in inhibition of proliferation of gastric cancer cells. 1MAB362 is a chimeric monoclonal antibody of IgG1 subtype directed against CLDN 18.2. 1MAB362 recognizes the first extracellular domain of CLDN 18.2 with high affinity and specificity and does not bind to any other claudin family member including the closely related splice variant 1 of Claudin 18.

(56) CD151 (Cluster of differentiation 151, Tspan24, PETA-3, SFA-1); human gene from the Raph blood group. The protein encoded by CD151 gene is a member of the transmembrane 4 superfamily, also known as the tetraspanin family. Most of these members are cell-surface proteins that are characterized by the presence of four hydrophobic domains. The proteins mediate signal transduction events that play a role in the regulation of cell development, activation, growth and motility. This encoded protein is a cell surface glycoprotein that is known to complex with integrins and other transmembrane 4 superfamily proteins. It is involved in cellular processes including cell adhesion and may regulate integrin trafficking and/or function. This protein enhances cell motility, invasion and metastasis of cancer cells. Multiple alternatively spliced transcript variants that encode the same protein have been described for this gene (Berdichevski F., *J. Cell Sci.*, 2002, **114**(Pt 23):4143-51; Ashman, L.K., *J. Biol. Regul. Homeost. Agents*, 2003, **16**(3):223-6; Sincock, P.M., *et al.*, *J. Histochem. Cytochem.*, 1997, **45**(4):515-25; Fitter S, *et al.*, *Biochim. Biophys.*

Acta., 1998, **1398**(1):75–85; Sincock, P.M., *et al.*, *J. Cell Sci.*, 1999, **112**(Pt 6):833–44; Sterk, L.M., *et al.*, *J. Cell Biol.*, 2000, **149**(4):969–82; Zhang, X.A., *Mol. Biol. Cell.*, 2002, **13**(1):1–11).

(57) ITGaV (integrin alpha-V, CD51; MSK8; VNRA; VTNR); a protein in
 5 humans is encoded by the ITGAV gene (Sosnoski, D. M., *et al.*, *J. Clin. Invest.*, 1988, **81**(6):1993–8). ITGAV encodes integrin alpha chain V. Integrins are heterodimeric integral membrane proteins composed of an alpha chain and a beta chain. Alpha V undergoes post-translational cleavage to yield disulfide-linked heavy and light chains that combine with multiple integrin beta chains to form different integrins. Among the known associating beta
 10 chains (beta chains 1,3,5,6, and 8; 'ITGB1', 'ITGB3', 'ITGB5', 'ITGB6', and 'ITGB8'), each can interact with extracellular matrix ligands; the alpha V beta 3 integrin, perhaps the most studied of these, is referred to as the Vitronectin receptor (VNR). In addition to adhesion, many integrins are known to facilitate signal transduction. Overexpression of the ITGAV gene is associated with progression and spread of colorectal cancer (Waisberg, J., *et al.*,
 15 *Anticancer Res.*, 2014, **34**(10):5599–607) and prostate cancer (Cooper, C. R., *et al.*, *Neoplasia*, 2002, **4**(3):191–4). Monoclonal antibodies intetumumab and abituzumab target this protein which is found on some tumor cells (Élez, E., *et al.*, *Ann. Oncology*, 2015, **26**(1):132–40).

Further exemplary antigens include, but are not limited to: (58) Nectin-4; (59)
 20 FGFR2; (60) FGFR3; (61) gpNMB; (62) GUCY2C; (63) CLDN6; (64) cMET; (65) CEACAM4; (66) CEACAM5; (67) CD29; (68) CD37; (69) CD352; (70) CD248; (71) MUC1; (72) CD123; (73) BCMA; (74) ADAM9; (75) 5T4; (76) P-cadherin; (77) CA9; (78) CD138; (79) CD166; (80) CD71; (81) CD22; (82) CD20; (83) CD74; (84) DLL3; (85) Folate Receptor alpha; (86) ITGa2; (87) ITGa3; (88) LAMP1; (89) LIV-1; (90) MMP14; (91)
 25 LRCC15; (92) MSLN; (93) PMSA; (94) PRLR; (95) PTK7; (96) SLAMF7; (97) SCL44A4; (98) SLTRK5; (99) TM4SF1; and (100) TROP2.

In certain embodiments of the present invention, TAA are selected from the group consisting of TROP2, HER2, CD33, CD70, MUC1/CanAg, MUC16, CD151 and ITGaV.

EXEMPLARY ANTIBODIES (e.g., anti-Trop2 antibodies)

30 Certain embodiments of the invention provide an antibody or fragment thereof (e.g., antigen binding domain). In certain embodiments, the antibody or fragment thereof is not conjugated to a payload drug. In certain embodiments, the antibody or fragment thereof is

conjugated to a payload drug. For example, the antibody drug conjugate of the invention comprises an antibody or fragment thereof.

Included in the scope of the embodiments of the invention are functional variants of the antibody or antigen binding domain described herein. The term “functional variant” as
5 used herein refers to an antibody having an antigen binding domain with substantial or significant sequence identity or similarity to a parent antibody or antigen binding domain, which functional variant retains the biological activity of the antibody or antigen binding domain of which it is a variant. Functional variants encompass, for example, those variants of the antibody or antigen binding domain described herein (the parent antibody or antigen
10 binding domain) that retain the ability to recognize target cells expressing a TAA, such as Trop2, to a similar extent, the same extent, or to a higher extent, as the parent antibody or antigen binding domain.

In reference to the antibody or antigen binding domain, the functional variant can, for instance, be at least about 30%, about 50%, about 75%, about 80%, about 85%, about 90%,
15 about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or more identical in amino acid sequence to the antibody or antigen binding domain. In certain embodiments, the antibody or antigen binding domain or functional variant thereof can be at least about 75% (e.g., 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%)
20 or more identical in amino acid sequence to the antibody or antigen binding domain described herein.

A functional variant can, for example, comprise the amino acid sequence of the parent antibody or antigen binding domain with at least one conservative amino acid substitution. Alternatively, or additionally, the functional variants can comprise the amino acid sequence
25 of the parent antibody or antigen binding domain with at least one non-conservative amino acid substitution. In this case, it is preferable for the non-conservative amino acid substitution to not interfere with or inhibit the biological activity of the functional variant. The non-conservative amino acid substitution may enhance the biological activity of the functional variant, such that the biological activity of the functional variant is increased as
30 compared to the parent antibody or antigen binding domain.

The antibodies, including the antibody drug conjugate of the invention, include Fc engineered variants. In some embodiments, the mutations in the Fc region that result in modulated binding to one or more Fc receptors can include one or more of the following mutations: SD (S239D), SDIE (S239D/I332E), SE (S267E), SELF (S267E/L328F), SDIE

(S239D/I332E), SDIEAL (S239D/I332E/A330L), GA (G236A), ALIE (A330L/I332E), GASDALIE (G236A/S239D/A330L/I332E), V9 (G237D/P238D/P271G/A330R), and V11 (G237D/P238D/H268D/P271G/A330R), and/or one or more mutations at the following amino acids: E345R, E233, G237, P238, H268, P271, L328 and A330. Additional Fc region
5 modifications for modulating Fc receptor binding are described in, for example, U.S. Patent Application Publication 2016/0145350 and U.S. Patents 7,416,726 and 5,624,821, which are hereby incorporated by reference in their entireties herein.

The antibodies, including the antibody drug conjugate of the invention, include glycan variants, such as afucosylation. In some embodiments, the Fc region of the binding agents are
10 modified to have an altered glycosylation pattern of the Fc region compared to the native non-modified Fc region.

Amino acid substitutions of the inventive antibody or antigen binding domains can be conservative amino acid substitutions. Conservative amino acid substitutions are known in the art, and include amino acid substitutions in which one amino acid having certain physical
15 and/or chemical properties is exchanged for another amino acid that has the same or similar chemical or physical properties. For instance, the conservative amino acid substitution can be an acidic/negatively charged polar amino acid substituted for another acidic/negatively charged polar amino acid (e.g., Asp or Glu), an amino acid with a nonpolar side chain substituted for another amino acid with a nonpolar side chain (e.g., Ala, Gly, Val, Ile, Leu,
20 Met, Phe, Pro, Trp, Cys, Val, etc.), a basic/positively charged polar amino acid substituted for another basic/positively charged polar amino acid (e.g., Lys, His, Arg, etc.), an uncharged amino acid with a polar side chain substituted for another uncharged amino acid with a polar side chain (e.g., Asn, Gln, Ser, Thr, Tyr, etc.), an amino acid with a beta-branched side-chain substituted for another amino acid with a beta-branched side-chain (e.g., Ile, Thr, and Val), an
25 amino acid with an aromatic side-chain substituted for another amino acid with an aromatic side chain (e.g., His, Phe, Trp, and Tyr), etc.

The antibody or antigen binding domain can consist essentially of the specified amino acid sequence or sequences described herein, such that other components, e.g., other amino acids, do not materially change the biological activity of the antibody or antigen binding
30 domain functional variant.

Methods for generating antibodies are described in, for example, Köhler and Milstein, *Eur. J. Immunol.*, 5: 511-519 (1976); Harlow and Lane (eds.), *Antibodies: A Laboratory Manual*, CSH Press (1988); and Janeway et al. (eds.), *Immunobiology, 9th Ed.*, Garland Publishing, New York, NY (2017). In certain embodiments, a human or chimeric antibody or

antibody fragment can be generated using a transgenic animal (e.g., a mouse) wherein one or more endogenous immunoglobulin genes are replaced with one or more human immunoglobulin genes. Examples of transgenic mice wherein endogenous antibody genes are effectively replaced with human antibody genes include, but are not limited to, the

5 Medarex HUMAB-MOUSE™, the Kirin TC MOUSE™, and the Kyowa Kirin KM-MOUSE™ (see, e.g., Lonberg, *Nat. Biotechnol.*, 23(9): 1117-25 (2005), and Lonberg, *Handb. Exp. Pharmacol.*, 181: 69-97 (2008)). A humanized antibody can be generated using any suitable method known in the art (see, e.g., An, Z. (ed.), *Therapeutic Monoclonal Antibodies: From Bench to Clinic*, John Wiley & Sons, Inc., Hoboken, New Jersey (2009)),

10 including, e.g., grafting of non-human CDRs onto a human antibody scaffold (see, e.g., Kashmiri et al., *Methods*, 36(1): 25-34 (2005); and Hou et al., *J. Biochem.*, 144(1): 115-120 (2008) and use of phage display (see, e.g., Fellouse, et al., *Journal of Molecular Biology*, 373(4): 924-940 (2007) and Glanville, et al., *PNAS*, 106(48): 20216-20221 (2009)).

In an exemplary embodiment, the antibody or ADC of the invention comprise an

15 antibody that comprises an antigen binding domain that specifically recognizes and binds human and non-human primate Trop2 (also known as Tumor associated calcium signal transducer 2, also see NCBI accession numbers NP_002344 and XP_005543292).

In some embodiments, the Trop2 binding agent (e.g., antibody or fragment thereof) binds to human Trop2, for example, a protein comprising SEQ ID NO: 38. However, binding

20 agents that bind to any Trop2 homolog or paralog also are encompassed. In some embodiments, the Trop2 protein comprises at least about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more sequence identity to SEQ ID NO: 38. In some embodiments, the binding agent binds human Trop2 and cynomolgus Trop2; or human,

25 cynomolgus, and mouse Trop2.

30 MARGPGLAPPPLRLPLLLLVLAAVTGHTAAQDNCTCPTNKMTVCSPDGPGRRCQCRLGSGMAVDCSTLT
SKCLLLKARMSAPKNARTLVRPSEHALVDNDGLYDPCDPEGFRFKARQCNTSVWCVCVNSVGVRRTDKGD
LSLRCDLVTRTHHILIDLRRPTAGAFNHSDLDLAEPLRRLFRERYRLHPKFVAAVHYEQPTIQIELRQNTS
QKAAGDVIDGDAAYYFERDIKGESLFQGRGGLDLVRGEPLQVERTLIYYLDEIPPKFSMKRLTAGLIAV
IVVVVVALVAGMAVLVITNRRKSGKYKKVEIKELGELRKEPSL

SEQ ID NO: 38

A method is provided for delivering a payload to a cell expressing Trop2 comprising administering to the cell, or mammal comprising the cell, an ADC comprising an anti-Trop2 antibody covalently attached to a payload (e.g., via a linker or without a linker).

The invention also provides a Trop2 binding agent (e.g., antibody or fragment thereof) comprising an immunoglobulin heavy chain variable region polypeptide and an immunoglobulin light chain variable region polypeptide.

5 The Trop2 binding agent specifically binds Trop2. The binding specificity of the agent allows for targeting Trop2 expressing cells, for instance, to deliver therapeutic payloads to such cells.

In some embodiments, the Trop2 binding agent binds Trop2 without substantially inhibiting or preventing Trop2 from binding to one or more of its ligand(s) (e.g., claudin 1, claudin 7, or IGF-1). However, in other embodiments, the Trop2 binding agent can
10 completely or partially block (inhibit or prevent) binding of Trop2 to one or more of its binding partners such as the fibronectin receptor $\alpha 5 \beta 1$ (integrin $\alpha 5 \beta 1$), such that the antibody can be used to inhibit Trop2 mediated cancer cell migration or metastasis (Trerotola M. et al., Cancer Research, 2013, May 15;73(10):3155-67), or may completely or partially block cis- or trans-dimerization of the Trop2 receptor's ectodomains (Sun M. et al., iScience, 2021, Oct
15 22; 24(10): 103190).

The antibody or antigen-binding antibody fragment can be monospecific for Trop2, or can be bispecific or multi-specific. For instance, in bivalent or multivalent antibodies or antibody fragments, the binding domains can be different, targeting different epitopes of the same antigen, or targeting different antigens. Methods of constructing multivalent binding
20 constructs are known in the art. Bispecific and multispecific antibodies are known in the art. Furthermore, a diabody, triabody, or tetrabody can be provided, which is a dimer, trimer, or tetramer of polypeptide chains each comprising a V_H connected to a V_L by a peptide linker that is too short to allow pairing between the V_H and V_L on the same polypeptide chain, thereby driving the pairing between the complementary domains on different V_H - V_L
25 polypeptide chains to generate a multimeric molecule having two, three, or four functional antigen binding sites. Also, bis-scFv fragments, which are small scFv fragments with two different variable domains can be generated to produce bispecific bis-scFv fragments capable of binding two different epitopes. Fab dimers (Fab2) and Fab trimers (Fab3) can be produced using genetic engineering methods to create multispecific constructs based on Fab fragments.

30 The Trop2-binding agent also can be an antibody conjugate. In this respect, the Trop2-binding agent can be a conjugate of (1) an antibody, an alternative scaffold, or fragments thereof, and (2) a protein or non-protein moiety. For example, the Trop2 binding agent can be conjugated to a peptide, a fluorescent molecule, chemotherapeutic, a radioisotope, or other cytotoxic payload agent.

The Trop2-binding agent can be, or can be obtained from, a human antibody, a non-human antibody, a humanized antibody, or a chimeric antibody, or corresponding antibody fragments. A “chimeric” antibody is an antibody or fragment thereof typically comprising human constant regions and non-human variable regions. A “humanized” antibody is a
5 monoclonal antibody typically comprising a human antibody scaffold with non-human origin amino acids or sequences in at least one CDR (e.g., 1, 2, 3, 4, 5, or all six CDRs).

Furthermore, in some embodiments, the Trop2-binding agent provided herein may cause cellular internalization of Trop2 or the binding agent complex upon binding to Trop2 on the cell surface. Without wishing to be bound by any particular theory or mechanism of
10 action, it is believed that certain Trop2-binding agents according to this embodiment may cause Trop2 internalization upon binding, and remain bound to Trop2 during internalization resulting in internalization of the binding agent along with Trop2. Cellular internalization of Trop2 and bound Trop2 binding agent can be determined by any suitable method, such as a method described herein, or assaying for persistence on the cell surface and/or detection of
15 internalized antibodies. In some embodiments, the Trop2 binding agent internalizes strongly enough that at least about 25% (e.g., at least about 35%, at least about 50%, at least about 75%, or at least about 90%) of the Trop2 binding agent that binds Trop2 on the cell surface is internalized (e.g., using a surface persistence assay, about 75% or less, about 65% or less, about 50% or less, about 25% or less or about 10% or less of Trop2 binding agent molecules
20 bound to Trop2 on the cell surface at the beginning of the assay remain bound at the end of the assay).

In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises one or more (e.g., 1, 2, 3, 4, 5, or 6) complementarity determining region (CDRs) as described herein (e.g., as described in Tables
25 S1-S6).

In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises an immunoglobulin heavy chain variable region polypeptide as described herein, and/or an immunoglobulin light chain variable region polypeptide as described herein.

30 In certain embodiments, the immunoglobulin heavy chain variable region polypeptide of the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises

a complementarity determining region 1 (HCDR1) comprising an amino acid sequence that is at least 75% (e.g., 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%,

86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%) identical to NYGMN (SEQ ID NO: 14),

a complementarity determining region 2 (HCDR2) comprising an amino acid sequence that is at least 75% (e.g., 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 5 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%) identical to WINTKTGEPTYAEEFKG (SEQ ID NO: 16) or WINTKTGEPTYAQEFTG (SEQ ID NO: 21), and

a complementarity determining region 3 (HCDR3) comprising an amino acid sequence that is at least 75% (e.g., 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 10 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%) identical to GYGSSYWFYFDV (SEQ ID NO: 18).

In certain embodiments, the immunoglobulin light chain variable region polypeptide of the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises

15 a complementarity determining region 1 (LCDR1) comprising an amino acid sequence that is at least 75% (e.g., 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%) identical to KASQDVSIAVA (SEQ ID NO: 27),

a complementarity determining region 2 (LCDR2) comprising an amino acid 20 sequence that is at least 75% (e.g., 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%) identical to SASYRYT (SEQ ID NO: 29), and

a complementarity determining region 3 (LCDR3) comprising an amino acid sequence that is at least 75% (e.g., 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 25 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%) identical to QQHYITPLT (SEQ ID NO: 31).

In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises

an immunoglobulin heavy chain variable region polypeptide comprising a 30 complementarity determining region 1 (HCDR1) comprising an amino acid sequence that is at least 80%, 85%, 90%, or 95% identical to NYGMN (SEQ ID NO: 14), a complementarity determining region 2 (HCDR2) comprising an amino acid sequence that is at least 80%, 85%, 90%, or 95% identical to WINTKTGEPTYAEEFKG (SEQ ID NO: 16) or WINTKTGEPTYAQEFTG (SEQ ID NO: 21), and a complementarity determining region 3

(HCDR3) comprising an amino acid sequence that is at least 80%, 85%, 90%, or 95% identical to GGYGSSYWFYFDV (SEQ ID NO: 18); and/or

an immunoglobulin light chain variable region polypeptide comprising a complementarity determining region 1 (LCDR1) comprising an amino acid sequence that is
5 at least 80%, 85%, 90%, or 95% identical to KASQDVSIABA (SEQ ID NO: 27), a complementarity determining region 2 (LCDR2) comprising an amino acid sequence that is at least 80%, 85%, 90%, or 95% identical to SASYRYT (SEQ ID NO: 29), and a complementarity determining region 3 (LCDR3) comprising an amino acid sequence that is at least 80%, 85%, 90%, or 95% identical to QQHYITPLT (SEQ ID NO: 31).

10 In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises

an immunoglobulin heavy chain variable region polypeptide comprising a complementarity determining region 1 (HCDR1) comprising an amino acid sequence that is at least 90% identical to NYGMN (SEQ ID NO: 14), a complementarity determining region 2
15 (HCDR2) comprising an amino acid sequence that is at least 90% identical to WINTKTGEPTYAQEFTG (SEQ ID NO: 21), and a complementarity determining region 3 (HCDR3) comprising an amino acid sequence that is at least 90% identical to GGYGSSYWFYFDV (SEQ ID NO: 18); and/or

an immunoglobulin light chain variable region polypeptide comprising a complementarity determining region 1 (LCDR1) comprising an amino acid sequence that is at least 90% identical to KASQDVSIABA (SEQ ID NO: 27), a complementarity determining region 2 (LCDR2) comprising an amino acid sequence that is at least 90% identical to SASYRYT (SEQ ID NO: 29), and a complementarity determining region 3 (LCDR3) comprising an amino acid sequence that is at least 90% identical to QQHYITPLT (SEQ ID
25 NO: 31).

In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises

an immunoglobulin heavy chain variable region polypeptide comprising a complementarity determining region 1 (HCDR1) comprising NYGMN (SEQ ID NO: 14), a
30 complementarity determining region 2 (HCDR2) comprising WINTKTGEPTYAEEFKG (SEQ ID NO: 16) or WINTKTGEPTYAQEFTG (SEQ ID NO: 21), and a complementarity determining region 3 (HCDR3) comprising GGYGSSYWFYFDV (SEQ ID NO: 18); and/or

an immunoglobulin light chain variable region polypeptide comprising a complementarity determining region 1 (LCDR1) comprising KASQDVSIABA (SEQ ID NO:

27), a complementarity determining region 2 (LCDR2) comprising SASYRYT (SEQ ID NO: 29), and a complementarity determining region 3 (LCDR3) comprising QQHYITPLT (SEQ ID NO: 31).

5 In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises an immunoglobulin heavy chain variable region polypeptide comprising a complementarity determining region 1 (HCDR1) comprising SEQ ID NO: 14, a complementarity determining region 2 (HCDR2) comprising SEQ ID NO: 16, and a complementarity determining region 3 (HCDR3) comprising SEQ ID NO: 18.

10 In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises an immunoglobulin heavy chain variable region polypeptide comprising a complementarity determining region 1 (HCDR1) comprising SEQ ID NO: 14, a complementarity determining region 2 (HCDR2) comprising SEQ ID NO: 21, and a complementarity determining region 3 (HCDR3) comprising SEQ ID NO: 18.

15 In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises an immunoglobulin light chain variable region polypeptide comprising a complementarity determining region 1 (LCDR1) comprising SEQ ID NO: 27, a complementarity determining region 2 (LCDR2) comprising SEQ ID NO: 29, and a complementarity determining region 3 (LCDR3) comprising SEQ ID NO: 31.

20 In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises one or more complementarity determining regions (CDRs) and/or Framework region (FRs) as described herein (e.g., as described in Tables S1-S6).

25 In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises one or more (e.g., 1, 2, 3, 4, 5, 6, 7 or 8) Framework region (FRs) as described herein (e.g., as described in Tables S1-S6).

30 In particular embodiments, the binding agent comprises an immunoglobulin heavy chain polypeptide and an immunoglobulin light chain polypeptide, wherein the immunoglobulin heavy chain polypeptide comprises a first framework region, a second framework region, a third framework region, and/or a fourth framework region; and/or the immunoglobulin light chain polypeptide comprises a first framework region, a second framework region, a third framework region, and/or a fourth framework region; and/or the immunoglobulin heavy chain polypeptide and light chain polypeptide comprises any combination of the framework regions listed in Tables S1-S6.

In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises an immunoglobulin heavy chain variable region polypeptide comprising an amino acid sequence that is at least 80% (e.g., 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%) identical to any one of SEQ ID NOs: 1-4, and/or an immunoglobulin light chain variable region polypeptide comprising an amino acid sequence that is at least 80% (e.g., 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%) identical to any one of SEQ ID NOs: 5-6.

In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises an immunoglobulin heavy chain variable region polypeptide comprising an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to any one of SEQ ID NOs: 1-4, and/or an immunoglobulin light chain variable region polypeptide comprising an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to any one of SEQ ID NOs: 5-6.

In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises an immunoglobulin heavy chain variable region polypeptide comprising an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 4, and/or an immunoglobulin light chain variable region polypeptide comprising an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to any one of SEQ ID NOs: 5-6.

In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises an immunoglobulin heavy chain variable region polypeptide comprising an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to any one of SEQ ID NOs: 1-4, and/or an immunoglobulin light chain variable region polypeptide comprising an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO:6.

In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises an immunoglobulin heavy chain variable region polypeptide comprising an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to any one of SEQ ID NO: 2 or 4, and/or an immunoglobulin light chain variable region polypeptide comprising an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO:6.

In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises an immunoglobulin heavy chain variable region polypeptide comprising an amino acid sequence of any one of SEQ ID NOs: 1-4, and/or an immunoglobulin light chain variable region polypeptide comprising an amino acid sequence of any one of SEQ ID NOs: 5-6.

In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises an immunoglobulin heavy chain variable region polypeptide comprising an amino acid sequence of SEQ ID NO: 2 or 4, and/or an immunoglobulin light chain variable region polypeptide comprising an amino acid sequence of any one of SEQ ID NOs: 5-6.

In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises an immunoglobulin heavy chain variable region polypeptide comprising an amino acid sequence of SEQ ID NO: 4, and/or an immunoglobulin light chain variable region polypeptide comprising an amino acid sequence of any one of SEQ ID NOs: 5-6.

In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises an immunoglobulin heavy chain variable region polypeptide comprising an amino acid sequence of SEQ ID NO: 1, and/or an immunoglobulin light chain variable region polypeptide comprising an amino acid sequence of SEQ ID NO: 5.

In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises an immunoglobulin heavy chain variable region polypeptide comprising an amino acid sequence of SEQ ID NO: 1, and/or an immunoglobulin light chain variable region polypeptide comprising an amino acid sequence of SEQ ID NO: 6.

In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises an immunoglobulin heavy chain variable region polypeptide comprising an amino acid sequence of SEQ ID NO: 2, and/or an immunoglobulin light chain variable region polypeptide comprising an amino acid sequence of SEQ ID NO: 6.

In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises an immunoglobulin heavy chain variable region polypeptide comprising an amino acid sequence of SEQ ID NO: 3, and/or an

immunoglobulin light chain variable region polypeptide comprising an amino acid sequence of SEQ ID NO:6.

In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises an immunoglobulin heavy chain variable
5 region polypeptide comprising an amino acid sequence of SEQ ID NO: 4, and/or an immunoglobulin light chain variable region polypeptide comprising an amino acid sequence of SEQ ID NO:6.

In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises an immunoglobulin heavy chain variable
10 region polypeptide comprising an amino acid sequence of SEQ ID NO: 2 or 4, and/or an immunoglobulin light chain variable region polypeptide comprising an amino acid sequence of SEQ ID NO:5.

In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises an immunoglobulin heavy chain variable
15 region polypeptide comprising an amino acid sequence of SEQ ID NO: 2, and/or an immunoglobulin light chain variable region polypeptide comprising an amino acid sequence of SEQ ID NO:5.

In certain embodiments, the Trop2-binding agent (e.g., antibody or fragment thereof such as the antigen binding domain) comprises an immunoglobulin heavy chain variable
20 region polypeptide comprising an amino acid sequence of SEQ ID NO: 4, and/or an immunoglobulin light chain variable region polypeptide comprising an amino acid sequence of SEQ ID NO:5.

In certain embodiments, the Trop2-binding agent (e.g., antibody) comprises a human IgG1, IgG2, IgG3, or IgG4 Fc domain. In certain embodiments, the Trop2-binding agent
25 (e.g., antibody) comprises a human IgG1 Fc domain. In certain embodiments, the Trop2-binding agent (e.g., antibody) comprises an immunoglobulin heavy chain polypeptide comprising a human IgG1, IgG2, IgG3, or IgG4 Fc region polypeptide. In certain embodiments, the Trop2-binding agent (e.g., antibody) comprises an immunoglobulin heavy chain polypeptide comprising a human IgG1 Fc region polypeptide.

30 In certain embodiments, the Trop2-binding agent (e.g., antibody) comprises a human kappa light chain polypeptide. In certain embodiments, the Trop2-binding agent (e.g., antibody) comprises a human lambda light chain polypeptide.

In certain embodiments, the Trop2-binding agent (e.g., antibody) comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence that is at least

80% (e.g., 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%) identical to any one of SEQ ID NOs: 7, 9, 10, 11, or 34-37, and/or an immunoglobulin light chain polypeptide comprising an amino acid sequence that is at least 80% (e.g., 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%) identical to any one of SEQ ID NOs: 8 or 12.

In certain embodiments, the Trop2-binding agent (e.g., antibody) comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to any one of SEQ ID NOs: 7, 9, 10, 11, or 34-37, and/or an immunoglobulin light chain polypeptide comprising an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to any one of SEQ ID NOs: 8 or 12.

In certain embodiments, the Trop2-binding agent (e.g., antibody) comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to any one of SEQ ID NOs: 7, 9, 10, 11, or 34-37, and/or an immunoglobulin light chain polypeptide comprising an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO:8.

In certain embodiments, the Trop2-binding agent (e.g., antibody) comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to any one of SEQ ID NOs: 7, 10, 35, or 37, and/or an immunoglobulin light chain polypeptide comprising an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO:8.

In certain embodiments, the Trop2-binding agent (e.g., antibody) comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence that is at least 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 7 or 37, and/or an immunoglobulin light chain polypeptide comprising an amino acid sequence that is at least 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO:8 or 12.

In certain embodiments, the Trop2-binding agent (e.g., antibody) comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence of any one of SEQ ID NOs: 7, 9, 10, 11, or 34-37, and/or an immunoglobulin light chain polypeptide comprising an amino acid sequence of any one of SEQ ID NOs: 8 or 12.

In certain embodiments, the Trop2-binding agent (e.g., antibody) comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence of any one of SEQ ID NOs: 7 or 37, and/or an immunoglobulin light chain polypeptide comprising an amino acid sequence of any one of SEQ ID NOs: 8 or 12.

- 5 In certain embodiments, the Trop2-binding agent (e.g., antibody) comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence of any one of SEQ ID NOs: 7, 10, 35, or 37, and/or an immunoglobulin light chain polypeptide comprising an amino acid sequence of any one of SEQ ID NOs: 8 or 12.

- 10 In certain embodiments, the Trop2-binding agent (e.g., antibody) comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence of SEQ ID NO: 9 or 34, and/or an immunoglobulin light chain polypeptide comprising an amino acid sequence of SEQ ID NO:8.

- 15 In certain embodiments, the Trop2-binding agent (e.g., antibody) comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence of SEQ ID NO: 10 or 35, and/or an immunoglobulin light chain polypeptide comprising an amino acid sequence of SEQ ID NO:8.

- 20 In certain embodiments, the Trop2-binding agent (e.g., antibody) comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence of SEQ ID NO: 11 or 36, and/or an immunoglobulin light chain polypeptide comprising an amino acid sequence of SEQ ID NO:8.

- In certain embodiments, the Trop2-binding agent (e.g., antibody) comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence of SEQ ID NO: 7 or 37, and/or an immunoglobulin light chain polypeptide comprising an amino acid sequence of SEQ ID NO:8.

- 25 In certain embodiments, the Trop2-binding agent (e.g., antibody) comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence of any one of SEQ ID NO: 7, 10, 35, or 37, and/or an immunoglobulin light chain polypeptide comprising an amino acid sequence of SEQ ID NO:12.

- 30 In certain embodiments, the Trop2-binding agent (e.g., antibody) comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence of SEQ ID NO: 10 or 35, and/or an immunoglobulin light chain polypeptide comprising an amino acid sequence of SEQ ID NO:12.

In certain embodiments, the Trop2-binding agent (e.g., antibody) comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence of SEQ ID

NO: 7 or 37, and/or an immunoglobulin light chain polypeptide comprising an amino acid sequence of SEQ ID NO:12.

In an embodiment of the invention, the Trop2-targeting antibody or antigen binding domain comprises the Variable heavy chain (VH) of SEQ ID NO:1

5 QIQLVQSGHEVKKPGASVKVSCASGYTFTNYGMNWVRQAPGQGLEWMGWINTK
TGEPTYAEEFKGRFVFSLETSASTAYLQISSLKAEDMAMYFCGRGGYGSSYWFYFDV
WGQGTTVTVSS (SEQ ID NO:1).

In an embodiment of the invention, the Trop2-targeting antibody or antigen binding domain comprises one or more heavy chain CDR (complementarity determining region) or
10 heavy chain framework (HFR) sequences of SEQ ID NO. 13-19.

Region	Sequence Fragment	Residues	Length	SEQ ID NO.
HFR1	QIQLVQSGHEVKKPGASVKVSCASGYTFT	1 - 30	30	13
CDR-H1	NYGMN	31 - 35	5	14
HFR2	WVRQAPGQGLEWMG	36 - 49	14	15
CDR-H2	WINTKTGEPTYAEEFKG	50 - 66	17	16
HFR3	RFVFSLETSASTAYLQISSLKAEDMAMYFCGR	67 - 98	32	17
CDR-H3	GGYGSSYWFYFDV	99 - 110	12	18
HFR4	WGQGTTVTVSS	111- 121	11	19

Table S1.

In an embodiment of the invention, the Trop2-targeting antibody or antigen binding domain comprises the Variable heavy chain (VH) of SEQ ID NO:2

15 QVQLVQSGHEVKKPGASVKVSCASGYTFTNYGMNWVRQAPGQGLEWMGWINTK
TGEPTYAQEFTGRFVFSLDTSVSTAYLQISSLKAEDMAVYFCGRGGYGSSYWFYFDV
WGQGTTVTVSS (SEQ ID NO:2).

In an embodiment of the invention, the Trop2-targeting antibody or antigen binding domain comprises one or more heavy chain CDR (complementarity determining region) or heavy chain framework (HFR) sequences of SEQ ID NO. 14, 15, 18, 19, 20, 21 or 22.

Region	Sequence Fragment	Residues	Length	SEQ ID NO.
HFR1	QVQLVQSGHEVKKPGASVKVSCASGYTFT	1 - 30	30	20
CDR-H1	NYGMN	31 - 35	5	14
HFR2	WVRQAPGQGLEWMG	36 - 49	14	15
CDR-H2	WINTKTGEPTYAQEFTG	50 - 66	17	21
HFR3	RFVFSLDTSVSTAYLQISSLKAEDMAVYFCGR	67 - 98	32	22
CDR-H3	GGYGSSYWFYFDV	99 - 110	12	18
HFR4	WGQGTTVTVSS	111- 121	11	19

20 **Table S2.**

In an embodiment of the invention, the Trop2-targeting antibody or antigen binding domain comprises the Variable heavy chain (VH) of SEQ ID NO:3

QVQLVQSGHELKKPGASVKVSCASGYTFTNYGMNWVRQAPGQGLEWMGWINTK
TGEPTYAQEFTGRFVFLDTSVSTAYLQISSLKAEDMAVYYCGRGGYGSSYWFYFDV
WGQGTTTVTVSS (SEQ ID NO:3).

- In an embodiment of the invention, the Trop2-targeting antibody or antigen binding
5 domain comprises one or more heavy chain CDR (complementarity determining region) or
heavy chain framework (HFR) sequences of SEQ ID NO. 14, 15, 18, 19, 21, 23 or 24.

Region	Sequence Fragment	Residues	Length	SEQ ID NO.
HFR1	QVQLVQSGHELKKPGASVKVSCASGYTFT	1 - 30	30	23
CDR-H1	NYGMN	31 - 35	5	14
HFR2	WVRQAPGQGLEWMG	36 - 49	14	15
CDR-H2	WINTKTGEPTYAQEFTG	50 - 66	17	21
HFR3	RFVFLDTSVSTAYLQISSLKAEDMAVYYCGR	67 - 98	32	24
CDR-H3	GGYGSSYWFYFDV	99 - 110	12	18
HFR4	WGQGTTTVTVSS	111- 121	11	19

Table S3.

- In an embodiment of the invention, the Trop2-targeting antibody or antigen binding
domain comprises the Variable heavy chain (VH) of SEQ ID NO:4
10 QVQLVQSGHEVKKPGASVKVSCASGYTFTNYGMNWVRQAPGQGLEWMGWINTK
TGEPTYAQEFTGRFVFLDTSVSTAYLQISSLKAEDTAVYFCGRGGYGSSYWFYFDV
WGQGTTTVTVSS (SEQ ID NO:4).

- In an embodiment of the invention, the Trop2-targeting antibody or antigen binding
domain comprises one or more heavy chain CDR (complementarity determining region) or
15 heavy chain framework (HFR) sequences of SEQ ID NO. 14, 15, 18, 19, 20, 21 or 25.

Region	Sequence Fragment	Residues	Length	SEQ ID NO.
HFR1	QVQLVQSGHEVKKPGASVKVSCASGYTFT	1 - 30	30	20
CDR-H1	NYGMN	31 - 35	5	14
HFR2	WVRQAPGQGLEWMG	36 - 49	14	15
CDR-H2	WINTKTGEPTYAQEFTG	50 - 66	17	21
HFR3	RFVFLDTSVSTAYLQISSLKAEDTAVYFCGR	67 - 98	32	25
CDR-H3	GGYGSSYWFYFDV	99 - 110	12	18
HFR4	WGQGTTTVTVSS	111- 121	11	19

Table S4.

- In an embodiment of the invention, the Trop2-targeting antibody or antigen binding
domain comprises the Variable light chain (VL) of SEQ ID NO:5
DIQMTQSPSSLSASVGDRVTITCKASQDVSI AVAWYQQKPGKAPK VLIYSASYRYTG
20 VPDRFSGSGSGTDFTFTISSLQPEDIAVYYCQQHYITPLTFGGGTKVEIKR (SEQ ID
NO:5).

In an embodiment of the invention, the Trop2-targeting antibody or antigen binding domain comprises one or more light chain CDR (complementarity determining region) or light chain framework (LFR) sequences of SEQ ID NO. 26-32.

Region	Sequence Fragment	Residues	Length	SEQ ID NO.
LFR1	DIQMTQSPSSLSASVGDRVTITC	1 - 23	23	26
CDR-L1	KASQDVSIAVA	24 - 34	11	27
LFR2	WYQQKPGKAPKVLIIY	35 - 49	15	28
CDR-L2	SASYRYT	50 - 56	7	29
LFR3	GVPDRFSGSGSGTDFTFTISLQPEDIAVYYC	57 - 88	32	30
CDR-L3	QQHYITPLT	89 - 97	9	31
LFR4	FGGGTKVEIKR	98- 108	11	32

Table S5.

5 In an embodiment of the invention, the Trop2-targeting antibody or antigen binding domain comprises the Variable light chain (VL) of SEQ ID NO:6
 DIQMTQSPSSLSASVGDRVTITCKASQDVSIAWYQQKPGKAPKVLIIYSASYRYTG
 VPSRFSGSGSGTDFTFTISLQPEDIAVYYCQQHYITPLTFGGGTKVEIKR (SEQ ID
 NO:6).

10 In an embodiment of the invention, the Trop2-targeting antibody or antigen binding domain comprises one or more light chain CDR (complementarity determining region) or light chain framework (LFR) sequences of SEQ ID NO. 26-29 or 31-33.

Region	Sequence Fragment	Residues	Length	SEQ ID NO.
LFR1	DIQMTQSPSSLSASVGDRVTITC	1 - 23	23	26
CDR-L1	KASQDVSIAVA	24 - 34	11	27
LFR2	WYQQKPGKAPKVLIIY	35 - 49	15	28
CDR-L2	SASYRYT	50 - 56	7	29
LFR3	GVPDRFSGSGSGTDFTFTISLQPEDIAVYYC	57 - 88	32	33
CDR-L3	QQHYITPLT	89 - 97	9	31
LFR4	FGGGTKVEIKR	98- 108	11	32

Table S6.

In an embodiment of the invention, the LFR4 region comprises FGGGTKVEIK.

15 In an embodiment of the invention, the Trop2-targeting antibody or antigen binding domain comprises the Variable light chain (VL) of SEQ ID NO:39
 DIQMTQSPSSLSASVGDRVTITCKASQDVSIAWYQQKPGKAPKVLIIYSASYRYTG
 VPDRFSGSGSGTDFTFTISLQPEDIAVYYCQQHYITPLTFGGGTKVEIK (SEQ ID
 NO:39).

20 In an embodiment of the invention, the Trop2-targeting antibody or antigen binding domain comprises the Variable light chain (VL) of SEQ ID NO:40

DIQMTQSPSSLSASVGDRVTITCKASQDVSIAVAWYQQKPGKAPKVLISASRYRTG
VPSRFSGSGSGTDFTFTISSLQPEDIAVYYCQQHYITPLTFGGGTKVEIK (SEQ ID
NO:40).

In an embodiment of the invention, the Trop2-targeting antibody comprises the heavy
5 chain of SEQ ID NO:9
QIQLVQSGHEVKKPGASVKVSCASGYTFTNYGMNWVRQAPGQGLEWMGWINTK
TGEPTYAEEFKGRFVFSLETSASTAYLQISSLKAEDMAMYFCGRGGYGSSYWFYFDV
WGQGTTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
TSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD
10 KTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK
TISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSVMSVHEALHNHYTQKSLSLSPGK
(SEQ ID NO:9).

15 In an embodiment of the invention, the Trop2-targeting antibody comprises the heavy
chain of SEQ ID NO:10
QVQLVQSGHEVKKPGASVKVSCASGYTFTNYGMNWVRQAPGQGLEWMGWINTK
TGEPTYAQEFTGRFVFSLDTSVSTAYLQISSLKAEDMAVYFCGRGGYGSSYWFYFDV
WGQGTTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
20 TSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD
KTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK
TISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSVMSVHEALHNHYTQKSLSLSPGK
25 (SEQ ID NO:10).

In an embodiment of the invention, the Trop2-targeting antibody comprises the heavy
chain of SEQ ID NO:11
QVQLVQSGHEVKKPGASVKVSCASGYTFTNYGMNWVRQAPGQGLEWMGWINTK
TGEPTYAQEFTGRFVFSLDTSVSTAYLQISSLKAEDMAVYYCGRGGYGSSYWFYFDV
30 WGQGTTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
TSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD
KTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK
TISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY

KTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
(SEQ ID NO:11).

In an embodiment of the invention, the Trop2-targeting antibody comprises the heavy chain of SEQ ID NO:7

5 QVQLVQSGHEVKKPGASVKVSCKASGYTFTNYGMNWVRQAPGQGLEWMGWINTK
TGEPTYAQEFTGRFVFSLDTSVSTAYLQISSLKAEDTAVYFCGRGGYGSSYWFYFDVW
GQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT
SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD
KTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
10 DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK
TISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
(SEQ ID NO:7).

15 In an embodiment of the invention, the c-terminus of the heavy chain polypeptide is not lysine residue.

In an embodiment of the invention, the Trop2-targeting antibody comprises the heavy chain of SEQ ID NO:34

QIQLVQSGHEVKKPGASVKVSCKASGYTFTNYGMNWVRQAPGQGLEWMGWINTK
TGEPTYAEEFKGRFVFSLETSASTAYLQISSLKAEDMAMYFCGRGGYGSSYWFYFDV
20 WGQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD
KTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK
TISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
25 KTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
(SEQ ID NO:34).

In an embodiment of the invention, the Trop2-targeting antibody comprises the heavy chain of SEQ ID NO:35

QVQLVQSGHEVKKPGASVKVSCKASGYTFTNYGMNWVRQAPGQGLEWMGWINTK
30 TGEPTYAQEFTGRFVFSLDTSVSTAYLQISSLKAEDMAVYFCGRGGYGSSYWFYFDV
WGQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD
KTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK

TISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSVMSHEALHNHYTQKSLSLSPG
(SEQ ID NO:35).

5 In an embodiment of the invention, the Trop2-targeting antibody comprises the heavy
chain of SEQ ID NO:36
QVQLVQSGHEKKPGASVKVSCKASGYTFTNYGMNWVRQAPGQGLEWMGWINTK
TGEPTYAQEFTGRFVFLDTSVSTAYLQISLKAEDMAVYYCGRGGYGSSYWFYFDV
WGQGTITVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
TSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD
10 KTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK
TISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSVMSHEALHNHYTQKSLSLSPG
(SEQ ID NO:36).

15 In an embodiment of the invention, the Trop2-targeting antibody comprises the heavy
chain of SEQ ID NO:37
QVQLVQSGHEVKKPGASVKVSCKASGYTFTNYGMNWVRQAPGQGLEWMGWINTK
TGEPTYAQEFTGRFVFLDTSVSTAYLQISLKAEDTAVYFCGRGGYGSSYWFYFDVW
GQGTITVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT
20 SGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD
KTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK
TISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSVMSHEALHNHYTQKSLSLSPG
25 (SEQ ID NO:37).

In an embodiment of the invention, the Trop2-targeting antibody comprises the light
chain of SEQ ID NO:12
DIQMTQSPSSLSASVGDRVTITCKASQDVSIAVAWYQQKPGKAPKVLIIYSASYRYTG
VPDRFSGSGSGTDFTFTISSLQPEDIAVYYCQQHYITPLTFGGGTKVEIKRTVAAPSVFI
30 FPPSDEQLKSGTASVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDY
SLSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO:12).

In an embodiment of the invention, the Trop2-targeting antibody comprises the light
chain of SEQ ID NO:8

DIQMTQSPSSLSASVGDRVTITCKASQDVSIAVAWYQQKPGKAPKVLIIYSASYRYTG
VPSRFSGSGSGTDFTFTISSLQPEDIAVYYCQQHYITPLTFGGGKTKVEIKRTVAAPSVFI
FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTY
SLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO:8).

- 5 In an embodiment of ADC of the invention, the Trop2-targeting antibody comprises the CDR regions, FR regions, H and/or L variable regions, heavy chain, and/or light chain of hRS7 (i.e., mAb of Sacituzumab govitecan, also referred to as IMMU-132) as described in US Patent 7,238,785 and US Patent 10,130,626, which are incorporated by reference herein.

- In an embodiment of ADC of the invention, the Trop2-targeting antibody (hRS7)
10 comprises Heavy chain sequence:
QIQLVQSGHEVKKPGASVKVSCKASGYTFTNYGMNWVRQAPGQGLEWMG
WINTKTGEPTYAEFEKGRFVFSLETSASTAYLQISLKAEDMAMYFCGRGGYGYSSYW
YFDVWGQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
SGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEP
15 KSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP
APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQP
ENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSQSVMEALHNHYTQKSLSL
SPGK (SEQ ID NO:40),

- 20 and Light Chain sequence:

- DIQMTQSPSSLSASVGDRVTITCKASQDVSIAVAWYQQKPGKAPKVLIIYSASY
RYTGVPDRFSGSGSGTDFTFTISSLQPEDIAVYYCQQHYITPLTFGGGKTKVEIKRTVAA
PSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSK
DSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO:41).

- 25 In an embodiment of ADC of the invention, the Trop2-targeting antibody comprises the CDR regions, FR regions, H and/or L variable regions, heavy chain, and/or light chain of T6-16 as described in US Patent 10,202,461, which is incorporated by reference herein.

- In an embodiment of ADC of the invention, the Trop2-targeting antibody (T6-16)
comprises Heavy chain sequence:

- 30 EVQLQQSGPELVKPGASVKISCKASGYTFTDYNMHWVKQSHGKNLEWIGYI
YPYNGGTGYNQRFKSRATMTVDKSSSTAYMELRSLTSDDSAVYYCAREDYGSSPSY
AMDYWGQGTSTVIVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
SGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEP
KSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF

NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP
APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQP
ENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSL
SPGK (SEQ ID NO:42),

5 and Light Chain sequence:

DVVMTQTPLSLPVSLGDQASISCRSSQSLVHGNGNTYLHWYLQKPGQSPKLLI
YKVSNRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYFCSQTTHVPTFGSGTKLEIK
RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVT
EQDSKDSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID

10 NO:43).

In an embodiment of ADC of the invention, the Trop2-targeting antibody comprises the CDR regions, FR regions, H and/or L variable regions, heavy chain, and/or light chain of DS1062a as described in International Patent application WO2020/240467, which is incorporated by reference herein.

15 In an embodiment of ADC of the invention, the Trop2-targeting antibody (TINA) comprises Heavy chain sequence:

QVQLVQSGAEVKKPGASVKVSCKASGYTFTTAGMQWVRQAPGQGLEWMG
WINTHSGVPKYAEDFKGRVTISADTSTSTAYLQLSSLKSEDTAVYYCARSFGSSYW
YFDVWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
20 SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEP
KSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP
APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQP
ENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSL

25 SPGK (SEQ ID NO:44),

and Light Chain sequence:

DIQMTQSPSSLSASVGDRVITITCKASQDVSTAVAWYQQKPGKAPKLLIYSAS
YRYTGVPSTRFSGSGSGTDFTLTISLQPEDFAVYYCQQHYITPLTFGQGTKLEIKRTVA
APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDS
30 KDSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO:45).

Antibody Fragments

In certain embodiments, an antibody provided herein is an antibody fragment. Antibody fragments include, but are not limited to, Fab, Fab', Fab'-SH, F(ab')₂, Fv, and scFv fragments,

and other fragments described below. For a review of certain antibody fragments, see Hudson et al. *Nat. Med.* 9:129-134 (2003). For a review of scFv fragments, see, e.g., Pluckthün, in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds., (Springer-Verlag, New York), pp. 269-315 (1994); see also WO 93/16185; and U.S. Patent Nos. 5,571,894 and 5,587,458. For discussion of Fab and F(ab')₂ fragments comprising salvage receptor binding epitope residues and having increased in vivo half-life, see U.S. Patent No. 5,869,046.

Diabodies are antibody fragments with two antigen-binding sites that may be bivalent or bispecific. See, for example, EP 404,097; WO 1993/01161; Hudson et al., *Nat. Med.* 9:129-134 (2003); and Hollinger et al., *Proc. Natl. Acad. Sci. USA* 90: 6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson et al., *Nat. Med.* 9:129-134 (2003).

Single-domain antibodies are antibody fragments comprising all or a portion of the heavy chain variable domain or all or a portion of the light chain variable domain of an antibody. In certain embodiments, a single-domain antibody is a human single-domain antibody (Domantis, Inc., Waltham, MA; see, e.g., U.S. Patent No. 6,248,516 B1).

Antibody fragments can be made by various techniques, including but not limited to proteolytic digestion of an intact antibody as well as production by recombinant host cells (e.g. *E. coli* or phage), as described herein. Chimeric and Humanized Antibodies

In certain embodiments, an antibody provided herein is a chimeric antibody. Certain chimeric antibodies are described, e.g., in U.S. Patent No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)). In one example, a chimeric antibody comprises a non-human variable region (e.g., a variable region derived from a mouse, rat, hamster, rabbit, or non-human primate, such as a monkey) and a human constant region. In a further example, a chimeric antibody is a "class switched" antibody in which the class or subclass has been changed from that of the parent antibody. Chimeric antibodies include antigen-binding fragments thereof.

In certain embodiments, a chimeric antibody is a humanized antibody. Typically, a non-human antibody is humanized to reduce immunogenicity to humans, while retaining the specificity and affinity of the parental non-human antibody. Generally, a humanized antibody comprises one or more variable domains in which HVRs, e.g., CDRs, (or portions thereof) are derived from a non-human antibody, and FRs (or portions thereof) are derived from human antibody sequences. A humanized antibody optionally will also comprise at least a portion of a human constant region. In some embodiments, some FR residues in a humanized antibody are substituted with corresponding residues from a non-human antibody (e.g., the antibody from which the HVR residues are derived), e.g., to restore or improve antibody specificity or affinity.

Humanized antibodies and methods of making them are reviewed, e.g., in Almagro and Fransson, *Front. Biosci.* 13:1619-1633 (2008), and are further described, e.g., in Riechmann et

al., *Nature* 332:323-329 (1988); Queen et al., *Proc. Nat'l Acad. Sci. USA* 86:10029-10033 (1989); US Patent Nos. 5, 821,337, 7,527,791, 6,982,321, and 7,087,409; Kashmiri et al., *Methods* 36:25-34 (2005) (describing SDR (a-CDR) grafting); Padlan, *Mol. Immunol.* 28:489-498 (1991) (describing "resurfacing"); Dall'Acqua et al., *Methods* 36:43-60 (2005) (describing "FR shuffling"); and Osbourn et al., *Methods* 36:61-68 (2005) and Klimka et al., *Br. J. Cancer*, 83:252-260 (2000) (describing the "guided selection" approach to FR shuffling).

Human framework regions that may be used for humanization include but are not limited to: framework regions selected using the "best-fit" method (see, e.g., Sims et al. *J. Immunol.* 151:2296 (1993)); framework regions derived from the consensus sequence of human antibodies of a particular subgroup of light or heavy chain variable regions (see, e.g., Carter et al. *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); and Presta et al. *J. Immunol.*, 151:2623 (1993)); human mature (somatically mutated) framework regions or human germline framework regions (see, e.g., Almagro and Fransson, *Front. Biosci.* 13:1619-1633 (2008)); and framework regions derived from screening FR libraries (see, e.g., Baca et al., *J. Biol. Chem.* 272:10678-10684 (1997) and Rosok et al., *J. Biol. Chem.* 271:22611-22618 (1996)).

Human Antibodies

In certain embodiments, an antibody provided herein is a human antibody. Human antibodies can be produced using various techniques known in the art. Human antibodies are described generally in van Dijk and van de Winkel, *Curr. Opin. Pharmacol.* 5: 368-74 (2001) and Lonberg, *Curr. Opin. Immunol.* 20:450-459 (2008).

Human antibodies may be prepared by administering an immunogen to a transgenic animal that has been modified to produce intact human antibodies or intact antibodies with human variable regions in response to antigenic challenge. Such animals typically contain all or a portion of the human immunoglobulin loci, which replace the endogenous immunoglobulin loci, or which are present extrachromosomally or integrated randomly into the animal's chromosomes. In such transgenic mice, the endogenous immunoglobulin loci have generally been inactivated. For review of methods for obtaining human antibodies from transgenic animals, see Lonberg, *Nat. Biotech.* 23:1117-1125 (2005). See also, e.g., U.S. Patent Nos. 6,075,181 and 6,150,584 describing XENOMOUSE™ technology; U.S. Patent No. 5,770,429 describing HUMAB® technology; U.S. Patent No. 7,041,870 describing K-M MOUSE® technology, and U.S. Patent Application Publication No. US 2007/0061900, describing VELOCIMOUSE® technology). Human variable regions from intact antibodies generated by such animals may be further modified, e.g., by combining with a different human constant region.

Human antibodies can also be made by hybridoma-based methods. Human myeloma and mouse-human heteromyeloma cell lines for the production of human monoclonal antibodies have

been described. (See, e.g., Kozbor *J. Immunol.*, 133: 3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987); and Boerner et al., *J. Immunol.*, 147: 86 (1991).) Human antibodies generated via human B-cell hybridoma technology are also described in Li et al., *Proc. Natl. Acad. Sci. USA*,
5 103:3557-3562 (2006). Additional methods include those described, for example, in U.S. Patent No. 7,189,826 (describing production of monoclonal human IgM antibodies from hybridoma cell lines) and Ni, *Xiandai Mianyixue*, 26(4):265-268 (2006) (describing human-human hybridomas). Human hybridoma technology (Trioma technology) is also described in Vollmers and Brandlein, *Histology and Histopathology*, 20(3):927-937 (2005) and Vollmers and Brandlein, *Methods and*
10 *Findings in Experimental and Clinical Pharmacology*, 27(3):185-91 (2005).

Human antibodies may also be generated by isolating Fv clone variable domain sequences selected from human-derived phage display libraries. Such variable domain sequences may then be combined with a desired human constant domain. Techniques for selecting human antibodies from antibody libraries are described below.

15

Library-Derived Antibodies

Antibodies of the invention may be isolated by screening combinatorial libraries for antibodies with the desired activity or activities. For example, a variety of methods are known in the art for generating phage display libraries and screening such libraries for antibodies
20 possessing the desired binding characteristics. Such methods are reviewed, e.g., in Hoogenboom et al. in *Methods in Molecular Biology* 178:1-37 (O'Brien et al., ed., Human Press, Totowa, NJ, 2001) and further described, e.g., in the McCafferty et al., *Nature* 348:552-554; Clackson et al., *Nature* 352: 624-628 (1991); Marks et al., *J. Mol. Biol.* 222: 581-597 (1992); Marks and Bradbury, in *Methods in Molecular Biology* 248:161-175 (Lo, ed., Human Press, Totowa, NJ,
25 2003); Sidhu et al., *J. Mol. Biol.* 338(2): 299-310 (2004); Lee et al., *J. Mol. Biol.* 340(5): 1073-1093 (2004); Fellouse, *Proc. Natl. Acad. Sci. USA* 101(34): 12467-12472 (2004); and Lee et al., *J. Immunol. Methods* 284(1-2): 119-132(2004).

In certain phage display methods, repertoires of VH and VL genes are separately cloned by polymerase chain reaction (PCR) and recombined randomly in phage libraries, which can then
30 be screened for antigen-binding phage as described in Winter et al., *Ann. Rev. Immunol.*, 12: 433-455 (1994). Phage typically display antibody fragments, either as single-chain Fv (scFv) fragments or as Fab fragments. Libraries from immunized sources provide high-affinity antibodies to the immunogen without the requirement of constructing hybridomas. Alternatively, the naive repertoire can be cloned (e.g., from human) to provide a single source of antibodies to a
35 wide range of non-self and also self antigens without any immunization as described by Griffiths

et al., *EMBO J.* 12: 725-734 (1993). Finally, naive libraries can also be made synthetically by cloning unrearranged V-gene segments from stem cells, and using PCR primers containing random sequence to encode the highly variable CDR3 regions and to accomplish rearrangement *in vitro*, as described by Hoogenboom and Winter, *J. Mol. Biol.*, 227: 381-388 (1992). Patent publications describing human antibody phage libraries include, for example: US Patent No. 5,750,373, and US Patent Publication Nos. 2005/0079574, 2005/0119455, 2005/0266000, 2007/0117126, 2007/0160598, 2007/0237764, 2007/0292936, and 2009/0002360.

Antibodies or antibody fragments isolated from human antibody libraries are considered human antibodies or human antibody fragments herein.

Multispecific Antibodies

In certain embodiments, an antibody provided herein is a multispecific antibody, *e.g.* a bispecific antibody. Multispecific antibodies are monoclonal antibodies that have binding specificities for at least two different sites. In certain embodiments, bispecific antibodies may bind to two different epitopes of the same target. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express the target. Bispecific antibodies can be prepared as full length antibodies or antibody fragments.

Techniques for making multispecific antibodies include, but are not limited to, recombinant co-expression of two immunoglobulin heavy chain-light chain pairs having different specificities (see Milstein and Cuello, *Nature* 305: 537 (1983)), WO 93/08829, and Traunecker et al., *EMBO J.* 10: 3655 (1991)), and “knob-in-hole” engineering (*see, e.g.*, U.S. Patent No. 5,731,168). The term “knob-into-hole” or “KnH” technology as used herein refers to the technology directing the pairing of two polypeptides together *in vitro* or *in vivo* by introducing a protuberance (knob) into one polypeptide and a cavity (hole) into the other polypeptide at an interface in which they interact. For example, KnHs have been introduced in the Fc:Fc binding interfaces, CL:CH1 interfaces or VH/VL interfaces of antibodies (see, *e.g.*, US 2011/0287009, US2007/0178552, WO 96/027011, WO 98/050431, Zhu et al., 1997, *Protein Science* 6:781-788, and WO2012/106587). In some embodiments, KnHs drive the pairing of two different heavy chains together during the manufacture of multispecific antibodies. For example, multispecific antibodies having KnH in their Fc regions can further comprise single variable domains linked to each Fc region, or further comprise different heavy chain variable domains that pair with similar or different light chain variable domains. KnH technology can be also be used to pair two different receptor extracellular domains together or any other polypeptide sequences that comprises different target recognition sequences (*e.g.*, including affibodies, peptibodies and other Fc fusions).

The term “knob mutation” as used herein refers to a mutation that introduces a protuberance (knob) into a polypeptide at an interface in which the polypeptide interacts with another polypeptide. In some embodiments, the other polypeptide has a hole mutation.

The term “hole mutation” as used herein refers to a mutation that introduces a cavity (hole) into a polypeptide at an interface in which the polypeptide interacts with another polypeptide. In some embodiments, the other polypeptide has a knob mutation.

A brief nonlimiting discussion is provided below.

A “protuberance” refers to at least one amino acid side chain which projects from the interface of a first polypeptide and is therefore positionable in a compensatory cavity in the adjacent interface (i.e. the interface of a second polypeptide) so as to stabilize the heteromultimer, and thereby favor heteromultimer formation over homomultimer formation, for example. The protuberance may exist in the original interface or may be introduced synthetically (e.g., by altering nucleic acid encoding the interface). In some embodiments, nucleic acid encoding the interface of the first polypeptide is altered to encode the protuberance. To achieve this, the nucleic acid encoding at least one “original” amino acid residue in the interface of the first polypeptide is replaced with nucleic acid encoding at least one “import” amino acid residue which has a larger side chain volume than the original amino acid residue. It will be appreciated that there can be more than one original and corresponding import residue. The side chain volumes of the various amino residues are shown, for example, in Table 1 of US2011/0287009. A mutation to introduce a “protuberance” may be referred to as a “knob mutation.”

In some embodiments, import residues for the formation of a protuberance are naturally occurring amino acid residues selected from arginine (R), phenylalanine (F), tyrosine (Y) and tryptophan (W). In some embodiments, an import residue is tryptophan or tyrosine. In some embodiment, the original residue for the formation of the protuberance has a small side chain volume, such as alanine, asparagine, aspartic acid, glycine, serine, threonine or valine.

A “cavity” refers to at least one amino acid side chain which is recessed from the interface of a second polypeptide and therefore accommodates a corresponding protuberance on the adjacent interface of a first polypeptide. The cavity may exist in the original interface or may be introduced synthetically (e.g. by altering nucleic acid encoding the interface). In some embodiments, nucleic acid encoding the interface of the second polypeptide is altered to encode the cavity. To achieve this, the nucleic acid encoding at least one “original” amino acid residue in the interface of the second polypeptide is replaced with DNA encoding at least one “import” amino acid residue which has a smaller side chain volume than the original amino acid residue. It will be appreciated that there can be more than one original and corresponding import residue. In some embodiments, import residues for the formation of a cavity are naturally occurring amino

acid residues selected from alanine (A), serine (S), threonine (T) and valine (V). In some embodiments, an import residue is serine, alanine or threonine. In some embodiments, the original residue for the formation of the cavity has a large side chain volume, such as tyrosine, arginine, phenylalanine or tryptophan. A mutation to introduce a “cavity” may be referred to as a “hole mutation.”

The protuberance is “positionable” in the cavity which means that the spatial location of the protuberance and cavity on the interface of a first polypeptide and second polypeptide respectively and the sizes of the protuberance and cavity are such that the protuberance can be located in the cavity without significantly perturbing the normal association of the first and second polypeptides at the interface. Since protuberances such as Tyr, Phe and Trp do not typically extend perpendicularly from the axis of the interface and have preferred conformations, the alignment of a protuberance with a corresponding cavity may, in some instances, rely on modeling the protuberance/cavity pair based upon a three-dimensional structure such as that obtained by X-ray crystallography or nuclear magnetic resonance (NMR). This can be achieved using widely accepted techniques in the art.

In some embodiments, a knob mutation in an IgG1 constant region is T366W (EU numbering). In some embodiments, a hole mutation in an IgG1 constant region comprises one or more mutations selected from T366S, L368A and Y407V (EU numbering). In some embodiments, a hole mutation in an IgG1 constant region comprises T366S, L368A and Y407V (EU numbering).

In some embodiments, a knob mutation in an IgG4 constant region is T366W (EU numbering). In some embodiments, a hole mutation in an IgG4 constant region comprises one or more mutations selected from T366S, L368A, and Y407V (EU numbering). In some embodiments, a hole mutation in an IgG4 constant region comprises T366S, L368A, and Y407V (EU numbering).

Multi-specific antibodies may also be made by engineering electrostatic steering effects for making antibody Fc-heterodimeric molecules (WO 2009/089004A1); cross-linking two or more antibodies or fragments (*see, e.g.*, US Patent No. 4,676,980, and Brennan et al., *Science*, 229: 81 (1985)); using leucine zippers to produce bi-specific antibodies (*see, e.g.*, Kostelny et al., *J. Immunol.*, 148(5):1547-1553 (1992)); using “diabody” technology for making bispecific antibody fragments (*see, e.g.*, Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993)); and using single-chain Fv (sFv) dimers (*see, e.g.* Gruber et al., *J. Immunol.*, 152:5368 (1994)); and preparing trispecific antibodies as described, *e.g.*, in Tutt et al. *J. Immunol.* 147: 60 (1991).

Engineered antibodies with three or more functional antigen binding sites, including “Octopus antibodies,” are also included herein (*see, e.g.* US 2006/0025576A1).

The antibody or fragment herein also includes a “Dual Acting FAB” or “DAF” comprising an antigen binding site that binds to the target as well as another, different antigen (*see*, US 2008/0069820, for example).

5 Antibody Variants

 In certain embodiments, amino acid sequence variants of the antibodies provided herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of an antibody may be prepared by introducing appropriate modifications into the nucleotide sequence encoding the antibody, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics, *e.g.*, antigen-binding.

15 Substitution, Insertion, and Deletion Variants

 In certain embodiments, antibody variants having one or more amino acid substitutions are provided. Sites of interest for substitutional mutagenesis include the HVRs and FRs. Conservative substitutions are shown in Table 1 under the heading of “preferred substitutions.” More substantial changes are provided in Table 1 under the heading of “exemplary substitutions,” and as further described below in reference to amino acid side chain classes. Amino acid substitutions may be introduced into an antibody of interest and the products screened for a desired activity, *e.g.*, retained/improved antigen binding, decreased immunogenicity, or improved ADCC or CDC.

25

TABLE 1

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn; Glu	Asn
Glu (E)	Asp; Gln	Asp

Original Residue	Exemplary Substitutions	Preferred Substitutions
Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucine	Leu
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine	Leu

Amino acids may be grouped according to common side-chain properties:

- (1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;
- (2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;
- 5 (3) acidic: Asp, Glu;
- (4) basic: His, Lys, Arg;
- (5) residues that influence chain orientation: Gly, Pro;
- (6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions will entail exchanging a member of one of these classes
10 for another class.

One type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (*e.g.* a humanized or human antibody). Generally, the resulting variant(s) selected for further study will have modifications (*e.g.*, improvements) in certain biological properties (*e.g.*, increased affinity, reduced immunogenicity) relative to the parent
15 antibody and/or will have substantially retained certain biological properties of the parent antibody. An exemplary substitutional variant is an affinity matured antibody, which may be conveniently generated, *e.g.*, using phage display-based affinity maturation techniques such as

those described herein. Briefly, one or more HVR residues are mutated and the variant antibodies displayed on phage and screened for a particular biological activity (*e.g.* binding affinity).

Alterations (*e.g.*, substitutions) may be made in HVRs, *e.g.*, to improve antibody affinity. Such alterations may be made in HVR “hotspots,” *i.e.*, residues encoded by codons that undergo mutation at high frequency during the somatic maturation process (*see, e.g.*, Chowdhury, *Methods Mol. Biol.* 207:179-196 (2008)), and/or SDRs (a-CDRs), with the resulting variant VH or VL being tested for binding affinity. Affinity maturation by constructing and reselecting from secondary libraries has been described, *e.g.*, in Hoogenboom et al. in *Methods in Molecular Biology* 178:1-37 (O’Brien et al., ed., Human Press, Totowa, NJ, (2001).) In some embodiments of affinity maturation, diversity is introduced into the variable genes chosen for maturation by any of a variety of methods (*e.g.*, error-prone PCR, chain shuffling, or oligonucleotide-directed mutagenesis). A secondary library is then created. The library is then screened to identify any antibody variants with the desired affinity. Another method to introduce diversity involves HVR-directed approaches, in which several HVR residues (*e.g.*, 4-6 residues at a time) are randomized. HVR residues involved in antigen binding may be specifically identified, *e.g.*, using alanine scanning mutagenesis or modeling. CDR-H3 and CDR-L3 in particular are often targeted.

In certain embodiments, substitutions, insertions, or deletions may occur within one or more HVRs so long as such alterations do not substantially reduce the ability of the antibody to bind antigen. For example, conservative alterations (*e.g.*, conservative substitutions as provided herein) that do not substantially reduce binding affinity may be made in HVRs. Such alterations may be outside of HVR “hotspots” or SDRs. In certain embodiments of the variant VH and VL sequences provided above, each HVR either is unaltered, or contains no more than one, two or three amino acid substitutions.

A useful method for identification of residues or regions of an antibody that may be targeted for mutagenesis is called “alanine scanning mutagenesis” as described by Cunningham and Wells (1989) *Science*, 244:1081-1085. In this method, a residue or group of target residues (*e.g.*, charged residues such as arg, asp, his, lys, and glu) are identified and replaced by a neutral or negatively charged amino acid (*e.g.*, alanine or polyalanine) to determine whether the interaction of the antibody with antigen is affected. Further substitutions may be introduced at the amino acid locations demonstrating functional sensitivity to the initial substitutions. Alternatively, or additionally, a crystal structure of an antigen-antibody complex is used to identify contact points between the antibody and antigen. Such contact residues and neighboring residues may be targeted or eliminated as candidates for substitution. Variants may be screened to determine whether they contain the desired properties.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal methionyl residue. Other insertional variants of the antibody molecule include the fusion to the N- or C-terminus of the antibody to an enzyme (e.g. for ADEPT) or a polypeptide which increases the serum half-life of the antibody.

Glycosylation variants

In certain embodiments, an antibody provided herein is altered to increase or decrease the extent to which the antibody is glycosylated. Addition or deletion of glycosylation sites to an antibody may be conveniently accomplished by altering the amino acid sequence such that one or more glycosylation sites is created or removed.

Where the antibody comprises an Fc region, the carbohydrate attached thereto may be altered. Native antibodies produced by mammalian cells typically comprise a branched, biantennary oligosaccharide that is generally attached by an N-linkage to Asn297 of the CH2 domain of the Fc region. *See, e.g.,* Wright et al. *TIBTECH* 15:26-32 (1997). The oligosaccharide may include various carbohydrates, e.g., mannose, N-acetyl glucosamine (GlcNAc), galactose, and sialic acid, as well as a fucose attached to a GlcNAc in the “stem” of the biantennary oligosaccharide structure. In some embodiments, modifications of the oligosaccharide in an antibody of the invention may be made in order to create antibody variants with certain improved properties.

In one embodiment, antibody variants are provided having a carbohydrate structure that lacks fucose attached (directly or indirectly) to an Fc region. For example, the amount of fucose in such antibody may be from 1% to 80%, from 1% to 65%, from 5% to 65% or from 20% to 40%. The amount of fucose is determined by calculating the average amount of fucose within the sugar chain at Asn297, relative to the sum of all glycostructures attached to Asn 297 (e. g. complex, hybrid and high mannose structures) as measured by MALDI-TOF mass spectrometry, as described in WO 2008/077546, for example. Asn297 refers to the asparagine residue located at about position 297 in the Fc region (Eu numbering of Fc region residues); however, Asn297 may also be located about ± 3 amino acids upstream or downstream of position 297, i.e., between positions 294 and 300, due to minor sequence variations in antibodies. Such fucosylation variants may have improved ADCC function. *See, e.g.,* US Patent Publication Nos. US 2003/0157108 (Presta, L.); US 2004/0093621 (Kyowa Hakko Kogyo Co., Ltd). Examples of publications related to “defucosylated” or “fucose-deficient” antibody variants include: US 2003/0157108; WO 2000/61739; WO 2001/29246; US 2003/0115614; US 2002/0164328; US 2004/0093621; US

2004/0132140; US 2004/0110704; US 2004/0110282; US 2004/0109865; WO 2003/085119; WO 2003/084570; WO 2005/035586; WO 2005/035778; WO2005/053742; WO2002/031140; Okazaki et al. *J. Mol. Biol.* 336:1239-1249 (2004); Yamane-Ohnuki et al. *Biotech. Bioeng.* 87: 614 (2004). Examples of cell lines capable of producing defucosylated antibodies include Lec13
 5 CHO cells deficient in protein fucosylation (Ripka et al. *Arch. Biochem. Biophys.* 249:533-545 (1986); US Pat Appl No US 2003/0157108 A1, Presta, L; and WO 2004/056312 A1, Adams *et al.*, especially at Example 11), and knockout cell lines, such as alpha-1,6-fucosyltransferase gene, *FUT8*, knockout CHO cells (*see, e.g.*, Yamane-Ohnuki et al. *Biotech. Bioeng.* 87: 614 (2004); Kanda, Y. et al., *Biotechnol. Bioeng.*, 94(4):680-688 (2006); and WO2003/085107).

- 10 Antibodies variants are further provided with bisected oligosaccharides, *e.g.*, in which a biantennary oligosaccharide attached to the Fc region of the antibody is bisected by GlcNAc. Such antibody variants may have reduced fucosylation and/or improved ADCC function. Examples of such antibody variants are described, *e.g.*, in WO 2003/011878 (Jean-Mairet et al.); US Patent No. 6,602,684 (Umana et al.); and US 2005/0123546 (Umana *et al.*). Antibody
 15 variants with at least one galactose residue in the oligosaccharide attached to the Fc region are also provided. Such antibody variants may have improved CDC function. Such antibody variants are described, *e.g.*, in WO 1997/30087 (Patel et al.); WO 1998/58964 (Raju, S.); and WO 1999/22764 (Raju, S.).

Fc region variants

- 20 In certain embodiments, one or more amino acid modifications may be introduced into the Fc region of an antibody provided herein, thereby generating an Fc region variant. The Fc region variant may comprise a human Fc region sequence (*e.g.*, a human IgG1, IgG2, IgG3 or IgG4 Fc region) comprising an amino acid modification (*e.g.* a substitution) at one or more amino acid positions.
- 25 In certain embodiments, the invention contemplates an antibody variant that possesses some but not all effector functions, which make it a desirable candidate for applications in which the half life of the antibody *in vivo* is important yet certain effector functions (such as complement and ADCC) are unnecessary or deleterious. *In vitro* and/or *in vivo* cytotoxicity assays can be conducted to confirm the reduction/depletion of CDC and/or ADCC activities. For
 30 example, Fc receptor (FcR) binding assays can be conducted to ensure that the antibody lacks FcγR binding (hence likely lacking ADCC activity), but retains FcRn binding ability. The primary cells for mediating ADCC, NK cells, express Fc(RIII only, whereas monocytes express Fc(RI, Fc(RII and Fc(RIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457-492 (1991). Non-limiting examples

of *in vitro* assays to assess ADCC activity of a molecule of interest is described in U.S. Patent No. 5,500,362 (*see, e.g.* Hellstrom, I. et al. *Proc. Nat'l Acad. Sci. USA* 83:7059-7063 (1986)) and Hellstrom, I et al., *Proc. Nat'l Acad. Sci. USA* 82:1499-1502 (1985); 5,821,337 (see Bruggemann, M. et al., *J. Exp. Med.* 166:1351-1361 (1987)). Alternatively, non-radioactive assays methods
5 may be employed (*see, for example*, ACTIT[™] non-radioactive cytotoxicity assay for flow cytometry (CellTechnology, Inc. Mountain View, CA; and CytoTox 96[®] non-radioactive cytotoxicity assay (Promega, Madison, WI). Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo, e.g.*, in a animal
10 model such as that disclosed in Clynes et al. *Proc. Nat'l Acad. Sci. USA* 95:652-656 (1998). C1q binding assays may also be carried out to confirm that the antibody is unable to bind C1q and hence lacks CDC activity. *See, e.g.*, C1q and C3c binding ELISA in WO 2006/029879 and WO 2005/100402. To assess complement activation, a CDC assay may be performed (*see, for example*, Gazzano-Santoro *et al.*, *J. Immunol. Methods* 202:163 (1996); Cragg, M.S. et al., *Blood*
15 101:1045-1052 (2003); and Cragg, M.S. and M.J. Glennie, *Blood* 103:2738-2743 (2004)). FcRn binding and *in vivo* clearance/half life determinations can also be performed using methods known in the art (*see, e.g.*, Petkova, S.B. et al., *Int'l. Immunol.* 18(12):1759-1769 (2006)).

In some embodiments, one or more amino acid modifications may be introduced into the Fc portion of the antibody provided herein in order to increase IgG binding to the neonatal Fc
20 receptor. In certain embodiments, the antibody comprises the following three mutations according to EU numbering: M252Y, S254T, and T256E (the "YTE mutation") (US Patent No. 8,697,650; *see also* Dall'Acqua et al., *Journal of Biological Chemistry* 281(33):23514-23524 (2006). In certain embodiments, the YTE mutation does not affect the ability of the antibody to bind to its cognate antigen. In certain embodiments, the YTE mutation increases the antibody's
25 serum half-life compared to the native (i.e., non-YTE mutant) antibody. In some embodiments, the YTE mutation increases the serum half-life of the antibody by 3-fold compared to the native (i.e., non-YTE mutant) antibody. In some embodiments, the YTE mutation increases the serum half-life of the antibody by 2-fold compared to the native (i.e., non-YTE mutant) antibody. In some embodiments, the YTE mutation increases the serum half-life of the antibody by 4-fold
30 compared to the native (i.e., non-YTE mutant) antibody. In some embodiments, the YTE mutation increases the serum half-life of the antibody by at least 5-fold compared to the native (i.e., non-YTE mutant) antibody. In some embodiments, the YTE mutation increases the serum half-life of the antibody by at least 10-fold compared to the native (i.e., non-YTE mutant) antibody. *See, e.g.*, US Patent No. 8,697,650; *see also* Dall'Acqua et al., *Journal of Biological*
35 *Chemistry* 281(33):23514-23524 (2006).

In certain embodiments, the YTE mutant provides a means to modulate antibody-dependent cell-mediated cytotoxicity (ADCC) activity of the antibody. In certain embodiments, the YTEO mutant provides a means to modulate ADCC activity of a humanized IgG antibody directed against a human antigen. See, e.g., US Patent No. 8,697,650; see also Dall'Acqua et al.,
5 Journal of Biological Chemistry 281(33):23514-23524 (2006).

In certain embodiments, the YTE mutant allows the simultaneous modulation of serum half-life, tissue distribution, and antibody activity (e.g., the ADCC activity of an IgG antibody). See, e.g., US Patent No. 8,697,650; see also Dall'Acqua et al., Journal of Biological Chemistry 281(33):23514-23524 (2006).

10 Antibodies with reduced effector function include those with substitution of one or more of Fc region residues 238, 265, 269, 270, 297, 327 and 329 (U.S. Patent No. 6,737,056). Such Fc mutants include Fc mutants with substitutions at two or more of amino acid positions 265, 269, 270, 297 and 327, including the so-called "DANA" Fc mutant with substitution of residues 265 and 297 to alanine (US Patent No. 7,332,581).

15 In certain embodiments, the proline at position 329 (EU numbering) (P329) of a wild-type human Fc region is substituted with glycine or arginine or an amino acid residue large enough to destroy the proline sandwich within the Fc/Fc gamma receptor interface, that is formed between the P329 of the Fc and tryptophane residues W87 and W110 of FcγRIII (Sondermann et al.: Nature 406, 267-273 (20 July 2000)). In a further embodiment, at least one further amino acid
20 substitution in the Fc variant is S228P, E233P, L234A, L235A, L235E, N297A, N297D, or P331S and still in another embodiment said at least one further amino acid substitution is L234A and L235A of the human IgG1 Fc region or S228P and L235E of the human IgG4 Fc region, all according to EU numbering (U.S. Patent No. 8,969,526 which is incorporated by reference in its entirety).

25 In certain embodiments, a polypeptide comprises the Fc variant of a wild-type human IgG Fc region wherein the polypeptide has P329 of the human IgG Fc region substituted with glycine and wherein the Fc variant comprises at least two further amino acid substitutions at L234A and L235A of the human IgG1 Fc region or S228P and L235E of the human IgG4 Fc region, and wherein the residues are numbered according to the EU numbering (U.S. Patent No. 8,969,526
30 which is incorporated by reference in its entirety). In certain embodiments, the polypeptide comprising the P329G, L234A and L235A (EU numbering) substitutions exhibit a reduced affinity to the human FcγRIIIA and FcγRIIA, for down-modulation of ADCC to at least 20% of the ADCC induced by the polypeptide comprising the wildtype human IgG Fc region, and/or for down-modulation of ADCP (U.S. Patent No. 8,969,526 which is incorporated by reference in its
35 entirety).

In a specific embodiment the polypeptide comprising an Fc variant of a wildtype human Fc polypeptide comprises a triple mutation: an amino acid substitution at position Pro329, a L234A and a L235A mutation according to EU numbering (P329 / LALA) (U.S. Patent No. 8,969,526 which is incorporated by reference in its entirety). In specific embodiments, the polypeptide comprises the following amino acid substitutions: P329G, L234A, and L235A according to EU numbering.

Certain antibody variants with improved or diminished binding to FcRs are described. (See, e.g., U.S. Patent No. 6,737,056; WO 2004/056312, and Shields et al., *J. Biol. Chem.* 9(2): 6591-6604 (2001).)

In certain embodiments, an antibody variant comprises an Fc region with one or more amino acid substitutions which improve ADCC, e.g., substitutions at positions 298, 333, and/or 334 of the Fc region (EU numbering of residues).

In some embodiments, alterations are made in the Fc region that result in altered (*i.e.*, either improved or diminished) C1q binding and/or Complement Dependent Cytotoxicity (CDC), e.g., as described in US Patent No. 6,194,551, WO 99/51642, and Idusogie et al. *J. Immunol.* 164: 4178-4184 (2000).

Antibodies with increased half lives and improved binding to the neonatal Fc receptor (FcRn), which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., *J. Immunol.* 117:587 (1976) and Kim et al., *J. Immunol.* 24:249 (1994)), are described in US2005/0014934A1 (Hinton et al.). Those antibodies comprise an Fc region with one or more substitutions therein which improve binding of the Fc region to FcRn. Such Fc variants include those with substitutions at one or more of Fc region residues: 238, 256, 265, 272, 286, 303, 305, 307, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424 or 434, e.g., substitution of Fc region residue 434 (US Patent No. 7,371,826).

See also Duncan & Winter, *Nature* 322:738-40 (1988); U.S. Patent No. 5,648,260; U.S. Patent No. 5,624,821; and WO 94/29351 concerning other examples of Fc region variants.

Cysteine engineered antibody variants

In certain embodiments, it may be desirable to create cysteine engineered antibodies, e.g., a “THIOMAB™” or TDC, in which one or more residues of an antibody are substituted with cysteine residues. In particular embodiments, the substituted residues occur at sites of the antibody that are available for conjugation. By substituting those residues with cysteine, reactive thiol groups are thereby positioned at accessible sites of the antibody and may be used to conjugate the antibody to other moieties, such as drug moieties or linker-drug moieties, to create an immunoconjugate, as described further herein. In certain embodiments, any one or more of the

following residues may be substituted with cysteine: K149 (Kabat numbering) of the light chain; V205 (Kabat numbering) of the light chain; A118 (EU numbering) of the heavy chain; A140 (EU numbering) of the heavy chain; L174 (EU numbering) of the heavy chain; Y373 (EU numbering) of the heavy chain; and S400 (EU numbering) of the heavy chain Fc region. In specific
 5 embodiments, the antibodies described herein comprise the HC-A140C (EU numbering) cysteine substitution. In specific embodiments, the antibodies described herein comprise the LC-K149C (Kabat numbering) cysteine substitution. In specific embodiments, the antibodies described herein comprise the HC-A118C (EU numbering) cysteine substitution. Cysteine engineered antibodies may be generated as described, *e.g.*, in U.S. Patent No. 7,521,541.

10 Antibody Derivatives

In certain embodiments, an antibody provided herein may be further modified to contain additional nonproteinaceous moieties that are known in the art and readily available. The moieties suitable for derivatization of the antibody include but are not limited to water soluble polymers. Non-limiting examples of water soluble polymers include, but are not limited to, polyethylene
 15 glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (*e.g.*,
 20 glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water. The polymer may be of any molecular weight, and may be branched or unbranched. The number of polymers attached to the antibody may vary, and if more than one polymer is attached, they can be the same or different molecules. In general, the number and/or type of polymers used for derivatization can be
 25 determined based on considerations including, but not limited to, the particular properties or functions of the antibody to be improved, whether the antibody derivative will be used in a therapy under defined conditions, etc.

In another embodiment, conjugates of an antibody and nonproteinaceous moiety that may be selectively heated by exposure to radiation are provided. In one embodiment, the
 30 nonproteinaceous moiety is a carbon nanotube (Kam et al., *Proc. Natl. Acad. Sci. USA* 102: 11600-11605 (2005)). The radiation may be of any wavelength, and includes, but is not limited to, wavelengths that do not harm ordinary cells, but which heat the nonproteinaceous moiety to a temperature at which cells proximal to the antibody-nonproteinaceous moiety are killed.

Recombinant Methods and Compositions

Antibodies may be produced using recombinant methods and compositions, *e.g.*, as described in U.S. Patent No. 4,816,567. In one embodiment, isolated nucleic acid encoding an antibody described herein is provided. Such nucleic acid may encode an amino acid sequence comprising the VL and/or an amino acid sequence comprising the VH of the antibody (*e.g.*, the light and/or heavy chains of the antibody). In a further embodiment, one or more vectors (*e.g.*, expression vectors) comprising such nucleic acid are provided. In a further embodiment, a host cell comprising such nucleic acid is provided. In one such embodiment, a host cell comprises (*e.g.*, has been transformed with): (1) a vector comprising a nucleic acid that encodes an amino acid sequence comprising the VL of the antibody and an amino acid sequence comprising the VH of the antibody, or (2) a first vector comprising a nucleic acid that encodes an amino acid sequence comprising the VL of the antibody and a second vector comprising a nucleic acid that encodes an amino acid sequence comprising the VH of the antibody. In one embodiment, the host cell is eukaryotic, *e.g.* a Chinese Hamster Ovary (CHO) cell or lymphoid cell (*e.g.*, Y0, NS0, Sp20 cell). In one embodiment, a method of making an antibody is provided, wherein the method comprises culturing a host cell comprising a nucleic acid encoding the antibody, as provided above, under conditions suitable for expression of the antibody, and optionally recovering the antibody from the host cell (or host cell culture medium).

For recombinant production of an antibody, nucleic acid encoding an antibody, *e.g.*, as described above, is isolated and inserted into one or more vectors for further cloning and/or expression in a host cell. Such nucleic acid may be readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody).

Suitable host cells for cloning or expression of antibody-encoding vectors include prokaryotic or eukaryotic cells described herein. For example, antibodies may be produced in bacteria, in particular when glycosylation and Fc effector function are not needed. For expression of antibody fragments and polypeptides in bacteria, *see, e.g.*, U.S. Patent Nos. 5,648,237, 5,789,199, and 5,840,523. (See also Charlton, *Methods in Molecular Biology*, Vol. 248 (B.K.C. Lo, ed., Humana Press, Totowa, NJ, 2003), pp. 245-254, describing expression of antibody fragments in *E. coli*.) After expression, the antibody may be isolated from the bacterial cell paste in a soluble fraction and can be further purified.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for antibody-encoding vectors, including fungi and yeast strains whose glycosylation pathways have been “humanized,” resulting in the production of an antibody with a partially or fully human glycosylation pattern. See Gerngross, *Nat. Biotech.* 22:1409-1414 (2004), and Li et al., *Nat. Biotech.* 24:210-215 (2006).

Suitable host cells for the expression of glycosylated antibody are also derived from multicellular organisms (invertebrates and vertebrates). Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains have been identified which may be used in conjunction with insect cells, particularly for transfection of *Spodoptera frugiperda* cells.

5 Plant cell cultures can also be utilized as hosts. *See, e.g.*, US Patent Nos. 5,959,177, 6,040,498, 6,420,548, 7,125,978, and 6,417,429 (describing PLANTIBODIES™ technology for producing antibodies in transgenic plants).

Vertebrate cells may also be used as hosts. For example, mammalian cell lines that are adapted to grow in suspension may be useful. Other examples of useful mammalian host cell
 10 lines are monkey kidney CV1 line transformed by SV40 (COS-7); human embryonic kidney line (293 or 293 cells as described, *e.g.*, in Graham et al., *J. Gen Virol.* 36:59 (1977); baby hamster kidney cells (BHK); mouse sertoli cells (TM4 cells as described, *e.g.*, in Mather, *Biol. Reprod.* 23:243-251 (1980); monkey kidney cells (CV1); African green monkey kidney cells (VERO-76); human cervical carcinoma cells (HELA); canine kidney cells (MDCK; buffalo rat liver cells
 15 (BRL 3A); human lung cells (W138); human liver cells (Hep G2); mouse mammary tumor (MMT 060562); TRI cells, as described, *e.g.*, in Mather et al., *Annals N.Y. Acad. Sci.* 383:44-68 (1982); MRC 5 cells; and FS4 cells. Other useful mammalian host cell lines include Chinese hamster ovary (CHO) cells, including DHFR⁻ CHO cells (Urlaub et al., *Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); and myeloma cell lines such as Y0, NS0 and Sp2/0. For a review of certain
 20 mammalian host cell lines suitable for antibody production, *see, e.g.*, Yazaki and Wu, *Methods in Molecular Biology*, Vol. 248 (B.K.C. Lo, ed., Humana Press, Totowa, NJ), pp. 255-268 (2003).

ANTIBODY-DRUG CONJUGATES (ADC)

The antibody-drug conjugate (ADC) compounds of the invention comprise an
 25 antibody specific for a tumor-associated antigen covalently attached, linked to a potent toxin

It is to be understood that where more than one nucleophilic lysine nitrogen (*e.g.*, sidechain amine nitrogen) or cysteine thiol group of an antibody reacts with a drug-linker intermediate or linker reagent, then the resulting product is a mixture of ADC compounds with a distribution of one or more drug moieties attached to an antibody. The average number
 30 of drugs per antibody (DAR) may be calculated from the mixture by a dual ELISA antibody assay, which is specific for antibody and specific for the drug. Individual ADC molecules may be identified in the mixture by mass spectroscopy and separated by HPLC, *e.g.* hydrophobic interaction chromatography (*see, e.g.*, McDonagh et al (2006) *Prot. Engr. Design & Selection* 19(7):299-307; Hamblett et al (2004) *Clin. Cancer Res.* 10:7063-7070;

Hamblett, K.J., et al. "Effect of drug loading on the pharmacology, pharmacokinetics, and toxicity of an anti-CD30 antibody-drug conjugate," Abstract No. 624, American Association for Cancer Research, 2004 Annual Meeting, March 27-31, 2004, Proceedings of the AACR, Volume 45, March 2004; Alley, S.C., et al. "Controlling the location of drug attachment in antibody-drug conjugates," Abstract No. 627, American Association for Cancer Research, 2004 Annual Meeting, March 27-31, 2004, Proceedings of the AACR, Volume 45, March 2004). In certain embodiments, a homogeneous ADC with a single loading value may be isolated from the conjugation mixture by electrophoresis or chromatography.

A linker is a bifunctional or multifunctional compound which can be used to link an active agent (e.g., a drug) and an antibody to form an antibody-drug conjugate (ADC). Such conjugates are useful, for example, in the formation of immunoconjugates directed against tumor associated antigens. Such conjugates allow the selective delivery of cytotoxic drugs to tumor cells. In an ADC, the linker serves to attach the toxin to the antibody. The linker can also link an active agent to a reactive group, wherein the reactive group can react with an antibody to form the ADC.

The "Linker" (L) is a bifunctional or multifunctional moiety that can be used to link one or more toxin drug moieties to an antibody (Ab) to form the antibody-drug conjugate (ADC) of Formula III. In some embodiments, antibody-drug conjugates (ADC) can be prepared using a linker having reactive functionalities for covalently attaching to the drug and to the antibody. For example, in some embodiments, a side chain amine of a lysine of an antibody (Ab) can form a bond with a reactive functional group of a linker or a drug-linker intermediate to make an ADC. In another example, a cysteine thiol of an antibody (Ab) can form a bond with a reactive functional group of a linker or a drug-linker intermediate of to make an ADC.

The Linker L is covalently bound via one reactive site of a bifunctional compound, leaving the other functional site available for subsequent attachment to an antibody. The Linker L is a moiety having 1-200 non-hydrogen atoms selected from C, N, O, S, or halogen, and optionally incorporates ether, oxo, carboxyl, carboxamide, carboxamidyl, urethanyl, branched, cyclic, unsaturated, amino acid, heterocyclic, aromatic or heteroaromatic moieties. Linker L may be unbranched or branched, flexible or rigid, short or long and may incorporate any combination of moieties as deemed useful. In some embodiments, at least a portion of the linker L may have a polyalkylene oxide polymeric region, which may enhance solubility of the compound. In some embodiments, the Linker L may have a repeating unit of ethylene glycol, and may have a number of repeating ethylene glycol units of about 1 to about 25, or

any number there between. In some embodiments, L may include about 1 to about 4, about 3 to about 20, about 4 to about 15, about 5 to about 12 or about 6 to about 10 ethylene glycol units.

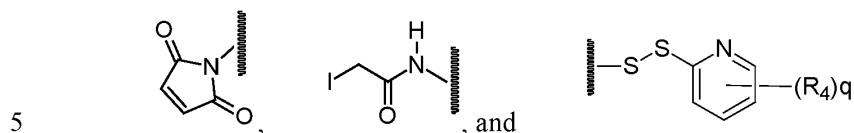
5 In some embodiments, at least a portion of Linker L may include one or more amino acid moieties which may provide enhanced solubility for the compound or may provide amino acid sequences to enhance target binding, enhance compatibility with a target binding agent, or enhance target binding recognition. In other embodiments, the Linker L may include one or more amino acid moieties that provide a suitable substrate motif for a protease. Such embodiments include amino acids selected from glycine, alanine, phenylalanine, lysine, 10 arginine, valine, and citrulline. When a set of amino acid moieties are incorporated into the linker L that provide a substrate motif specific for a selected protease, the cytotoxic drug compound may be released from a target bound conjugate to provide localized cytotoxic effects. Such substrate motifs are known in the art and may be incorporated into the Linker L as desired to provide selective release from the target bound conjugate. This selectivity can 15 be based on known presence of a desired protease within the localized delivery region of the drug-antibody conjugate. Other polymeric types of moieties may be incorporated in the Linker L, such as polyacids, polysaccharides, or polyamines. Other moieties such as substituted aromatic or heteroaromatic moieties may be used to enhance rigidity or provide synthetically accessible sites on substituents therein for linking to reactive moieties or to the 20 compound.

The other second functional site of Linker L is available for subsequent attachment to an antibody, thus covalently bonding the toxin of the invention to an antibody via a linker. This second reactive site is, for example, an electrophilic group that is reactive to a nucleophilic group present on an antibody unit (e.g., an antibody). Useful nucleophilic 25 groups on an antibody include but are not limited to, sulfhydryl, hydroxyl and amino groups. The heteroatom of the nucleophilic group of an antibody is reactive to an electrophilic group on a linker unit and forms a covalent bond to a linker unit. Useful electrophilic groups include, but are not limited to, maleimide and haloacetamide groups. The electrophilic group provides a convenient site for antibody attachment.

30 In another embodiment, a linker unit has a reactive site which has a nucleophilic group that is reactive to an electrophilic group present on an antibody. Useful electrophilic groups on an antibody include, but are not limited to, aldehyde and ketone carbonyl groups. The heteroatom of a nucleophilic group of a linker unit can react with an electrophilic group on an antibody and form a covalent bond to the antibody. Useful nucleophilic groups on a

linker unit include, but are not limited to, hydrazide, oxime, amino, hydrazine, thiosemicarbazone, hydrazine carboxylate, and arylhydrazide.

The electrophilic group on an antibody provides a convenient site for attachment to a linker unit. In some embodiments, the electrophilic groups include:



where the wavy lines indicate the attachments to L, and

R^4 is NO_2 , Cl, F, CN, or Br, and q is 0, 1, or 2.

Amino functional groups are also useful reactive sites for a linker unit because they can react with carboxylic acid, or activated esters of a compound to form an amide linkage.

10 Typically, the peptide-based compounds of the invention can be prepared by forming a peptide bond between two or more amino acids and/or peptide fragments.

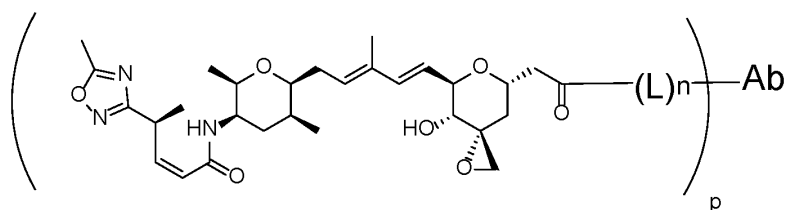
In one aspect, a linker has a functionality that is capable of reacting with a free cysteine present on an antibody to form a covalent bond. Nonlimiting examples of such reactive functionalities include maleimide, haloacetamides, α -haloacetyl, pyridyl disulfide, activated
15 esters such as succinimide esters, N-hydroxysuccinimide, 4-nitrophenyl esters, pentafluorophenyl esters, tetrafluorophenyl esters, anhydrides, acid chlorides, sulfonyl chlorides, isocyanates, and isothiocyanates. *See, e.g.*, the conjugation method at page 766 of Klussman, *et al.*, *Bioconjugate Chemistry*, 2004, **15**(4):765-773, and the Examples herein.

In some embodiments, a linker has a functionality that is capable of reacting with an
20 electrophilic group present on an antibody. Examples of such electrophilic groups include, but are not limited to, aldehyde and ketone carbonyl groups. In some embodiments, a heteroatom of the reactive functionality of the linker can react with an electrophilic group on an antibody and form a covalent bond to an antibody unit. Nonlimiting examples of reactive functionalities include, but are not limited to, hydrazide, oxime, amino, hydrazine,
25 thiosemicarbazone, hydrazine carboxylate, and arylhydrazide.

A linker may comprise one or more linker components, including but not limited to, a
stretcher unit, a peptidomimetic unit, a peptide unit, and a spacer unit. *See J. Lu., et al. (Int. J. Mol. Sci.*, 2016, **17**:561). Exemplary linker components and linker reagents include, but
are not limited to, p-amino benzoic acid-valine-citrulline ("PABA-val-cit"), 6-
30 maleimidocaproyl ("MC"), maleimidopropanoyl ("MP"), valine-citrulline ("val-cit" or "vc"), valine-alanine ("val-ala" or "va"), alanine-phenylalanine ("ala-phe"), phenylalanine-lysine

- (phe-lys), glycine-phenylalanine-leucine-glycine (“GFLG”), p-aminobenzyloxycarbonyl (“PAB”), N-succinimidyl 4-(2-pyridylthio) pentanoate (“SPP”), 4-(N-maleimidomethyl) cyclohexane-1 carboxylate (“MCC”), 4-(N-maleimidomethyl)cyclohexanecarboxylic acid N-hydroxysuccinimide ester (“SMCC”), N-succinimidyl 4-(2-pyridyldithio)butanoate (“SPDB”), and sulfo-N-succinimidyl 4-(2-pyridyldithio)butyrate (“sulfo-SPDB”). Various linker components are known in the art, which are described herein. Exemplary linker components include, but are not limited to, “VC-2xPEG”, “VA-2xPEG”, and “VC-8xPEG”, and “VA-8xPEG”, where a valine-citrulline unit is attached to two or more ethyleneoxy units (PEG), for example, two to 10 PEG units.
- A linker may be a “cleavable linker,” facilitating release of a drug or toxin. Nonlimiting examples of cleavable linkers include acid-labile linkers (e.g., comprising hydrazone), protease-sensitive (e.g., peptidase-sensitive) linkers, photolabile linkers, or disulfide-containing linkers (Chari, *et al.*, *Cancer Research*, 1992, **52**:127-131; U.S. Patent No. 5,208,020). Further examples of embodiments of linkers are described in U.S. Patent No. 7,498,298, which is expressly incorporated herein by reference.

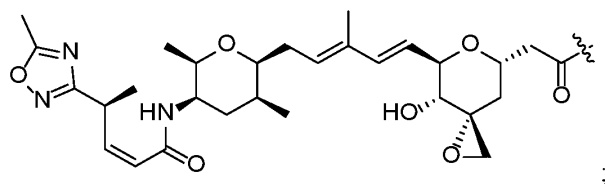
One embodiment provides an antibody-drug conjugate compound comprising an antibody covalently attached to a drug moiety directly (i.e., without a linker) or through a linker of Formula I:



20

I

or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein the drug moiety is:



L is a linker;

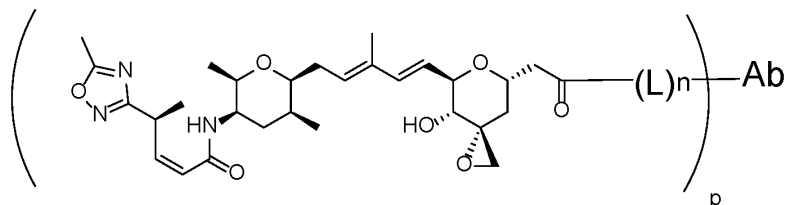
25

n is 0 or 1;

p is 1 to 10 (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10); and

Ab is an antibody.

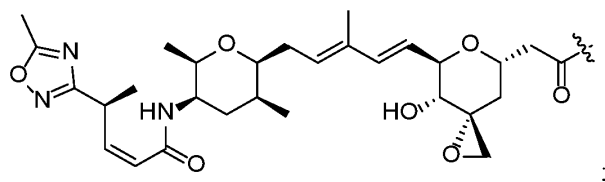
One embodiment provides an antibody-drug conjugate compound comprising an antibody covalently attached to a drug moiety directly (i.e., without a linker) or through a linker of Formula I:



5

I

or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein the drug moiety is:



L is a linker;

10

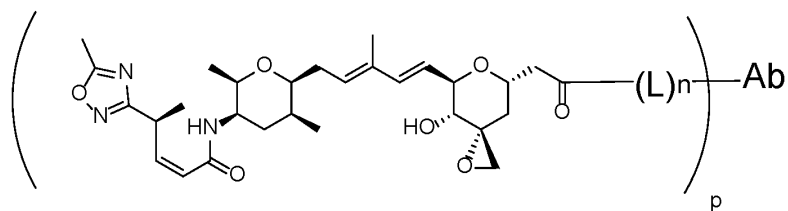
n is 0 or 1;

p is 1 to 10 (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10); and

Ab is an antibody that binds to Trop2.

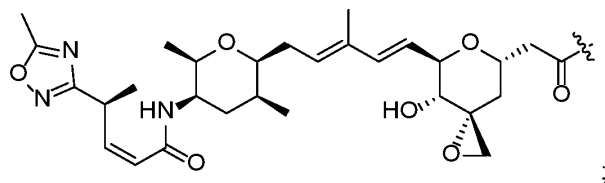
One embodiment provides an antibody-drug conjugate compound comprising an antibody covalently attached to a drug moiety directly (i.e., without a linker) or through a linker of Formula I:

15



I

or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein the drug moiety is:



20

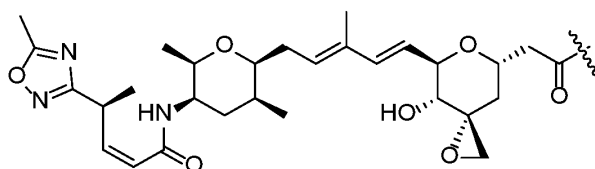
L is a linker;

n is 0 or 1;

p is 1 to 10 (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10); and

Ab is an antibody that binds to Trop2.

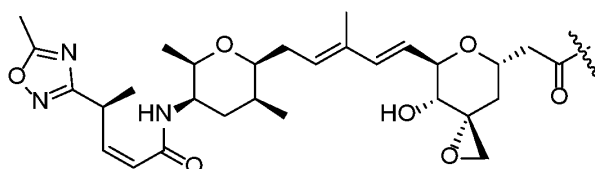
- 5 One embodiment provides an antibody-drug conjugate comprising an anti-Trop2 antibody covalently attached to one or more a drug moieties of formula II:



II

or a salt thereof (e.g., a pharmaceutically acceptable salt thereof).

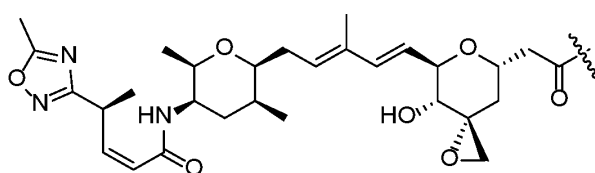
- 10 One embodiment provides an antibody-drug conjugate comprising an antibody covalently attached to one or more a drug moieties of formula II:



II

or a salt thereof (e.g., a pharmaceutically acceptable salt thereof).

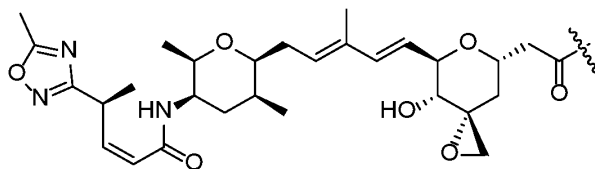
- 15 One embodiment provides an antibody-drug conjugate (or a mixture of antibody-drug conjugates) comprising an anti-Trop2 antibody covalently attached to one or more a drug moieties of formula II:



II

- 20 or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein the average DAR (drug antibody ratio) is 1-15, 1-12, 1-10, 1-8, 1-6, 1-5, 1-4, 1-3, 2-15, 2-12, 2-10, 2-8, 2-6, 2-5, 2-4, 2-3, 3-15, 3-12, 3-10, 3-8, 3-6, 3-5, or 3-4.

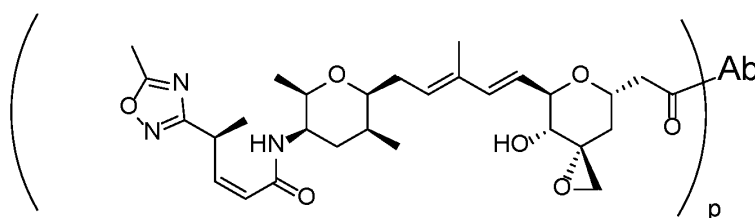
One embodiment provides an antibody-drug conjugate (or a mixture of antibody-drug conjugates) comprising an antibody covalently attached to one or more a drug moieties of formula II:



II

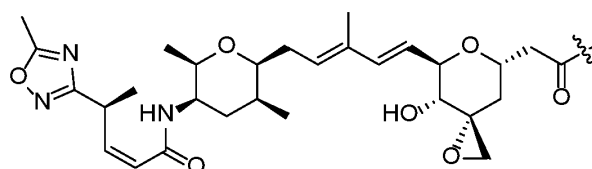
or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein the average DAR (drug antibody ratio) is 1-15, 1-12, 1-10, 1-8, 1-6, 1-5, 1-4, 1-3, 2-15, 2-12, 2-10, 2-8, 2-6, 2-5, 2-4, 2-3, 3-15, 3-12, 3-10, 3-8, 3-6, 3-5, or 3-4.

One embodiment provides an antibody-drug conjugate compound comprising an antibody covalently attached to a drug moiety directly of Formula Ia:



Ia

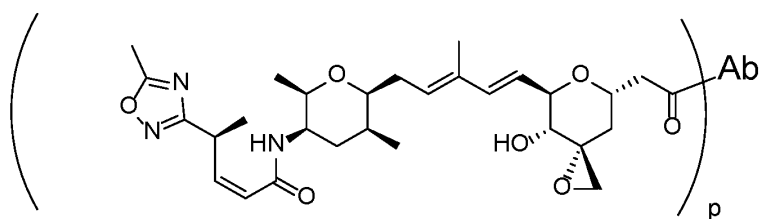
or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein the drug moiety is:



p is 1-20 or 1 to 10 (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10); and

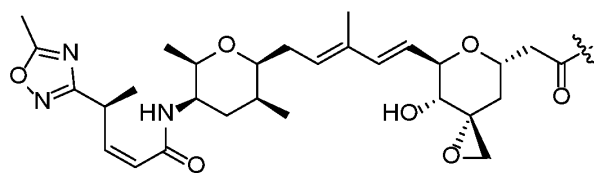
Ab is an anti-Trop2 antibody.

One embodiment provides an antibody-drug conjugate compound comprising an antibody covalently attached to a drug moiety directly of Formula Ia:



Ia

or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein the drug moiety is:

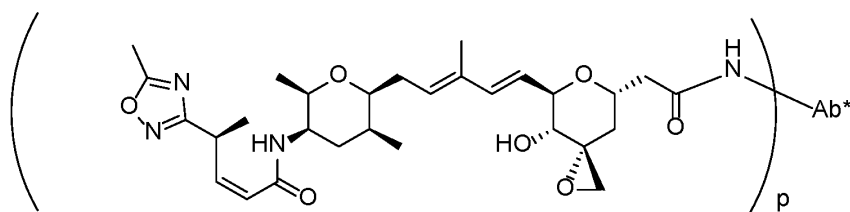


5

p is 1-20 or 1 to 10 (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10); and

Ab is antibody.

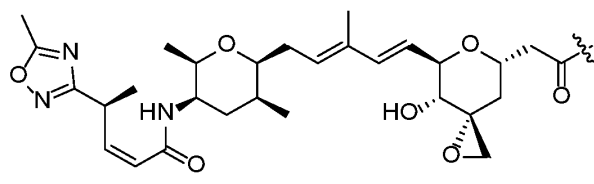
One embodiment provides an antibody-drug conjugate compound comprising an antibody covalently attached to a drug moiety directly of Formula Ib:



10

Ib

or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein the drug moiety is:

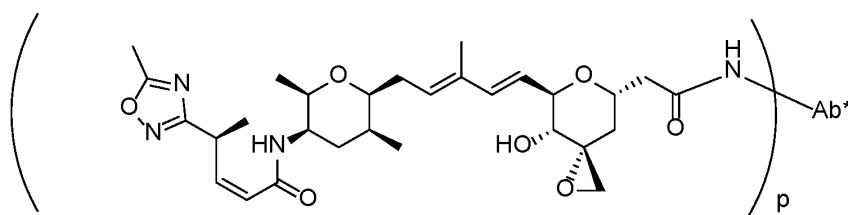


15

p is 1-20 or 1 to 10 (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10); and

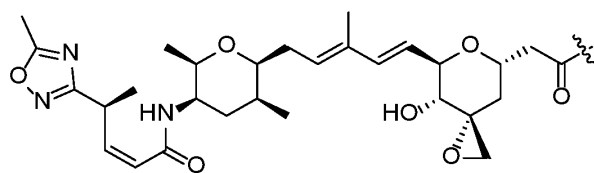
-NHAb* is an anti-Trop2 antibody, wherein the each -NH- is a sidechain amine of a lysine residue of the antibody.

One embodiment provides an antibody-drug conjugate compound comprising an antibody covalently attached to a drug moiety directly of Formula Ib:



Ib

or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein the drug moiety is:



5

p is 1-20 or 1 to 10 (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10); and

-NHAb* is an antibody, wherein the each -NH- is a sidechain amine of a lysine residue of the antibody.

In one embodiment L is 0.

10 In one embodiment L is absent (i.e., n is 0).

In one embodiment antibody is attached to the drug moiety by a sidechain nitrogen of a lysine residue of the antibody.

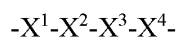
In one embodiment antibody is attached to the drug moiety by a sidechain sulfur of a cysteine residue of the antibody.

15 In one embodiment antibody is attached to the drug moiety by a sidechain nitrogen of an amino acid residue of the antibody.

In one embodiment antibody is attached to the drug moiety by a sidechain sulfur of an amino acid residue of the antibody.

In one embodiment n is 1.

20 In one embodiment the linker L has the formula



wherein,

25 X^1 is a bond, $-N(R^a)-$, $-N(R^a)-N(R^a)-$, $-O-$, $-N-O-$, $-S-$, $-C(=O)-$, $-C(=O)N(R^a)$, $C(=S)N(R^a)-$, $-C(=O)O-$, $-C(=O)S-$, $-N(R^a)SO_2-$, $-OC(=O)N(R^a)-$, $-(R^a)HC(=O)N(R^a)-$, or $-N(R^a)C(=S)N(R^a)-$, wherein R^a is H or $(C_1-C_6)alkyl$;

X^2 is a bond or a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain having from 1 to 30 carbon atoms, wherein one or more of the carbon

atoms is optionally replaced by (-O-), (-S-), -N(R^b)-, a divalent 6-10 membered aryl or a divalent 5-20 membered heteroaryl wherein R^b is H or (C₁-C₆)alkyl, wherein the hydrocarbon chain is optionally substituted on carbon with one or more substituents selected from the group consisting of halo, hydroxy, mercapto, oxo(=O), and thioxo(=S), and wherein the

5 divalent 6-10 membered aryl or a divalent 5-20 membered heteroaryl is optionally substituted with one more halo or (C₁-C₆)alkyl;

X³ is a bond or a peptide (e.g., a peptide comprising 1, 2, 3, 4, 5, or 6 amino acid residues); and

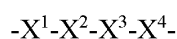
X⁴ is a bond or a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain having from 1 to 30 carbon atoms, wherein one or more of the carbon

10 atoms is optionally replaced by (-O-),

(-S-), or -N(R^c)- wherein R^c is H or (C₁-C₆)alkyl, wherein the hydrocarbon chain is optionally substituted on carbon with one or more substituents selected from the group consisting of halo, hydroxy, mercapto, oxo(=O), and thioxo(=S);

15 wherein at least one of X¹, X², or X³ is not a bond.

In one embodiment L has the formula



wherein,

X¹ is a bond, -N(R^a)-, -N(R^a)-N(R^a)-, -O-, -N-O-, -S-, -C(=O)-, -C(=O)N(R^a),

20 C(=S)N(R^a)-, -C(=O)O-, -C(=O)S-, -N(R^a)SO₂-, -OC(=O)N(R^a)-, -(R^a)HC(=O)N(R^a)-, or -N(R^a)C(=S)N(R^a)-, wherein R^a is H or (C₁-C₆)alkyl;

X² is a bond or a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain having from 1 to 30 carbon atoms, wherein one or more of the carbon atoms is optionally replaced by (-O-), (-S-), -N(R^b)-, a divalent 6-10 membered aryl or a

25 divalent 5-20 membered heteroaryl wherein R^b is H or (C₁-C₆)alkyl, wherein the hydrocarbon chain is optionally substituted on carbon with one or more substituents selected from the group consisting of halo, hydroxy, mercapto, oxo(=O), and thioxo(=S), and wherein the divalent 6-10 membered aryl or a divalent 5-20 membered heteroaryl is optionally substituted with one more halo or (C₁-C₆)alkyl;

30 X³ is a bond or a peptide (e.g., a peptide comprising 1, 2, 3, 4, 5, or 6 amino acid residues); and

X⁴ is a bond or a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain having from 1 to 30 carbon atoms, wherein one or more of the carbon atoms is optionally replaced by (-O-), (-S-), or -N(R^c)- wherein R^c is H or (C₁-C₆)alkyl,

wherein the hydrocarbon chain is optionally substituted on carbon with one or more substituents selected from the group consisting of halo, hydroxy, mercapto, oxo(=O), and thioxo(=S).

In one embodiment L has the formula



wherein,

X^1 is a bond, $-N(R^a)-$, $-N(R^a)-N(R^a)-$, $-O-$, $-N-O-$, $-S-$, $-C(=O)-$, $-C(=O)N(R^a)$, $C(=S)N(R^a)-$, $-C(=O)O-$, $-C(=O)S-$, $-N(R^a)SO_2-$, $-OC(=O)N(R^a)-$, $-(R^a)HC(=O)N(R^a)-$, or $-N(R^a)C(=S)N(R^a)-$, wherein R^a is H or (C_1-C_6) alkyl;

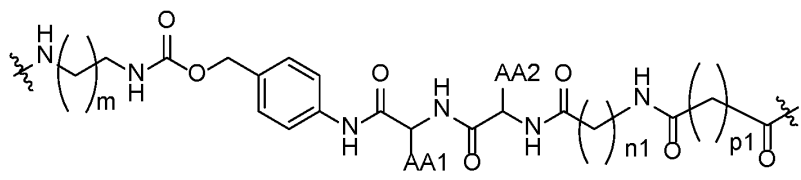
X^2 is a bond or a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain having from 1 to 30 carbon atoms, wherein one or more of the carbon atoms is optionally replaced by $(-O-)$, $(-S-)$, $-N(R^b)-$, a divalent 6-10 membered aryl or a divalent 5-20 membered heteroaryl wherein R^b is H or (C_1-C_6) alkyl, wherein the hydrocarbon chain is optionally substituted on carbon with one or more substituents selected from the group consisting of halo, hydroxy, mercapto, oxo(=O), and thioxo(=S), and wherein the divalent 6-10 membered aryl or a divalent 5-20 membered heteroaryl is optionally substituted with one more halo or (C_1-C_6) alkyl;

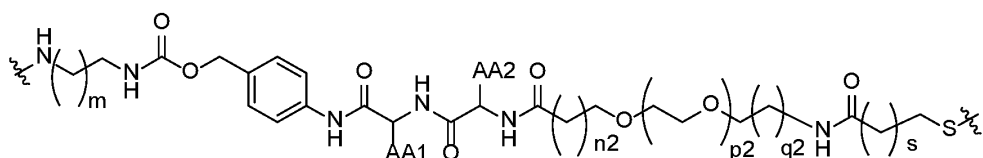
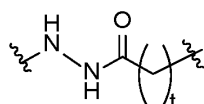
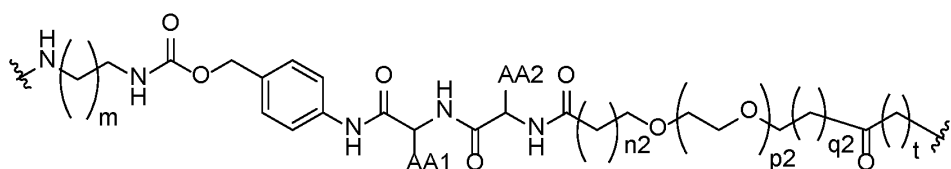
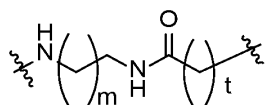
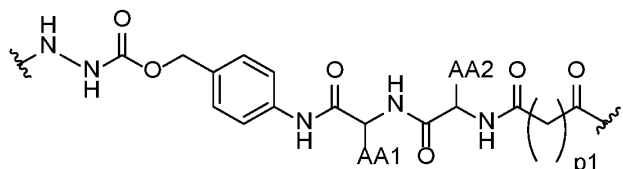
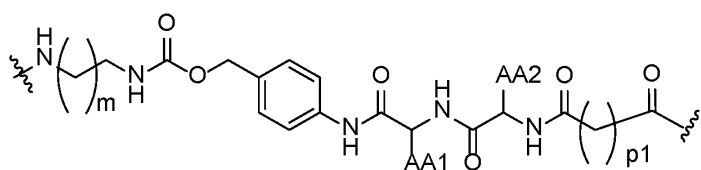
X^3 is a bond or a peptide (e.g., a peptide comprising 1, 2, 3, 4, 5, or 6 amino acid residues); and

X^4 is a bond or a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain having from 1 to 60 carbon atoms (or 2-50, 2-40, 5-60, 5-50, 5-0, 5-30, or 5-20 carbon atoms), wherein one or more of the carbon atoms is optionally replaced by $(-O-)$, $(-S-)$, or $-N(R^c)-$ wherein R^c is H or (C_1-C_6) alkyl, wherein the hydrocarbon chain is optionally substituted on carbon with one or more substituents selected from the group consisting of halo, hydroxy, mercapto, oxo(=O), and thioxo(=S);

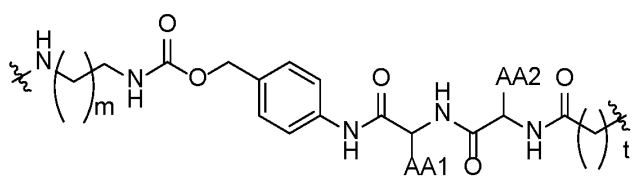
wherein at least one of X^1 , X^2 , or X^3 is not a bond.

In one embodiment n is 0 (i.e., L is absent) or n is 1 and L is a linker selected from the group consisting of:





, and



wherein:

- each m is independently 1, 2, 3, or 4;
- each n1 is independently 1, 2, 3, 4, 5, or 6;
- each p1 is independently 1, 2, 3, 4, 5, or 6;
- each t is independently 1, 2, 3, or 4;
- each n2 is independently 1, 2, 3, 4, 5, or 6;
- each p2 is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;

each q2 is independently 1, 2, 3, 4, 5, or 6;

each s is independently 1, 2, 3, or 4; and

each AA1 and AA2 are independently a sidechain of an amino acid.

In one embodiment

5 each m is independently 1, 2, 3, 4, 5, or 6;

each n1 is independently 1, 2, 3, 4, 5, 6, 7 or 8;

each p1 is independently 1, 2, 3, 4, 5, 6, 7 or 8;

each t is independently 1, 2, 3, 4, 5, 6, 7 or 8;

each n2 is independently 1, 2, 3, 4, 5, 6, 7 or 8;

10 each p2 is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12;

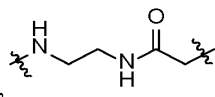
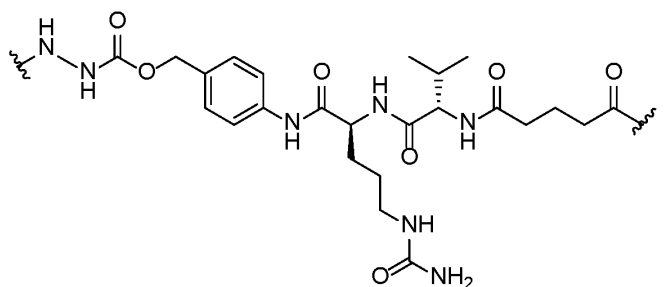
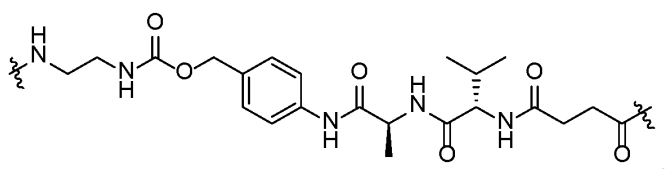
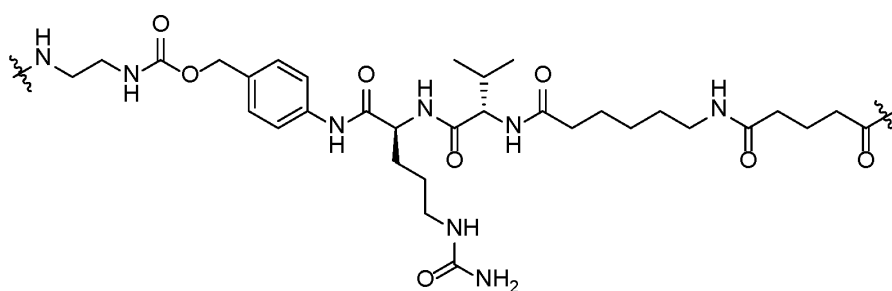
each q2 is independently 1, 2, 3, 4, 5, or 6;

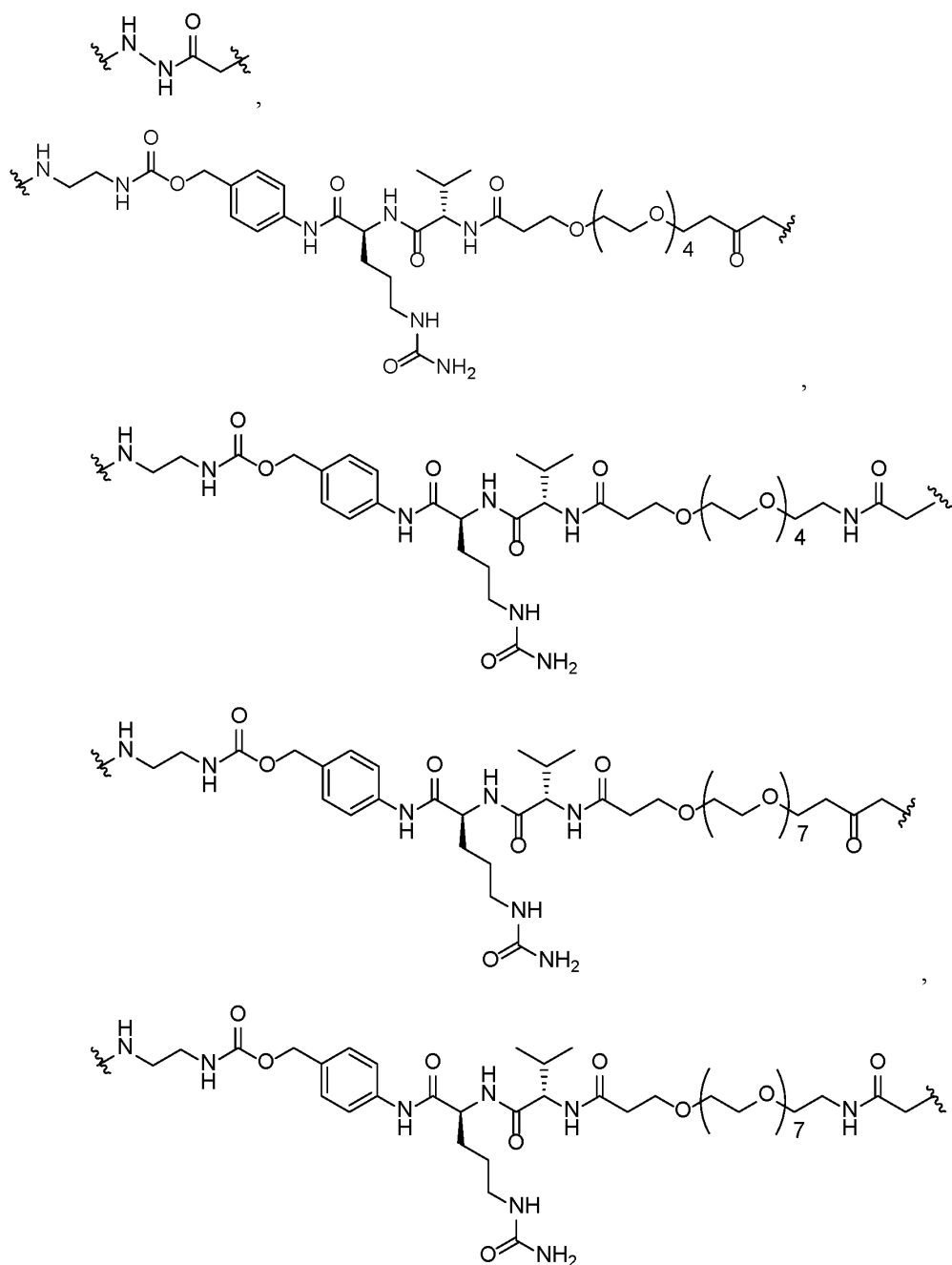
each s is independently 1, 2, 3, 4, 5, or 6; and

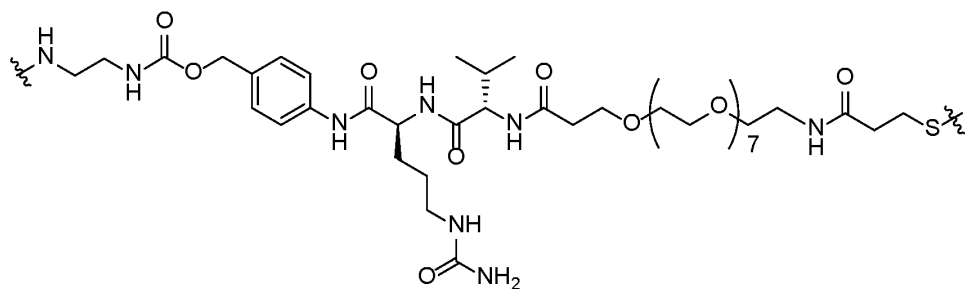
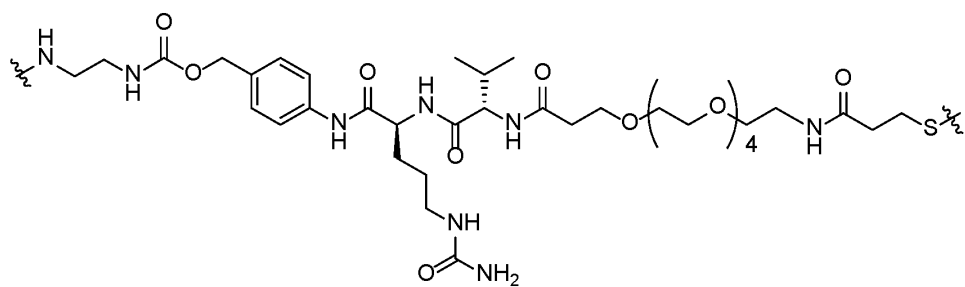
each AA1 and AA2 are independently a sidechain of an amino acid.

In one embodiment n is 0 (i.e., L is absent) or n is 1 and L is a linker selected from the

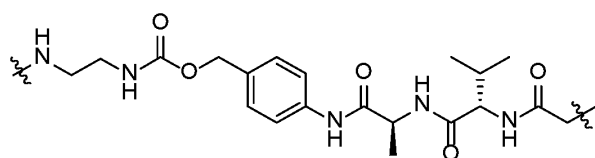
15 group consisting of:



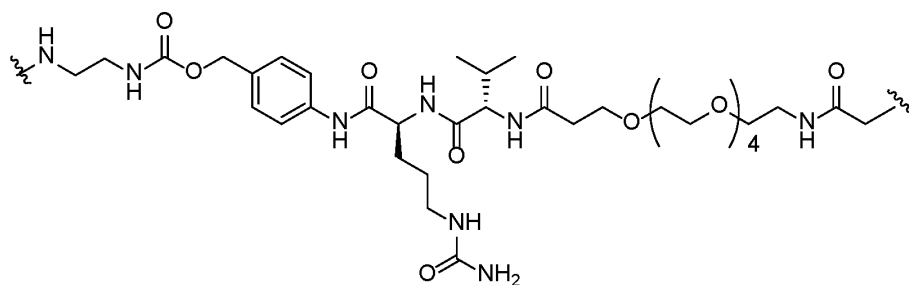




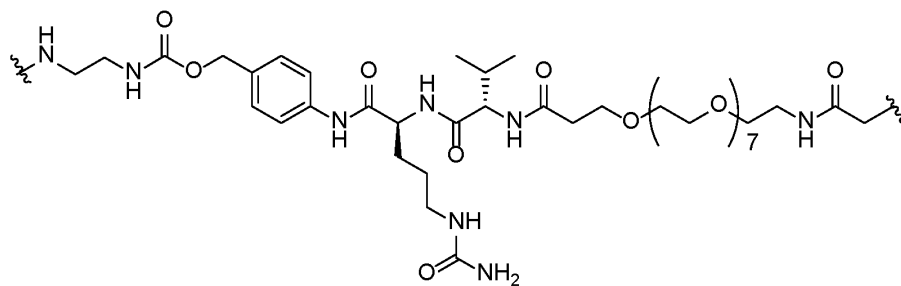
and



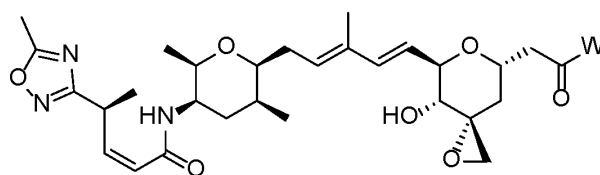
- 5 In one embodiment n is 0 (i.e., L is absent) or n is 1 and L is a linker selected from the group consisting of:



and



One embodiment provides a compound of formula II



II

or a salt thereof,

wherein

5 W is a leaving group or -L-X;

L is a linker; and

X is a reactive group.

In one embodiment W is a leaving group.

In one embodiment the leaving group is halo, -O(C₁-C₆)alkyl, -Oaryl, -Oheteroaryl
10 or -Oheterocyclyl, wherein the -O(C₁-C₆)alkyl, -Oaryl, Oheteroaryl or Oheterocyclyl are
each optionally substituted with one or more substituents independently selected from the
group consisting of oxo, halo, (C₁-C₆)alkyl and -O(C₁-C₆)alkyl,

In one embodiment W together with the carbonyl group to which it is attached forms
an activated ester.

15 In one embodiment W is -Ophenyl optionally substituted with one or more halo or
2,5, dioxopyrrolidinyl.

In one embodiment W is -Ophenyl optionally substituted with one or more halo.

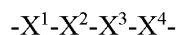
In one embodiment W is -Ophenyl optionally substituted with one or more fluoro.

In one embodiment W is a 2,3,5,6,-tetrafluorophenoxy.

20 In one embodiment W is 2,5, dioxopyrrolidinyl.

In one embodiment W is L-X .

In one embodiment L has the formula



wherein,

25 X¹ is a bond, -N(R^a)-, -N(R^a)-N(R^a)-, -O-, -N-O-, -S-, -C(=O)-, -C(=O)N(R^a),
C(=S)N(R^a)-, -C(=O)O-, -C(=O)S-, -N(R^a)SO₂-, -OC(=O)N(R^a)-, -(R^a)HC(=O)N(R^a)-,
or -N(R^a)C(=S)N(R^a)-, wherein R^a is H or (C₁-C₆)alkyl;

X² is a bond or a divalent, branched or unbranched, saturated or unsaturated,
hydrocarbon chain having from 1 to 30 carbon atoms, wherein one or more of the carbon
30 atoms is optionally replaced by (-O-), (-S-), -N(R^b)-, a divalent 6-10 membered aryl or a
divalent 5-20 membered heteroaryl wherein R^b is H or (C₁-C₆)alkyl, wherein the hydrocarbon

chain is optionally substituted on carbon with one or more substituents selected from the group consisting of halo, hydroxy, mercapto, oxo(=O), and thioxo(=S), and wherein the divalent 6-10 membered aryl or a divalent 5-20 membered heteroaryl is optionally substituted with one more halo or (C₁-C₆)alkyl;

5 X³ is a bond or a peptide (e.g., a peptide comprising 1, 2, 3, 4, 5, or 6 amino acid residues); and

X⁴ is a bond or a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain having from 1 to 30 carbon atoms, wherein one or more of the carbon atoms is optionally replaced by (-O-),

10 (-S-), or -N(R^c)- wherein R^c is H or (C₁-C₆)alkyl, wherein the hydrocarbon chain is optionally substituted on carbon with one or more substituents selected from the group consisting of halo, hydroxy, mercapto, oxo(=O), and thioxo(=S);

wherein at least one of X¹, X², or X³ is not a bond.

In one embodiment L has the formula

15 $-X^1-X^2-X^3-X^4-$

wherein,

X¹ is a bond, -N(R^a)-, -N(R^a)-N(R^a)-, -O-, -N-O-, -S-, -C(=O)-, -C(=O)N(R^a), C(=S)N(R^a)-, -C(=O)O-, -C(=O)S-, -N(R^a)SO₂-, -OC(=O)N(R^a)-, -(R^a)HC(=O)N(R^a)-, or -N(R^a)C(=S)N(R^a)-, wherein R^a is H or (C₁-C₆)alkyl;

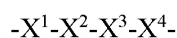
20 X² is a bond or a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain having from 1 to 30 carbon atoms, wherein one or more of the carbon atoms is optionally replaced by (-O-), (-S-), -N(R^b)-, a divalent 6-10 membered aryl or a divalent 5-20 membered heteroaryl wherein R^b is H or (C₁-C₆)alkyl, wherein the hydrocarbon chain is optionally substituted on carbon with one or more substituents selected from the group consisting of halo, hydroxy, mercapto, oxo(=O), and thioxo(=S), and wherein the divalent 6-10 membered aryl or a divalent 5-20 membered heteroaryl is optionally substituted with one more halo or (C₁-C₆)alkyl;

X³ is a bond or a peptide (e.g., a peptide comprising 1, 2, 3, 4, 5, or 6 amino acid residues); and

30 X⁴ is a bond or a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain having from 1 to 30 carbon atoms, wherein one or more of the carbon atoms is optionally replaced by (-O-), (-S-), or -N(R^c)- wherein R^c is H or (C₁-C₆)alkyl, wherein the hydrocarbon chain is optionally substituted on carbon with one or more substituents selected from the group consisting of halo, hydroxy, mercapto, oxo(=O), and

thioxo(=S).

In one embodiment L has the formula



wherein,

- 5 X^1 is a bond, $-N(R^a)-$, $-N(R^a)-N(R^a)-$, $-O-$, $-N-O-$, $-S-$, $-C(=O)-$, $-C(=O)N(R^a)$, $C(=S)N(R^a)-$, $-C(=O)O-$, $-C(=O)S-$, $-N(R^a)SO_2-$, $-OC(=O)N(R^a)-$, $-(R^a)HC(=O)N(R^a)-$, or $-N(R^a)C(=S)N(R^a)-$, wherein R^a is H or (C_1-C_6) alkyl;

- X^2 is a bond or a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain having from 1 to 30 carbon atoms (or 2-30, 2-20, 5-30, or 5-20 carbon
10 atoms), wherein one or more of the carbon atoms is optionally replaced by $(-O-)$, $(-S-)$, $-N(R^b)-$, a divalent 6-10 membered aryl or a divalent 5-20 membered heteroaryl wherein R^b is H or (C_1-C_6) alkyl, wherein the hydrocarbon chain is optionally substituted on carbon with one or more substituents selected from the group consisting of halo, hydroxy, mercapto, oxo(=O), and thioxo(=S), and wherein the divalent 6-10 membered aryl or a divalent 5-20
15 membered heteroaryl is optionally substituted with one more halo or (C_1-C_6) alkyl;

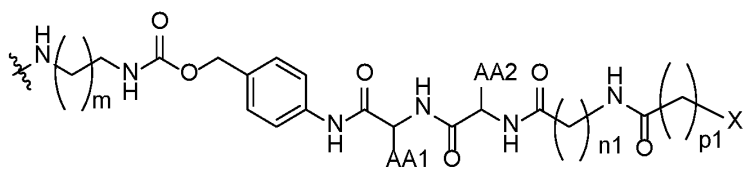
X^3 is a bond or a peptide (e.g., a peptide comprising 1, 2, 3, 4, 5, or 6 amino acid residues); and

- X^4 is a bond or a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain having from 1 to 60 (or 2-50, 2-40, 5-60, 5-50, 5-40, 5-30, or 5-20 carbon
20 atoms), wherein one or more of the carbon atoms is optionally replaced by $(-O-)$, $(-S-)$, or $-N(R^c)-$ wherein R^c is H or (C_1-C_6) alkyl, wherein the hydrocarbon chain is optionally substituted on carbon with one or more substituents selected from the group consisting of halo, hydroxy, mercapto, oxo(=O), and thioxo(=S);

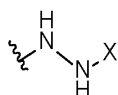
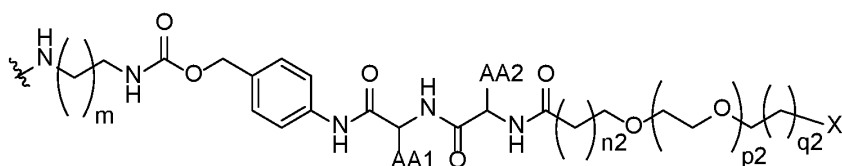
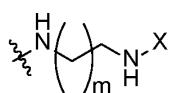
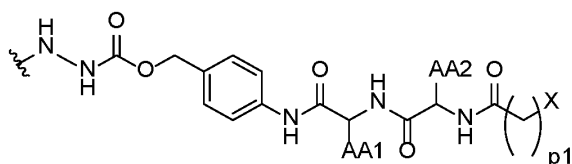
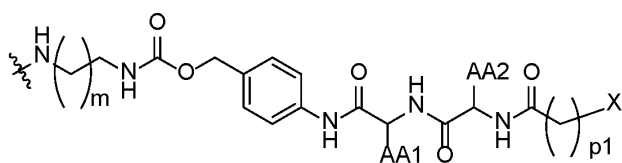
wherein at least one of X^1 , X^2 , or X^3 is not a bond.

- 25 In one embodiment the amino acid residue is a residue of a natural amino acid or citrulline. In one embodiment the amino acid residue is a residue of a natural amino acid or citrulline or a stereoisomer thereof. In one embodiment the amino acid residue is an alanine, glycine, valine or citrulline residue.

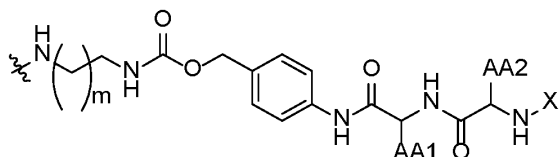
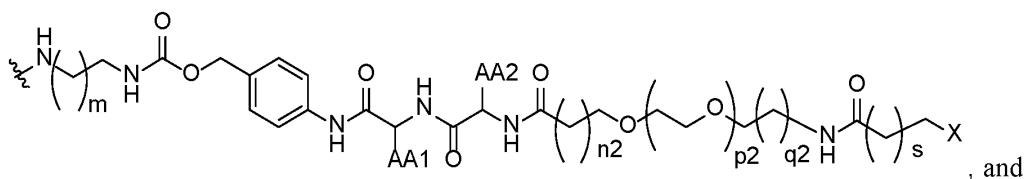
In one embodiment W is selected from the group consisting of .



30



5



wherein:

each m is independently 1, 2, 3, or 4;

10 each n1 is independently 1, 2, 3, 4, 5, or 6;

each p1 is independently 1, 2, 3, 4, 5, or 6;

each t is independently 1, 2, 3, or 4;

each n2 is independently 1, 2, 3, 4, 5, or 6;

each p2 is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;

15 each q2 is independently 1, 2, 3, 4, 5, or 6;

each s is independently 1, 2, 3, or 4; and

each AA1 and AA2 are independently a sidechain of an amino acid.

In one embodiment

each m is independently 1, 2, 3, 4, 5, or 6;

each n1 is independently 1, 2, 3, 4, 5, 6, 7 or 8;

5 each p1 is independently 1, 2, 3, 4, 5, 6, 7 or 8;

each t is independently 1, 2, 3, 4, 5, 6, 7 or 8;

each n2 is independently 1, 2, 3, 4, 5, 6, 7 or 8;

each p2 is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12;

each q2 is independently 1, 2, 3, 4, 5, or 6;

10 each s is independently 1, 2, 3, 4, 5, or 6; and

each AA1 and AA2 are independently a sidechain of an amino acid.

In one embodiment the side chain of an amino acid is a side chain of a natural amino acid or citrulline. In one embodiment each sidechain of an amino acid (AA1 and AA2) is independently H, methyl, isopropyl, or $-(CH_2)_3-NH-C(=O)-NH_2$.

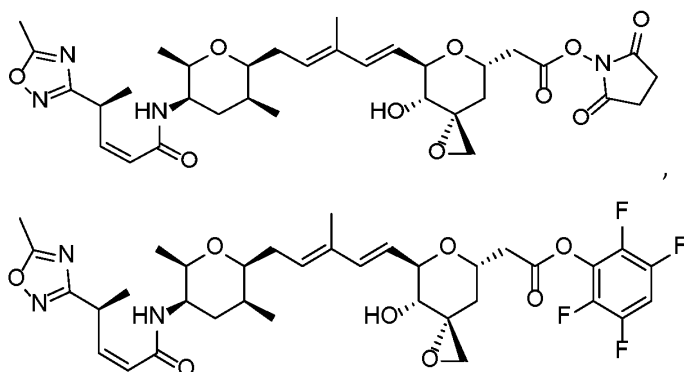
15 In one embodiment each X is independently selected from an ester, activated ester, haloacetamide, halo, and disulfanylheteroaryl.

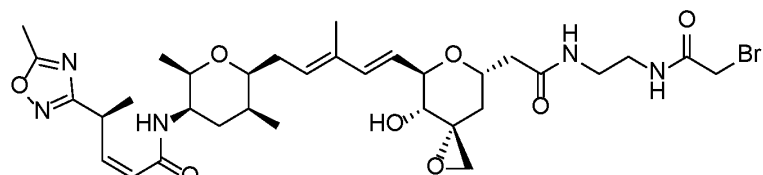
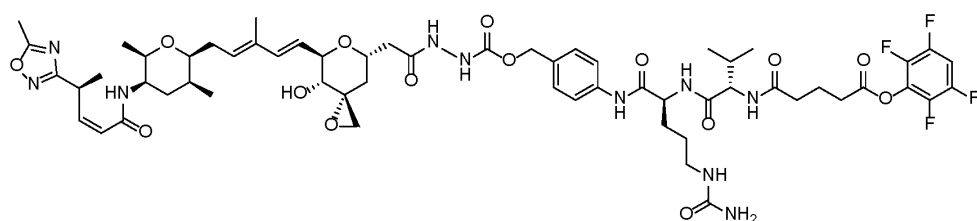
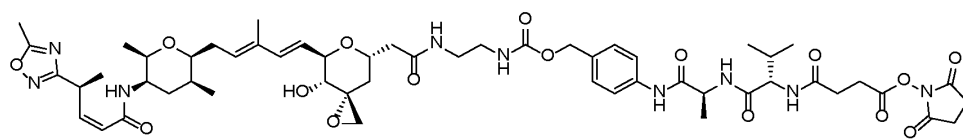
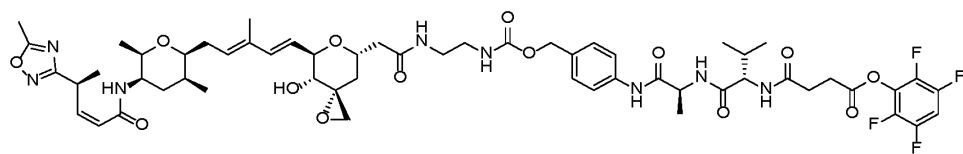
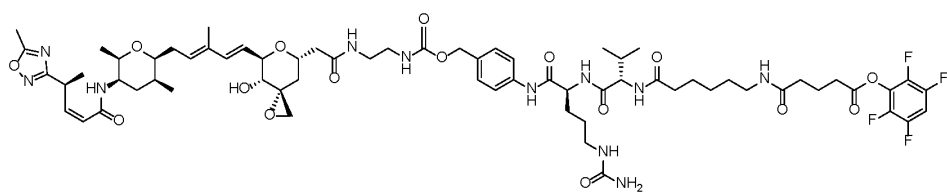
In one embodiment each X is selected from phenoxy carbonyl, 2,5-dioxopyrrolidinyl, disulfanylpiperidinyl, and bromoacetyl, wherein the phenoxy carbonyl is optionally substituted with one or more (e.g., 1, 2, 3, 4, or 5) halo, nitro, 20 or (C_1-C_6) alkyl.

In one embodiment each X is selected from 2,3,5,6-tetrafluorophenoxy carbonyl, 2,5-dioxopyrrolidinyl, disulfanylpiperidinyl, and bromoacetyl.

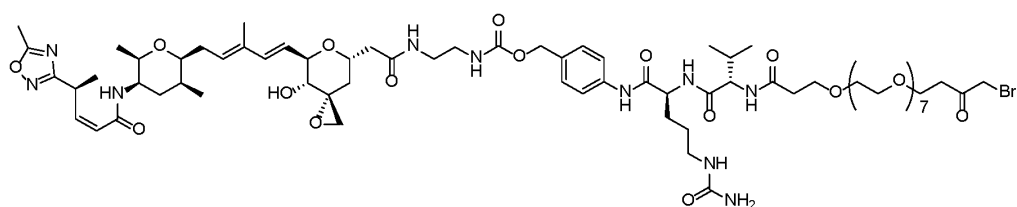
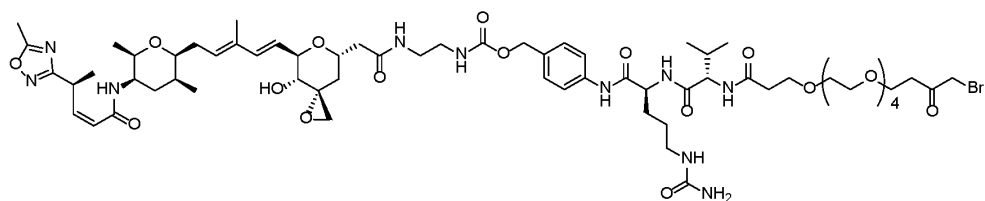
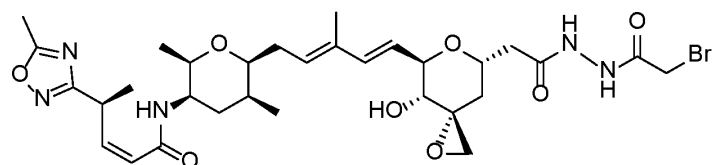
In one embodiment each X is selected from 2,3,5,6-tetrafluorophenoxy carbonyl and bromoacetyl,

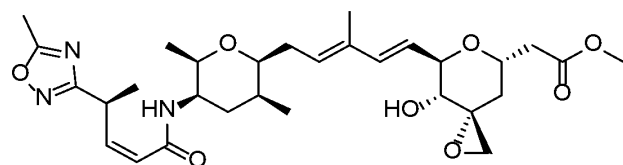
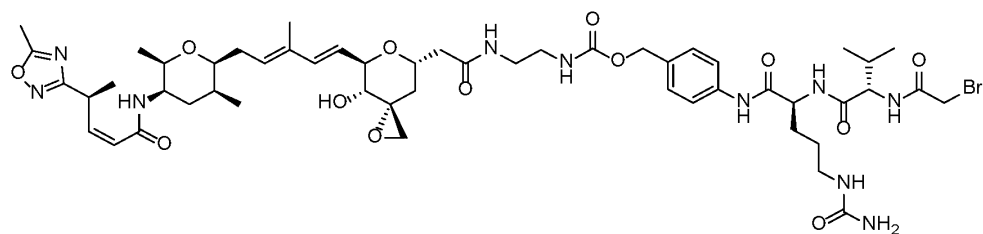
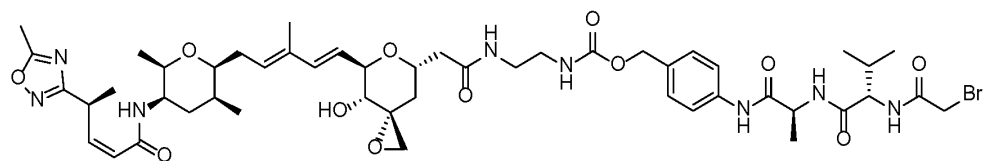
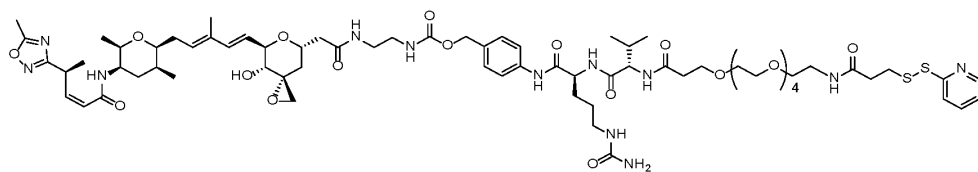
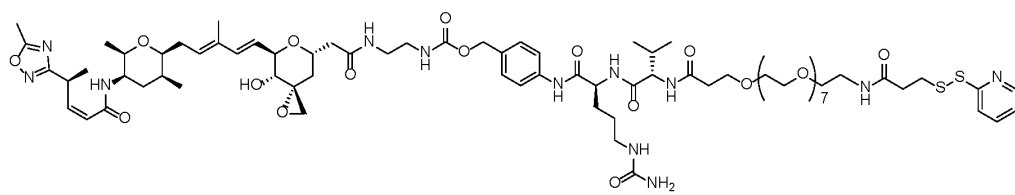
25 One embodiment provides a compound selected from the group consisting of





5

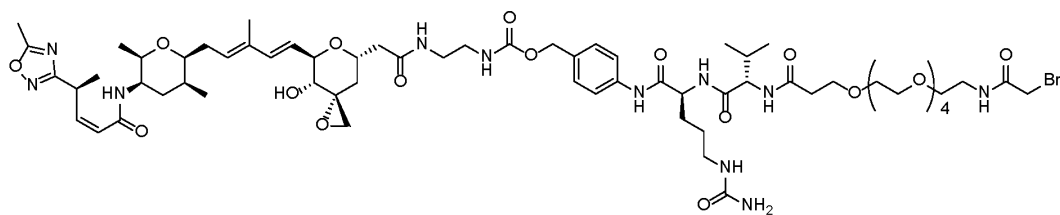




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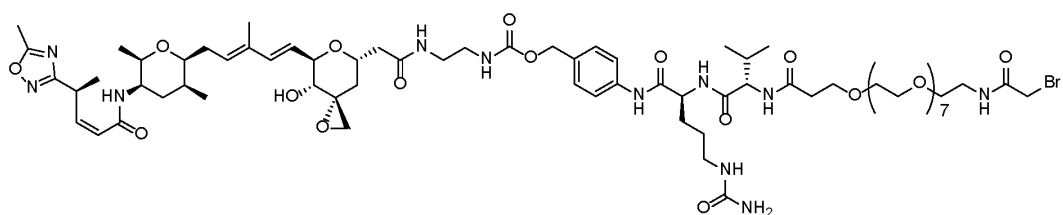
or a salt thereof.

One embodiment provides a compound selected from the group consisting of



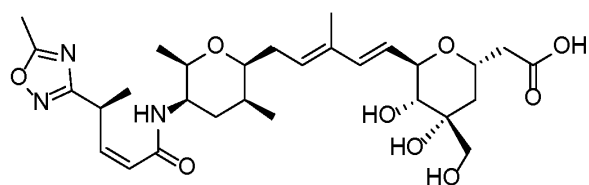
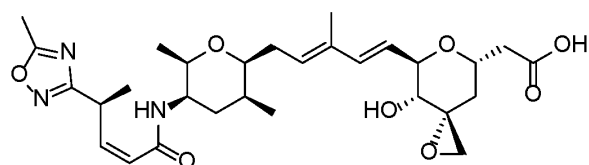
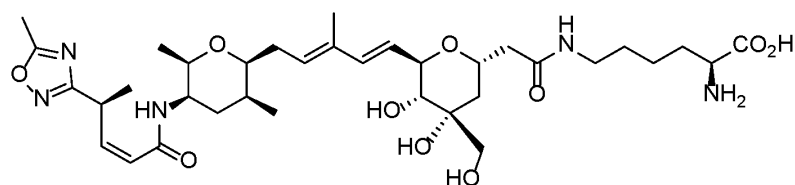
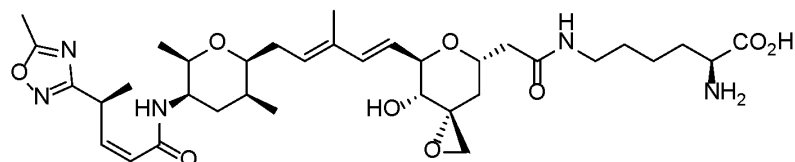
10

and

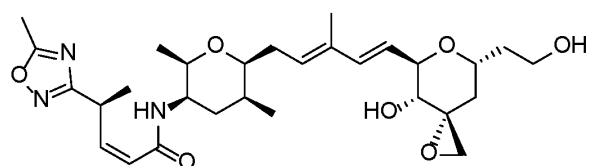


or a salt thereof.

One embodiment provides a compound selected from the group consisting of



, and



or a salt thereof (e.g., a pharmaceutically acceptable salt thereof).

In one embodiment p is 1, 2, 3, 4, or 5.

In one embodiment p is 1, 2, 3, 4, 5, 6, 7, or 8.

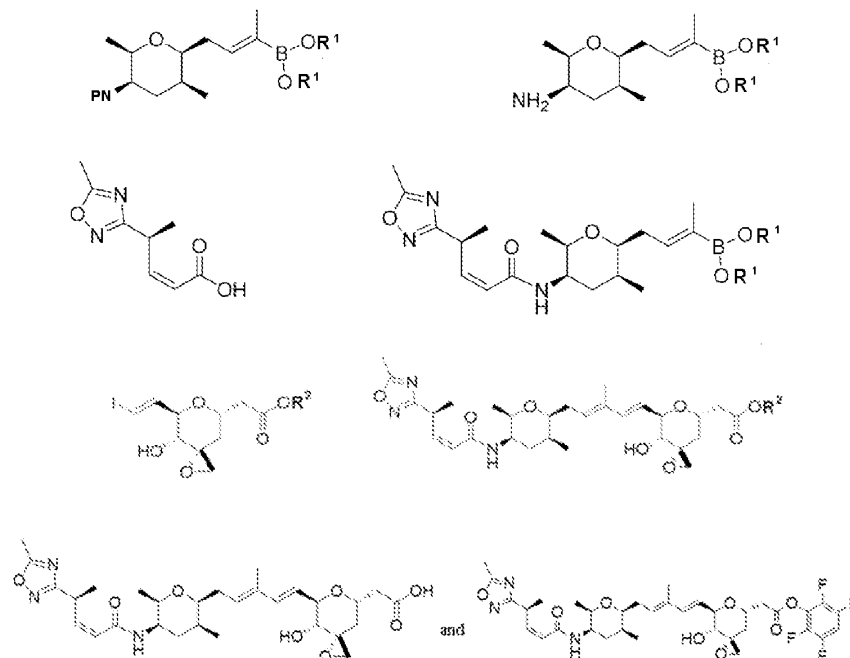
In one embodiment p is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

In one embodiment provides a mixture of the antibody-drug conjugate compounds of formula I, wherein the average drug loading per antibody in the mixture of antibody-drug conjugate compounds is about 1 to about 5.

Provided below are certain non-limiting embodiments of the invention. It is to be understood that two or more embodiments may be combined.

One embodiment provides a compound selected from the group consisting of:

5



wherein:

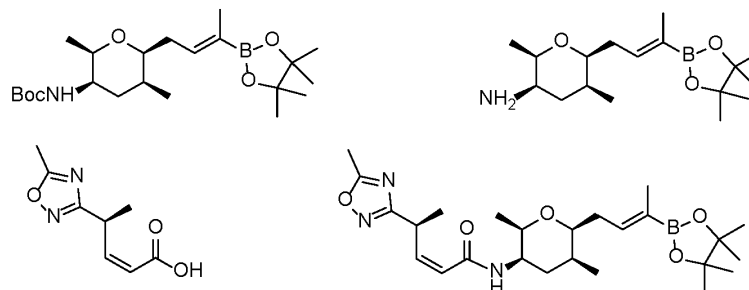
P is an amine nitrogen protecting group;

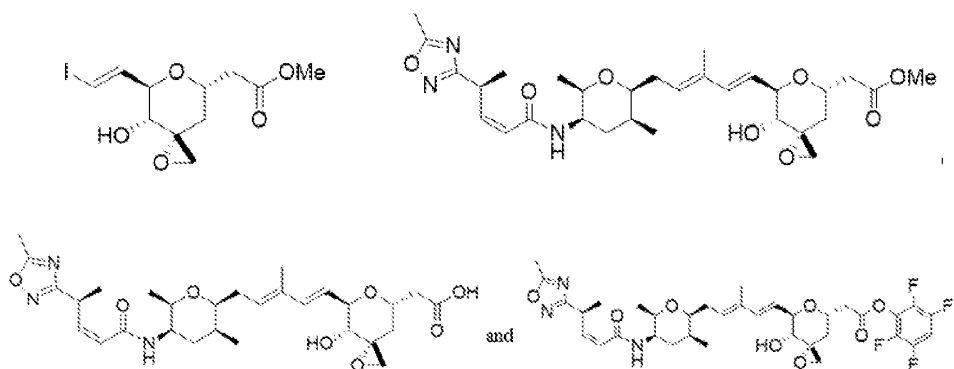
10 each R^1 is independently a (C₁-C₆)alkyl, or two R^1 groups together with the atoms to which they are attached form a heterocyclyl optionally substituted with one or more (C₁-C₆)alkyl; and

R^2 is a (C₁-C₆)alkyl;

or a salt thereof.

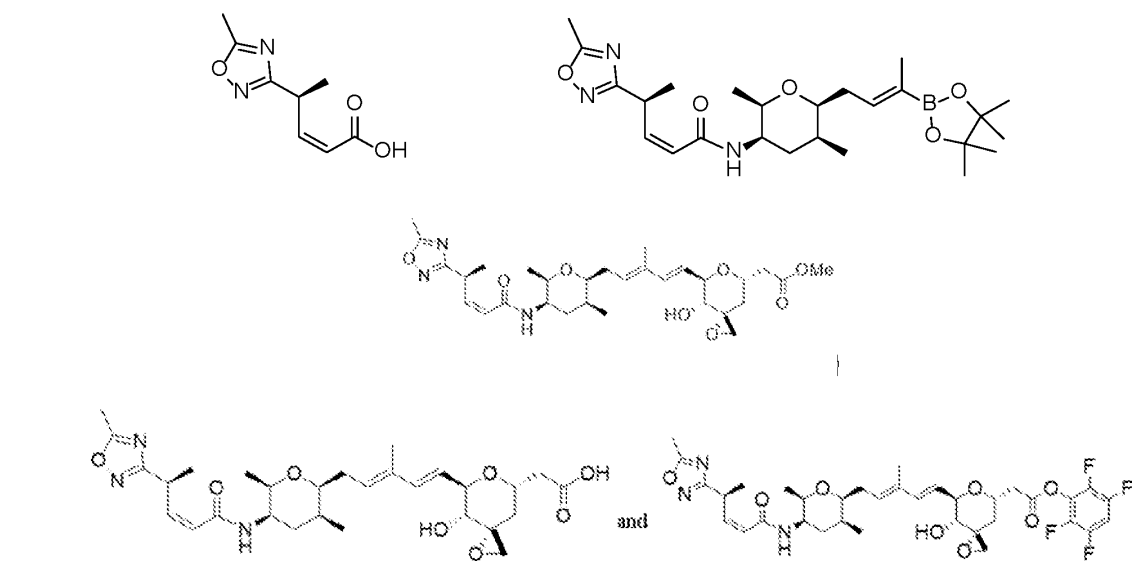
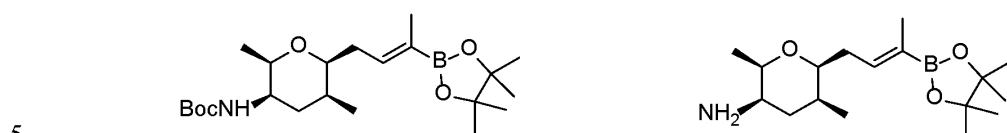
15 One embodiment provides a compound selected from the group consisting of:





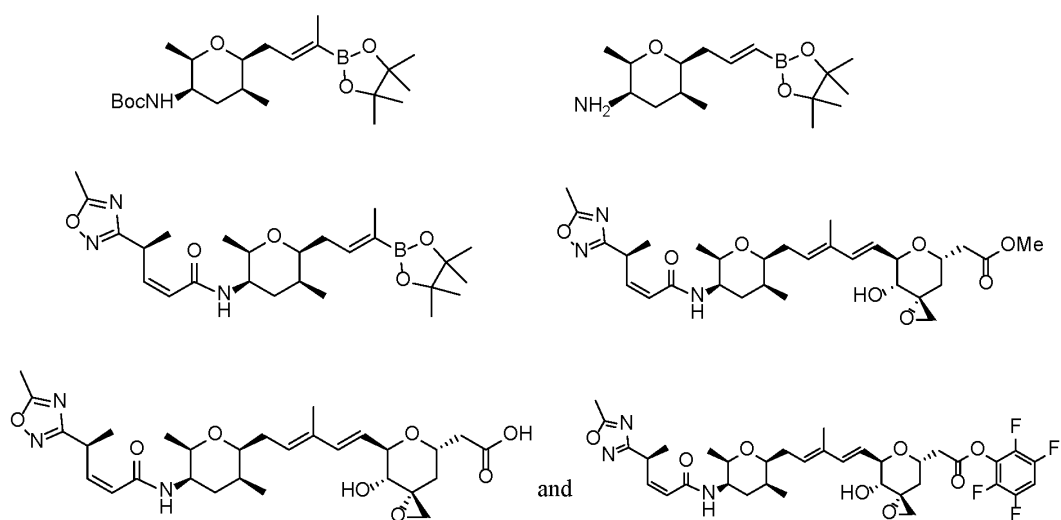
or a salt thereof.

One embodiment provides a compound selected from the group consisting of:



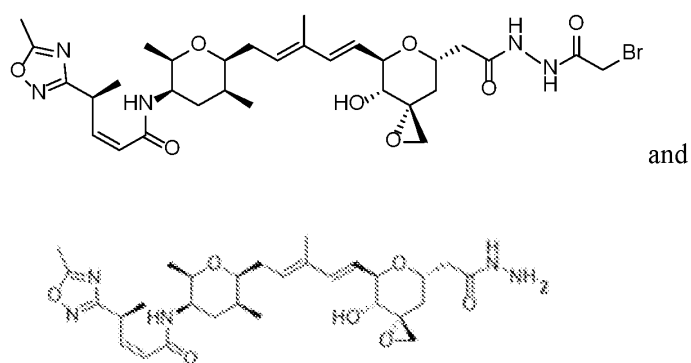
10 or a salt thereof.

One embodiment provides a compound selected from the group consisting of:



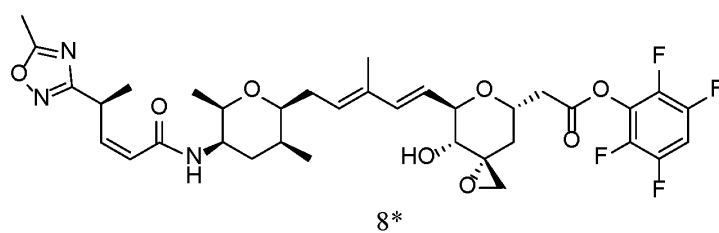
or a salt thereof.

One embodiment provides a compound selected from the group consisting of:

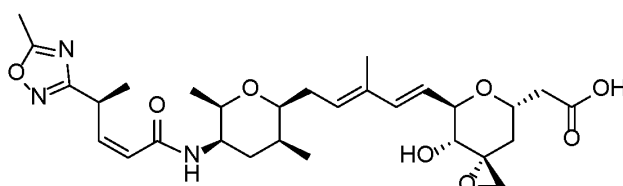


5 or a salt thereof.

One embodiment provides a method for preparing a compound of formula 8*:



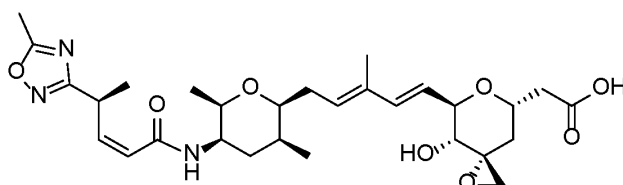
or a salt thereof, comprising converting a corresponding compound of formula 7*:



7*

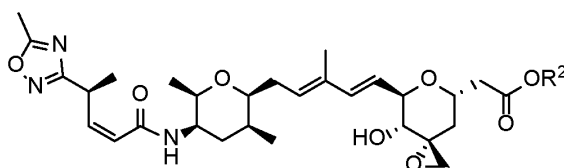
or a salt thereof, to the compound of formula 8 or a salt thereof.

One embodiment provides a method for preparing a compound of formula 7*:



7*

- 5 or a salt thereof, comprising converting a corresponding compound of formula 6a*:

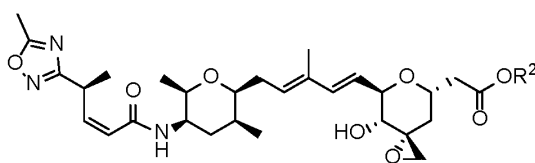


6a*

or a salt thereof, to the compound of formula 7* or a salt thereof,

wherein R² is a (C₁-C₆)alkyl.

- One embodiment provides a method, wherein the compound of formula 7* or a salt thereof is prepared, comprising converting a corresponding compound of formula 6a*:
- 10

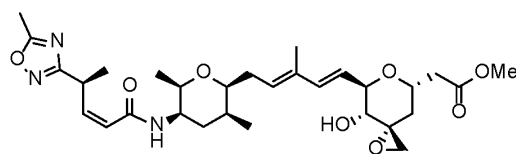


6a*

or a salt thereof, to the compound of formula 7* or a salt thereof,

wherein R² is a (C₁-C₆)alkyl.

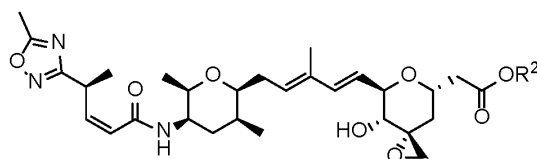
In one embodiment the compound of formula 6a* is a compound of formula 6*:



6*

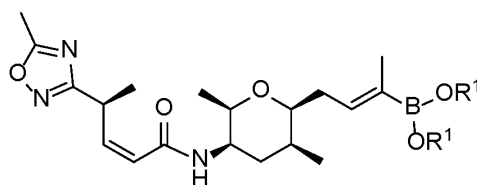
or a salt thereof.

One embodiment provides a method for preparing a compound of formula 6a*:



6a*

- 5 or a salt thereof, comprising converting a corresponding compound of formula 5a*:



5a*

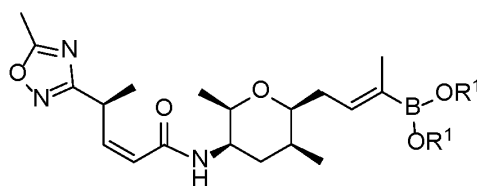
or a salt thereof, to the compound of formula 6a* or a salt thereof,

wherein:

- each R¹ is independently a (C₁-C₆)alkyl, or two R¹ groups together with the atoms to
 10 which they are attached form a heterocyclyl optionally substituted with one or more (C₁-C₆)alkyl; and

R² is a (C₁-C₆)alkyl.

One embodiment provides a method, wherein the compound of formula 6a* or a salt thereof is prepared, comprising converting a corresponding compound of formula 5a*:

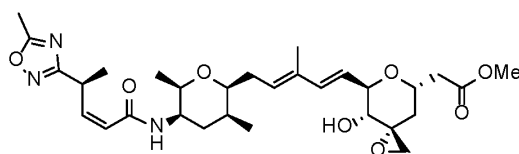


5a*

or a salt thereof, to the compound of formula 6a* or a salt thereof,

wherein each R¹ is independently a (C₁-C₆)alkyl, or two R¹ groups together with the atoms to which they are attached form a heterocyclyl optionally substituted with one or more (C₁-C₆)alkyl.

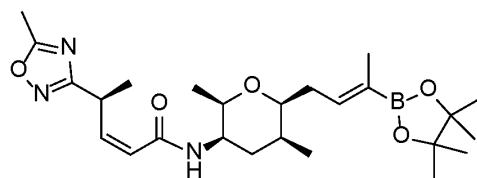
In one embodiment the compound of formula 6a* is a compound of formula 6*:



6*

or a salt thereof.

In one embodiment the the compound of formula 5a* is a compound of formula 5*:

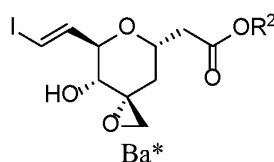


5*

10

or a salt thereof.

One embodiment provides a method, wherein the compound of formula 6a* is prepared, comprising reacting the compound of formula 5a* or a salt thereof, with a compound of formula Ba:



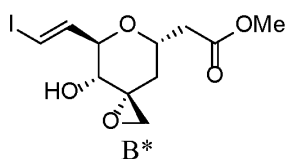
Ba*

15

or a salt thereof, to provide the compound of formula 6a*,

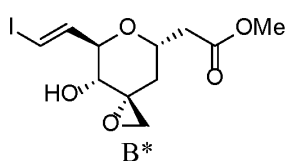
wherein R^2 is a (C₁-C₆)alkyl.

In one embodiment the compound of formula Ba* is a compound of formula B*:



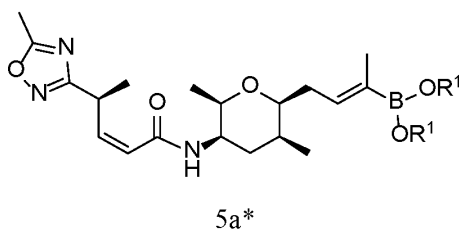
or a salt thereof.

- 5 One embodiment provides a method, wherein the compound of formula 6* is prepared, comprising reacting the compound of formula 5a* or a salt thereof, with a compound of formula B*:

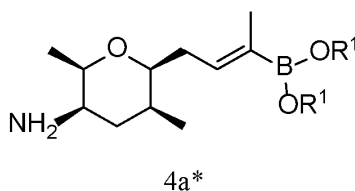


or a salt thereof, to provide the compound of formula 6*.

- 10 One embodiment provides a method for preparing a compound of formula 5a*:

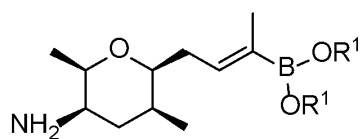


or a salt thereof, comprising converting a corresponding compound of formula 4a*:



- 15 or a salt thereof, to the compound of formula 5a or a salt thereof, wherein each R^1 is independently a (C₁-C₆)alkyl, or two R^1 groups together with the atoms to which they are attached form a heterocyclyl optionally substituted with one or more (C₁-C₆)alkyl.

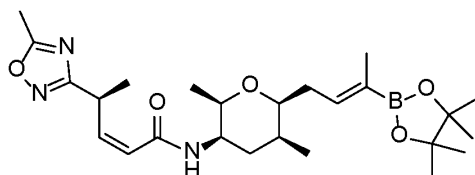
- 20 One embodiment provides a method, wherein the compound of formula 5a* or a salt thereof is prepared, comprising converting a corresponding compound of formula 4a*:



4a*

or a salt thereof, to the compound of formula 5a* or a salt thereof.

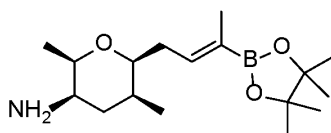
In one embodiment the compound of formula 5a* is a compound of formula 5*:



5*

5

or a salt thereof, and the compound of formula 4a* is a compound of formula 4*:

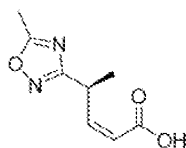


4*

or a salt thereof.

One embodiment provides a method, wherein the compound of formula 5a* is prepared, comprising reacting the compound of formula 4a* or salt thereof with a compound of formula

10

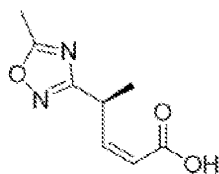


A*

or salt thereof, to provide the compound of formula 5a* or a salt thereof.

15

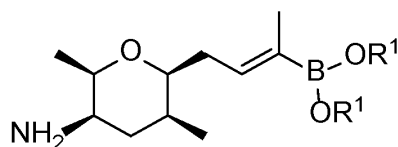
One embodiment provides a method for preparing a compound of formula 5*, comprising reacting the compound of formula 4* or salt thereof, with a compound of formula A*:



A*

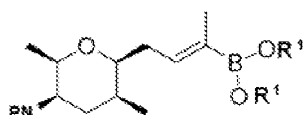
or salt thereof, to provide the compound of formula 5* or a salt thereof.

One embodiment provides a method for preparing a compound of formula 4a*:



4a*

or a salt thereof, comprising converting a corresponding compound of formula 3a*:



3a*

or a salt thereof, to the compound of formula 4a* or a salt thereof,

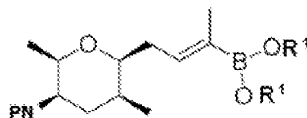
wherein:

P is an amine nitrogen protecting group; and

each R¹ is independently a (C₁-C₆)alkyl, or two R¹ groups together with the atoms to which they are attached form a heterocyclyl optionally substituted with one or more (C₁-

C₆)alkyl.

One embodiment provides a method, wherein the compound of formula 4a* or a salt thereof is prepared, comprising converting a corresponding compound of formula 3a*:

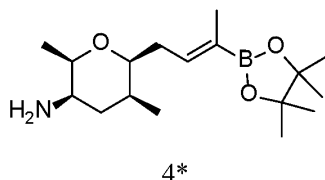


3a*

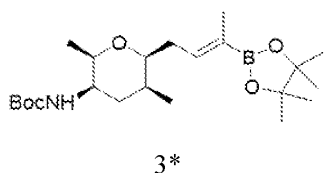
or a salt thereof, to the compound of formula 4a* or a salt thereof,

wherein each R^1 is independently a (C₁-C₆)alkyl, or two R^1 groups together with the atoms to which they are attached form a heterocyclyl optionally substituted with one or more (C₁-C₆)alkyl.

In one embodiment the compound of formula 4a* or a salt thereof is a compound of
 5 formula 4*:



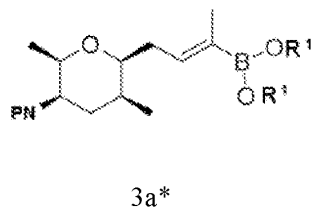
or a salt thereof, and the compound of formula 3a* or a salt thereof is a compound of formula
 3*:



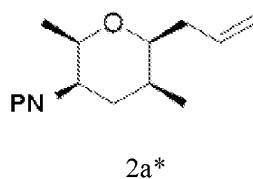
10

or a salt thereof.

One embodiment provides a method for preparing a compound of formula 3a*:



15 or a salt thereof, comprising converting a corresponding compound of formula 2a*:

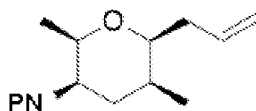


or a salt thereof, to the compound of formula 3a* or a salt thereof,
 wherein:

20 P is an amine nitrogen protecting group; and

each R¹ is independently a (C₁-C₆)alkyl or two R¹ groups together with the atoms to which they are attached form a heterocyclyl optionally substituted with one or more (C₁-C₆)alkyl.

One embodiment provides a method, wherein the compound of formula 3a* or a salt thereof is prepared, comprising converting a corresponding compound of formula 2a*:

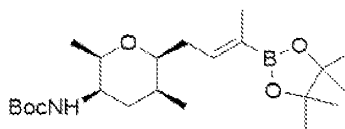


2a*

or a salt thereof, to the compound of formula 3a* or a salt thereof,

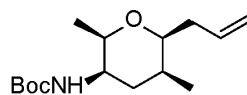
wherein P is an amine nitrogen protecting group.

In one embodiment the compound of formula 3a* or a salt thereof, is a compound of formula 3*:



3

or a salt thereof, and the compound of formula 2a* is a compound of formula 2:



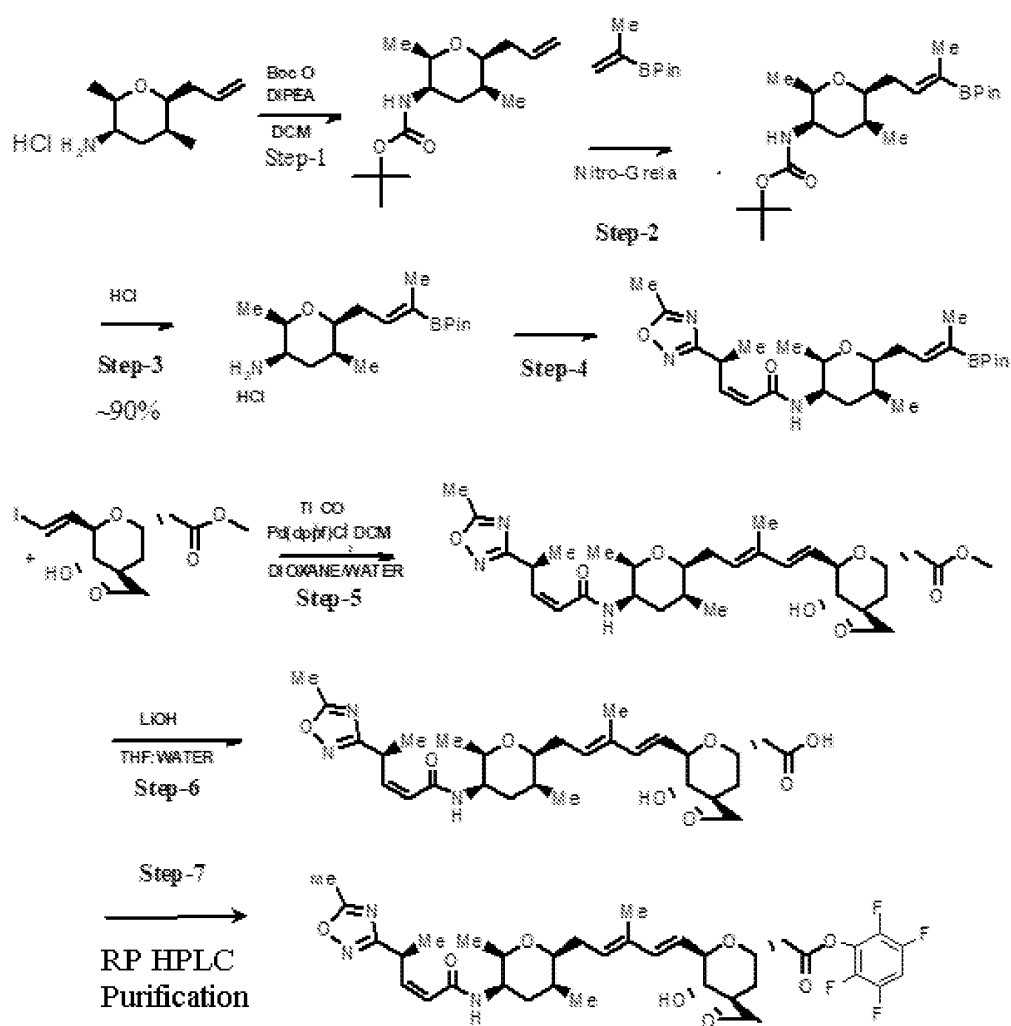
2*

or a salt thereof.

It is to be understood that 2 or more of the above described embodiments may be combined.

A compound described herein or a salt thereof can be prepared as illustrated in Scheme 1 or Scheme 2.

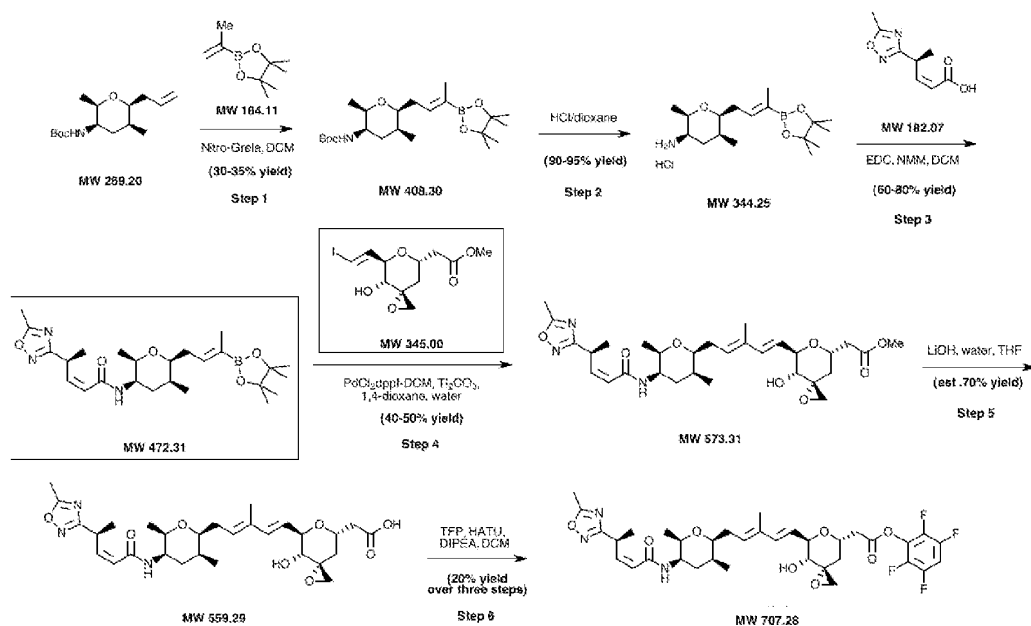
Scheme 1



5

10

Scheme 2



PHARMACEUTICAL FORMULATIONS

- 5 Pharmaceutical formulations of therapeutic antibody-drug conjugates (ADC) of the invention are typically prepared for parenteral administration, i.e. bolus, intravenous, intratumor injection with a pharmaceutically acceptable parenteral vehicle and in a unit dosage injectable form. An antibody-drug conjugate (ADC) having the desired degree of purity is optionally mixed with pharmaceutically acceptable diluents, carriers, excipients or
- 10 stabilizers (Remington's Pharmaceutical Sciences (1980) 16th edition, Osol, A. Ed.), in the form of a lyophilized formulation or an aqueous solution.

ANTIBODY-DRUG CONJUGATE METHODS OF TREATMENT

- It is contemplated that the antibody-drug conjugates (ADC) of the present invention
- 15 may be used to treat various diseases or disorders, e.g. characterized by the overexpression of a tumor antigen. Exemplary conditions or hyperproliferative disorders include benign or malignant solid tumors and hematological disorders such as leukemia and lymphoid malignancies.

- The antibody-drug conjugates (ADC) of the invention may be administered by any
- 20 route appropriate to the condition to be treated. The ADC will typically be administered

parenterally, i.e. infusion, subcutaneous, intramuscular, intravenous, intradermal, intrathecal and epidural.

Generally, the disease or disorder to be treated is a hyperproliferative disease such as cancer. Examples of cancer to be treated herein include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include squamous cell cancer (e.g. epithelial squamous cell cancer), lung cancer including small-cell lung cancer, non-small cell lung cancer, 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.

Certain embodiments of the invention provide a method for treating cancer in a patient in need thereof, comprising administering a therapeutically effective amount of an antibody described herein (e.g., anti-Trop2 antibody), or the pharmaceutical composition comprising the antibody, to the patient in need thereof. In certain embodiments, the antibody is conjugated to payload drug. In certain embodiments, the antibody is a naked antibody or fragment thereof (that is not conjugated to payload drug). In certain embodiments, the pharmaceutical composition comprises the naked antibody and does not comprise antibody conjugated with payload drug. In certain embodiments, the antibody may modulate (e.g., reduce) the target antigen (e.g., Trop2) level on the cell membrane surface, for example, the antibody may induce internalization of the target antigen. In certain embodiments, the antibody may modulate (e.g., reduce) the target antigen mediated signaling, for example, the antibody may block the binding between the target antigen (e.g., Trop2) and its endogenous ligand(s). In certain embodiments, the antibody may modulate (e.g., enhance) ADCC and/or CDC activity towards the cell expressing the target antigen.

Certain embodiments of the invention provide a method for treating cancer in a patient in need thereof, comprising administering a therapeutically effective amount of an antibody drug conjugate compound described herein, or the pharmaceutical composition comprising the ADC, to the patient in need thereof.

In certain embodiments, the cancer is selected from lung cancer (e.g., non-small cell lung cancer), pancreatic cancer, esophageal cancer, gastric cancer, bladder cancer, ovarian cancer, breast cancer, uterine cancer, and head and neck cancer.

In certain embodiments, the cancer is selected from lung cancer (e.g., non-small cell lung cancer, lung adenocarcinoma, and large cell lung carcinoma), pancreatic cancer, esophageal cancer, gastric cancer, bladder cancer, ovarian cancer, breast cancer, and uterine cancer.

In certain embodiments, the patient has Trop2 positive tumor cells.

In certain embodiments, the patient has tumor cells comprising one or more mutation that is a loss-of-function mutation in a tumor suppressor gene, and/or a gain-of-function mutation in an oncogenic driver gene and/or amplification of an oncogenic driver gene.

In certain embodiments, the patient has tumor cell(s) that comprises one or more genetic aberration or mutation as listed in Table B3.

In certain embodiments, the patient has tumor cell(s) that comprises one or more mutation in a gene selected from the group consisting of KRAS, BRAF, EGFR, ERBB2, ERBB3, APC, CNGA2, ARID1A, SLC34A2, ROS1, FGFR3, TACC3, BAIAP2L1, PIK3CA, PIK3R1, BRCA2, PTEN, STK11, CDKN2A, SMAD4, TP53, UGT2B17, SMARCA2, and SMARCA4.

In certain embodiments, the patient has tumor cell(s) that comprises one or more mutation in a gene selected from the group consisting of ATM, NF1, AKT1/2, CLPTM1L, and PVT1, NSD1 and MRE11A.

In certain embodiments, the one or more mutation comprises a point mutation.

In certain embodiments, the one or more mutation comprises a mutation selected from the group consisting of KRas G12V, BRAF G466V, EGFR L861Q, PIK3CA E545K, APC R1450*, PIK3R1 R386fs, PIK3R1 R639ter, TP53 V218, ARID1A L649fs/R693ter and SMARCA4 G1162C.

In certain embodiments, the one or more mutation comprises a gain-of-function mutation.

In certain embodiments, the patient has tumor cell that comprises one or more mutation in a gene selected from the group consisting of KRAS, BRAF, EGFR, SLC34A2, ROS1, FGFR3, TACC3, BAIAP2L1, PIK3CA, PIK3R1, and BRCA2.

In certain embodiments, the one or more mutation comprises a loss-of-function mutation.

In certain embodiments, the subject (e.g., patient) has tumor cell that comprises one or more mutation in a gene selected from the group consisting of PTEN, STK11, CDKN2A, SMAD4, and TP53.

5 In certain embodiments, the one or more mutation comprises fusion mutation of two genes.

In certain embodiments, the one or more mutation comprise fusion mutation selected from the group consisting of SLC34A2-ROS1, FGFR3-TACC3, and FGFR3-BAIAP2L1.

10 In certain embodiments, the one or more mutation comprises a BRCA-deficient mutation, FGFR3 gain-of-function mutation, TP53 loss-of-function mutation, or SMARCA4 loss-of-function mutation.

In certain embodiments, the one or more mutation is copy number variations (CNV).

In certain embodiments, one or more copy number variations (CNV) comprises a partial or total loss of PTEN, STK11, CDKN2A, CNGA2, SMAD4, UGT2B17; or gain/amplification of ERBB2, ERBB3, PIK3CA.

15 In certain embodiments, the patient has HPV positive tumor cells. In certain embodiments, the patient has HPV39 positive tumor cells.

In certain embodiments, the patient is unresponsive to standard-of-care (SOC) therapy. The term “standard-of-care” or “SOC” refers to treatment that is accepted by medical experts as a proper treatment for a certain type of disease (e.g., cancer) and that is
20 widely used by medical experts in the relevant field as likely best practice, and/or standard therapy.

In certain embodiments, the patient (e.g., having tumor cells) unresponsive to standard-of-care may show resistance to MEK or BET inhibitor therapy (e.g., in Lung adenocarcinoma), anti-EGFR therapy (e.g., in NSCLC), or multi-kinase inhibitor therapies,
25 such as Sorafenib or AZ628 inhibitors (e.g., in Gastric cancer).

For the prevention or treatment of disease, the appropriate dosage of an ADC will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the molecule is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion
30 of the attending physician. The molecule is suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease, about 1 µg/kg to 15 mg/kg (e.g. 0.1-20 mg/kg) of molecule is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by

continuous infusion. A typical daily dosage might range from about 1 µg/kg to 100 mg/kg or more, depending on the factors mentioned above. An exemplary dosage of ADC to be administered to a patient is in the range of about 0.1 to about 10 mg/kg of patient weight.

Antibodies or immunoconjugates of the invention can be used either alone or in
5 combination with other agents in a therapy. For instance, an antibody or immunoconjugate of the invention may be co-administered with at least one additional therapeutic agent.

Such combination therapies noted above encompass combined administration (where two or more therapeutic agents are included in the same or separate formulations), and separate administration, in which case, administration of the antibody or immunoconjugate of
10 the invention can occur prior to, simultaneously, and/or following, administration of the additional therapeutic agent and/or adjuvant. Antibodies or immunoconjugates of the invention can also be used in combination with radiation therapy.

An antibody or immunoconjugate of the invention (and any additional therapeutic agent) can be administered by any suitable means, including parenteral, intrapulmonary, and
15 intranasal, and, if desired for local treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. Dosing can be by any suitable route, e.g. by injections, such as intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic. Various dosing schedules including but not limited to single or multiple administrations over
20 various time-points, bolus administration, and pulse infusion are contemplated herein.

Antibodies or immunoconjugates of the invention would be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of
25 the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The antibody or immunoconjugate need not be, but is optionally formulated with one or more agents currently used to prevent or treat the disorder in question. The effective amount of such other agents depends on the amount of antibody or immunoconjugate present in the formulation, the type of disorder or treatment, and other
30 factors discussed above. These are generally used in the same dosages and with administration routes as described herein, or about from 1 to 99% of the dosages described herein, or in any dosage and by any route that is empirically/clinically determined to be appropriate.

For the prevention or treatment of disease, the appropriate dosage of an antibody or immunoconjugate of the invention (when used alone or in combination with one or more other additional therapeutic agents) will depend on the type of disease to be treated, the type of antibody or immunoconjugate, the severity and course of the disease, whether the antibody or immunoconjugate is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody or immunoconjugate, and the discretion of the attending physician. The antibody or immunoconjugate is suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease, about 1 µg/kg to 15 mg/kg (e.g. 0.1mg/kg-10mg/kg) of antibody or immunoconjugate can be an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. One typical daily dosage might range from about 1 µg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment would generally be sustained until a desired suppression of disease symptoms occurs. One exemplary dosage of the antibody or immunoconjugate would be in the range from about 0.05 mg/kg to about 10 mg/kg. Thus, one or more doses of about 0.5 mg/kg, 2.0 mg/kg, 4.0 mg/kg or 10 mg/kg (or any combination thereof) may be administered to the patient. Such doses may be administered intermittently, e.g. every week or every three weeks (e.g. such that the patient receives from about two to about twenty, or e.g. about six doses of the antibody). An initial higher loading dose, followed by one or more lower doses may be administered. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

ARTICLES OF MANUFACTURE

In another embodiment of the invention, an article of manufacture, or “kit”, containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, blister pack, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds an antibody-drug conjugate (ADC) composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an ADC. The

label or package insert indicates that the composition is used for treating the condition of choice, such as cancer. Alternatively, or additionally, the article of manufacture may further comprise a second (or third) container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

EXAMPLES

Certain exemplary embodiments of anti-Trop2 antibody drug conjugate invention described herein may be referred to as Trop2 PH1 ADCs or PH Trop2 ADCs, wherein a new payload (e.g., PH1 or compound 3A) is conjugated to a Peak Trop2 antibody, also referred to as PH antibody (e.g., M2.8 mAb). For example, certain embodiments of an exemplary M2.8 mAb conjugated with PH1 payload is referred to as M2.8 PH1 in Example 4 or M2.8 L22 ADCs in Example 12. The Thailanstatin (PH1) payload is also referred to as PH1-3, or A13d2Th.

Certain exemplary embodiments of anti-Trop2 antibody drug conjugate invention described herein may comprise a new payload (e.g., Thailanstatin (PH1)) described herein that is conjugated to a known antibody. The known antibody may be hRS7 (also referred to as Sacituzumab, namely the antibody unit of IMMU-132). The known antibody may be TINA (also referred to as DS1062a or Datopotamab, namely the antibody unit of DS-1062). The known antibody may be 2G10 (namely the antibody unit of LCB84). The known antibody may be T6-16.

Example 1. Trop2 ADC linker screening.

The efficacy of various linker-toxins as ADC were evaluated in this Example. The antibody is the anti-TROP2 hRS7 (Sacituzumab, the antibody of IMMU-132) and the toxin being evaluated is Thailanstatin (PH1, also referred to as PH1-3, or A13d2Th). L2 (NHS) and L22 (TFP) are conjugated via noncleavable lysine and may release the same catabolite intracellularly after the ADC is digested within the cell. L92 (Val-Ala TFP) is conjugated via a cleavable lysine and may release a different catabolite. L18 is conjugated via non-cleavable cysteine and may release a different catabolite from L2/22.

This Example evaluates the efficacy of linker toxin combinations on N87 gastric carcinoma that expresses high levels of Trop2 and Her2 and is therefore susceptible to Trop2-ADC or Her2-ADC inhibition. Athymic mice bearing pre-implanted N87 tumors were i.v. treated with 3 mg/kg of various linker toxin ADCs and efficacy read as a function of tumor volume over time. DAR 4/8 versions of Trop2 PH1 ADC were included in the experiment. At 3 mg/kg, anti-Trop2 ADCs were the most efficacious when conjugated on random lysines with non-cleavable linkers L-2 (77% TGI) and L-22 (82% TGI). The slightly higher activity of L-22 (DAR 4.5) linker over L-2 (DAR 3.65) Trop2 PH1 ADC may be due to higher DAR during conjugation, see Figure 1.

Using a L-2 linker, Trop2 PH1 ADCs were more efficacious as a DAR8 vs a DAR4 species (88% vs 77% TGI). While the DAR8 ADC may be more efficacious than the DAR4 species in such model of this Example, one needs also balance the stability and toxicity of the former for therapeutic index considerations.

At the same dose, the activity of above anti-Trop2 ADCs were comparable to Kadcylla, which targets Her2-expressing cells and is an approved drug for therapy of Gastric Carcinoma.

In comparison, anti-Trop2 ADCs made by coupling the same toxin to random lysines using a cleavable linker L-92 or to cysteine using a non-cleavable linker L-18 showed lower efficacy of about 68% and 65% TGI, respectively. Overall, in this Example, the lysine conjugates with non-cleavable linker have highest potency.

Example 2. Antibody binding profile and binding affinity for Trop2

This Example relates to the characterization and analysis of anti-Trop2 antibodies as naked mAbs. In particular, binding affinity/binding profile were also compared with mAbs TINA and hRS7, respectively.

Binding affinity and binding profiles were determined by Biolayer Interferometry (BLI). Binding assays were performed on Octet Red instrument at 25°C. Antibodies were loaded onto anti-human IgG Fc capture biosensors for 300 seconds. Ligand-loaded sensors were dipped into a 3-fold series dilution (starting at 300 nM in 0.1% BSA, 1X PBS, 0.02% Tween20, and 0.05% NaN₃) of Trop2 for 200 seconds followed by dissociation for 400 seconds. Kinetic constants were calculated using a monovalent (1:1) binding model. PH antibodies (e.g., M2.1, M2.2., M2.8) and mAbs TINA and hRS7 were produced from CHO cells for comparison in binding affinity towards Trop2. Binding analysis of select PH

Example 4. Efficient conjugation of linker toxin to Trop2 mAb and characterization of ADCs (DAR2 or DAR4 versions)

Conjugation of PH1 to PH antibody (e.g., M2.8)

- 5 M2.8 antibody was buffer exchanged to 50 mM borate/50 mM NaCl pH 8.5 buffer (BB) in Amicon concentrators (30 kDa, regenerated cellulose). Antibody in BB was carefully adjusted to 10 mg/mL with additional BB then cooled on ice until 4°C. PH1L2 was added at varying molar excesses of 2.2, 2.6, 4.2, or 4.8 to the antibody and quickly mixed in. The reaction was kept overnight at 4°C to ensure the conjugation reaction went to completion.
- 10 Conjugated M2.8 PH1 was harvested, and buffer exchanged into phosphate buffered saline (PBS) with the above-mentioned method and finally filtered through a 0.22 µm PES filter. DAR analysis of conjugates revealed molar excesses of 2.2, 2.6, 4.2, and 4.8 gave rise to experimental DARs of 1.6, 2.0, 3.3 and 3.6, respectively. The successful molar excesses of experimental DARs in smaller batch conjugations were used to drive conjugations for larger
- 15 batches. Plus/ minus 0.5 of the target DAR was considered acceptable. For example, an experimental DAR of 3.5-4.5 was released for testing as a DAR4 ADC. At 10-25mg scale, molar excesses of 5, 6 and 8 PH1 were used to drive conjugations in order to achieve a DAR of 4.

- 20 High-performance liquid chromatography

ADCs diluted in PBS were run on a size exclusion column (SEC) with the Agilent 1100 HPLC machine. Readouts taken at 280 nm with the diode-array detection were used to identify the protein and if there are aggregates. Similarly, ADCs were run on a hydrophobic interaction column (HIC) HPLC.

25

Mass spectrometry

ADCs were characterized with electrospray ionization liquid chromatography mass spectrometry.

Results of M2.8 PH1 (DAR2 or DAR4 versions) characterization

- 30 Upon conjugation of the M2.8 antibody with PH1 payload, M2.8 PH1 was characterized on both HIC (Figure 6, A and D) and by SEC (Figure 6, B and E). Figure 6A and 6D (DAR2 or DAR4 versions) show the analytes eluting out around the 2.2-minute time point with subsequently multiple peaks afterwards forming a single large peak, indicating a heterogenous ADC population conjugated at different lysines with an increasing number of

PH1 payloads. Given the closeness of various populations of PH1 on M2.8, mass spectrometry (MS) was used to clarify the exact average drug-to-antibody ratio (DAR) of M2.8 PH1 population. ESI-MS provided the breakdown in distribution of each DAR population (Figure 6C or Figure 6F, for DAR2 or DAR4 versions), where a range from 0 to 4 DAR (DAR 2) or 1 to 7 DAR (DAR 4) was observed. The average DAR of each population of DAR2 version or DAR4 version is about 1.7 or 3.6, respectively. Thus, M2.8 PH1 ADCs can be controlled to yield minimal aggregation (<1.5% as measured by SEC-HPLC) and reproducibly yield a DAR of 1.5-2 or 3.5-4.0 (by LC-MS). The conjugation efficiency of M2.8 with PH1 can be verified reliably by ESI-MS.

Example 5. Cellular internalization of Trop2 mABs and ADCs

Internalization assay was performed with Trop2 mAbs: hRS7, M2.1, M2.3, T6-16, TINA. Control Ab used was HuLys 11 targeting chicken lysozyme. hRS7 and TINA appear to internalize more rapidly, but T6-16 shows the highest internalization percentage (Figure 7). M2.1 and M2.8 ADCs showed similar internalization (accumulation) rates in cells (Figure 8).

Method

Percent internalization was calculated based on fraction internalized at time x minus fraction internalized at start, and then multiplied by 100 using formula: $\frac{[V(tx)/M(tx)] - [V(t0)/M(t0)]}{[V(t0)/M(t0)]} \times 100$, where V and M are average Geometric Mean Fluorescence Intensity signals (GMFI) of Alexa 488- conjugated mAbs or ADCs bound to antigen-expressing cells; and quenching photo-bleaches the fluorescent-labeled antigen-antibody complexes on the cell surface leaving signal available from only the internalized complexes. V(t0) and M(t0) represent the amount of pulse-labeled receptor-mAb complexes at start time t0, at the beginning of the chase, that were quenched and unquenched, respectively. V(tx) and M(tx) represent corresponding quenched and unquenched fluorescence values from mAb-labeled receptors initiated in parallel at time t0, but quenched after a period x, tx, respectively. The GMFIs of V(tx) and M(tx) represent amounts of quenched and unquenched complexes at the end of the chase periods. Samples were quenched at time intervals of x=0.5, 3 and 6 hours

Cells were trypsinized and plated the night before (50-100k cells/well). Plates, media, and 1X PBS were pre-chilled on ice for 15 mins. Cells were washed 1x with cold PBS followed by aspiration of PBS. Abs were diluted to 1.5 ug/ml in pre-chilled indicator free media. Diluted primary Abs were transferred to wells at 200 ul/well. Cells were incubated for

30 min on ice. Cells were washed 1x with cold PBS followed by aspiration of PBS (step repeated). Quenching antibody was diluted as described above in pre-chilled indicator free media.

For T0 plate, quenching antibody was transferred at 200 ul/well to time point 0 plate.

- 5 Cells were incubated for 30 min on ice. Cells were washed 1x with cold PBS followed by aspiration of PBS. Cells were trypsinized and transferred to V-bottom polystyrene plate. After spinning 5 min at 1200 rpm, buffer was aspirated, and cell pellet was retained. Cells were washed 1x with cold PBS followed by spinning 5 min at 1200 rpm. Cell pellet was re-suspended in 200 ul indicator free media. Cell samples were read using the Guava Easycyte
- 10 Plus HT flow cytometer and data were analyzed using the Guava Cytosoft software suite.

- For all other time point plates, 200 uL of prewarmed media was added to wells of all remaining plates, which were placed in 37°C incubator for 0.5hr, 3hrs or 6hrs. Plate was removed from incubator and excess of prechilled PBS was added. Plate was placed on ice for 15 mins. Buffer was aspirated. Quenching antibody was transferred to each plate at 200
- 15 ul/well. Plate was incubated on ice for 30 min. Cells were washed 1x with cold PBS and then buffer was aspirated. Cells were trypsinized and transferred to V-bottom polystyrene plate. After spinning 5 min at 1200 rpm, buffer was aspirated, and cell pellet was retained. Cells were washed 1x with cold PBS followed by spinning 5 min at 1200 rpm. Cell pellet was re-suspended in 200 ul indicator free media. Cell samples were read using the Guava Easycyte
- 20 Plus HT flow cytometer and data were analyzed using the Guava Cytosoft software suite.

Example 6. Payload superiority of PH1 (when conjugated on IMMU-132 antibody hRS7) over SN-38 on IMMU-132 antibody

- 25 To determine whether PH Trop2 ADCs with Thailanstatin payloads have comparable (or better) activity than best-in-class anti-Trop2 ADC, IMMU-132 (SN-38 conjugated on hRS7 at DAR8), using the same hRS7 antibody but different payloads (PH1 vs SN-38), the tumor inhibition efficacy of IMMU-132 at its efficacious dose of 10mpk QW x2 was compared with the efficacy of PH1 payload ADC at lower dose (1mpk QW x3 or 3mpk QW
- 30 x2) and lower DAR (DAR4). The number of mice with regressed tumors within the cohort is represented as a fraction of the total.

Results

PH1 demonstrated significant TGI as payload on hRS7 mAb. The hRS7 mAb, that is part of first-in-class IMMU-132 (Sacituzumab Govitecan), demonstrated significantly better anti-tumor efficacy with a PH1 payload at DAR4 than the clinically approved SN38 conjugate at DAR8 (Figure 9), suggesting that PH1 payload ADC may provide superior efficacy at lower dose and/or lower DAR as compared to SN-38.

Conclusion

Using the same hRS7 mAb, ADC conjugated with PH1 payload demonstrated significantly better activity than SN38-containing IMMU-132, even though the IMMU-132 was conjugated to SN38 at DAR8 and PH1 was conjugated at DAR 4. When administered at 3mpk QWx2, ADC with PH1 payload exhibited greater TGI than IMMU-132 at 10mpk QWx2. ADC with PH1 payload also performed better in suboptimal dose of 1mpk as compared to IMMU-132 at 1mpk.

Method

N87 gastric carcinoma cells express high levels of endogenous Trop2 protein. IMMU-132 is currently the first-in-class (FIC) Trop2 ADC using the mAb hRS7 conjugated to the irinotecan metabolite payload SN-38 at a drug-antibody ratio (DAR) of 8. It is a standard-of-care (SOC) for treatment of triple negative breast cancer (TNBC), and is currently being tested in the clinic for activity against gastric cancer that expresses elevated levels of Trop2. In pre-clinical gastric cancer model N87, 10mpk QWx2 was established as the efficacious dose of IMMU-132.

ADCs were tested for the treatment of subcutaneous NCI-N87 gastric Cancer Xenograft Model in female BALB/c nude Mice.

Cell Culture

The NCI-N87 tumor cell line was maintained in vitro in RPMI1640 medium supplemented with 10% fetal bovine serum at 37 °C in an atmosphere of 5% CO₂ in air. The tumor cells were routinely sub-cultured twice weekly by trypsin-EDTA treatment. The cells growing in an exponential growth phase were harvested and counted for tumor inoculation.

Tumor Inoculation

Each mouse was inoculated subcutaneously in the right flank region with tumor cells (1×10^7) in 0.1 ml of PBS mixed with Matrigel (1:1) for tumor development.

Randomization

5 The randomization was started when the mean tumor size reached approximately 195.81 mm³. All animals were randomly allocated to study groups. Randomization was performed based on “Matched distribution” method (StudyDirector™ software, version 3.1.399.19).

Observation and Data Collection

10 After tumor cells inoculation, the animals were checked daily for morbidity and mortality. During routine monitoring, the animals were checked for any effects of tumor growth and treatments on behavior such as mobility, food and water consumption, body weight gain/loss (Body weights were measured twice per week after randomization), eye/hair matting and any other abnormalities. Mortality and observed clinical signs were recorded for
15 individual animals in detail.

 Tumor volumes were measured twice per week after randomization in two dimensions using a caliper, and the volume was expressed in mm³ using the formula: $V = (L \times W \times W)/2$, where V is tumor volume, L is tumor length (the longest tumor dimension) and W is tumor width (the longest tumor dimension perpendicular to L). Dosing as well as tumor
20 and body weight measurements were conducted in a Laminar Flow Cabinet. The body weights and tumor volumes were measured by using StudyDirector™ software (version 3.1.399.19).

ADC administration

25 The treatment was initiated on the same day of randomization (day 7) per study design.

 Tumor growth inhibition (TGI): TGI% is an indication of antitumor activity, and expressed as: $TGI (\%) = 100 \times (1 - T/C)$. T and C are the mean tumor volume (or weight) of the treated and control groups, respectively, on a given day.

30 Statistical analysis of the difference in mean tumor volume among the groups was conducted using the methods below: Use the data collected on the last observation day for every single group in despite of diverse individual termination date.

 To compare tumor volumes of different groups at a pre-specified day, Bartlett's test was first used to check the assumption of homogeneity of variance across all groups. When

the p-value of Bartlett's test was ≥ 0.05 , a one-way ANOVA was performed to test overall equality of means across all groups. If the p-value of the one-way ANOVA was < 0.05 , a post hoc testing was performed by running Tukey's HSD (honest significant difference) tests for all pairwise comparisons, and Dunnett's tests for comparing each treatment group with the vehicle group. When the p-value of Bartlett's test was < 0.05 , a Kruskal-Wallis test was performed to test overall equality of medians among all groups. If the p-value the Kruskal-Wallis test was < 0.05 , a post hoc testing was performed by running Conover's non-parametric test for all pairwise comparisons or for comparing each treatment group with the vehicle group, both with single-step p-value adjustment.

In addition, pairwise comparisons were performed without multiple comparison correction and nominal/uncorrected p-values were reported directly from Welch's t-test or Mann-Whitney U test. Specifically, Bartlett's test was first used to check the assumption of homogeneity of variance for a pair of groups. When the p-value of Bartlett's test was ≥ 0.05 , a Welch's t-test was performed, otherwise a Mann-Whitney U test was performed to obtain nominal p-values.

All statistical analyses had been done in R-a language and environment for statistical computing and graphics (version 3.3.1). All tests were two-sided unless otherwise specified, and p-values of < 0.05 were regarded as statistically significant.

Example 7. superiority of Trop-2 PH1 ADC (M2.1) over IMMU-132

To determine whether PH Trop2 ADC (M2.1 mAb) with Thailanstatin (PH1) payload have comparable (or better) activity than best-in-class anti-Trop2 ADC, IMMU-132, the tumor inhibition efficacy of IMMU-132 at its efficacious dose of 10mpk QW x2 was compared with the efficacy of PH ADC of M2.1 mAb conjugated with PH1 payload at lower dose (3mpk QW x2) and lower DAR (DAR2 or DAR4). Suboptimal dose at 1mpk QW x3 comparison was also investigated. The number of mice with regressed tumors within the cohort is represented as a fraction of the total.

Method

N87 gastric carcinoma cells express high levels of endogenous Trop2 protein. IMMU-132 is currently the best-in-class (BIC) Trop2 ADC using the mAB hRS7 conjugated to the irinotecan metabolite payload SN-38 at a drug-antibody ratio (DAR) of 8. It is a standard-of-care (SOC) for treatment of triple negative breast cancer (TNBC), and is currently being

tested in the clinic for activity against gastric cancer that expresses elevated levels of Trop2. In pre-clinical gastric cancer model N87, 10mpk QWx2 was established as the efficacious dose of IMMU-132.

5 Results

When durability of the TGI was assessed, PH Trop2 ADC (mAb M2.1 conjugated with PH1 payload) performed significantly better than IMMU-132 at preventing tumor growth. The results support the use of M2.1 PH Trop2 mAb Thailanstatin ADC at DAR2 or DAR4 for further assessment as a candidate. PH1 payload demonstrated significantly
10 improved efficacy on PH mAb (M2.1) as compared to the SN38-payload containing IMMU-132 (Figure 10).

Conclusion

PH Trop2 ADCs conjugated with PH1 payload demonstrated significantly better
15 activity than SN38-containing IMMU-132, even though IMMU-132 was conjugated at DAR8 while PH Trop2 ADCs were conjugated at DARs 4 and 2. When administered at 3mpk QWx2, PH Trop2 ADCs exhibited greater TGIs than IMMU-132 at 10mpk QWx2.

When PH Trop2 ADCs conjugated with PH1 payload were compared at 1mpk QWx3 dose in a dose-matched suboptimal setting, efficacy of PH1 payload conjugated Trop2 ADCs
20 was higher than IMMU-132, which is largely inactive at this dose, whereas PH1-Trop2 ADCs (M2.1 conjugated with PH1 at DAR2 or DAR4) have about 45-70% TGI. At suboptimal dose, M2.1 ADCs exhibited better efficacy at DAR4 vs DAR2. However, when dosed sufficiently high such as 3mpk QWx2, there was no significant difference in early anti-tumor efficacies or tumor re-growth properties between M2.1 DAR4 vs DAR2
25 ADCs.

Example 8. Trop2 ADCs with PH1 payload regress tumors

The same model was used as described in Examples 6-7. PH mAbs M2.1 and M2.3
30 were conjugated with PH1 payload at DAR 4 or DAR 2. Anti-Trop2 mAbs T6-16 and hRS7 were conjugated with PH1 payload at DAR4. The number of mice with regressed tumors within the cohort is represented as a fraction of the total. Cohorts with differences in tumor volume significant at $p < 0.05$ with the isotype control anti-RSV PH1 ADC is indicated.

All tested Trop2 ADCs with PH1 payload regress individual tumors at 3 mg/kg (Figure 11). All anti-Trop2 ADCs conjugated with PH1 payload demonstrated significantly improved efficacy than SN38-containing IMMU-132, even though IMMU-132 was conjugated at higher DAR 8 and was administered at higher dose of 10mpk.

5

Example 9. Superiority of Trop2 ADCs with PH1 payload over IMMU-132 in suboptimal dose

10 The same model was used as described in Examples 6-7. PH mAbs M2.1 and M2.3 were conjugated with PH1 payload at DAR 4 or DAR 2. Anti-Trop2 mAbs TINA, T6-16 and hRS7 were conjugated with PH1 payload at DAR4.

When all Trop2 ADCs with PH1 were compared at 1mpk QWx3 dose in a dose-matched suboptimal setting, efficacy of all PH1 payload conjugated Trop2 ADCs was higher. 15 IMMU-132 at this dose is largely inactive, whereas all PH1-Trop2 ADCs have 45-70% TGI (Figure 12). M2.1 PH1 showed higher TGI than M2.3 PH1. For M2.1 PH1 and M2.3 PH1, DAR4 showed higher TGI than DAR2.

Example 10. Trop2 PH1 ADC (M2.8 PH1) is a potent inhibitor of cancers of multiple origins. 20

To evaluate ADC cytotoxicity against multiple cancer indications, an unbiased cytotoxicity screen against a panel of 70 cell lines was performed (Table B1). Trop2 PH1 ADC activity was observed in 30 cell lines representing 9 indications (Table B2). Of these, IC50s of 27 cell lines were lower than 100nM. Single digit nanomolar inhibition was observed in 19 cell lines 25 from 8 indications. A representative list of 25 cell lines with single digit or double digit IC50s is shown in Figure 13 (also see Figures 14A-14C for representative assay using NCI-N87 cells, BxPC3 cells, or RT112/84 cells).

On the other hand, Trop2 PH1 ADC shows lower off-target toxicity towards Trop2 negative cells as compared to IMMU-132 (see Figures 14D-14F for assay using 786-0 cells, 30 BJ Normal human fibroblast cells, or HS27 Normal human fibroblast cells). Furthermore, Trop2 PH1 ADC efficacy was observed in cancers with oncogenic drivers, such as KRas G12V, BRAF G466V (sorafenib resistance), EGFR L861Q, SLC34A2-ROS1 fusion, FGFR3 GOF or FGFR3 fusions (both FGFR3-TACC3 and FGFR3-BAIAP2L1 fusions), PI-

3K signaling mutations (PIK3CA E545K and PIK3R1 R386fs/R639ter), BRCA2-deficient (familial), and HPV39-positive (Viral) cancers.

In addition, Trop2 PH1 ADC efficacy was observed in cancers with loss of function of tumor suppressors, such as PTEN, STK11, CDKN2A, SMAD4, TP53 LOF or TP53 V218
5 mutation. Trop2 PH1 ADC efficacy was observed in cancers with UGT2B17 loss of function. Trop2 PH1 ADC efficacy was also observed in cancers with compensatory activation due to loss of SMARCA2, SMARCA4 G1162C or LOF.

Methods

10 The objective of this Example is to explore the potential effect of ADC on cell viability of 70 cancer cell lines. The 50% inhibitory concentration (IC₅₀) were determined in cancer cell lines using CellTiter-Glo luminescent cell viability assay after incubation with different concentrations of ADC or control.

Each cell line was treated with ADC, a standard chemotherapy drug as reference
15 control and culture medium as vehicle control. A549 and Calu-6 are negative control cell lines that do not express the protein target Trop2. All remaining cell lines express some of the target protein Trop2 on RNA and/or protein levels.

All the cells were cultured in the media supplemented with 10-15% FBS, at a temperature of 37°C, 5% CO₂ and 95% humidity. CellTiter-Glo® Luminescent Cell Viability
20 Assay (Cat# G7572, Promega) was used.

Determination of the half maximal inhibition concentration IC₅₀

1. Harvest cells during the logarithmic growth period and count cell number using Count-star.
- 25 2. Adjust cell concentrations to 4.44×10^4 cells/mL with respective culture medium.
3. Add 90 µL cell suspensions to two 96-well plates (plates A and B) with the final cell density of 4×10^3 cells/well (cell concentration may be adjusted according to the data base or density optimization assay).
- 4a. Next day: For the plates of T0 reading:
30 1) Add 10 µL culture medium to each well of plate A for T0 reading.
2) Equilibrate the plate and its content at RT for approximately 30 min.
3) Add 50 µL CellTiter-Glo reagent to each well.
4) Mix content for 5 min on an orbital shaker to induce cell lysis.

- 5) Allow the plate to incubate at RT for 20 min to stabilize luminescent signal.
- 6) Record luminescence (T0) using EnVision Multi Label Reader.
- 4b. For the plates of test reading:
 - 1) Prepare 10×solution of ADC (Top working concentration: 200 nM of ADC in media with 10-fold serial dilutions to achieve 9 dose levels.
 - 2) Prepare 10×reference control solutions Cisplatin (Top working concentration: 100 μM in media with 3.16-fold serial dilutions to achieve 9 dose levels.
 - 3) Dispense 10 μL (10×) drug solution of both ADC and reference control in each well (triplicate for each drug concentration) of the plate B.
 - 4) Incubate the test plate B for 120 hours in the humidified incubator at 37°C with 5% CO₂.
 - 5) Equilibrate the plate and its content at RT for approximately 30 min.
 - 6) Add 50 μL CellTiter-Glo reagent to each well.
 - 7) Mix contents for 5 min on an orbital shaker to induce cell lysis.
 - 8) Allow the plates to incubate at RT for 20 min to stabilize luminescent signal.
 - 9) Record luminescence (T5) using EnVision Multi Label Reader.

Data Analysis

The data were displayed or analyzed using GraphPad Prism 5.0. In order to calculate absolute IC₅₀ (EC₅₀), a dose-response curve will be fitted using nonlinear regression model with a sigmoidal dose response. The formula for calculating surviving rate is shown below and the absolute IC₅₀ (EC₅₀) will be calculated according to the dose-response curve generated by GraphPad.

The surviving rate (%) = $(\text{Lum}_{\text{Test article}} - \text{Lum}_{\text{Medium control}}) / (\text{Lum}_{\text{Non-treated}} - \text{Lum}_{\text{Medium control}}) \times 100\%$.

Table B1. cytotoxicity screen against a panel of 70 cell lines

Cell Line Name	Tissue Origin
HT-1376	Bladder
RT112/84	Bladder
RT4	Bladder
SW-780	Bladder
HCC 1806	Breast
HCC 1937	Breast
HCC 1954	Breast
HCC 38	Breast
HDQ-P1	Breast
JIMT-1	Breast

KPL-4	Breast
MCF7	Breast
MX-1	Breast
KYSE-150	HN/Esophagus
KYSE-270	HN/Esophagus
KYSE-410	HN/Esophagus
T.Tn	HN/Esophagus
OE19	HN/Esophagus
C666-1	HN/Nose
FaDu	HN/Pharynx
CAL-27	HN/Tongue
OSC-19	HN/Tongue
SCC-15	HN/Tongue
SCC-4	HN/Tongue
SCC-9	HN/Tongue
COLO 205	Large intestine/Colorectum
LS 123	Large intestine/Colorectum
SNU-C5	Large intestine/Colorectum
A549	Lung
Calu-6	Lung
EBC-1	Lung
HCC 78	Lung
HCC 827	Lung
HCC 95	Lung
NCI-H1435	Lung
NCI-H1650	Lung
NCI-H1666	Lung
NCI-H1781	Lung
NCI-H1869	Lung
NCI-H2009	Lung
NCI-H2126	Lung
NCI-H2170	Lung
NCI-H292	Lung
NCI-H441	Lung
NCI-H596	Lung
PC-9	Lung
SNU-2535	Lung
Caov-3	Ovary
COV644	Ovary
OVCAR-3	Ovary
BxPC-3	Pancreas
Capan-2	Pancreas
CFPAC-1	Pancreas
HPAF-II	Pancreas
HuP-T4	Pancreas
KATO III	Stomach
MKN45	Stomach
MKN74	Stomach
NCI-N87	Stomach
OCUM-2D	Stomach
SCH	Stomach
SNU-216	Stomach
YCC-2	Stomach
Ca Ski	Uterus
HEC-265	Uterus
Ishikawa	Uterus
ME-180	Uterus
RL95-2	Uterus

DoTc2 4510	Uterus/Cervix
MS751	Uterus/Cervix

Table B2. cytotoxicity data on a representative list of 30 responsive cell lines

Cell lines	Cell line type	Trop2 PH1 IC50 (nM)
NCI-H1666	Lung 1	0.56
NCI-H292	Lung 2	1.63
NCI-H1435	Lung 3	1.88
NCI-H1650	Lung 4	3.35
NCI-H441	Lung 5	3.69
NCI-H2126	Lung 6	3.78
HCC 78	Lung 7	4.65
NCI-H1781	Lung 8	54.10
Capan-2	Pancreatic 1	1.21
BxPC-3	Pancreatic 2	1.5
HuP-T4	Pancreatic 3	7.52
HPAF-II	Pancreatic 4	86.53
CFPAC-1	Pancreatic 5	181.31
KYSE-270	Esophageal 1	1.21
NCI-N87	Gastric 1	1.32
MKN74	Gastric 2	4.03
SCH	Gastric 3	10.55
SW-780	Bladder 1	1.36
RT4	Bladder 2	1.77
RT112/84	Bladder 3	1.97
Caov-3	Ovarian 1	3.25
HCC 1954	Breast 1	7.77
JIMT-1	Breast 2	12.10
KPL-4	Breast 3	12.3
MCF7	Breast 4	191.48
RL95-2	Uterine 1	9.68
ME-180	Uterine 3	27.4
DoTc2 4510	Uterine 2	23.82
MS751	Uterine 4	41.23
SCC-4	Head & Neck 1	198.85

- 5 Table B3. Representative cell lines that are responsive to anti-Trop2 PH1 ADC and mutations of these cell lines.

No.	Cell lines	Cell line type	Genetic aberration / mutation
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1	NCI-H1666	Lung 1 (NSCLC)	Harbors G466V BRAF mutation may result in hyperactive ERK signaling. EGFR therapy- resistant. Additional efficacy due to ATM heterozygosity which causes synthetic lethality with MEK inhibitors such as trametinib
2	NCI-H292	Lung 2 (NSCLC)	No ALK, RET or ROS driver mutations. WT EGFR
3	NCI-H1435	Lung 3 (LuAd)	SMARCA4 G1162C mutations. NF1-mutant. Not responsive to MEK inhibitors or BET inhibitors.
4	NCI-H1650	Lung 4 (NSCLC)	Homozygous PTEN loss mutations- resistant to erlotinib.
5	NCI-H441	Lung 5 (LuAd)	KRASG12V mutation
6	NCI-H2126	Lung 6 (Large Cell)	SMARCA4 loss of function. Loss of STK11 tumor suppressor
7	HCC 78	Lung 7 (LuAd)	SLC34A2-ROS1 fusion receptor
8	Capan-2	Pancreatic 1	KRASG12V mutation
9	BxPC-3	Pancreatic 2	KRAS WT, P53+, CDKN2A CNV loss, SMAD4 CNV loss
10	HuP-T4	Pancreatic 3	KRASG12V mutation, P53+, CDKN2A CNV loss, SMAD4+
11	KYSE-270	Esophageal 1	SMARCA2 LOF, L861Q EGFR mutation
12	NCI-N87	Gastric 1	ERBB2 amplification, SMAD4 CNV loss, UGT2B17 loss
13	MKN74	Gastric 2	BRAF G466V (resistant to sorafenib and AZ628). APC nonsense mutation R1450*
14	SCH	Gastric 3	TP53 loss, ERBB3 amplification, FGFR3 GOF, UGT2B17 loss
15	SW-780	Bladder 1	FGFR3-BAIAP2L1 fusion, CDKN2A loss, UGT2B17 loss
16	RT4	Bladder 2	FGFR3-TACC3 fusion
17	RT112/84	Bladder 3	FGFR3-TACC3 fusion
18	Caov-3	Ovarian 1	TP53 mutation, PIK3CA amplification, PTEN gain, AKT1/2 gain
19	HCC 1954	Breast 1	Translocation (p15.33;q24.21), CLPTM1L and PVT1, NSD1 and MRE11A truncation, TP53 deletion, CNGA2 deletion
20	KPL-4	Breast 2	Her2+
21	RL95-2	Uterine 1	TP53 V218 mutation, PIK3R1 R386fs/R639ter, ARID1A L649fs/R693ter, PTEN T321fs-ter
22	DoTc2 4510	Uterine 2	BRCA2-deficient
23	ME-180	Uterine 3	HPV39-positive, PIK3CA E545K mutation

Example 12 Durable regression in Trop2 positive gastric xenograft model

In this Example, using subcutaneous NCI-N87 gastric cancer xenograft model in female BALB/c nude Mice, the efficacy of Trop2 PH1 ADCs (M2.8 mAb conjugated with PH1 payload, also referred to as M2.8 L22 ADCs) at DAR2 and DAR4 were tested against Trodelvy (pharmaceutical-grade IMMU-132) which was used for human clinical trials and subsequently approved for human use. Even at a lower dose (3mpk vs 10mpk) and DAR (2 or 4, vs 8), Trop2 PH1 ADCs outperformed pharmaceutical-grade IMMU-132.

10 Methods

Cell Culture

The NCI-N87 tumor cell line was maintained in vitro in RPMI1640 medium supplemented with 10% fetal bovine serum at 37 °C in an atmosphere of 5% CO₂ in air. The tumor cells were routinely sub-cultured twice weekly by trypsin-EDTA treatment. The cells growing in an exponential growth phase were harvested and counted for tumor inoculation.

Tumor Inoculation

Each mouse was inoculated subcutaneously in the right flank region with tumor cells (1×10^7) in 0.1 ml of PBS mixed with Matrigel (1:1) for tumor development.

Randomization

The randomization was started when the mean tumor size reached approximately 192.40 mm³. 100 mice were enrolled in the study. All animals were randomly allocated to 12 study groups. Randomization was performed based on "Matched distribution" method (StudyDirector™ software, version 3.1.399.19).

Observation and Data Collection

After tumor cells inoculation, the animals were checked daily for morbidity and mortality. During routine monitoring, the animals were checked for any effects of tumor growth and treatments on behavior such as mobility, food and water consumption, body weight gain/loss (Body weights were measured twice per week after randomization), eye/hair matting and any other abnormalities. Mortality and observed clinical signs were recorded for individual animals in detail.

Tumor volumes were measured twice per week after randomization in two dimensions using a caliper, and the volume was expressed in mm³ using the formula: $V = (L \times W \times W)/2$, where V is tumor volume, L is tumor length (the longest tumor dimension) and

W is tumor width (the longest tumor dimension perpendicular to L). Dosing as well as tumor and body weight measurements were conducted in a Laminar Flow Cabinet.

The treatment was initiated on the same day of randomization (day 6) per study design.

- 5 Tumor growth inhibition (TGI): TGI% is an indication of antitumor activity and expressed as: $TGI (\%) = 100 \times (1 - T/C)$. T and C are the mean tumor volume (or weight) of the treated and control groups, respectively, on a given day.

- Statistical analysis of the difference in mean tumor volume among the groups was conducted using the methods below: Use the data collected on the last observation day for every single group in despite diverse individual termination date.

Statistical Analysis

- To compare tumor volumes of different groups at a pre-specified day, Bartlett's test was first used to check the assumption of homogeneity of variance across all groups. When the p-value of Bartlett's test was ≥ 0.05 , a one-way ANOVA was performed to test overall equality of means across all groups. If the p-value of the one-way ANOVA was < 0.05 , a post hoc testing was performed by running Tukey's HSD (honest significant difference) tests for all pairwise comparisons, and Dunnett's tests for comparing each treatment group with the vehicle group. When the p-value of Bartlett's test was < 0.05 , a Kruskal-Wallis test was performed to test overall equality of medians among all groups. If the p-value the Kruskal-Wallis test was < 0.05 , post hoc testing was performed by running Conover's non-parametric test for all pairwise comparisons or for comparing each treatment group with the vehicle group, both with single-step p-value adjustment. In addition, pairwise comparisons were performed without multiple comparison correction and nominal/uncorrected p-values directly from Welch's t-test or Mann-Whitney U test were reported. Specifically, Bartlett's test was first used to check the assumption of homogeneity of variance for a pair of groups. When the p-value of Bartlett's test was ≥ 0.05 , a Welch's t-test was performed, otherwise a Mann-Whitney U test was performed, to obtain nominal p-values.

- All statistical analyses had been done in R-a language and environment for statistical computing and graphics (version 3.3.1). All tests were two-sided unless otherwise specified, and p-values of < 0.05 were regarded as statistically significant.

Results

In a Trop2 positive tumor model (nude mice bearing human NCI-N87 gastric tumors with expression level IHC3+), mice were dosed at dosing regimen of QW for 2 weeks

(QWx2). Horizontal dotted line indicates mean tumor volume of 200 mm³ size at which treatment was initiated. Tumor shrinkage below this line was considered regression. Tumor sizes were monitored.

5 Early efficacy for M2.8 L22 ADC DAR4 manifests as about 90% TGI and was observed from D21 through D41. Although, the TGI by Trodelvy was ~80%, there was no statistical difference in the inhibitions caused by the 2 agents during this early period.

Long term tumor regression (TR) was observed in 50% of treated mice over a period of about 5 months in the group treated with Trop2 PH1 (DAR4) ADC at 3mg/kg (Figure 16). Stable disease was observed in 50% of treated mice over a period of about 5 months in the
10 group treated with Trop2 PH1 (DAR2) ADC at 3mg/kg. Both treatment groups of Trop2 PH1 outperformed the treatment group of IMMU-132* at lower DAR (2 or 4 vs 7.6) and at lower dose (3mg/kg vs 10 mg/kg). IMMU-132* represents clinical grade IMMU-132 (Sacituzumab govitecan).

15 Conclusion

M2.8 L22 ADCs exhibit potent anti-tumor efficacy against NCI-N87 gastric carcinoma model.

Over a period of 5 months (160 days), Trodelvy (clinical grade IMMU-132) showed fewer tumor regressions (2/10) below implanted size of 200mm³ and rebounded sooner than
20 M2.8 L22 ADC (DAR4) which exhibited tumor regressions in 50% of animals treated. At DAR2, stable disease was observed in animals treated with M2.8 L22 ADC as 50% of tumors failed to grow beyond 400mm³.

Since the antibodies for both agents, hRS7 and M2.8 compete for the same binding site or/ epitopes (Figure 4), the difference is attributable to superior payload efficacy of PH1
25 over SN-38.

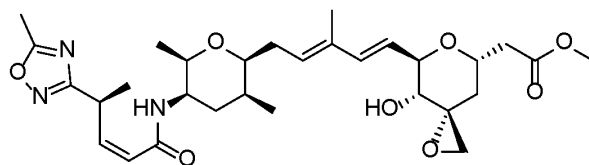
Example 14 Production of anti-Trop2 antibodies (e.g., M2.8, etc.)

Geneblocks for SEQ ID NOs.1 –6 were generated with differing restriction enzyme
30 cutting sites to ensure consistent insertion into desired vectors for expression of heavy chain and light chain. For initial expression, variable region genes (VH or VL) were inserted into pcDNA3.3 vectors for transient expression (in HEK293 cells) of antibodies (having variable regions as described herein and constant regions of hIgG1/Ck). Similarly, stable cell lines

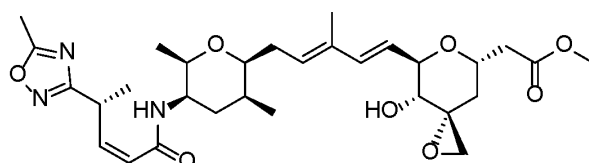
were made using Lonza's GS Xceed™ expression system and vectors per manufacturer's instruction.

Example 15

- 5 The diastereomeric mixture of compounds 1A and 1B (International Patent Application Publication Number WO 20019/060398) were separated using chiral chromatography and isolated as pure diastereomers.



Compound 1A



Compound 1B

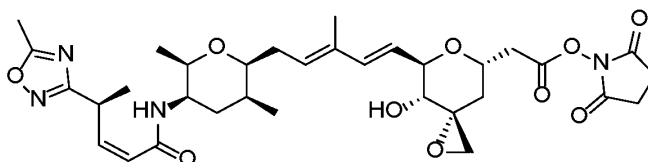
10 It was unexpectedly discovered that compound 1A (also referred to as ThA13D2) was significantly more active than compound 1B (also referred to as ThA13D1). Table 3 shows the activity of compound 1A versus compound 1B and the diastereomeric mixture (also called

15 ThA13) on various cell lines.

Table X3 (IC₅₀ nM)

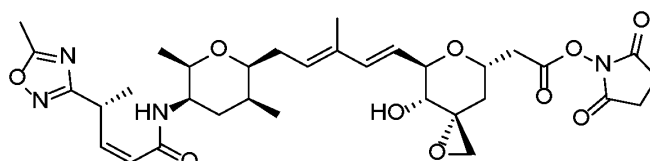
	ThA13	Compound 1A	Compound 1B
HCT116	3.4	0.8	16.2
DLD1	11.4	3.8	125.3
MCF7	2.2	0.8	8.8
HT29	7.6	3.1	22.6

The diastereomeric mixture of compounds 2A and 2B were also separated using chiral chromatography and isolated as pure diastereomers.



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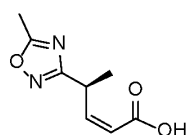
Compound 2A



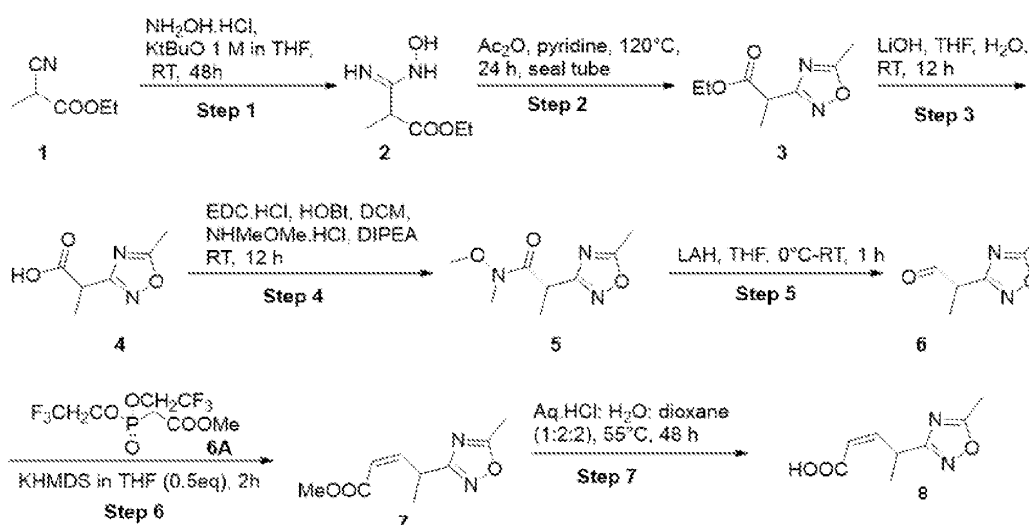
Compound 2B

It was discovered that compound 2A (also called ThA13D2L2) was significantly more active than compound 2B (also called ThA13D1L2). Figure 17 shows the activity of compound 2A versus compound 2B and the diastomeric mixture (also called ThA13L2) on cell viability for HCT116.

Example 16. Preparation of intermediate A



Intermediate A



Step 1: Synthesis of ethyl 3-(hydroxyl amino)-3-imino-2-methylpropanoate (2).

To a stirred solution of ethyl 2-cyanopropanoate (20 g, 15.7 mmol) in THF (500 mL) was added a solution of K^tBuO (314 mL, 31.4 mmol, 1 M in THF) at 0°C. After 10 min hydroxyl amine hydrochloride (27 g, 39.3 mmol) was added & the reaction mixture was stirred at room temperature for 48 h. The progress of the reaction was monitored by TLC. TLC indicated SM got consumed. TLC system (50% EtOAc in Hexane, R_f: 0.1, KMnO₄ active).

The reaction mass evaporated under reduced pressure, residue was purified by silica column using 40% ethyl acetate in hexane as an eluent. The pure fractions were collected and evaporated under reduced pressure to get ethyl 3-(hydroxyl amino)-3-imino-2-methylpropanoate (5.5 g, 21.8%) as colourless oil.

- 5 ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 1.17 (t, *J* = 7.15 Hz, 3 H), 1.24 (d, *J* = 7.15 Hz, 3 H), 3.10 - 3.20 (m, 1 H), 3.92 - 4.23 (q, 2 H), 5.31 - 5.45 (br, 2 H).
LCMS: 43.4% (161.13, M+H), RT = 0.37 min. and 49.48% (161.13, M+H), RT = 0.41 min

Step 2: Synthesis of ethyl 2-(5-methyl-1, 2, 4-oxadiazol-3-yl) propanoate (3)

- 10 To a stirred solution of ethyl 3-(hydroxyl amino)-3-imino-2-methylpropanoate (5.5 g, 34.3 mmol) in pyridine (50 mL) was added acetic anhydride (9.7 mL, 10.3 mmol) in seal tube at room temperature. The reaction mixture was heated at 120°C for 24 h, progress of the reaction was monitored by TLC. TLC indicated SM got consumed. TLC system (50% EtOAc in Hexane, Rf: 0.5, KMnO₄ active).

- 15 The reaction mass was evaporated under reduced pressure, residue was purified by silica column using 20% ethyl acetate in hexane as an eluent. The pure fractions were collected and evaporated under reduced pressure to get ethyl 2-(5-methyl-1, 2, 4-oxadiazol-3-yl) propanoate (4.5 g, 71.4%) as yellow coloured oil.
¹H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 1.26 (t, *J* = 7.09 Hz, 3 H), 1.58 - 1.62 (d, 3
20 H), 2.61 (s, 3 H), 3.89 - 4.00 (q, 1 H), 4.15 - 4.26 (q, 2 H).
LCMS: 86.56% (185.22, M+H), RT = 1.48 min.

Step 3: Synthesis of 2-(5-Methyl-1, 2, 4-oxadiazol-3-yl) propanoic acid (4).

- 25 To a stirred solution of ethyl 2-(5-methyl-1, 2, 4-oxadiazol-3-yl) propanoate (4.5 g, 24.4 mmol) in MeOH:THF:H₂O (45 ml, 4:4:1) was added LiOH.H₂O (4 g, 9.7 mmol) at 0°C and stirred the reaction mixture at room temperature for 12 h. The progress of the reaction was monitored by TLC. TLC indicated SM got consumed. TLC system (50% EtOAc in Hexane, Rf: 0.1, KMnO₄ active).

- 30 The reaction mass evaporated under reduced pressure, pH of residue was adjusted ~1 using 6N HCl (~10 ml) then extracted with ethyl acetate (25 ml x 2). The combined organic layer was dried using Na₂SO₄ and concentrated under reduced pressure to get 2-(5-methyl-1, 2, 4-oxadiazol-3-yl) propanoic acid (3.5 g, 92%) as colourless oil.
¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 1.41 (d, *J* = 7.23 Hz, 3 H), 2.57 (s, 3 H), 3.88 - 3.97 (q, 1 H), 12.65 - 12.82 (br s, 1 H).

LCMS: 94.38% (157.1, M+H), RT = 1.05 min.

Step 4: Synthesis of N-methoxy-N-methyl-2-(5-methyl-1, 2, 4-oxadiazol-3-yl) propanamide (5).

5 To a stirred solution of 2-(5-methyl-1, 2, 4-oxadiazol-3-yl) propanoic acid (3.5 g, 22.4 mmol) in DCM (100 ml), EDC.HCl (6.4 g, 33.6 mmol), HOBt (4.5 g, 33.6 mmol) DIPEA (11.7 ml, 67.2 mmol) were added at 0°C. After 10 min, N, O-dimethyl hydroxyl amine hydrochloride (3.2 g, 33.6 mmol) was added at 0°C. The reaction mixture was stirred at room temperature for 12 h. The progress of the reaction was monitored by TLC. TLC indicated SM
10 got consumed. TLC system (50% EtOAc in Hexane, Rf: 0.2, KMnO₄ active).

The reaction mass was diluted with DCM (40 mL) and washed with saturated solution of sodium bicarbonate (30 ml). The organic layer was separated and dried over sodium sulphate. The organic layer was evaporated under reduced pressure and obtained 5 g of crude N-methoxy-N-methyl-2-(5-methyl-1, 2, 4-oxadiazol-3-yl) propanamide. The crude compound
15 was purified by silica column using 40% ethyl acetate in hexane as an eluent. The pure fractions were collected and evaporated under reduced pressure to get N-methoxy-N-methyl-2-(5-methyl-1, 2, 4-oxadiazol-3-yl) propanamide (2.2 g, 50%) as colourless oil.

¹H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 1.57 (d, *J* = 7.03 Hz, 3 H), 2.60 (s, 3 H), 3.26 (s, 3 H), 3.72 (s, 3 H), 4.34 - 4.42 (q, 1 H).

20 LCMS: 96.65% (200.16, M+H), RT = 1.21 min.

Step 5: 2 Synthesis of 2-(5-methyl-1, 2, 4-oxadiazol-3-yl) propanal (6).

To a stirred solution of LAH (380 mg, 10 mmol) in THF (10 ml) was added N-methoxy-N-methyl-2-(5-methyl-1, 2, 4-oxadiazol-3-yl) propanamide (1 g, 50 mmol) in THF (20 ml)
25 slowly at 0°C. The reaction mixture was stirred at room temperature for 1 h. The progress of the reaction was monitored by TLC. TLC indicated SM got consumed. TLC system (40% EtOAc in Hexane, Rf: 0.3, KMnO₄ active and 2, 4-DNP slightly active).

The reaction mixture was quenched using 1 N HCl (~3 ml) at 0°C and stirred for 10 min and added ethyl acetate (20 ml). The reaction mixture was filtered through celite bed. The
30 filtrate was dried using sodium sulphate and minimized volume up to 4 mL (~800 mg, crude) of 2-(5-methyl-1, 2, 4-oxadiazol-3-yl) propanal. As such crude 2-(5-methyl-1, 2, 4-oxadiazol-3-yl) propanal used for Step 6.

ESMS: 40.4% (141.1, M+H), RT = 2.24 min. (Crude)

Step 6: Synthesis of methyl (Z)-4-(5-methyl-1, 2, 4-oxadiazol-3-yl) pent-2-enoate (7).

To a stirred solution of 18-crown-6-ether (7.5 g, 28.5 mmol) in dry THF (30 ml) was added methyl 2-(bis(2,2,2-trifluoroethoxy) phosphoryl) acetate (1.3 ml, 6.2 mmol) at 0°C followed by added 1 M KHMDS in THF (6.2 ml, 6.2 mmol) at -78°C. The reaction mixture maintained at -78°C for 30 min then added 2-(5-methyl-1, 2, 4-oxadiazol-3-yl) propanal (~800 mg) in dry THF (10 ml) at -78°C and allow the reaction mixture at room temperature gradually & stirred at same temperature for 2 h. The progress of the reaction was monitored by TLC. TLC indicated SM got consumed. TLC system (30% EtOAc in Hexane, Rf: 0.5, KMnO₄ active).

The reaction mixture was quenched using H₂O (30 ml) and extracted in ethyl acetate (50 ml x 2). The combined organic layer was dried over sodium sulphate and concentrated under reduced pressure to get 600 mg of crude methyl (Z)-4-(5-methyl-1, 2, 4-oxadiazol-3-yl) pent-2-enoate. The crude compound was purified by silica column using 10% ethyl acetate in hexane as an eluent. The pure fractions were collected and evaporated under reduced pressure to get methyl (Z)-4-(5-methyl-1, 2, 4-oxadiazol-3-yl) pent-2-enoate (420 mg, 59% pure by LCMS, 37%) as yellow coloured oil.

¹H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 1.44 - 1.48 (d, 3 H), 2.57 (s, 3 H) 3.74 (s, 3 H), 5.01 - 5.10 (q, 1 H), 5.92 - 5.89 (d, *J* = 10 Hz, 1 H), 6.35 - 6.46 (dd, *J* = 10 Hz, 1 H).

LCMS: 59% (197.21, M+H), RT = 1.57 min.

Z geometric isomer confirm by JJ coupling constant value of olefin bond: 10 Hz

Step 7: Synthesis of (Z)-4-(5-methyl-1, 2, 4-oxadiazol-3-yl) pent-2-enoic acid (8).

Methyl (Z)-4-(5-methyl-1, 2, 4-oxadiazol-3-yl) pent-2-enoate (550 mg, 28 mmol, crude 80% pure by LCMS) was added in dioxane (10 ml) & 2 N HCl (5 ml). The reaction mixture was heated at 55 °C for 48 h. The progress of the reaction was monitored by TLC. TLC indicated SM got consumed. TLC system (30% EtOAc in Hexane, Rf: 0.1, KMnO₄ active).

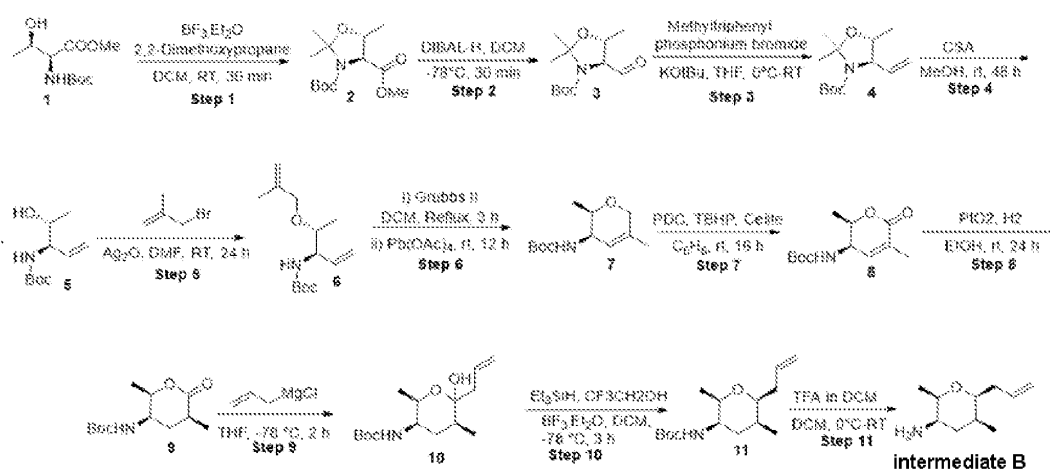
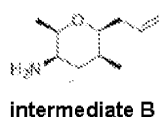
The reaction mixture was concentrated under reduced pressure and purified using Prep HPLC to get pure (Z)-4-(5-methyl-1, 2, 4-oxadiazol-3-yl) pent-2-enoic acid (138 mg, 28.6%) as colourless oil.

¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 1.33 (d, *J* = 7.02 Hz, 3 H), 2.55 (s, 3 H), 4.86 - 5.08 (q, 1 H), 5.84 (dd, *J* = 11.40 Hz, 1 H), 6.24 (m, *J* = 10.52 Hz, 1 H), 11.64 - 12.96 (br s, 1 H). LCMS: 98.59% (183.21, M+H), RT = 1.4 min.

Step 8: Synthesis of

The racemic mixture of methyl (Z)-4-(5-methyl-1, 2, 4-oxadiazol-3-yl) pent-2-enoic acid (8) from step 7 was subjected to chiral chromatography to provide the desired enantiomer (intermediate A).

5 Example 17. Preparation of intermediate B



Step 1: Synthesis of (4S, 5R)-3-tert-butyl 4-methyl 2, 2, 5-trimethyloxazolidine-3, 4-dicarboxylate (2).

2,2-Dimethoxypropane (159.1 mL, 1285 mmol) was added to a stirred solution of (2S,3R)-methyl 2-(tert-butoxycarbonylamino)-3-hydroxybutanoate (150 g, 642 mmol) in DCM (1500 mL) at 23 °C, and boron trifluoride diethyl etherate (3.96 mL, 32.13 mmol) was added. The reaction mixture was then stirred at 23 °C for 30 min. The progress of the reaction was monitored by TLC. TLC system (50 % EtOAc in Hexane, R_f = 0.6, PMA visible). After completion of the reaction, quenched with brine (1000 ml). The organic layer was separated, dried over anhydrous sodium sulphate, filtered and concentrated in vacuo to get crude compound. The crude residue was purified by flash chromatography (1 to 5% EtOAc in hexanes) on silica (100-200 mesh) to afford (4S, 5R)-3-tert-butyl 4-methyl 2, 2, 5-trimethyloxazolidine-3, 4-dicarboxylate (145 g, 82.85%) as a colourless oil. ¹H NMR (400

MHz, CHLOROFORM-d) δ ppm 1.36 - 1.42 (m, 9 H) 1.48 (s, 3 H) 1.54 - 1.67 (m, 6 H) 3.76 (s, 3 H) 3.86 - 4.02 (m, 1 H) 4.07 - 4.19 (m, 1 H).

Step 2: Synthesis of (4S, 5R)-tert-butyl 4-formyl-2, 2, 5-trimethyloxazolidine-3-carboxylate (3).

To a stirred solution of (4S,5R)-tert-butyl 4-formyl-2,2,5-trimethyloxazolidine-3-carboxylate (130 g, 475.6mmol) in dry toluene (750 mL) at -78 °C was added DIBAL-H (1 Min toluene) (713 mL, 713 mmol). After the addition, the mixture was stirred at -78 °C for another 10 min and quenched with by slow addition of cold MeOH (220 mL) at -78 °C. The resulting white emulsion was poured into ice-cold 1N HCl (2000 mL) with stirring over 15 min and the aqueous mixture was then extracted with EtOAc (1000 mL x 2), dried over anhydrous sodium sulphate, filtered and concentrated in vacuo to get crude compound. TLC system (30 % EtOAc in Hexane, R_f =0.55, 2, 4-DNP visible) The crude residue was purified by flash chromatography (1 to 5% EtOAc in hexanes) on silica (100-200 mesh) to afford (4S, 5R)-tert-butyl 4-formyl-2, 2, 5-trimethyloxazolidine-3-carboxylate (100 g, 86.95%) as a colourless oil. ^1H NMR (400 MHz, CHLOROFORM-d) δ ppm 1.36 (d, J =6.10 Hz, 3 H) 1.40 - 1.52 (m, 9 H) 1.57 - 1.72 (m, 6 H) 3.67 - 3.84 (m, 1 H) 4.02 - 4.10 (m, 1 H) 9.36 - 9.47 (m, 1 H).

Step 3: Synthesis of (4R, 5R)-tert-butyl 2, 2, 5-trimethyl-4-vinyloxazolidine-3-carboxylate (4).

To a solution of methyltriphenylphosphonium bromide (293 g, 822 mmol) in THF (750 mL) at 0 °C was added KOtBu (92 g, 822 mmol) in one portion. The resulting mixture was stirred for an additional one hour at the same temperature. After 1 h at the same temperature, (4S, 5R)-tert-butyl 4-formyl-2, 2, 5-trimethyloxazolidine-3-carboxylate (100 g, 411 mmol) in THF (250 mL) was added and the mixture was stirred at 50 °C for 12 h. The progress of the reaction was monitored by TLC. TLC system (10 % EtOAc in Hexane, R_f =0.5, PMA visible). After cooling to 23 °C, the reaction mixture was quenched with saturated NH_4Cl solution (1000 ml), extracted with EtOAc (1000 mL x 2). The combined organic layers were washed with brine (1000 mL), dried over anhydrous sodium sulphate, filtered and concentrated in vacuum to get crude compound. The crude residue was purified by flash chromatography (1 to 5% EtOAc in hexanes) on silica (100-200 mesh) to afford (4R, 5R)-tert-butyl 2, 2, 5-trimethyl-4-vinyloxazolidine-3-carboxylate (80 g, 80.80%) as a colourless oil. ^1H NMR (400 MHz, CHLOROFORM-d) δ ppm 1.28 (d, J =5.99 Hz, 3 H) 1.38

- 1.49 (m, 9 H) 1.51 (s, 3 H) 1.56 - 1.63 (m, 3 H) 3.72 (br d, $J=1.31$ Hz, 1 H) 3.78 - 3.86 (m, 1 H) 5.16 (br d, $J=7.85$ Hz, 2 H) 5.66 (br d, $J=6.43$ Hz, 1 H).

Step 4: Synthesis of tert-butyl (3R, 4R)-4-hydroxypent-1-en-3-ylcarbamate (5).

5 To a stirred solution of (4R, 5R)-tert-butyl 2, 2, 5-trimethyl-4-vinyloxazolidine-3-carboxylate (80 g, 331.4 mmol) in MeOH (2.0 L) at 23 °C, was added camphor sulfonic acid (7.7 g, 33.14 mmol). The reaction mixture was stirred at 23 °C for 48 h. The progress of the reaction was monitored by TLC. TLC system (40 % EtOAc in Hexane, $R_F=0.25$, PMA visible). After completion, the reaction mixture was quenched with saturated NaHCO₃ solution (1000 ml) and most of the solvent was removed in vacuo. The aqueous residue was
10 extracted with EtOAc (1000 mL x 2). The combined organic layers were washed with brine (1000 mL), dried over anhydrous sodium sulphate, filtered and concentrated in vacuo to get crude compound. The crude residue was purified by flash chromatography (10 → 40% EtOAc in hexanes) on silica (100-200 mesh) to afford tert-butyl (3R, 4R)-4-hydroxypent-1-en-3-ylcarbamate (58 g, 86.95%) as a colourless oil.

¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 1.23 (d, $J=6.32$ Hz, 3 H) 1.46 (s, 9 H) 1.94 (br s, 1 H) 3.87 (br dd, $J=5.99, 3.05$ Hz, 1 H) 4.02 - 4.12 (m, 1 H) 4.87 (br s, 1 H) 5.21 - 5.26 (m, 1 H) 5.28 - 5.31 (m, 1 H) 5.84 (ddd, $J=17.19, 10.49, 5.12$ Hz, 1 H); LCMS: 94.36% (202.14, M+H), RT: 1.50 min.

20

Step 5: Synthesis of tert-butyl (3R, 4R)-4-(2-methylallyloxy) pent-1-en-3-ylcarbamate (6).

To stirred solution of tert-butyl (3R, 4R)-4-hydroxypent-1-en-3-ylcarbamate 5 (40 g, 198.73 mmol) in DMF (250 mL) at 23 °C, was added methallyl bromide (107.3 g, 795.9 mmol). The flask was wrapped with aluminium foil and Silver oxide (69 g, 298 mmol) was
25 added. The reaction mixture was stirred at 23 °C for 24 h. The progress of the reaction was monitored by TLC. TLC system (10 % EtOAc in Hexane, $R_F=0.4$, PMA visible). After 24 h at 23 °C, the reaction mixture was diluted with ether (500 mL), filtered through Celite and washed with additional ether (500 mL). The filtrate was then extracted with water (5 × 500 mL), brine (50 mL), dried over anhydrous Na₂SO₄, filtered and concentrated in vacuo. The
30 crude residue was purified by flash chromatography (2.5 → 10% EtOAc in hexanes) on silica (100-200 mesh) to afford tert-butyl (3R, 4R)-4-(2-methylallyloxy) pent-1-en-3-ylcarbamate 6 (40.5 g, 79.88%) as a colorless oil.

¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 1.16 (d, $J=6.32$ Hz, 3 H) 1.45 (s, 9 H) 1.68 - 1.77 (m, 3 H) 3.51 - 3.63 (m, 1 H) 3.75 - 3.86 (m, 1 H) 3.89 - 4.01 (m, 1 H) 4.12 (br d, $J=7.08$

Hz, 1 H) 4.79 - 4.91 (m, 2 H) 4.94 (d, $J=0.87$ Hz, 1 H) 5.09 - 5.25 (m, 2 H) 5.89 (ddd, $J=17.22, 10.46, 5.45$ Hz, 1 H); LCMS: 78.48% (256.33, M+H), RT: 2.69 min.

Step 6: Synthesis of tert-butyl ((2R, 3R)-2, 5-dimethyl-3, 6-dihydro-2H-pyran-3-yl) carbamate (7).

To a stirred solution of tert-butyl ((3R,4R)-4-((2-methylallyl)oxy)pent-1-en-3-yl)carbamate 6 (35 g, 137 mmol) in benzene (500 mL) was added Grubb's 2nd generation catalyst (2.3 g, 2.74 mmol) at 23 °C under a nitrogen atmosphere. The reaction was then refluxed for 3 h. After cooling to 23 °C, Lead tetra acetate (1.8 g, 4.11 mmol) was added and stirred for an additional 12 h at 23 °C. TLC system (10 % EtOAc in Hexane, $R_F=0.3$, PMA visible). The solvent was then removed in vacuo and the crude residue was purified by flash chromatography (2.5 → 10% EtOAc in hexanes) on silica (100-200 mesh) to afford tert-butyl ((2R, 3R)-2,5-dimethyl-3,6-dihydro-2H-pyran-3-yl)carbamate 7 (26 g, 83.60%) as a white solid. ¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 1.16 - 1.22 (m, 3 H) 1.44 (s, 9 H) 1.61 (s, 3 H) 3.57 - 3.66 (m, 1 H) 3.86 - 3.94 (m, 1 H) 3.97 (s, 1 H) 3.98 - 4.06 (m, 1 H) 4.60 (br d, $J=9.66$ Hz, 1 H) 5.56 - 5.62 (m, 1 H); LCMS: 94.00% (228.29, M+H), RT: 2.35 min.

Step 7: Synthesis of tert-butyl ((2R, 3R)-2, 5-dimethyl-6-oxo-3, 6-dihydro-2H-pyran-3-yl) carbamate (8).

To a stirred solution of tert-butyl ((2R,3R)-2,5-dimethyl-3,6-dihydro-2H-pyran-3-yl)carbamate 7 (30 g, 132.1 mmol) in benzene (1200 mL) at 23 °C was added Celite (120 g), pyridinium dichromate (99.3 g, 364.3 mmol) and tert-butyl hydroperoxide (70 wt% in water, 47.5 mL, 528.6 mmol). After 5 h at 23 °C, the reaction mixture was filtered through Celite, washed with ethyl acetate (500 mL), dried over anhydrous Na₂SO₄, filtered and concentrated in vacuo. TLC system (30 % EtOAc in Hexane, $R_F=0.4$, UV visible). The crude residue was purified by flash chromatography (7.5 → 30% EtOAc in hexanes) on silica gel (100-200 mesh) to afford tert-butyl ((2R, 3R)-2, 5-dimethyl-6-oxo-3, 6-dihydro-2H-pyran-3-yl) carbamate 8 (18 g, 56.60%) as a white solid. ¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 1.38 (d, $J=6.44$ Hz, 3 H) 1.42 - 1.49 (m, 9 H) 1.92 - 1.97 (m, 3 H) 4.21 - 4.31 (m, 1 H) 4.51 - 4.65 (m, 2 H) 6.63 (dd, $J=6.32, 1.43$ Hz, 1 H); LCMS: 96.91% (242.17, M+H), RT: 1.68 min.

Step 8: Synthesis of tert-butyl ((2R, 3R, 5S)-2, 5-dimethyl-6-oxotetrahydro-2H-pyran-3-yl) carbamate (9).

To a stirred solution of tert-butyl ((2R, 3R)-2, 5-dimethyl-6-oxo-3, 6-dihydro-2H-pyran-3-yl) carbamate (25 g, 103.61 mmol) in ethanol (500 mL) at 23 °C was added PtO₂ (470 mg, 2.07 mmol) and the atmosphere in the reaction flask was changed to hydrogen. The reaction mixture was stirred at 23 °C for 24 h. TLC system (30 % EtOAc in Hexane, R_F=0.35, PMA visible). After 24 h at 23 °C, the reaction mixture was filtered through filter paper and the solvent was removed in vacuo to afford tert-butyl ((2R,3R,5S)-2,5-dimethyl-6-oxotetrahydro-2H-pyran-3-yl)carbamate 9 (25g, 99%) as a white solid. ¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 1.16 - 1.25 (m, 4 H) 1.29 - 1.38 (m, 5 H) 1.39 - 1.54 (m, 12 H) 2.49 - 2.71 (m, 2 H) 4.03 - 4.19 (m, 1 H) 4.41 - 4.55 (m, 1 H) 4.64 - 4.82 (m, 1 H).

10

Step 9: Synthesis of tert-butyl ((2R, 3R, 5S)-6-allyl-6-hydroxy-2, 5-dimethyltetrahydro-2H-pyran-3-yl) carbamate (10).

To a stirred solution of tert-butyl ((2R,3R,5S)-2,5-dimethyl-6-oxotetrahydro-2H-pyran-3-yl)carbamate 9 (25 g, 102.7 mmol) in THF (300 mL) at -78 °C was added allylmagnesium chloride (1.4 M solution in THF, 146 mL, 205.4 mmol) down the flask sides under a nitrogen atmosphere. After 1.5 h at the same temperature, the reaction was quenched with saturated aqueous NH₄Cl (500 mL). The aqueous residue was extracted with EtOAc (2 × 500 mL). The combined organic layers were washed with brine (500 mL), dried over anhydrous Na₂SO₄, filtered and concentrated in vacuo. TLC system (40 % EtOAc in Hexane, R_F=0.25, PMA visible). The crude residue was purified by flash chromatography (10 → 50% EtOAc in hexanes) on silica (100-200 mesh) to afford tert-butyl ((2R, 3R, 5S)-6-allyl-6-hydroxy-2, 5-dimethyltetrahydro-2H-pyran-3-yl)carbamate 10 (20 g, 68.25%) as a pale yellow oil. ¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 1.13 (d, J=7.2 Hz, 3 H) 1.16 (d, J=6.3 Hz, 3 H) 1.45 (s, 9 H) 1.93 - 2.03 (m, 1 H) 2.10 - 2.29 (m, 1 H) 2.68 - 2.80 (m, 1 H) 3.24 - 3.39 (m, 2 H) 3.65 - 3.76 (m, 1 H) 4.71 (br d, J=9.06 Hz, 1 H) 5.05 - 5.22 (m, 2 H) 5.78 - 6.02 (m, 1 H).

25

Step 10. Synthesis of tert-butyl ((2R, 3R, 5S, 6S)-6-allyl-2, 5-dimethyltetrahydro-2H-pyran-3-yl)carbamate (11).

To a stirred solution of tert-butyl ((2R, 3R, 5S)-6-allyl-6-hydroxy-2, 5-dimethyltetrahydro-2H-pyran-3-yl)carbamate 10 (5 g, 17.54 mmol) in DCM (50 mL) was added 2,2,2-trifluoroethanol (10 mL, 140.3 mmol) and triethylsilane (28 mL, 175.4 mmol) at 23 °C under a nitrogen atmosphere. The reaction was cooled to -78 °C and BF₃•OEt₂ complex (8.6 mL, 70.16 mmol) was added slowly down the flask side. After an additional 3 h

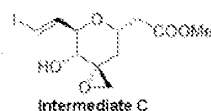
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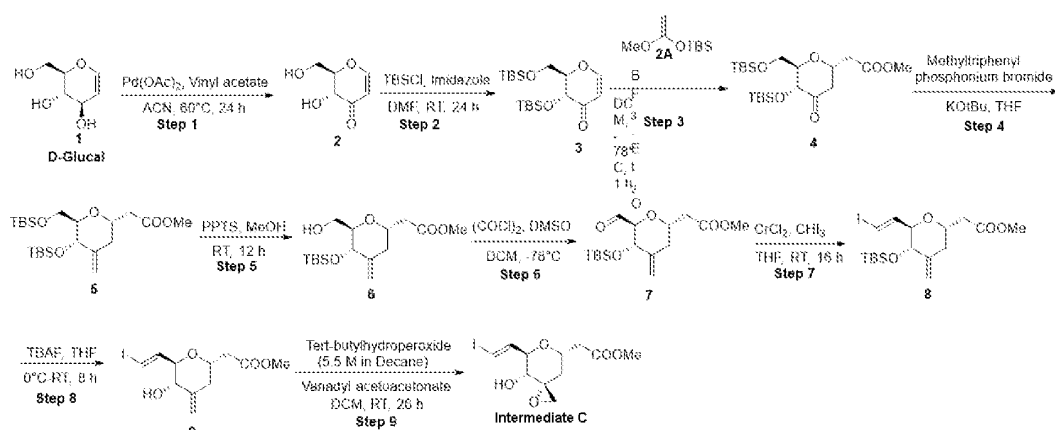
at the same temperature, saturated aqueous NaHCO_3 (100 mL) was added at -78°C , and the layers were separated. The aqueous residue was extracted with EtOAc (3×50 mL). The combined organic layers were washed with brine (100 mL), dried over anhydrous Na_2SO_4 , filtered, and concentrated under reduced pressure. TLC system (10 % EtOAc in Hexane, $R_F=0.3$, PMA visible). The resulting residue was purified by flash chromatography (2 → 12.5% EtOAc in hexanes) on silica gel (100-200 mesh) to afford tert-butyl ((2R, 3R, 5S, 6S)-6-allyl-2, 5-dimethyltetrahydro-2H-pyran-3-yl) carbamate 11 (1.8 g, 38.29%) as colourless oil. ^1H NMR (400 MHz, CHLOROFORM-d) δ ppm 0.98 - 1.07 (m, 3 H) 1.10 - 1.21 (m, 3 H) 1.45 (d, $J=1.43$ Hz, 9 H) 1.68 - 1.81 (m, 1 H) 1.95 (br d, $J=2.74$ Hz, 2 H) 2.03 - 2.20 (m, 1 H) 2.24 - 2.41 (m, 1 H) 3.37 - 3.68 (m, 3 H) 4.78 (br d, $J=9.06$ Hz, 1 H) 4.98 - 5.15 (m, 2 H) 5.71 - 5.88 (m, 1 H); LCMS: 60.33% (270.33, $\text{M}+\text{H}$), RT: 2.74 min.

Step 11: Synthesis of tert-butyl ((2R, 3R, 5S, 6S)-2, 5-dimethyl-6-((E)-3-methyl-4-oxobut-2-en-1-yl) tetrahydro-2H-pyran-3-yl) carbamate (intermediate B).

To a stirred solution of tert-butyl ((2R,3R,5S,6S)-6-allyl-2,5-dimethyltetrahydro-2H-pyran-3 yl)carbamate (900 mg, 3.345 m moles) in DCM (10 mL), stirred at 0°C a solution of TFA in DCM (10 mL) was added and then slowly allowed to reach to RT over 2 h when TLC indicated completion of the reaction (TLC system: 30% EtOAc in pet ether, R_f : 0.1, PMA active). Reaction mixture was concentrated under reduced pressure at 28°C to get the crude product which was further dried over high vacuum to get 1-(((2R,3R,5S,6S)-6-allyl-2,5-dimethyltetrahydro-2H-pyran-3-yl)-14-azanyl)-2,2,2-trifluoroethan-1-one as a light brown liquid (1 g crude) which was taken forward in subsequent reaction without further purification. ^1H NMR (400 MHz, CHLOROFORM-d) δ ppm 5.71 - 5.84 (m, 1 H) 4.99 - 5.16 (m, 2 H) 3.71 - 3.82 (m, 1 H) 3.51 - 3.64 (m, 1 H) 3.32 - 3.45 (m, 1 H) 2.25 - 2.45 (m, 1 H) 2.06 - 2.21 (m, 3 H) 1.86 - 1.97 (m, 1 H) 1.23 - 1.34 (m, 4 H) 0.99 - 1.10 (m, 4 H); LCMS: 90.2% (170.2, $\text{M}+\text{H}$), RT: 2.59 min.

Example 18. Preparation of intermediate C.





Step 1: Synthesis of (2R, 3R)-3-hydroxy-2-(hydroxymethyl)-2H-pyran-4(3H)-one (2).

A mixture of compound 1 (137.5 g, 0.941 mole), Pd (OAc)₂ (5.9 g, 0.026 mole) in ACN (550 ml) was added vinyl acetate (250 g, 2.907 mole) at RT. The reaction mixture was stirred at 60 °C for 24 h. The reaction mixture was filtered through a celite and washed with EtOAc (100 ml). The solvent was concentrated under reduced pressure to get residue. The residue was recrystallization from a mixture of acetone (68 ml), EtOAc (68 ml). Afford a (2R, 3R)-3-hydroxy-2-(hydroxymethyl)-2H-pyran-4(3H)-one (75 g, 55%) as an off white solid. TLC system (EtOAc, RF=0.39, UV active). ¹H NMR (400 MHz, DMSO-d₆) δ = 7.60 (d, J = 5.9 Hz, 1H), 5.61 (d, J = 4.9 Hz, 1H), 5.30 (d, J = 5.4 Hz, 1H), 5.00 (t, J = 5.6 Hz, 1H), 4.16 - 4.06 (m, 2H), 3.82 - 3.76 (m, 1H), 3.73 - 3.66 (m, 1H).

Step 2: Synthesis of (2R, 3R)-3-(tert-butyldimethylsilyloxy)-2-((tert-butyldimethylsilyloxy)methyl)-2H-pyran-4(3H)-one (3).

To a solution of compound (2R, 3R)-3-hydroxy-2-(hydroxymethyl)-2H-pyran-4(3H)-one (100 g, 0.69 mole) in DMF (1900 ml), were added TBS-Cl (277 g, 2.65 mole), Imidazole (140.9 g, 2.07 mole) at RT. The reaction mixture was stirred at RT for 24 h. The progress of the reaction was monitored by TLC. TLC indicated SM was consumed. TLC system (5% EtOAc in Pet Ether, RF=0.5, UV active). After completion of reaction, the reaction mixture was diluted with diethyl ether (2500 ml) and washed with water (1000 ml), brine (1000 ml). The diethyl ether layer was dried over anhydrous sodium sulphate. Then filtered, concentrated, crude formed. Crude purified by column chromatography (100-200 silica gel/eluent 2% EtOAc in Pet Ether) to give (2R, 3R)-3-(tert-butyldimethylsilyloxy)-2-((tert-butyldimethylsilyloxy)methyl)-2H-pyran-4(3H)-one (200 g, 77.5%) as an off white solid. ¹H NMR (400 MHz, CHLOROFORM-d) δ = 7.30 - 7.25 (m, 1H), 5.30 (d, J = 5.8 Hz, 1H), 4.45

- 4.40 (m, 1H), 4.18 (td, J = 2.6, 12.2 Hz, 1H), 3.99 (d, J = 2.8 Hz, 2H), 0.91 (d, J = 2.5 Hz, 18H), 0.23 (s, 3H), 0.11 - 0.05 (m, 6H); LCMS: 99.48% (373.37, M+H), RT: 3.47 min.

Step 3: Synthesis of methyl 2-((2S, 5R, 6R)-5-(tert-butyldimethylsilyloxy)-6-((tert-butyldimethylsilyloxy) methyl)-4-oxotetrahydro-2H-pyran-2-yl) acetate (4).

To a stirred solution of (2R,3R)-3-(tert-butyldimethylsilyloxy)-2-((tert-butyldimethylsilyloxy)methyl)-2H-pyran-4(3H)-one (55 g, 0.147 mole) in DCM(2000 ml) was added tert-butyl(1-methoxyvinyl)dimethylsilane (55 g, 0.292 mole) at RT. The reaction mixture was stirred at RT for 2 h. After BF₃.Et₂O (63.25 g, 3.03 mole) was added to the reaction mixture at -78 °C. The reaction mixture was stirred at -78 °C for 1 h. The progress of the reaction was monitored by TLC. TLC indicated SM was consumed. TLC system (15% EtOAc in Pet Ether, RF=0.5, PMA active). The reaction mixture was quenched with saturated NH₄Cl (500 ml) and extracted with DCM (2x250 ML). The DCM layer was washed with saturated NaHCO₃ (500 ml), brine (500 ml). The DCM layer was dried over anhydrous sodium sulphate. Then mixture was filtered, concentrated, and crude formed. The crude material was purified by column chromatography (100-200 silica gel/eluent 3% EtOAc in Pet Ether) to give methyl 2-((2S, 5R, 6R)-5-(tert-butyldimethylsilyloxy)-6-((tert-butyldimethylsilyloxy) methyl)-4-oxotetrahydro-2H-pyran-2-yl) acetate (25 g, 37.9%) as a transparent gum. ¹H NMR (400 MHz, CHLOROFORM-d) δ = 4.92 - 4.72 (m, 1H), 4.33 (d, J = 8.8 Hz, 1H), 3.91 - 3.78 (m, 2H), 3.74 - 3.58 (m, 4H), 2.83 - 2.60 (m, 2H), 2.52 - 2.38 (m, 2H), 0.99 - 0.79 (m, 18H), 0.15 (s, 3H), 0.11 - 0.01 (m, 9H)

Step 4: Synthesis of methyl 2-((2S, 5S, 6R)-5-(tert-butyldimethylsilyloxy)-6-((tert-butyldimethylsilyloxy) methyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (5).

To a suspension of methyltriphenylphosphoniumbromide (12 g, 0.033 mole) in THF (180 ml) at 0 °C, was added a THF (80 ml) solution of KOtBu (3.33 g, 0.029 mole). After 1 h, a THF (60 ml) solution of methyl 2-((2S, 5R, 6R)-5-(tert-butyldimethylsilyloxy)-6-((tert-butyldimethylsilyloxy) methyl)-4-oxotetrahydro-2H-pyran-2-yl) acetate (10 g, 0.022 mole) was added drop wise. Then reaction mixture allowed to 25 °C and stirred for 1 hr. The progress of the reaction was monitored by TLC. TLC indicated SM was consumed. TLC system (5% EtOAc in Pet Ether, RF=0.3, PMA active). The reaction mixture was quenched with saturated NH₄Cl (100 ml) and extracted with EtOAc (2x150 ml). The EtOAc layer was dried over anhydrous sodium sulphate. Then filtered, concentrated, crude formed. Crude purified by column chromatography (100-200 silica gel/eluent 5% EtOAc in Pet Ether) to

give methyl 2-((2S, 5S, 6R)-5-(tert-butyldimethylsilyloxy)-6-((tert-butyldimethylsilyloxy)methyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (4 g, 40.4%) as a Yellow colour liquid. ¹H NMR (400 MHz, CHLOROFORM-d) δ = 5.07 (s, 1H), 4.89 - 4.86 (m, 1H), 4.30 - 4.23 (m, 1H), 4.02 (d, J = 6.5 Hz, 1H), 3.75 - 3.66 (m, 5H), 3.47 (td, J = 4.7, 6.4 Hz, 1H),
5 2.65 (dd, J = 7.3, 15.0 Hz, 1H), 2.48 (dd, J = 6.8, 15.0 Hz, 1H), 2.42 - 2.28 (m, 2H), 0.95 - 0.84 (m, 18H), 0.12 - 0.01 (m, 12H)

Step 5: Synthesis of methyl 2-((2S, 5S, 6R)-5-(tert-butyldimethylsilyloxy)-6-(hydroxymethyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (6).

10 To a stirred solution of compound methyl 2-((2S, 5S, 6R)-5-(tert-butyldimethylsilyloxy)-6-((tert-butyldimethylsilyloxy)methyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (75.1 g, 0.168 mole) in MeOH (751 ml) was added PPTS (59.4 g, 0.236 mole) at RT. The reaction mixture was stirred at RT for 16 h. The progress of the reaction was monitored by TLC. TLC indicated SM was consumed. TLC system (25% EtOAc in Pet
15 Ether, RF=0.3, PMA active). The reaction mixture was quenched with saturated NaHCO₃ (1270 ml) and extracted with EtOAc (2x 1500 ml). The EtOAc layer was dried over anhydrous sodium sulphate. Then filtered, concentrated, crude formed. Crude purified by column chromatography (100-200 silica gel/eluent 25% EtOAc in Pet Ether) to methyl 2-
20 ((2S, 5S, 6R)-5-(tert-butyldimethylsilyloxy)-6-(hydroxymethyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (39 g, 69.8%) as a yellow color gum. ¹H NMR (400 MHz, CHLOROFORM-d) δ = 5.11 (s, 1H), 4.88 (s, 1H), 4.42 - 4.35 (m, 1H), 3.93 (d, J = 7.3 Hz, 1H), 3.71 - 3.64 (m, 5H), 3.56 - 3.50 (m, 1H), 2.73 (dd, J = 8.8, 15.4 Hz, 1H), 2.51 - 2.42 (m, 2H), 2.31 (dd, J = 3.8, 13.4 Hz, 1H), 2.15 (t, J = 6.4 Hz, 1H), 0.94 - 0.91 (m, 9H), 0.10 - 0.08 (m, 3H), 0.05 - 0.03 (m, 3H). LCMS: 80.17% (331.39, M+H), RT: 2.78 min

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Step 6: Synthesis of methyl 2-((2S, 5S, 6S)-5-(tert-butyldimethylsilyloxy)-6-formyl-4-methylenetetrahydro-2H-pyran-2-yl) acetate (7).

To a stirred solution of oxalyl chloride (17.2 g, 0.136 mole) in DCM (214 ml) at -78 °C was slowly added DMSO (21 g, 0.27 mole). The reaction mixture was stirred at -78 °C to
30 -60 °C for 20 min. Then a solution of compound to methyl 2-((2S, 5S, 6R)-5-(tert-butyldimethylsilyloxy)-6-(hydroxymethyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (30 g, 0.09 mole) in DCM (414 ml) was added drop wise. The reaction mixture was slowly warmed to -45 °C over 30 min. After TEA (52.8 g, 0.522 mole) was added at -45 °C and the reaction mixture was warmed to 0 °C over 10 min. The reaction progress was monitored by

TLC. TLC showed SM was consumed. TLC system (20% EtOAc in Pet Ether, RF=0.35, PMA active). The reaction mixture was quenched with saturated NH₄Cl (500 ml) and extracted with DCM (2x500 ML). The DCM layer was washed with brine (1000 ml). The DCM layer was dried over anhydrous sodium sulphate. Then filtered, concentrated, crude formed. Crude purified by column chromatography (100-200 silica gel/eluent 20% EtOAc in Pet Ether) to give methyl 2-((2S,5S,6S)-5-(tert-butyldimethylsilyloxy)-6-formyl-4-methylenetetrahydro-2H-pyran-2-yl)acetate (22 g, 73.8%) as a yellow color gum. ¹H NMR (400 MHz, CHLOROFORM-d) δ = 9.76 - 9.73 (m, 1H), 5.05 - 5.00 (m, 1H), 4.93 - 4.87 (m, 1H), 4.36 (d, J = 4.3 Hz, 1H), 4.29 - 4.20 (m, 1H), 4.12 - 4.08 (m, 1H), 3.73 - 3.67 (m, 4H), 2.78 - 2.69 (m, 1H), 2.55 - 2.40 (m, 1H), 2.27 - 2.20 (m, 2H), 0.96 - 0.87 (m, 9H), 0.12 - 0.02 (m, 6H); LCMS: 97.53% (329.34, M+H), RT: 2.60 min

Step 7: Synthesis of methyl 2-((2S, 5S, 6R)-5-((tert-butyldimethylsilyl) oxy)-6-((E)-2-iodovinyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (8).

To a stirred solution of methyl 2-((2S, 5S, 6S)-5-((tert-butyldimethylsilyl) oxy)-6-formyl-4-methylenetetrahydro-2H-pyran-2-yl) acetate (2 g, 6.088 m moles) in THF (140 mL), stirred at 26°C, CrCl₂ (4.489 g, 36.531 m moles) and CHI₃ (7.191 g, 18.265 m moles) were added and stirred at the same temperature for 16 h. TLC indicated completion of the reaction (TLC system: 20% of EtOAc in pet ether, Rf: 0.7, PMA active). Reaction mixture was diluted with ice-cold water (50 mL) and the product was extracted with EtOAc (2x50 mL). Combined organic layer was dried over Na₂SO₄ and concentrated to get the crude product (2.8 g) which was purified by normal phase chromatography using silica (100-200 mesh) and 4% of EtOAc in pet ether as an eluent. Collected fractions were evaporated under reduced pressure at 28°C to get methyl 2-((2S, 5S, 6R)-5-((tert-butyldimethylsilyl) oxy)-6-((E)-2-iodovinyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate as a pale yellow liquid (800 mg, Yield 28%). ¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 6.49 - 6.62 (m, 1 H) 6.31 - 6.44 (m, 1 H) 5.09 - 5.22 (m, 1 H) 4.83 - 4.96 (m, 1 H) 4.35 - 4.45 (m, 1 H) 3.84 - 3.92 (m, 1 H) 3.75 - 3.82 (m, 1 H) 3.65 - 3.72 (m, 3 H) 2.63 - 2.72 (m, 1 H) 2.42 - 2.55 (m, 2 H) 2.25 - 2.36 (m, 1 H) 0.87 - 0.96 (m, 9 H) -0.01 - 0.02 (m, 6 H); LCMS: 85.58% (453, M+H & 475 M+23 (Na adduct), RT: 2.8 min.

Step 8: Synthesis of methyl 2-((2S, 5S, 6R)-5-hydroxy-6-((E)-2-iodovinyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (9).

To a solution of methyl 2-((2S, 5S, 6R)-5-((tert-butyldimethylsilyl) oxy)-6-((E)-2-iodovinyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (800 mg, 1.768 m moles) in THF (40 mL), TBAF (1M in THF, 2.122 mL, 2.122 m moles) was added at 0°C then slowly allowed to reach to RT over 2 h when TLC indicated completion of the reaction (TLC system: 30% EtOAc in pet ether, Rf: 0.2, PMA active). Reaction mixture was quenched by adding NH₄Cl (Aq) (20 mL) and the product was extracted with EtOAc (2x20mL). Combined organic layer was dried over Na₂SO₄ and concentrated to get the crude product (2 g) which was purified by normal phase chromatography using silica (100-200 mesh) and 20% of the EtOAc in pet ether as an eluent. Collected fractions were evaporated to get methyl 2-((2S, 5S, 6R)-5-hydroxy-6-((E)-2-iodovinyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate as a pale yellow liquid (450 mg, Yield 76%). ¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 6.67 (br d, J=4.80 Hz, 2 H) 5.11 - 5.15 (m, 1 H) 4.95 - 5.00 (m, 1 H) 4.26 - 4.34 (m, 1 H) 4.08 - 4.16 (m, 1 H) 3.88 - 3.95 (m, 1 H) 3.66 - 3.73 (m, 3 H) 2.60 - 2.70 (m, 1 H) 2.44 - 2.51 (m, 1 H) 2.35 - 2.42 (m, 2 H) 1.96 - 2.00 (m, 1 H); LCMS: 89% (339.04, M+H), RT: 1.69 min

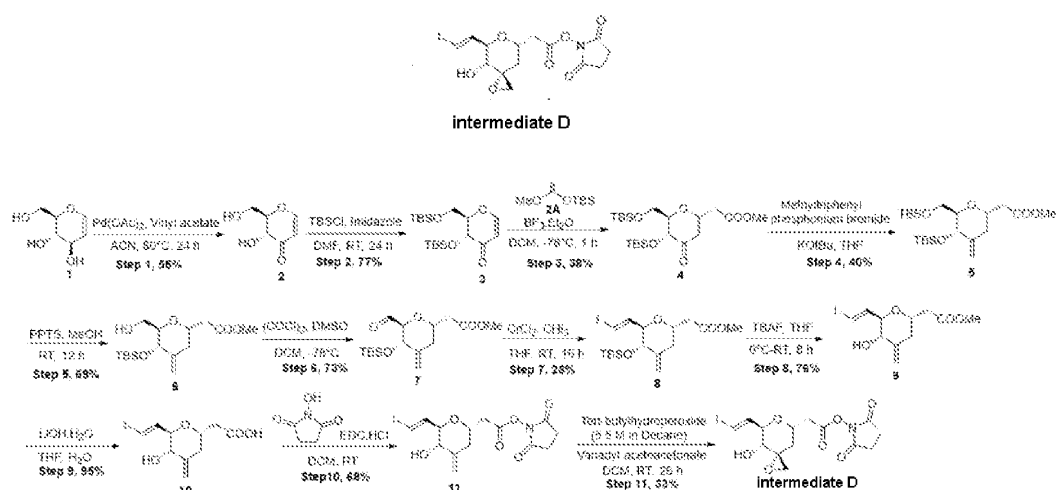
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Step 9: Synthesis of methyl 2-((3R, 5S, 7R, 8R)-8-hydroxy-7-((E)-2-iodovinyl)-1, 6-dioxaspiro [2.5] octan-5-yl) acetate (intermediate C).

To a solution of methyl 2-((2S, 5S, 6R)-5-hydroxy-6-((E)-2-iodovinyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (450 mg, 1.33 m moles) in DCM (27 mL), stirred at 0°C, V(acac)₂ (35.2 mg, 0.133 m moles) followed by ^{tert}BuOOH (5-6 M in decane, 0.53 mL, 2.66 m moles) was added and allowed to stir at RT for 4 h when TLC indicated completion of the reaction (TLC system: 50% EtOAc in pet ether, Rf: 0.3, PMA active). Reaction mixture was filtered through celite, washed with DCM (20 mL). The filtrate was concentrated to get the crude product (500 mg) which was purified by normal phase chromatography using silica (100-200 mesh) and 25% of EtOAc in pet ether as an eluent. Collected fractions were evaporated to get methyl 2-((3R, 5S, 7R, 8R)-8-hydroxy-7-((E)-2-iodovinyl)-1, 6-dioxaspiro [2.5] octan-5-yl) acetate as a pale yellow gum (220 mg, Yield 46%). ¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 6.70 (dd, J=14.60, 4.90 Hz, 1 H) 6.49 - 6.57 (m, 1 H) 4.55 (s, 1 H) 4.08 - 4.13 (m, 1 H) 3.47 - 3.52 (m, 1 H) 2.97 - 3.01 (m, 1 H) 2.88 - 2.96 (m, 1 H) 2.59 - 2.69 (m, 2 H) 2.18 (dd, J=14.17, 5.34 Hz, 1 H) 1.79 - 1.86 (m, 1 H) 1.68 - 1.76 (m, 1 H)

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Example 19. Preparation of intermediate D.



Step 1: Synthesis of (2R, 3R)-3-hydroxy-2-(hydroxymethyl)-2H-pyran-4(3H)-one (2).

- 5 A mixture of compound 1 (137.5 g, 0.941 mole), $\text{Pd}(\text{OAc})_2$ (5.9 g, 0.026 mole) in ACN (550 ml) was added vinyl acetate (250 g, 2.907 mole) at RT. The reaction mixture was stirred at 60°C for 24 h. The reaction mixture was filtered through a celite and washed with EtOAc (100 ml). The solvent was concentrated under reduced pressure to get residue. The residue was recrystallization from a mixture of acetone (68 ml), EtOAc (68 ml). Afford a
- 10 (2R, 3R)-3-hydroxy-2-(hydroxymethyl)-2H-pyran-4(3H)-one (75 g, 55%) as an off white solid. TLC system (EtOAc, $\text{RF}=0.39$, UV active). ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ = 7.60 (d, J = 5.9 Hz, 1H), 5.61 (d, J = 4.9 Hz, 1H), 5.30 (d, J = 5.4 Hz, 1H), 5.00 (t, J = 5.6 Hz, 1H), 4.16 - 4.06 (m, 2H), 3.82 - 3.76 (m, 1H), 3.73 - 3.66 (m, 1H).

- 15 Step 2: Synthesis of (2R, 3R)-3-(tert-butyldimethylsilyloxy)-2-((tert-butyldimethylsilyloxy)methyl)-2H-pyran-4(3H)-one (3):

- To a solution of compound (2R, 3R)-3-hydroxy-2-(hydroxymethyl)-2H-pyran-4(3H)-one (100 g, 0.69 mole) in DMF (1900 ml), were added TBS-Cl (277 g, 2.65 mole), Imidazole (140.9 g, 2.07 mole) at RT. The reaction mixture was stirred at RT for 24 h. The progress of
- 20 the reaction was monitored by TLC. TLC indicated SM was consumed. TLC system (5% EtOAc in Pet Ether, $\text{RF}=0.5$, UV active). After completion of reaction, the reaction mixture was diluted with diethyl ether (2500 ml) and washed with water (1000 ml), brine (1000 ml). The diethyl ether layer was dried over anhydrous sodium sulphate. Then filtered, concentrated, crude formed. Crude purified by column chromatography (100-200 silica
- 25 gel/eluent 2% EtOAc in Pet Ether) to give (2R, 3R)-3-(tert-butyldimethylsilyloxy)-2-((tert-

butyldimethylsilyloxy) methyl)-2H-pyran-4(3H)-one (200 g, 77.5%) as an off white solid. ¹H NMR (400 MHz, CHLOROFORM-d) δ = 7.30 - 7.25 (m, 1H), 5.30 (d, J = 5.8 Hz, 1H), 4.45 - 4.40 (m, 1H), 4.18 (td, J = 2.6, 12.2 Hz, 1H), 3.99 (d, J = 2.8 Hz, 2H), 0.91 (d, J = 2.5 Hz, 18H), 0.23 (s, 3H), 0.11 - 0.05 (m, 6H); LCMS: 99.48% (373.37, M+H), RT: 3.47 min.

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Step 3: Synthesis of methyl 2-((2S, 5R, 6R)-5-(tert-butyldimethylsilyloxy)-6-((tert-butyldimethylsilyloxy) methyl)-4-oxotetrahydro-2H-pyran-2-yl) acetate (4).

To a stirred solution of (2R,3R)-3-(tert-butyldimethylsilyloxy)-2-((tert-butyldimethylsilyloxy)methyl)-2H-pyran-4(3H)-one (55 g, 0.147 mole) in DCM(2000 ml) was added tert-butyl(1-methoxyvinyl)dimethylsilane (55 g, 0.292 mole) at RT. The reaction mixture was stirred at RT for 2 h. After BF₃.Et₂O (63.25 g, 3.03 mole) was added to the reaction mixture at -78 °C. The reaction mixture was stirred at -78 °C for 1 h. The progress of the reaction was monitored by TLC. TLC indicated SM was consumed. TLC system (15% EtOAc in Pet Ether, RF=0.5, PMA active). The reaction mixture was quenched with saturated NH₄Cl (500 ml) and extracted with DCM (2x250 ML). The DCM layer was washed with saturated NaHCO₃ (500 ml), brine (500 ml). The DCM layer was dried over anhydrous sodium sulphate. Then filtered, concentrated, crude formed. Crude purified by column chromatography (100-200 silica gel/eluent 3% EtOAc in Pet Ether) to give methyl 2-((2S, 5R, 6R)-5-(tert-butyldimethylsilyloxy)-6-((tert-butyldimethylsilyloxy) methyl)-4-oxotetrahydro-2H-pyran-2-yl) acetate (25 g, 37.9%) as a transparent gum. ¹H NMR (400 MHz, CHLOROFORM-d) δ = 4.92 - 4.72 (m, 1H), 4.33 (d, J = 8.8 Hz, 1H), 3.91 - 3.78 (m, 2H), 3.74 - 3.58 (m, 4H), 2.83 - 2.60 (m, 2H), 2.52 - 2.38 (m, 2H), 0.99 - 0.79 (m, 18H), 0.15 (s, 3H), 0.11 - 0.01 (m, 9H).

Step 4: Synthesis of methyl 2-((2S, 5S, 6R)-5-(tert-butyldimethylsilyloxy)-6-((tert-butyldimethylsilyloxy) methyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (5):

To a suspension of methyltriphenylphosphoniumbromide (12 g, 0.033 mole) in THF (180 ml) at 0 °C, was added a THF (80 ml) solution of KOtBu (3.33 g, 0.029 mole). After 1 h, a THF (60 ml) solution of methyl 2-((2S, 5R, 6R)-5-(tert-butyldimethylsilyloxy)-6-((tert-butyldimethylsilyloxy) methyl)-4-oxotetrahydro-2H-pyran-2-yl) acetate (10 g, 0.022 mole) was added drop wise. Then reaction mixture allowed to 25 °C and stirred for 1 hr. The progress of the reaction was monitored by TLC. TLC indicated SM was consumed. TLC system (5% EtOAc in Pet Ether, RF=0.3, PMA active). The reaction mixture was quenched with saturated NH₄Cl (100 ml) and extracted with EtOAc (2x150 ml). The EtOAc layer was

dried over anhydrous sodium sulphate. Then filtered, concentrated, crude formed. Crude purified by column chromatography (100-200 silica gel/eluent 5% EtOAc in Pet Ether) to give methyl 2-((2S, 5S, 6R)-5-(tert-butyldimethylsilyloxy)-6-((tert-butyldimethylsilyloxy)methyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (4 g, 40.4%) as a Yellow colour liquid. ¹H NMR (400 MHz, CHLOROFORM-d) δ = 5.07 (s, 1H), 4.89 - 4.86 (m, 1H), 4.30 - 4.23 (m, 1H), 4.02 (d, J = 6.5 Hz, 1H), 3.75 - 3.66 (m, 5H), 3.47 (td, J = 4.7, 6.4 Hz, 1H), 2.65 (dd, J = 7.3, 15.0 Hz, 1H), 2.48 (dd, J = 6.8, 15.0 Hz, 1H), 2.42 - 2.28 (m, 2H), 0.95 - 0.84 (m, 18H), 0.12 - 0.01 (m, 12H).

- 10 Step 5: Synthesis of methyl 2-((2S, 5S, 6R)-5-(tert-butyldimethylsilyloxy)-6-(hydroxymethyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (6).

To a stirred solution of compound methyl 2-((2S, 5S, 6R)-5-(tert-butyldimethylsilyloxy)-6-((tert-butyldimethylsilyloxy)methyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (75.1 g, 0.168 mole) in MeOH (751 ml) was added PPTS (59.4 g, 0.236 mole) at RT. The reaction mixture was stirred at RT for 16 h. The progress of the reaction was monitored by TLC. TLC indicated SM was consumed. TLC system (25% EtOAc in Pet Ether, RF=0.3, PMA active). The reaction mixture was quenched with saturated NaHCO₃ (1270 ml) and extracted with EtOAc (2x 1500 ml). The EtOAc layer was dried over anhydrous sodium sulphate. Then filtered, concentrated, crude formed. Crude purified by column chromatography (100-200 silica gel/eluent 25% EtOAc in Pet Ether) to methyl 2-((2S, 5S, 6R)-5-(tert-butyldimethylsilyloxy)-6-(hydroxymethyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (39 g, 69.8%) as a Yellow colour gum. ¹H NMR (400 MHz, CHLOROFORM-d) δ = 5.11 (s, 1H), 4.88 (s, 1H), 4.42 - 4.35 (m, 1H), 3.93 (d, J = 7.3 Hz, 1H), 3.71 - 3.64 (m, 5H), 3.56 - 3.50 (m, 1H), 2.73 (dd, J = 8.8, 15.4 Hz, 1H), 2.51 - 2.42 (m, 2H), 2.31 (dd, J = 3.8, 13.4 Hz, 1H), 2.15 (t, J = 6.4 Hz, 1H), 0.94 - 0.91 (m, 9H), 0.10 - 0.08 (m, 3H), 0.05 - 0.03 (m, 3H). LCMS: 80.17% (331.39, M+H), RT: 2.78 min.

Step 6: Synthesis of methyl 2-((2S, 5S, 6S)-5-(tert-butyldimethylsilyloxy)-6-formyl-4-methylenetetrahydro-2H-pyran-2-yl) acetate (7).

- 30 To a stirred solution of oxalyl chloride (17.2 g, 0.136 mole) in DCM (214 ml) at -78 °C was slowly added DMSO (21 g, 0.27 mole). The reaction mixture was stirred at -78 °C to -60 °C for 20 min. Then a solution of compound to methyl 2-((2S, 5S, 6R)-5-(tert-butyldimethylsilyloxy)-6-(hydroxymethyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (30 g, 0.09 mole) in DCM (414 ml) was added drop wise. The reaction mixture was slowly

warmed to -45 °C over 30 min. After TEA(52.8 g, 0.522 mole) was added at -45 °C and the reaction mixture was warmed to 0 °C over 10 min. The reaction progress was monitored by TLC. TLC showed SM was consumed. TLC system (20% EtOAc in Pet Ether, RF=0.35, PMA active). The reaction mixture was quenched with saturated NH₄Cl (500 ml) and
5 extracted with DCM (2x500 ML). The DCM layer was washed with brine (1000 ml). The DCM layer was dried over anhydrous sodium sulphate. Then filtered, concentrated, crude formed. Crude purified by column chromatography (100-200 silica gel/eluent 20% EtOAc in Pet Ether) to give methyl 2-((2S,5S,6S)-5-(tert-butyldimethylsilyloxy)-6-formyl-4-methylenetetrahydro-2H-pyran-2-yl)acetate (22 g , 73.8%) as a Yellow colour gum. ¹H NMR
10 (400 MHz, CHLOROFORM-d) δ = 9.76 - 9.73 (m, 1H), 5.05 - 5.00 (m, 1H), 4.93 - 4.87 (m, 1H), 4.36 (d, J = 4.3 Hz, 1H), 4.29 - 4.20 (m, 1H), 4.12 - 4.08 (m, 1H), 3.73 - 3.67 (m, 4H), 2.78 - 2.69 (m, 1H), 2.55 - 2.40 (m, 1H), 2.27 - 2.20 (m, 2H), 0.96 - 0.87 (m, 9H), 0.12 - - 0.02 (m, 6H); LCMS: 97.53% (329.34, M+H), RT: 2.60 min.

15 Step 7: Synthesis of methyl 2-((2S, 5S, 6R)-5-((tert-butyldimethylsilyl) oxy)-6-((E)-2-iodovinyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (8).

To a stirred solution of methyl 2-((2S, 5S, 6S)-5-((tert-butyldimethylsilyl) oxy)-6-formyl-4-methylenetetrahydro-2H-pyran-2-yl) acetate (2 g, 6.088 m moles) in THF (140 mL), stirred at 26°C, CrCl₂ (4.489 g, 36.531 m moles) and CHI₃ (7.191 g, 18.265 m moles)
20 were added and stirred at the same temperature for 16 h. TLC indicated completion of the reaction (TLC system: 20% of EtOAc in pet ether, Rf: 0.7, PMA active). Reaction mixture was diluted with ice-cold water (50 mL) and the product was extracted with EtOAc (2x50 mL). Combined organic layer was dried over Na₂SO₄ and concentrated to get the crude product (2.8 g) which was purified by normal phase chromatography using silica (100-200
25 mesh) and 4% of EtOAc in pet ether as an eluent. Collected fractions were evaporated under reduced pressure at 28°C to get methyl 2-((2S, 5S, 6R)-5-((tert-butyldimethylsilyl) oxy)-6-((E)-2-iodovinyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate as a pale yellow liquid (800 mg, Yield 28%). ¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 6.49 - 6.62 (m, 1 H) 6.31 - 6.44 (m, 1 H) 5.09 - 5.22 (m, 1 H) 4.83 - 4.96 (m, 1 H) 4.35 - 4.45 (m, 1 H) 3.84 - 3.92 (m, 1
30 H) 3.75 - 3.82 (m, 1 H) 3.65 - 3.72 (m, 3 H) 2.63 - 2.72 (m, 1 H) 2.42 - 2.55 (m, 2 H) 2.25 - 2.36 (m, 1 H) 0.87 - 0.96 (m, 9 H) -0.01 - 0.02 (m, 6 H); LCMS: 85.58% (453, M+H & 475 M+23 (Na adduct), RT: 2.8 min.

Step 8: Synthesis of methyl 2-((2S, 5S, 6R)-5-hydroxy-6-((E)-2-iodovinyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (9).

To a solution of methyl 2-((2S, 5S, 6R)-5-((tert-butyldimethylsilyl) oxy)-6-((E)-2-iodovinyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (800 mg, 1.768 m moles) in THF (40 mL), TBAF (1M in THF, 2.122 mL, 2.122 m moles) was added at 0°C then slowly allowed to reach to RT over 2 h when TLC indicated completion of the reaction (TLC system: 30% EtOAc in pet ether, Rf: 0.2, PMA active). Reaction mixture was quenched by adding NH₄Cl (Aq) (20 mL) and the product was extracted with EtOAc (2x20mL). Combined organic layer was dried over Na₂SO₄ and concentrated to get the crude product (2 g) which was purified by normal phase chromatography using silica (100-200 mesh) and 20% of the EtOAc in pet ether as an eluent. Collected fractions were evaporated to get methyl 2-((2S, 5S, 6R)-5-hydroxy-6-((E)-2-iodovinyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate as a pale yellow liquid (450 mg, Yield 76%). ¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 6.67 (br d, J=4.80 Hz, 2 H) 5.11 - 5.15 (m, 1 H) 4.95 - 5.00 (m, 1 H) 4.26 - 4.34 (m, 1 H) 4.08 - 4.16 (m, 1 H) 3.88 - 3.95 (m, 1 H) 3.66 - 3.73 (m, 3 H) 2.60 - 2.70 (m, 1 H) 2.44 - 2.51 (m, 1 H) 2.35 - 2.42 (m, 2 H) 1.96 - 2.00 (m, 1 H); LCMS: 89% (339.04, M+H), RT: 1.69 min.

Step 9: Synthesis of 2-((2S, 5S, 6R)-5-hydroxy-6-((E)-2-iodovinyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetic acid (10).

To a stirred solution of methyl 2-((2S,5S,6R)-5-hydroxy-6-((E)-2-iodovinyl)-4-methylenetetrahydro-2H-pyran-2-yl)acetate (4.35 g, 12.86 momoles) in THF (30 mL) and water (30 mL), LiOH.H₂O (2.165 g, 51.6 m moles) was added at 0°C and then slowly allowed to reach to RT over 3 h when TLC indicated completion of the reaction (TLC system: 50% of EtOAc in pet ether, Rf: 0.1, PMA active). Reaction mixture was neutralized using a solution of citric acid (Aq) at 0°C and extracted with EtOAc (3x40 mL). The organic layer was dried over Na₂SO₄ and concentrated at 30°C under reduced pressure to get 2-((2S,5S,6R)-5-hydroxy-6-((E)-2-iodovinyl)-4-methylenetetrahydro-2H-pyran-2-yl)acetic acid as a pale yellow gum (4.1 g crude) which was taken forward in subsequent reaction without further purification. ¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 6.53 - 6.66 (m, 2 H) 5.14 - 5.17 (m, 1 H) 5.01 (s, 1 H) 4.32 (dq, J=7.83, 5.71 Hz, 1 H) 4.09 - 4.16 (m, 1 H) 3.93 - 3.97 (m, 1 H) 2.71 (dd, J=15.65, 7.83 Hz, 1 H) 2.51 - 2.60 (m, 1 H) 2.43 (d, J=5.38 Hz, 2 H); LCMS: 87.45% (323, M-H), RT: 1.42 min.

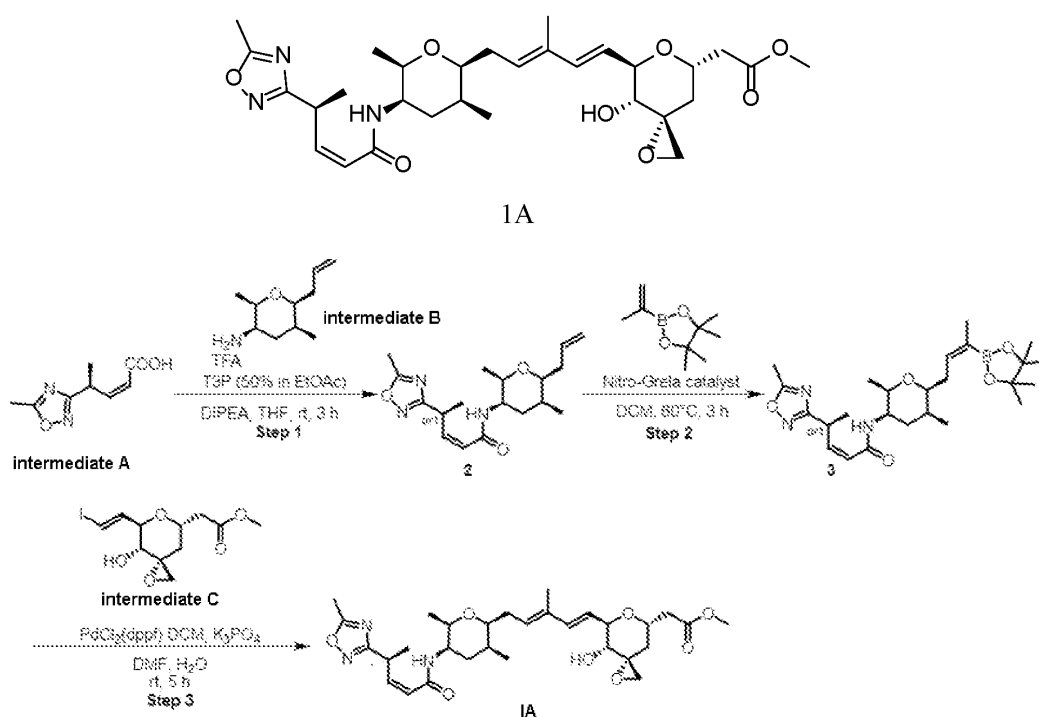
Step 10: Synthesis of 2, 5-dioxopyrrolidin-1-yl 2-((2S, 5S, 6R)-5-hydroxy-6-((E)-2-iodovinyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (11).

To a solution of 2-((2S,5S,6R)-5-hydroxy-6-((E)-2-iodovinyl)-4-methylenetetrahydro-2H-pyran-2-yl)acetic acid (4 g, 12.345 m moles) in DCM (80 mL), stirred at 25°C, EDC.HCl (3.559 g, 18.518 m moles) and N-hydroxy succinamide (2.131 g, 18.518 m moles) were added and stirred at the same temperature for 3 h when TLC indicated completion of the reaction (TLC system: 60% of EtOAc in pet ether, Rf: 0.5, UV and PMA active). Reaction mixture was concentrated under reduced pressure at 30°C. Obtained residue was purified by normal phase chromatography (by Combi-Flash) using 24 g of silica gel column and 40% of EtOAc in pet ether as an eluent. Collected fractions were evaporated to get 2, 5-dioxopyrrolidin-1-yl 2-((2S, 5S, 6R)-5-hydroxy-6-((E)-2-iodovinyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (3.5 g, Yield 68%) as a pale yellow gum. ¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 6.55 - 6.67 (m, 2 H) 5.16 (s, 1 H) 5.04 (s, 1 H) 4.30 - 4.40 (m, 1 H) 4.14 - 4.21 (m, 1 H) 3.92 - 4.00 (m, 1 H) 2.82 - 2.90 (m, 6 H) 2.51 - 2.59 (m, 1 H) 2.38 - 2.46 (m, 1 H) 2.27 - 2.34 (m, 1 H); LCMS: 93.7% (422.02, M+H), RT: 1.64 min.

Step 11: Synthesis of methyl 2-((3R, 5S, 7R, 8R)-8-hydroxy-7-((E)-2-iodovinyl)-1, 6-dioxaspiro [2.5] octan-5-yl) acetate (intermediate D).

To a solution of 2,5-dioxopyrrolidin-1-yl 2-((2S,5S,6R)-5-hydroxy-6-((E)-2-iodovinyl)-4-methylenetetrahydro-2H-pyran-2-yl)acetate (3.5 g, 8.31 m moles) in DCM (180 mL), stirred at 0°C, vanadyl acetylacetonate (330 mg, 1.245 m moles) followed by tBuOOH (4 mL, 5-6 M in decane, 20.75 m moles) were added and then slowly allowed to stir at RT for 4 h when TLC indicated completion of the reaction. Reaction mixture was filtered through celite, washed with DCM (20 mL). The filtrate was concentrated at 30°C to get the crude product (3.8 g) which was purified by normal phase silica gel chromatography (by Combi-Flash) using 24 g of silica gel column and 50% of EtOAc in pet ether as an eluent. Collected fractions were evaporated to get 2-((3R, 5S, 7R, 8R)-8-hydroxy-7-((E)-2-iodovinyl)-1, 6-dioxaspiro [2.5] octan-5-yl) acetate (1.2 g, Yield 33%) as an off white foamy solid. ¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 6.68 - 6.77 (m, 1 H) 6.59 - 6.66 (m, 1 H) 4.52 - 4.61 (m, 1 H) 4.08 - 4.13 (m, 1 H) 3.52 (dd, J=9.05, 7.52 Hz, 1 H) 3.13 - 3.23 (m, 1 H) 2.93 - 3.04 (m, 2 H) 2.67 - 2.72 (m, 1 H) 2.17 - 2.24 (m, 1 H) 1.93 - 1.99 (m, 1 H) 1.81 - 1.88 (m, 1 H); LCMS: 90.47% (438.14, M+H), RT: 1.49 min.

Example 20. Preparation of compound 1A



Step 1: Synthesis of (S,Z)-N-((2R,3R,5S,6S)-6-allyl-2,5-dimethyltetrahydro-2H-pyran-3-yl)-
 5 4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamide.

To a stirred solution of (S,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enoic acid (300
 mg 1.65 mmol) in THF (30 mL) was added (2R,3R,5S,6S)-6-allyl-2,5-dimethyltetrahydro-
 2H-pyran-3-amine (528 mg, 1.9 mmol), DIPEA (1279 mg, 9.9 mmol) and T3P (50% in
 EtOAc) (1575 mg, 4.95 mmol) at rt. The reaction mixture was stirred at rt for 3 h. The
 10 reaction was monitored by TLC. TLC showed SM was absent. TLC System: 50% EtOAc in
 hexane ($R_f=0.5$, UV visible). The reaction mixture was concentrated under reduced pressure
 to get 400 mg of crude compound as a brown colour gum. The crude compound was purified
 was purified by silica column chromatography using 30% ethyl acetate in hexane as an
 eluent. The pure fractions were collected and evaporated (Below 30°C) under reduced
 15 pressure to get (S,Z)-N-((2R,3R,5S,6S)-6-allyl-2,5-dimethyltetrahydro-2H-pyran-3-yl)-4-(5-
 methyl-1,2,4-oxadiazol-3-yl)pent-2-enamide as pale yellow gum. LCMS (SM-14644-111-
 1): 79.67%, RT: 2.84min, $[M+H]^+$: 334.48

Step 2: Synthesis of (S,Z)-N-((2R,3R,5S,6S)-2,5-dimethyl-6-((E)-3-(4,4,5,5-tetramethyl-
 20 1,3,2-dioxaborolan-2-yl)but-2-en-1-yl)tetrahydro-2H-pyran-3-yl)-4-(5-methyl-1,2,4-
 oxadiazol-3-yl)pent-2-enamide (1A).

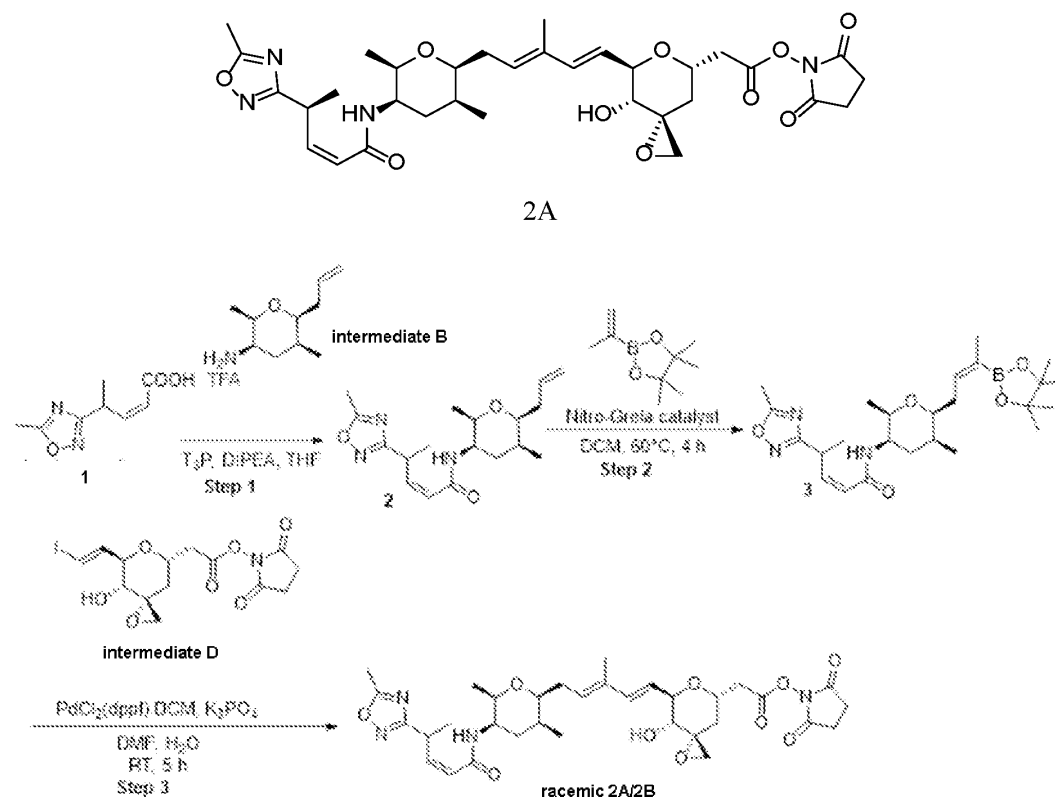
To a stirred solution of (S,Z)-N-((2R,3R,5S,6S)-6-allyl-2,5-dimethyltetrahydro-2H-pyran-3-yl)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamide (260 mg, 0.78 mmol) in DCM (20 mL) was added 4,4,5,5-tetramethyl-2-(prop-1-en-2-yl)-1,3,2-dioxaborolane (1311 mg, 7.8 mmol) and Nitro-Grela catalyst (104 mg, 0.16 mmol). The reaction mixture was stirred at 60 °C for 3 h. The progress of the reaction was monitored by TLC. TLC indicated SM was consumed. TLC system (30% EtOAc in Hexane, R_f: 0.3, UV visible). The reaction mixture was filtered through celite pad and the filtrate was evaporated to get crude compound. The crude compound was purified by flash silica column chromatography using 50% ethyl acetate in hexane as an eluent. The pure fractions were collected and evaporated under reduced pressure to get (S,Z)-N-((2R,3R,5S,6S)-2,5-dimethyl-6-((E)-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)but-2-en-1-yl)tetrahydro-2H-pyran-3-yl)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamide as a brown colour gum. LCMS (SM-14644-114-1): 60.12%, RT: 3.39min, [M+H]⁺: 474.61.

Step 3: Synthesis of methyl 2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((S,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetate.

To a stirred solution of (S,Z)-N-((2R,3R,5S,6S)-2,5-dimethyl-6-((E)-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)but-2-en-1-yl)tetrahydro-2H-pyran-3-yl)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamide (100 mg, 0.21 mmol) in DMF (6 mL) & water (0.4 mL) at rt, was added methyl 2-((3R,5S,7R,8R)-8-hydroxy-7-((E)-2-iodovinyl)-1,6-dioxaspiro[2.5]octan-5-yl)acetate (74.3 mg, 0.21 mmol) and degassed with argon for 20 min, then K₃PO₄ (89 mg, 0.42 mmol) & Pd(dppf)Cl₂-DCM (17 mg, 0.021 mmol) were added and the reaction was stirred at rt for 5 h. Reaction progress was monitored by TLC. TLC system: 80%EtOAc in Hexane (R_f: 0.3, UV visible). The reaction mixture was diluted with EtOAc (20 mL), washed with brine (20 mL) & cold water (2 x 20 mL), dried over anhydrous sodium sulphate, filtered and concentrated to get crude compound (SR-12580-124-A1) as a brown colour gum. The crude residue was purified by Prep. HPLC techniques to afford methyl 2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((S,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetate (SR-12580-124-A2) as an off-white solid. LCMS (SR-12580-124-A2): 98.03%, RT: 2.55min, [M+H]⁺: 574.72.

Example 21. Preparation of compound 2A

Compound 2A was prepared by the method described in the example, except that intermediate A of example 16 was used in place of the racemic compound 1.



5

Step 1: Synthesis of (Z)-N-((2R,3R,5S,6S)-6-allyl-2,5-dimethyltetrahydro-2H-pyran-3-yl)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamide.

To a stirred solution of (Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enoic acid (500 mg, 2.75 mmol) in THF (30 ml) was added (2R,3R,5S,6S)-6-allyl-2,5-dimethyltetrahydro-2H-pyran-3-amine (726.28 mg, 2.718.76 mmol), DIPEA (806.98 mg, 3.02 mmol) and T3P (50% in EtOAc) (2624.98 mg, 8.25 mmol). The reaction mixture was stirred for 16 hr. The reaction monitored by TLC. TLC showed SM was absent. TLC System: 50% EtOAc in pet ether. The reaction mixture was concentrated under reduced pressure to get 600 mg crude compound as a brown colour gum. The crude compound was purified by silica column using 30% ethyl acetate in hexane as an eluent. The pure fractions were collected and evaporated (Below 30°C) under reduced pressure to get (Z)-N-((2R,3R,5S,6S)-6-allyl-2,5-dimethyltetrahydro-2H-pyran-3-yl)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamide (450 mg, yield: 49%) as pale yellow gum.

¹H NMR (500 MHz, CHLOROFORM-d) δ ppm 6.36 - 6.49 (m, 1 H) 6.00 - 6.18 (m, 2 H) 5.73 - 5.86 (m, 2 H) 5.02 - 5.15 (m, 2 H) 3.96 (dtd, J=8.87, 4.61, 4.61, 2.67 Hz, 1 H) 3.62 - 3.69 (m, 1 H) 3.50 - 3.57 (m, 1 H) 2.53 - 2.59 (m, 3 H) 2.27 - 2.37 (m, 1 H) 2.06 - 2.17 (m, 1 H) 1.89 - 2.03 (m, 2 H) 1.72 - 1.82 (m, 1 H) 1.48 (t, J=6.56 Hz, 3 H) 1.23 - 1.33 (m, 1 H)
5 1.13 - 1.19 (m, 2 H) 1.08 - 1.12 (m, 2 H) 1.01 - 1.07 (m, 2 H) 0.90 - 0.99 (m, 3 H)

Step 2: Synthesis of (Z)-N-((2R,3R,5S,6S)-6-allyl-2,5-dimethyltetrahydro-2H-pyran-3-yl)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamide.

To a stirred solution of compound (Z)-N-((2R,3R,5S,6S)-6-allyl-2,5-dimethyltetrahydro-2H-pyran-3-yl)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamide in (450 mg, 1.35 mmol) DCM (50 mL) was added 4,4,5,5-tetramethyl-2-(prop-1-en-2-yl)-1,3,2-dioxaborolane (2269.4 mg, 13.5 mmol) and Nitro-Grela catalyst (181.17 mg, 0.27 mmol). The reaction mixture was stirred for 3 h at 60°C. The progress of the reaction was monitored by TLC. TLC indicated SM was consumed. TLC system (30% EtOAc in Hexane, Rf: 0.3, UV active). The reaction mixture was filtered through celite pad, filtrate was concentrated in vacuo to get crude compound.
10
15

The crude compound was purified by silica column using 40% ethyl acetate in hexane as an eluent. The pure fractions were collected and evaporated (Below 30°C) under reduced pressure to get (Z)-N-((2R,3R,5S,6S)-2,5-dimethyl-6-((E)-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)but-2-en-1-yl)tetrahydro-2H-pyran-3-yl)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamide (187 mg, yield: 29%) as brown color gum.
20

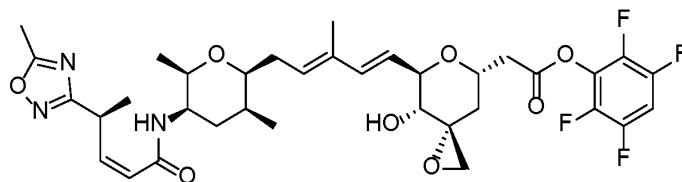
¹H NMR (500 MHz, CHLOROFORM-d) δ ppm 6.21 - 6.29 (m, 1 H) 6.10 - 6.19 (m, 1 H) 5.99 - 6.08 (m, 1 H) 5.78 - 5.87 (m, 1 H) 5.05 - 5.16 (m, 1 H) 4.85 - 4.95 (m, 1 H) 4.08 - 4.20 (m, 1 H) 3.89 - 4.01 (m, 1 H) 3.56 - 3.71 (m, 2 H) 2.56 (s, 3 H) 2.32 - 2.41 (m, 1 H) 2.21 - 2.31 (m, 1 H) 2.03 - 2.07 (m, 1 H) 1.88 - 1.96 (m, 3 H) 1.80 (br dd, J=14.50, 3.05 Hz, 1 H) 1.70 (br s, 2 H) 1.48 (br t, J=6.48 Hz, 4 H) 1.36 - 1.44 (m, 2 H) 1.21 - 1.31 (m, 19 H) 1.17 (br d, J=6.41 Hz, 2 H) 1.11 (br d, J=6.26 Hz, 2 H) 1.04 (br d, J=7.48 Hz, 2 H) 0.93 - 1.00 (m, 2 H)
25

Step 3: Synthesis of 2,5-dioxopyrrolidin-1-yl 2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetate.
30

To a stirred solution of (Z)-N-((2R,3R,5S,6S)-2,5-dimethyl-6-((E)-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)but-2-en-1-yl)tetrahydro-2H-pyran-3-yl)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamide (50 mg, 0.105 mmol) in DMF 94 mL & water (0.2 mL) at rt, was added 2,5-dioxopyrrolidin-1-yl 2-((3R,5S,7R,8R)-8-hydroxy-7-((E)-2-iodovinyl)-1,6-dioxaspiro[2.5]octan-5-yl)acetate (45 mg, 0.105 mmol) and degassed with argon for 20 min, then K₃PO₄ (33 mg, 0.157 mmol) & Pd(dppf)Cl₂-DCM (4.3 mg, 0.005 mmol) were added and the reaction was stirred at rt for 5 h. Reaction progress was monitored by TLC. TLC system: 80%EtOAc in Hexane (R_f: 0.2, UV visible). The reaction mixture was diluted with EtOAc (10 mL), washed with brine (20 mL) & cold water (2 x 20 mL), dried over anhydrous sodium sulphate, filtered and concentrated to get 50 mg of crude compound as a brown colour gum. The crude residue was purified by Prep. HPLC techniques, pure fractions were collected and lyophilized to afford 2,5-dioxopyrrolidin-1-yl 2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetate as a white solid.

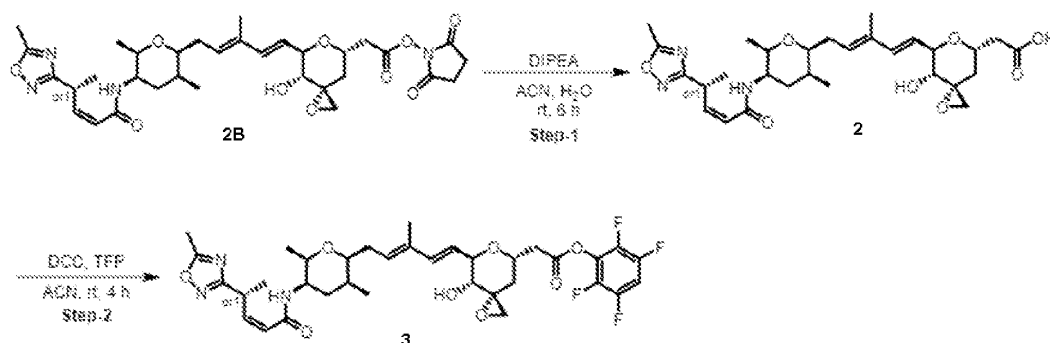
¹H NMR (400 MHz, CHLOROFORM-d) δ ppm Spectra was attached (compound is a diastereomeric mixture): LCMS (SR-12580-6-A3): 91.68% (36.46% + 55.22%), RT: 2.03 & 2.05 min, [M+H]⁺: 657.31.

20 Example 22. Preparation of compound 3A



3A

Compound 3A was prepared by the method described in the example, except that compound 2A of example 21 was used in place of compound 2B.



Step 1: Synthesis of 2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((R,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetic acid.

- 5 To a stirred solution of 2,5-dioxopyrrolidin-1-yl 2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((R,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetate (45 mg, 0.068 mmol) in Acetonitrile (1.0 mL) & water (0.5 mL) was added DIPEA (13 mg, 0.102 mmol) at rt. The reaction mixture was stirred at rt for 6
- 10 h. The reaction mixture was monitored by LCMS. Crude LCMS showed 57% of desired product mass. The reaction mixture was directly purified by Prep. HPLC purification. The pure fractions were collected at -78 °C and lyophilized to afford 2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((R,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-
- 15 dioxaspiro[2.5]octan-5-yl)acetic acid (8 mg, 21%) as a white solid.
LCMS (SR-12580-250-A3): 99.20%, 2.22min, M+H: 560.38.

- Step 2: Synthesis of 2,3,5,6-tetrafluorophenyl 2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((R,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-
- 20 dioxaspiro[2.5]octan-5-yl)acetate.

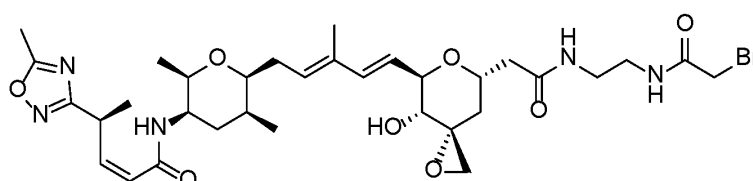
- To a stirred solution of 2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((R,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetic acid (16 mg, 0.03
- 25 mmol) in DMF (1.5 mL) was added 2,3,5,6-tetrafluorophenol (10 mg, 0.06 mmol) and DCC (9.3 mg, 0.045 mmol) stirred at rt for 4 h. The progress of the reaction was monitored by

LCMS. Crude LCMS showed 49% of desired product mass. The reaction mixture was directly purified by Prep. HPLC purification. The pure fractions were collected at -78 °C and lyophilized to afford 2,3,5,6-tetrafluorophenyl-2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((R,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetate (7 mg, 34.65%) as a white solid.

¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 6.96 - 7.05 (m, 1 H), 6.40 (d, J=15.74 Hz, 1 H), 6.14 (dd, J=11.32, 9.78 Hz, 1 H), 6.06 (br d, J=9.18 Hz, 1 H), 5.81 (dd, J=11.32, 0.83 Hz, 1 H), 5.65 (dd, J=15.74, 5.96 Hz, 1 H), 5.49 (br t, J=7.15 Hz, 1 H), 5.05 - 5.14 (m, 1 H), 4.60 - 4.67 (m, 1 H), 4.26 (t, J=6.62 Hz, 1 H), 3.95 - 4.00 (m, 1 H), 3.63 - 3.68 (m, 1 H), 3.58 (dd, J=8.40, 7.57 Hz, 1 H), 3.52 (td, J=7.21, 2.62 Hz, 1 H), 3.32 (dd, J=15.62, 8.34 Hz, 1 H), 2.98 - 3.06 (m, 2 H), 2.69 (d, J=4.53 Hz, 1 H), 2.56 (s, 3 H), 2.33 - 2.41 (m, 1 H), 2.16 - 2.29 (m, 2 H), 1.91 - 1.95 (m, 2 H), 1.73 - 1.86 (m, 6 H), 1.47 (d, J=7.03 Hz, 3 H), 1.16 (d, J=6.44 Hz, 3 H), 0.97 (d, J=7.39 Hz, 3 H).

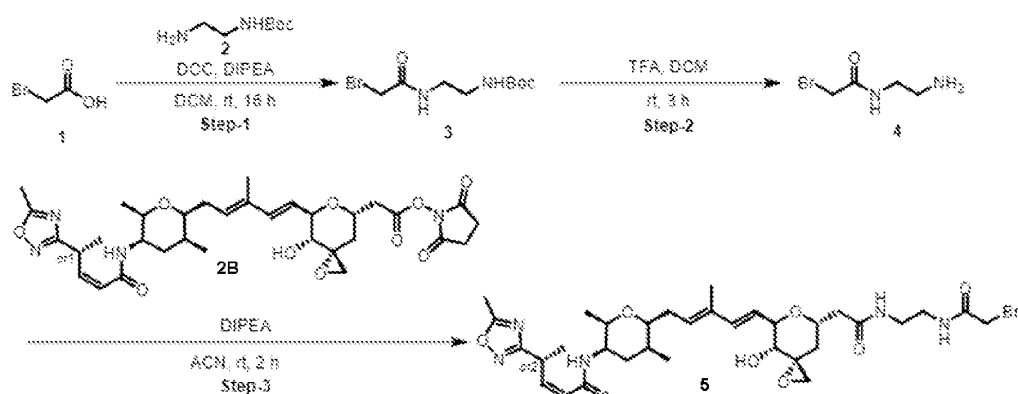
LCMS: 99.40%, RT: 2.93 min, M+H: 708.44.

Example 23. Preparation of compound 4A.



4A

Compound 4A was prepared by the method described in the example, except that compound 2A of example 21 was used in place of compound 2B.



Step 1: Synthesis of tert-butyl (2-(2-bromoacetamido)ethyl)carbamate.

To a stirred solution of 2-bromoacetic acid (2 g, 14.5 mmol) and tert-butyl (2-aminoethyl)carbamate (2.32 g, 14.5 mmol) in DCM (80 mL) was added DCC (2.98 g, 14.5 mmol) at 0 °C. The reaction was stirred at rt for 16 h. Progress of the reaction was monitored by TLC. The precipitated solid was filtered & the filtrate was concentrated under reduced pressure to get crude compound (SR-12580-176-A1). The crude residue was purified by flash column chromatography (50% to 100% EtOAc in Hexanes) on silica gel (100-200 mesh) to afford tert-butyl (2-(2-bromoacetamido) ethyl) carbamate (1.5 g, 38%) as a white solid.

¹H NMR (400 MHz, CHLOROFORM-d) δ ppm: 7.08 (br s, 1 H), 4.85 (br s, 1 H), 3.87 (s, 2 H), 3.38 - 3.42 (m, 2 H), 3.30 - 3.35 (m, 2 H), 1.45 (s, 9 H)

LCMS (SR-12580-176-A2): 91.20%, RT: 1.48 min, M+H: 281.00

Step 2: Synthesis of N-(2-aminoethyl)-2-bromoacetamide.

To a stirred solution of tert-butyl (2-(2-bromoacetamido)ethyl)carbamate (500 mg, 1.785 mmol) in DCM (10 mL) was added Trifluoro acetic acid (2 mL, 17.5 mmol) at room temperature. The reaction mixture was stirred at rt for 3 h. The reaction progress was monitored by TLC. TLC Mobile phase: 50% EtOAc in Hexane (Rf: 0.1, UV visible). The reaction mixture was concentrated under reduced pressure to afford N-(2-aminoethyl)-2-bromoacetamide (350 mg, TFA Salt) as a pale yellow gum.

¹H NMR (300 MHz, DMSO-d₆) δ ppm: 8.46 (br t, J=5.14 Hz, 1 H), 7.80 (br s, 2 H), 3.88 (s, 2 H), 3.27 - 3.40 (m, 2 H), 2.82 - 2.91 (m, 2 H).

LCMS (SR-12580-179-A2): 96.75%, 0.27 min, M+H: 181.04.

Step 3: Synthesis of (R,Z)-N-((2R,3R,5S,6S)-6-((2E,4E)-5-((3R,4R,5R,7S)-7-(2-((2-bromoacetamido)ethyl)amino)-2-oxoethyl)-4-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)-3-methylpenta-2,4-dien-1-yl)-2,5-dimethyltetrahydro-2H-pyran-3-yl)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamide.

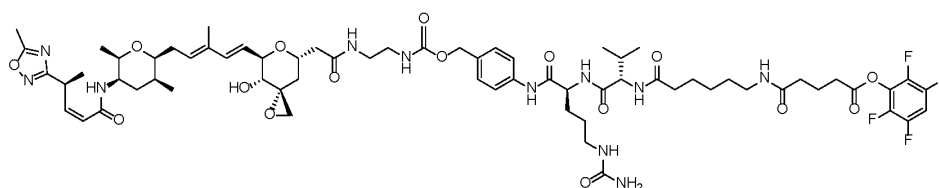
To a stirred solution of 2,5-dioxopyrrolidin-1-yl 2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((R,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetate (70 mg, 0.11 mmol) & N-(2-aminoethyl)-2-bromoacetamide (39 mg, 0.22 mmol) in acetonitrile (2 mL) was added DIPEA (21 mg, 0.165 mmol) at rt. The reaction mixture was stirred at rt for 2 h. The progress of the reaction was monitored by LCMS. Crude LCMS showed 49% of desired product mass. The reaction

mixture was directly purified by Prep. HPLC purification. The pure fractions were collected at -78 °C and lyophilized to afford (R,Z)-N-((2R,3R,5S,6S)-6-((2E,4E)-5-((3R,4R,5R,7S)-7-(2-((2-(2-bromoacetamido)ethyl)amino)-2-oxoethyl)-4-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)-3-methylpenta-2,4-dien-1-yl)-2,5-dimethyltetrahydro-2H-pyran-3-yl)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamide (16 mg, 21%) (SR-12580-300-A2) as a white solid.

¹H NMR (400 MHz, CHLOROFORM-d) δ ppm: 7.10 (br s, 1 H), 6.60 - 6.68 (m, 1 H), 6.37 (d, J=15.74 Hz, 1 H), 6.15 (dd, J=11.32, 9.78 Hz, 1 H), 6.04 (br d, J=9.06 Hz, 1 H), 5.81 (dd, J=11.44, 0.72 Hz, 1 H), 5.63 (dd, J=15.74, 6.44 Hz, 1 H), 5.49 - 5.55 (m, 1 H), 5.07 - 5.14 (m, 1 H), 4.39 - 4.43 (m, 1 H), 4.32 (t, J=6.02 Hz, 1 H), 3.95 - 4.00 (m, 1 H), 3.85 (s, 2 H), 3.65 - 3.69 (m, 1 H), 3.46 - 3.55 (m, 4 H), 3.34 - 3.40 (m, 2 H), 2.97 (d, J=4.53 Hz, 1 H), 2.72 (d, J=4.41 Hz, 1 H), 2.60 - 2.69 (m, 2 H), 2.56 (s, 3 H), 2.33 - 2.41 (m, 1 H), 2.18 - 2.26 (m, 1 H), 2.08 (d, J=6.91 Hz, 1 H), 1.90 - 1.96 (m, 4 H), 1.75 - 1.79 (m, 4 H), 1.47 (d, J=7.03 Hz, 3 H), 1.16 (d, J=6.44 Hz, 3 H), 0.97 - 1.00 (m, 3 H)

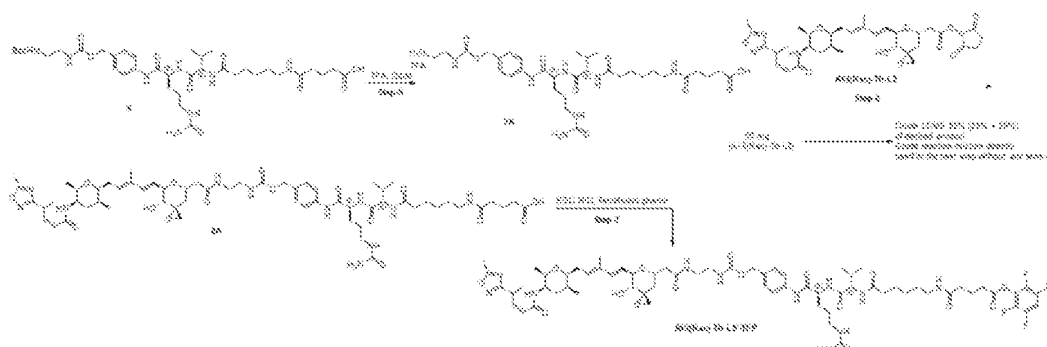
LCMS (SR-12580-300-A2): 95.44%, RT: 3.60min, M+H: 722.43.

Example 24. Preparation of compound 5A.



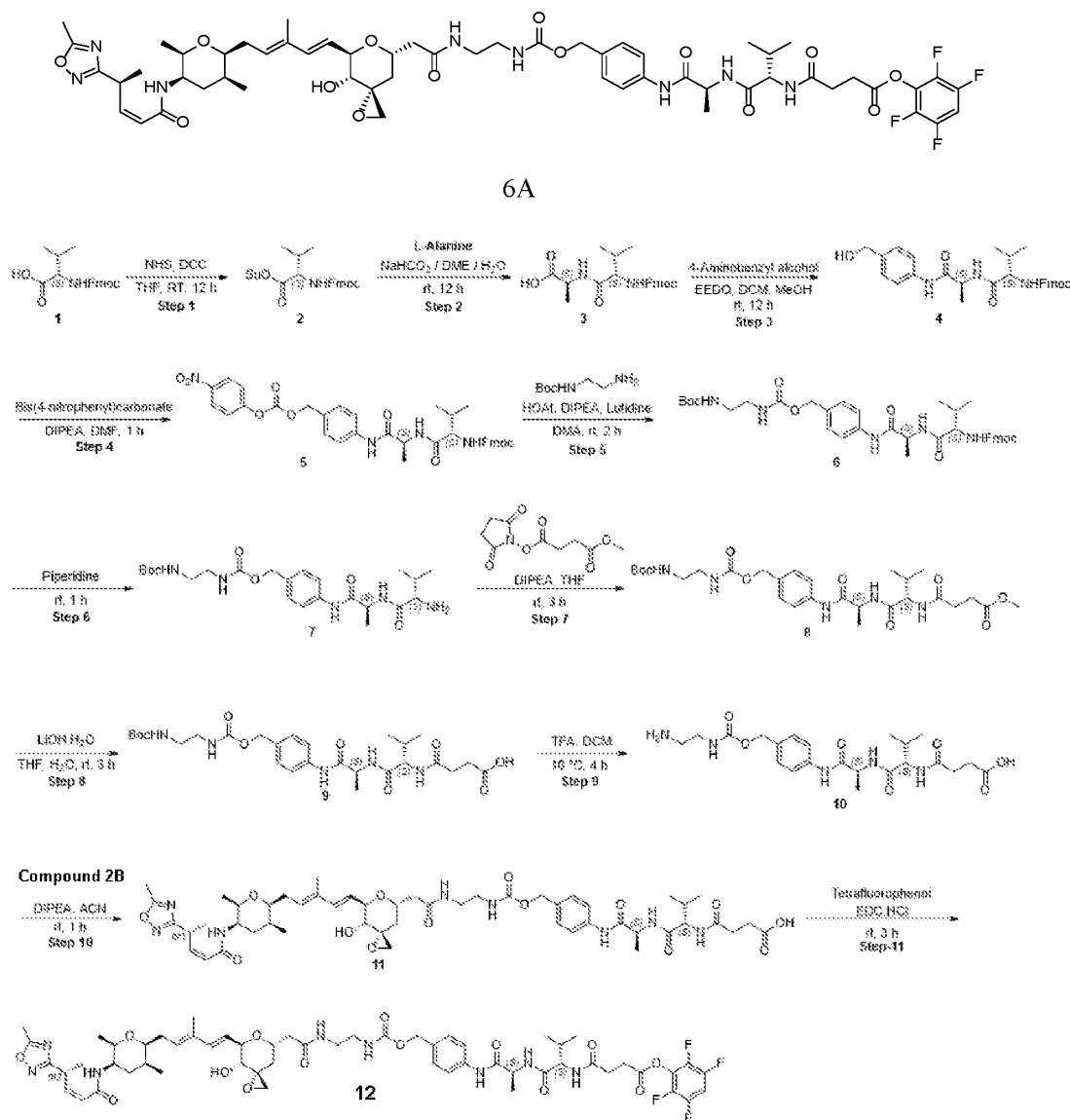
5A

Compound 5A was prepared according to the following synthetic scheme using compounds and intermediates described herein, and using synthetic procedures similar to the ones provided herein.



Example 25. Preparation of compound 6A.

Compound 6A was prepared by the method described in the example, except that compound 2A of example 21 was used in place of compound 2B.



Step 1: Synthesis of 2,5-dioxopyrrolidin-1-yl (((9H-fluoren-9-yl)methoxy)carbonyl)-L-valinate.

To a stirred solution of (((9H-fluoren-9-yl)methoxy)carbonyl)-L-valine (10 g, 29.5 mmol) and N-Hydroxysuccinimide (3.73 g, 32.45 mmol) in THF (200 mL) was added DCC (6.69 g, 32.45 mmol) in portions under ice-bath. After the addition the reaction mixture was stirred at room temperature for overnight. TLC system: 50% EtOAc in Hexane (Rf: 0.5, UV visible). The reaction mixture was cooled to -5 °C, filtered and washed with cold THF (50

mL), the filtrate was concentrated in vacuum to get crude compound. The crude residue was purified by trituration with MTBE and n-pentane to afford 2,5-dioxopyrrolidin-1-yl (((9H-fluoren-9-yl)methoxy)carbonyl)-L-valinate (12.2 g, 95.31%) as a white solid.

¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 7.77 (d, J=7.63 Hz, 2 H) 7.59 (br d, J=7.41 Hz, 2 H) 7.37 - 7.43 (m, 2 H) 7.29 - 7.35 (m, 2 H) 5.25 (br d, J=9.16 Hz, 1 H) 4.69 (dd, J=9.26, 4.80 Hz, 1 H) 4.45 (d, J=6.65 Hz, 2 H) 4.21 - 4.28 (m, 1 H) 3.21 (s, 1 H) 2.82 - 2.88 (m, 5 H) 2.29 - 2.40 (m, 1 H) 1.19 (s, 3 H) 1.06 (br dd, J=9.81, 6.98 Hz, 6 H).
LCMS (C3783-015-A2): 98.92% (437.27, M+H), RT: 2.61 min.

10 Step 2: Synthesis of (((9H-fluoren-9-yl)methoxy)carbonyl)-L-valyl-L-alanine.

Fmoc-Val-NHS (5 g, 11.5 mmol) in 30 mL of DME was added to a solution of L-Alanine (1.07 g, 12.07 mmol) in 20 mL of THF and NaHCO₃ (1.01 g, 12.07 mmol) in 30 mL of water. The mixture was stirred at room temperature overnight. TLC system: 10% MeOH in DCM (Rf: 0.1, UV visible). The reaction progress was monitored by TLC. Aqueous citric acid (150 mL of a 15% solution in water) was added and the precipitate was filtered and dried to get white solid. The resulting solid was triturated with 200 mL of diethyl ether and filtered to yield (((9H-fluoren-9-yl)methoxy)carbonyl)-L-valyl-L-alanine (3.0 g, 64%) as a white solid.

¹H NMR (400 MHz, DMSO-d₆) δ ppm: 12.27 - 12.53 (m, 1 H), 8.21 (br d, J=7.34 Hz, 1 H), 7.89 (d, J=7.34 Hz, 2 H), 7.75 (br t, J=6.60 Hz, 2 H), 7.38 - 7.44 (m, 3 H), 7.28 - 7.36 (m, 2 H), 4.14 - 4.32 (m, 4 H), 3.89 (dd, J=8.80, 7.34 Hz, 1 H), 1.97 (dq, J=13.63, 6.70 Hz, 1 H), 1.27 (d, J=7.34 Hz, 3 H), 0.88 (dd, J=13.69, 6.85 Hz, 6 H).

LCMS (SR-12580-168-A2): 92.75%, RT: 1.96 min, M+H: 410.94.

25 Step 3: Synthesis of (9H-fluoren-9-yl)methyl ((S)-1-(((S)-1-((4-(hydroxymethyl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate.

To a stirred solution of (((9H-fluoren-9-yl)methoxy)carbonyl)-L-valyl-L-alanine (3 g, 7.3 mmol) in a mixture of DCM and MeOH (2:1) (45 mL) was added 4-Aminobenzyl alcohol (1.07 g, 8.76 mmol) and EEDQ (3.6 g, 14.6 mmol). The reaction mixture was stirred at room temperature for overnight. Reaction progress was monitored by TLC. TLC system: 10% MeOH in DCM (Rf: 0.4, UV visible). The solvent was removed and to the residue was added diethyl ether (100 mL) and sonicated for 5 - 10 mins. The solid was filtered, washed with additional ether and dried to afford (9H-fluoren-9-yl)methyl ((S)-1-(((S)-1-((4-

(hydroxymethyl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate (3.0 g, 81%) as a white solid.

¹H NMR (400 MHz, DMSO-d₆) δ ppm: 9.92 (s, 1 H), 8.16 (d, J=6.85 Hz, 1 H), 8.15 - 8.18 (m, 1 H), 7.89 (d, J=7.34 Hz, 2 H), 7.73 - 7.76 (m, 2 H), 7.53 (br d, J=8.31 Hz, 2 H), 7.39 - 7.46 (m, 4 H), 7.30 - 7.34 (m, 2 H), 7.24 (d, J=8.31 Hz, 2 H), 5.08 - 5.11 (m, 1 H), 4.44 (s, 1 H), 4.28 - 4.31 (m, 1 H), 4.22 - 4.26 (m, 2 H), 3.90 (br d, J=8.31 Hz, 1 H), 1.97 - 2.02 (m, 1 H), 1.30 (d, J=7.34 Hz, 3 H), 0.84 - 0.90 (m, 6 H)

LCMS (SR-12580-172-A2): 94.86%, RT: 2.40 min, M+H: 516.27.

- 10 Step 4: Synthesis of (9H-fluoren-9-yl)methyl ((S)-3-methyl-1-(((S)-1-((4-((4-nitrophenoxy)carbonyl)oxy)methyl)phenyl)amino)-1-oxopropan-2-yl)amino)-1-oxobutan-2-yl)carbamate.

To a stirred solution of (9H-fluoren-9-yl)methyl ((S)-1-(((S)-1-((4-((hydroxymethyl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate (3 g, 5.8 mmol) and Bis(4-nitrophenyl)carbonate (3.52 g, 11.6 mmol) in DMF (20 mL) at 23 °C was added DIPEA (1.49 g, 11.6 mmol) and the mixture was stirred at room temperature for 1 hr. Reaction progress was monitored by TLC. TLC system: 10% MeOH in DCM (Rf: 0.5, UV visible). After completion, the reaction mixture was poured into ice-cooled water, precipitated solid was filtered and the solid was washed with diethyl ether (100 mL), dried to afford (9H-fluoren-9-yl)methyl ((S)-3-methyl-1-(((S)-1-((4-((4-nitrophenoxy)carbonyl)oxy)methyl)phenyl)amino)-1-oxopropan-2-yl)amino)-1-oxobutan-2-yl)carbamate (3.0 g, 77%) as an off-white solid.

¹H NMR (400 MHz, CHLOROFORM-d) δ ppm: 8.52 (br s, 1 H), 8.23 - 8.30 (m, 2 H), 7.76 (br d, J=7.83 Hz, 2 H), 7.50 - 7.65 (m, 4 H), 7.27 - 7.44 (m, 8 H), 6.40 (br d, J=6.36 Hz, 1 H), 5.24 (br s, 3 H), 4.58 - 4.69 (m, 1 H), 4.43 - 4.54 (m, 2 H), 4.20 (br t, J=6.36 Hz, 1 H), 3.94 - 4.03 (m, 1 H), 2.11 - 2.23 (m, 1 H), 1.46 (br d, J=6.85 Hz, 3 H), 0.94 (br dd, J=11.74, 5.38 Hz, 6 H).

LCMS (SR-12580-173-A2): 81.50%, RT: 2.32 min, M+H: 681.53.

- 30 Step 5: Synthesis of 4-((S)-2-((S)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl tert-butyl ethane-1,2-diyl dicarbamate.

To a stirred solution of (9H-fluoren-9-yl)methyl ((S)-3-methyl-1-(((S)-1-((4-((4-nitrophenoxy)carbonyl)oxy)methyl)phenyl)amino)-1-oxopropan-2-yl)amino)-1-oxobutan-2-yl)carbamate (3 g, 4.4 mmol) in DMA (30 mL) at 0 °C, HOAt (0.65 g, 4.84 mmol) and 2,6-

Lutidine (1.88 g, 17.6 mmol) were added and stirred for 5 min at 0 °C, then tert-butyl (2-aminoethyl)carbamate (0.77 g, 4.84 mmol) and DIPEA (1.70 g, 13.2 mmol) was added and allowed to stir at 23 °C for 2 h. Reaction progress was monitored by TLC. TLC system: 10% MeOH in DCM (Rf: 0.4, UV visible). After completion, the reaction mixture was poured into MTBE (200 mL) and stirred for 1 h, resulting solid was filtered, washed with MTBE and dried to afford 4-((S)-2-((S)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl tert-butyl ethane-1,2-diylldicarbamate(SR-12580-188-A2) (2.0 g, 65%) as a pale yellow solid.

¹H NMR (400 MHz, DMSO-d₆) δ ppm 9.95 - 10.04 (m, 1 H), 8.18 (d, J=6.87 Hz, 1 H), 7.89 (d, J=7.52 Hz, 2 H), 7.74 (t, J=7.03 Hz, 2 H), 7.57 (d, J=8.39 Hz, 2 H), 7.37 - 7.47 (m, 3 H), 7.24 - 7.35 (m, 4 H), 7.13 - 7.22 (m, 1 H), 6.80 (br d, J=5.01 Hz, 1 H), 4.89 - 5.01 (m, 2 H), 4.37 - 4.47 (m, 1 H), 4.18 - 4.34 (m, 3 H), 3.87 - 3.95 (m, 1 H), 2.91 - 3.05 (m, 4 H), 1.92 - 2.05 (m, 1 H), 1.37 (s, 9 H), 1.30 (d, J=7.08 Hz, 3 H), 0.87 (dd, J=12.53, 6.76 Hz, 6 H). LCMS (SR-12580-188-A2): 98.33%, RT: 2.21 min, M+H: 702.56.

15

Step 6: Synthesis of 4-((S)-2-((S)-2-amino-3-methylbutanamido)propanamido)benzyl tert-butyl ethane-1,2-diylldicarbamate.

To a stirred solution of 4-((S)-2-((S)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl tert-butyl ethane-1,2-diylldicarbamate (2 g, 2.9 mmol) in DMF (20 mL) at room temperature was added piperidine (1.23 g, 14.5 mmol) and the mixture was stirred at room temperature for 1 h. The reaction progress was monitored by TLC. TLC system: 10% MeOH in DCM (Rf: 0.1). The reaction mixture was poured into diethyl ether (100 mL) and the precipitated solid was filtered and dried to afford 4-((S)-2-((S)-2-amino-3-methylbutanamido)propanamido)benzyl tert-butyl ethane-1,2-diylldicarbamate (1.0 g, 73.5%) as a white solid.

¹H NMR (400 MHz, DMSO-d₆) δ ppm 10.05 (s, 1 H), 8.09 - 8.20 (m, 1 H), 7.57 (d, J=8.50 Hz, 2 H), 7.28 (d, J=8.50 Hz, 2 H), 7.18 (br s, 1 H), 6.72 - 6.85 (m, 1 H), 4.89 - 5.01 (m, 2 H), 4.41 - 4.52 (m, 1 H), 2.91 - 3.05 (m, 5 H), 1.85 - 1.98 (m, 1 H), 1.69 (br s, 2 H), 1.37 (s, 9 H), 1.30 (d, J=6.98 Hz, 3 H), 0.88 (d, J=6.87 Hz, 3 H), 0.78 (d, J=6.76 Hz, 3 H). LCMS: 98.63%, RT: 1.78 min, M+H: 480.73.

30

Step 7: Synthesis of methyl 4-(((S)-1-(((S)-1-((4-(10,10-dimethyl-3,8-dioxo-2,9-dioxo-4,7-diazaundecyl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)amino)-4-oxobutanoate.

To a stirred solution of 4-(((S)-2-(((S)-2-amino-3-methylbutanamido)propanamido)-benzyl tert-butyl ethane-1,2-diyl dicarbamate (900 mg, 1.878 mmol) & 2,5-dioxopyrrolidin-1-yl methyl succinate (516 mg, 2.25 mmol) in THF (20 mL) was added DIPEA (0.72 g, 0.5.63 mmol) at rt. The reaction mixture was stirred at rt for 3 h. The progress of the reaction was monitored by TLC. TLC system: 10%MeOH in DCM (Rf: 0.5, UV visible). The reaction mixture was concentrated in vacuum to get crude compound (1.3 g). The crude residue was triturated with diethyl ether to afford methyl 4-(((S)-1-(((S)-1-((4-(10,10-dimethyl-3,8-dioxo-2,9-dioxo-4,7-diazaundecyl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)amino)-4-oxobutanoate (1.0 g, 90%) as a pale yellow solid.

¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 8.41 - 8.47 (m, 1 H), 7.70 (d, J=8.61 Hz, 2 H), 7.29 (s, 2 H), 7.14 - 7.20 (m, 1 H), 6.06 - 6.13 (m, 1 H), 5.08 - 5.15 (m, 1 H), 5.04 (s, 2 H), 4.80 - 4.88 (m, 1 H), 4.65 - 4.75 (m, 1 H), 4.24 (dd, J=6.21, 4.47 Hz, 1 H), 3.67 - 3.76 (m, 2 H), 3.50 (s, 3 H), 3.44 - 3.49 (m, 1 H), 3.20 - 3.34 (m, 4 H), 3.15 (q, J=7.41 Hz, 2 H), 2.86 - 2.97 (m, 1 H), 2.64 (s, 9 H), 2.57 - 2.62 (m, 1 H), 2.39 - 2.56 (m, 4 H), 1.51 (d, J=7.30 Hz, 3 H), 1.43 (s, 7 H), 1.39 (d, J=6.65 Hz, 12 H), 1.21 (t, J=7.03 Hz, 1 H), 1.06 (d, J=6.98 Hz, 3 H), 1.00 (d, J=6.98 Hz, 3 H).

LCMS: 94.33%, RT: 1.73 min, M+H: 594.49.

Step 8: Synthesis of 4-(((S)-1-(((S)-1-((4-(10,10-dimethyl-3,8-dioxo-2,9-dioxo-4,7-diazaundecyl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)amino)-4-oxobutanoic acid.

To a stirred solution of methyl 4-(((S)-1-(((S)-1-((4-(10,10-dimethyl-3,8-dioxo-2,9-dioxo-4,7-diazaundecyl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)amino)-4-oxobutanoate (900 mg, 1.52 mmol) in THF (15 mL) water (5 mL) was added Lithium hydroxide monohydrate (255 mg, 6.08 mmol) at rt, reaction mixture stirred at rt for 3 h. The progress of the reaction monitored by TLC. TLC system: 10% Methanol in DCM (RF: 0.1, U.V visible). The reaction mixture was concentrated in vacuum to get crude compound. The residue was acidified with 15% citric acid solution and extracted with 10% Methanol in DCM (3 X 50 mL). The combined organic layers were dried over anhydrous sodium sulphate, filtered and concentrated under reduced pressure to afford 4-(((S)-1-(((S)-1-((4-(10,10-dimethyl-3,8-dioxo-2,9-dioxo-4,7-diazaundecyl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)amino)-4-oxobutanoic acid (800 mg, 91%) as an off white solid.

¹H NMR (400 MHz, DMSO-d₆) δ ppm: 9.80 - 9.89 (m, 1 H), 8.13 (d, J=6.91 Hz, 1 H), 7.93 (d, J=8.34 Hz, 1 H), 7.59 (d, J=8.58 Hz, 2 H), 7.28 (d, J=8.46 Hz, 2 H), 7.14 - 7.21 (m, 1 H), 6.71 - 6.85 (m, 1 H), 4.89 - 5.00 (m, 2 H), 4.38 (quin, J=7.06 Hz, 1 H), 4.17 (dd, J=8.11, 6.79 Hz, 1 H), 4.03 (q, J=7.07 Hz, 2 H), 2.91 - 3.06 (m, 4 H), 2.43 (s, 3 H), 1.93 - 2.02 (m, 4 H),
 5 1.34 - 1.40 (m, 9 H), 1.28 - 1.33 (m, 3 H), 1.22 - 1.26 (m, 1 H), 1.17 (t, J=7.15 Hz, 3 H), 0.86 (dd, J=14.19, 6.79 Hz, 6 H).
 LCMS: 91.6%, RT: 1.60 min, M+H: 580.26.

Step 9: Synthesis of 4-(((S)-1-(((S)-1-((4-(((2-aminoethyl)carbamoyl)oxy)methyl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)amino)-4-oxobutanoic acid.

To a stirred solution of 4-(((S)-1-(((S)-1-((4-((10,10-dimethyl-3,8-dioxo-2,9-dioxo-4,7-diazaundecyl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)amino)-4-oxobutanoic acid (400 mg, 0.69 mmol) in DCM (25 mL) was cooled to 0° C added TFA
 15 (1.179 g, 10.35 m.mol) and stirred for 4 h at 10° C. The progress of the reaction was monitored by TLC. TLC mobile phase: 15% methanol in DCM (RF: 0.1, U.V visible). Reaction mixture was concentrated in vacuum and azeotrope with diethyl ether (3 X 10 mL) to get crude compound as TFA salt. The Crude compound was purified through Prep HPLC techniques to afford 4-(((S)-1-(((S)-1-((4-(((2-aminoethyl)carbamoyl)oxy)methyl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)amino)-4-oxobutanoic acid (100 mg, 30%) as an off- white solid.
 20 ¹H NMR (300 MHz, DMSO-d₆) δ ppm: 10.60 - 10.76 (m, 1 H), 8.08 - 8.20 (m, 1 H), 7.45 - 7.97 (m, 6 H), 6.84 - 6.95 (m, 1 H), 6.23 - 6.35 (m, 1 H), 4.04 - 4.24 (m, 3 H), 3.53 - 3.72 (m, 2 H), 3.21 - 3.29 (m, 2 H), 3.17 (d, J=5.50 Hz, 2 H), 1.32 (br d, J=6.97 Hz, 9 H).
 25 LCMS: 96%, RT: 1.58 min, M+H: 480.35.

Step 10: Synthesis of 4-(((S)-1-(((S)-1-((4-(((2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((R,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetamido)ethyl)carbamoyl)oxy)methyl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)amino)-4-oxobutanoic acid.

To a stirred solution of 2,5-dioxopyrrolidin-1-yl 2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((R,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-

dioxaspiro[2.5]octan-5-yl)acetate (80 mg, 0.12 mmol) and 4-(((S)-1-(((S)-1-((4-(((2-aminoethyl)carbamoyl)oxy)methyl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)amino)-4-oxobutanoic acid (40.2 mg, 0.084 mmol) in DMF (4 mL) was added DIPEA (61.9 mg, 0.48 mmol) at rt. The reaction mixture was stirred at rt for 1 h. The progress of the reaction was monitored by LCMS. The crude reaction mixture was directly purified by Prep HPLC techniques to afford 4-(((S)-1-(((S)-1-((4-(((2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((R,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetamido)ethyl)carbamoyl)oxy)methyl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)amino)-4-oxobutanoic acid (30 mg, 24.19%) as an off white solid.

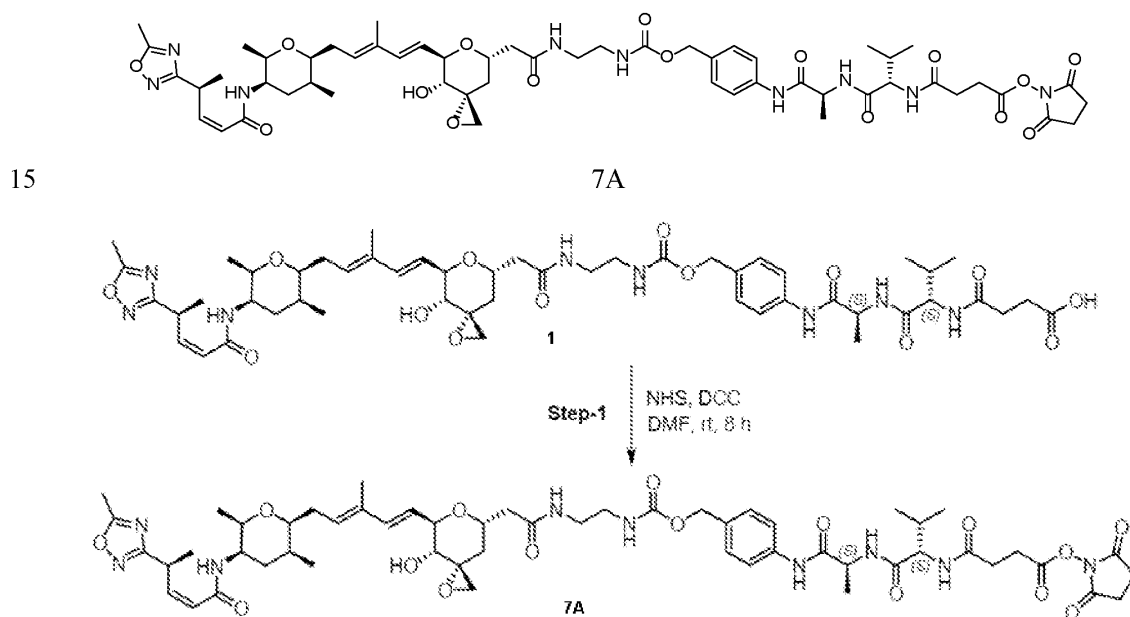
LCMS: 74%, RT: 3.73, M+H: 1021.69.

Step 11: Synthesis of 2,3,5,6-tetrafluorophenyl 4-(((S)-1-(((S)-1-((4-(((2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((R,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetamido)ethyl)carbamoyl)oxy)methyl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)amino)-4-oxobutanoate.

To a stirred solution of 4-(((S)-1-(((S)-1-((4-(((2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((R,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetamido)ethyl)carbamoyl)oxy)methyl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)amino)-4-oxobutanoic acid (30 mg, 0.03 mmol) in DMF (2 mL) added 2,3,5,6-tetrafluorophenol (10 mg, 0.06 mmol), EDC.HCl (11.5 mg, 0.06 mmol) was stirred for 3 h at rt. The progress of the reaction was monitored by LCMS. The reaction mass directly purified by Prep HPLC condition and fractions were collected at -78 °C & lyophilized to afford 2,3,5,6-tetrafluorophenyl 4-(((S)-1-(((S)-1-((4-(((2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((R,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetamido)ethyl)carbamoyl)oxy)methyl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)amino)-4-oxobutanoate (8 mg, 23.2%) as a white solid.

¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 8.44 - 8.56 (m, 1 H), 7.57 - 7.69 (m, 2 H), 7.29 (br d, J=8.46 Hz, 2 H), 6.93 - 7.05 (m, 1 H), 6.82 (br d, J=7.03 Hz, 1 H), 6.56 - 6.66 (m, 1 H), 6.27 - 6.42 (m, 2 H), 6.15 (dd, J=11.15, 9.95 Hz, 1 H), 5.99 - 6.08 (m, 1 H), 5.76 - 5.87 (m, 1 H), 5.60 (br dd, J=15.38, 6.08 Hz, 1 H), 5.48 (br t, J=6.44 Hz, 1 H), 5.26 (br s, 1 H),
 5.12 - 5.21 (m, 1 H), 5.00 - 5.08 (m, 2 H), 4.53 - 4.66 (m, 1 H), 4.21 - 4.41 (m, 3 H), 3.83 - 3.96 (m, 1 H), 3.54 - 3.63 (m, 1 H), 3.40 - 3.52 (m, 3 H), 3.20 - 3.37 (m, 3 H), 2.98 - 3.18 (m, 2 H), 2.92 (d, J=4.41 Hz, 1 H), 2.59 - 2.76 (m, 5 H), 2.46 - 2.55 (m, 2 H), 2.31 - 2.41 (m, 1 H), 2.13 - 2.30 (m, 2 H), 1.78 - 1.95 (m, 4 H), 1.69 - 1.77 (m, 5 H), 1.46 (d, J=6.91 Hz, 5 H), 1.36 (d, J=7.03 Hz, 3 H), 1.14 (d, J=6.44 Hz, 3 H), 0.98 (d, J=6.68 Hz, 3 H), 0.96 (s, 3 H),
 0.90 - 0.94 (m, 3 H).
 LCMS: RT: 4.69, M+H: 1169.74.

Example 26. Preparation of compound 7A.



Step 1: Synthesis of 2,5-dioxopyrrolidin-1-yl 4-(((S)-1-(((S)-1-((4-(((2-(2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((S,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetamido)ethyl)carbamoyl)oxy)methyl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)amino)-4-oxobutanoate.

20

To a stirred solution of 4-(((S)-1-(((S)-1-((4-(((2-(2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((S,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-

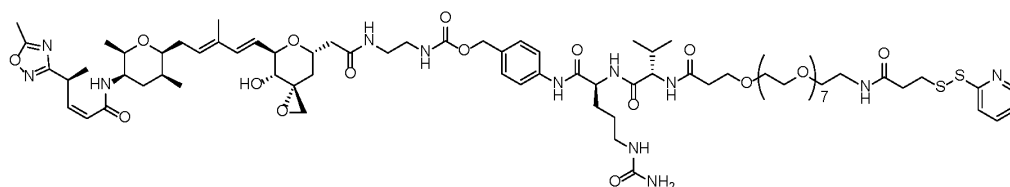
enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetamido) ethyl) carbamoyl oxy)methyl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)amino)-4-oxobutanoic acid (19 mg, 0.0029 mmol) in DMF (1.0 mL) was added DCC (1.8 mg, 0.0087 mmol), N-hydroxy succinate (1.7 mg, 0.014 m.mol) was stirred for 8 h at RT, reaction progress was monitored by LCMS. Crude LCMS showed 54% of desired product mass. The reaction mixture was directly purified by Prep. HPLC purification. The pure fractions were collected at -78 °C and lyophilized to afford 2,5-dioxopyrrolidin-1-yl 4-(((S)-1-(((S)-1-(((4-(((2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((S,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetamido)ethyl)carbamoyl)oxy)methyl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)amino)-4-oxobutanoate (4 mg, 18.2%) as a white solid.

¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 8.52 (s, 1 H), 7.61 (br d, J=8.28 Hz, 2 H), 7.29 (br d, J=8.28 Hz, 3 H), 6.88 (br d, J=7.08 Hz, 1 H), 6.62 (br d, J=4.47 Hz, 1 H), 6.42 (br d, J=6.87 Hz, 1 H), 6.32 (br d, J=15.80 Hz, 1 H), 6.15 (dd, J=11.23, 9.92 Hz, 1 H), 6.01 - 6.07 (m, 1 H), 5.81 (br d, J=11.23 Hz, 1 H), 5.60 (br dd, J=16.35, 5.78 Hz, 1 H), 5.49 (br t, J=6.38 Hz, 1 H), 5.27 (br t, J=5.23 Hz, 1 H), 5.12 - 5.20 (m, 1 H), 5.04 (s, 2 H), 4.60 - 4.69 (m, 1 H), 4.22 - 4.38 (m, 3 H), 3.87 - 3.93 (m, 1 H), 3.59 - 3.63 (m, 1 H), 3.43 - 3.49 (m, 3 H), 3.24 - 3.34 (m, 3 H), 2.91 - 3.03 (m, 3 H), 2.81 (s, 3 H), 2.56 - 2.71 (m, 9 H), 2.18 - 2.39 (m, 3 H), 1.81 - 1.93 (m, 3 H), 1.74 (s, 4 H), 1.43 - 1.49 (m, 6 H), 1.14 (d, J=6.32 Hz, 3 H), 0.94 - 1.00 (m, 9 H)

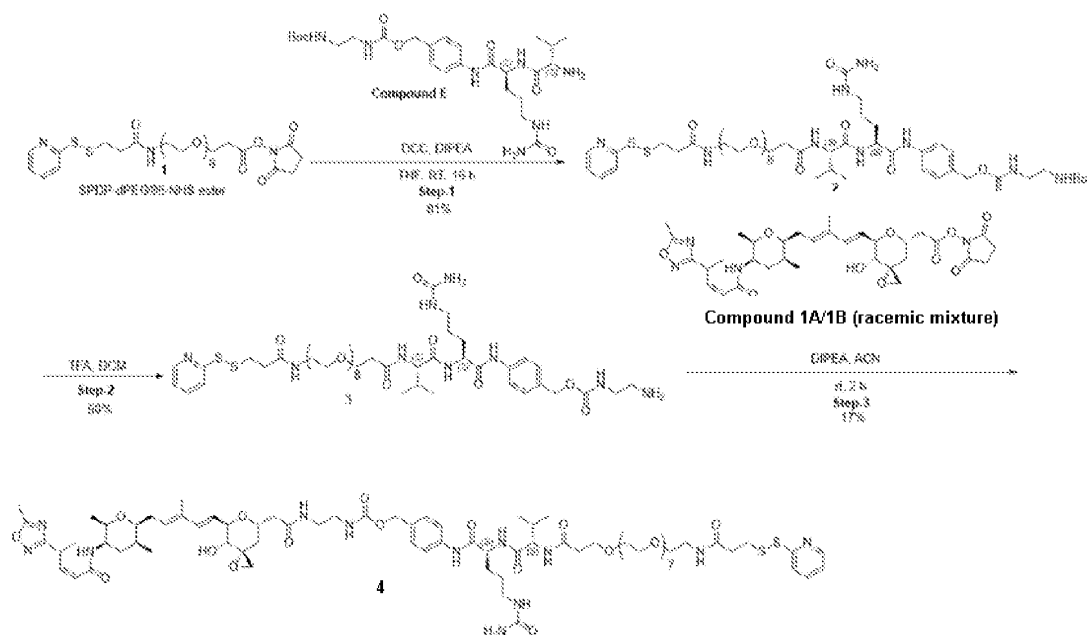
LCMS: 95.05% purity, M+H: 1118.76

25 Example 27. Preparation of Compound 8A.

Compound 8A was prepared by the method described in the example, except that compound 2A of example 20 was used in place of the racemate of 2A/2B.



8A



Step 1: Synthesis of tert-butyl (4-((2S,5S)-5-isopropyl-4,7,35-trioxo-37-(pyridin-2-yl)disulfanyl)-2-(3-ureidopropyl)-10,13,16,19,22,25,28,31-octaoxa-3,6,34-

5 triazaheptatriacontanamido)benzyl) ethane-1,2-diyl dicarbamate.

To a stirred solution of 4-((S)-2-((S)-2-amino-3-methylbutanamido)-5-ureidopentanamido)benzyl tert-butyl ethane-1,2-diyl dicarbamate (200 mg, 0.35 mmol) & 2,5-dioxopyrrolidin-1-yl 3-oxo-1-(pyridin-2-yl)disulfanyl)-7,10,13,16,19,22,25,28-octaoxa-4-azahentriacontan-31-oate (257 mg, 0.35 mmol) in THF (2 mL) at 23 °C was added DCC (108 mg, 0.525 mmol) and DIPEA (135 mg, 1.05 mmol). The reaction mixture was stirred at 23 °C for 16 h. The reaction progress was monitored by TLC. TLC system: 10% MeOH in DCM (R_f: 0.3, UV visible). After completion, diethyl ether (50 mL) was added to the reaction mixture, solid was filtered, dried under reduced pressure to give tert-butyl (4-((2S,5S)-5-isopropyl-4,7,35-trioxo-37-(pyridin-2-yl)disulfanyl)-2-(3-ureidopropyl)-10,13,16,19,22,25,28,31-octaoxa-3,6,34-triazaheptatriacontanamido)benzyl) ethane-1,2-diyl dicarbamate as an off-white gummy solid (330 mg, Yield: 81%).

¹H NMR (500 MHz, DMSO-d₆) δ ppm 9.94 - 10.01 (m, 1 H) 8.46 (br d, J=4.27 Hz, 1 H) 8.08 - 8.14 (m, 1 H) 7.99 - 8.05 (m, 1 H) 7.80 - 7.90 (m, 2 H) 7.73 - 7.79 (m, 1 H) 7.59 (br d, J=8.39 Hz, 2 H) 7.22 - 7.31 (m, 3 H) 7.18 (br s, 1 H) 6.76 - 6.83 (m, 1 H) 5.93 - 6.00 (m, 1 H) 5.36 - 5.44 (m, 2 H) 4.90 - 4.98 (m, 2 H) 4.34 - 4.42 (m, 1 H) 4.19 - 4.26 (m, 1 H) 3.56 - 3.64 (m, 2 H) 3.40 (br t, J=5.80 Hz, 2 H) 3.16 - 3.23 (m, 2 H) 2.91 - 3.06 (m, 9 H) 2.42 - 2.55 (m,

33 H) 2.34 - 2.41 (m, 2 H) 1.93 - 2.00 (m, 1 H) 1.64 - 1.74 (m, 1 H) 1.54 - 1.63 (m, 1 H) 1.41 - 1.47 (m, 1 H) 1.37 (s, 11 H) 0.84 (br dd, J=16.17, 6.71 Hz, 6 H).

Step 2: Synthesis of 4-((2S,5S)-5-isopropyl-4,7,35-trioxo-37-(pyridin-2-ylidisulfanyl)-2-(3-ureidopropyl)-10,13,16,19,22,25,28,31-octaoxa-3,6,34-triazaheptatriacontanamido)benzyl(2-aminoethyl)carbamate.

To a stirred solution of tert-butyl 4-((2S,5S)-5-isopropyl-4,7,35-trioxo-37-(pyridin-2-ylidisulfanyl)-2-(3-ureidopropyl)-10,13,16,19,22,25,28,31-octaoxa-3,6,34-triazaheptatriacontanamido)benzyl ethane-1,2-diyl dicarbamate (330 mg, 0.28 mmol) in DCM (10 mL) at 0 °C, was added TFA (0.47 g, 4.2 mmol) and the reaction was stirred at rt for 3 h. The progress of the reaction was monitored by TLC. TLC System: 10% MeOH in DCM (Rf: 0.05, UV visible). After completion of the reaction, concentrated under reduced pressure to give crude compound as a TFA salt. The crude compound was purified by Prep. HPLC techniques to afford 4-((2S,5S)-5-isopropyl-4,7,35-trioxo-37-(pyridin-2-ylidisulfanyl)-2-(3-ureidopropyl)-10,13,16,19,22,25,28,31-octaoxa-3,6,34-triazaheptatriacontanamido)benzyl (2-aminoethyl)carbamate (140 mg, yield: 47%) as a TFA salt.

¹H NMR (400 MHz, DMSO-d₆) δ ppm 9.95 - 10.00 (m, 1 H) 8.42 - 8.48 (m, 1 H) 8.08 - 8.15 (m, 1 H) 7.99 - 8.05 (m, 1 H) 7.80 - 7.90 (m, 2 H) 7.73 - 7.78 (m, 1 H) 7.59 (d, J=8.56 Hz, 2 H) 7.22 - 7.31 (m, 3 H) 7.17 (br t, J=5.38 Hz, 1 H) 5.94 - 6.01 (m, 1 H) 5.35 - 5.44 (m, 2 H) 4.90 - 4.97 (m, 2 H) 4.33 - 4.42 (m, 1 H) 4.19 - 4.25 (m, 1 H) 3.56 - 3.63 (m, 3 H) 3.45 - 3.53 (m, 30 H) 3.37 - 3.42 (m, 4 H) 3.16 - 3.23 (m, 3 H) 2.91 - 3.03 (m, 6 H) 2.56 (br t, J=6.42 Hz, 2 H) 2.30 - 2.42 (m, 2 H) 1.92 - 2.00 (m, 1 H) 1.89 (s, 2 H) 1.51 - 1.75 (m, 2 H) 1.29 - 1.49 (m, 2 H) 0.84 (dd, J=13.02, 6.79 Hz, 6 H)

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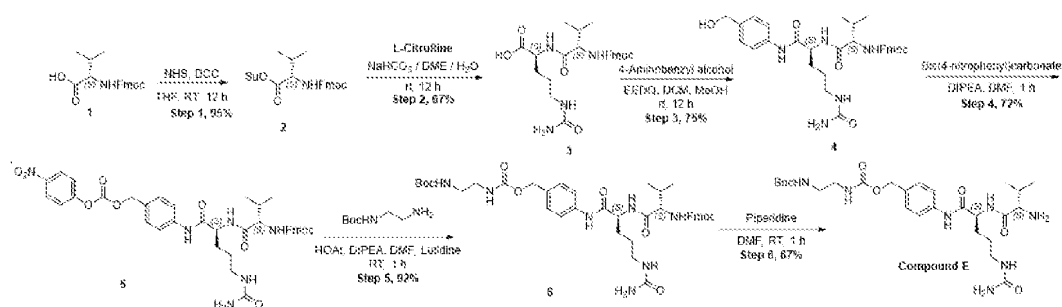
Step 3: Synthesis of 4-((2S,5S)-5-isopropyl-4,7,35-trioxo-37-(pyridin-2-ylidisulfanyl)-2-(3-ureidopropyl)-10,13,16,19,22,25,28,31-octaoxa-3,6,34-triazaheptatriacontanamido)benzyl (2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetamido)ethyl)carbamate.

To a stirred solution of 2,5-dioxopyrrolidin-1-yl 2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetate (25 mg, 0.038 mmol) & 4-((2S,5S)-5-isopropyl-4,7,35-

trioxo-37-(pyridin-2-yl)disulfanyl)-2-(3-ureidopropyl)-10,13,16,19,22,25,28,31-octaoxa-3,6,34-triazaheptatriacontanamido)benzyl (2-aminoethyl)carbamate (49 mg, 0.045 mmol) in acetonitrile (1.5 mL) was added DIPEA (14 mg, 0.114 mmol) at 0 °C. The reaction mixture was stirred at rt for 2 h. The progress of the reaction was monitored by TLC & LCMS. Crude LCMS showed 30% of desired product mass. The reaction mixture was directly purified by Prep. HPLC technique to afford 4-((2S,5S)-5-isopropyl-4,7,35-trioxo-37-(pyridin-2-yl)disulfanyl)-2-(3-ureidopropyl)-10,13,16,19,22,25,28,31-octaoxa-3,6,34-triazaheptatriacontanamido)benzyl (2-(2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetamido)ethyl)carbamate (11 mg, yield: 18%) as a white solid.

¹H NMR (500 MHz, CHLOROFORM-d) δ ppm 9.03 - 9.09 (m, 1 H) 8.45 - 8.50 (m, 1 H) 7.64 - 7.75 (m, 4 H) 7.52 - 7.60 (m, 2 H) 7.06 - 7.14 (m, 1 H) 6.68 - 6.75 (m, 1 H) 6.30 - 6.38 (m, 1 H) 6.03 (s, 1 H) 5.75 - 5.91 (m, 2 H) 5.63 (br dd, J=15.34, 5.87 Hz, 1 H) 5.49 - 5.56 (m, 1 H) 5.38 - 5.45 (m, 1 H) 5.12 - 5.20 (m, 2 H) 5.00 - 5.07 (m, 2 H) 4.63 - 4.73 (m, 1 H) 4.35 - 4.42 (m, 1 H) 4.31 (dt, J=16.82, 6.62 Hz, 2 H) 3.93 - 4.01 (m, 1 H) 3.82 - 3.90 (m, 1 H) 3.61 - 3.73 (m, 29 H) 3.52 - 3.60 (m, 8 H) 3.46 (q, J=5.09 Hz, 4 H) 3.23 - 3.34 (m, 5 H) 3.02 - 3.10 (m, 3 H) 2.91 (d, J=4.58 Hz, 1 H) 2.62 - 2.69 (m, 4 H) 2.55 - 2.61 (m, 6 H) 2.34 (s, 1 H) 2.20 - 2.31 (m, 2 H) 2.01 - 2.10 (m, 1 H) 1.89 - 1.98 (m, 2 H) 1.72 - 1.82 (m, 6 H) 1.50 (dd, J=6.79, 6.03 Hz, 5 H) 1.18 (d, J=6.41 Hz, 2 H) 1.10 - 1.14 (m, 2 H) 1.06 (d, J=7.48 Hz, 2 H) 0.95 - 1.04 (m, 9 H)

Preparation of compound E:



Step-1: 2, 5-dioxopyrrolidin-1-yl (((9H-fluoren-9-yl)methoxy)carbonyl)-L-valinate (1):

To a stirred solution of (((9H-fluoren-9-yl)methoxy)carbonyl)-L-valine (10 g, 29.5 mmol) and N-Hydroxysuccinamide (3.73 g, 32.45 mmol) in THF (200 mL) was added DCC (6.69 g, 32.45 mmol) in portions under ice-bath. After the addition the reaction mixture was

stirred at room temperature for overnight. TLC system: 50% EtOAc in Hexane (RF: 0.5, UV visible). The reaction mixture was cooled to -5 °C, filtered and washed with cold THF (50 mL), the filtrate was concentrated in vacuum to get crude compound. The crude residue was purified by trituration with MTBE and n-pentane to afford 2, 5-dioxopyrrolidin-1-yl (((9H-fluoren-9-yl)methoxy)carbonyl)-L-valinate (12.2 g, 95.31%) (C3783-015-A2) as a white solid. ¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 7.77 (d, J=7.63 Hz, 2 H) 7.59 (br d, J=7.41 Hz, 2 H) 7.37 - 7.43 (m, 2 H) 7.29 - 7.35 (m, 2 H) 5.25 (br d, J=9.16 Hz, 1 H) 4.69 (dd, J=9.26, 4.80 Hz, 1 H) 4.45 (d, J=6.65 Hz, 2 H) 4.21 - 4.28 (m, 1 H) 3.21 (s, 1 H) 2.82 - 2.88 (m, 5 H) 2.29 - 2.40 (m, 1 H) 1.19 (s, 3 H) 1.06 (br dd, J=9.81, 6.98 Hz, 6 H); LCMS (C3783-015-A2): 98.92% (437.27, M+H), RT: 2.61 min.

Step 2: (S)-2-((S)-2-(((9H-fluoren-9-yl) methoxy)carbonyl)amino)-3-methylbutanamido)-5-ureidopentanoic acid (3):

Fmoc-Val-NHS (15 g, 34.4 mmol) in 90 mL of DME was added to a solution of L-citrulline (6.32 g, 36.12 mmol) in 50 mL of THF and NaHCO₃ (3.03 g, 36.12 mmol) in 90 mL of water. The mixture was stirred at room temperature overnight. The reaction progress was monitored by TLC. TLC system: 10% MeOH in DCM (RF: 0.1, UV visible). Aqueous citric acid (450 mL of a 15% solution in water) was added and the mixture was extracted with 10% IPA/EtOAc (2 x 500 mL). The organic layers were washed with water (3 x 500 mL) and the solvent was evaporated under vacuum. The resulting solid was triturated with 500 mL of ethyl ether and filtered to yield the product. The resulting solid was triturated with 500 mL of diethyl ether and filtered to yield (S)-2-((S)-2-(((9H-fluoren-9-yl) methoxy) carbonyl) amino)-3-methylbutanamido)-5-ureidopentanoic acid (11.5 g, 67.64%) (C3783-023-A2) as a white solid. ¹H NMR (300 MHz, DMSO-d₆) δ ppm 12.33 - 12.64 (m, 1 H) 8.15 (br d, J=7.34 Hz, 1 H) 7.89 (d, J=7.70 Hz, 2 H) 7.60 - 7.80 (m, 2 H) 7.21 - 7.50 (m, 4 H) 5.93 (br t, J=5.69 Hz, 1 H) 5.37 (s, 2 H) 4.07 - 4.41 (m, 4 H) 3.92 (br dd, J=8.80, 6.97 Hz, 1 H) 3.20 - 3.47 (m, 6 H) 2.95 (q, J=6.24 Hz, 2 H) 1.89 - 2.07 (m, 1 H) 1.48 - 1.79 (m, 2 H) 1.30 - 1.47 (m, 2 H) 1.00 - 1.14 (m, 1 H) 0.87 (dd, J=9.35, 6.79 Hz, 6 H); LCMS (C3783-023-A2): 97.37% (497.06, M+H), RT: 1.75 min.

Step 3: (9H-fluoren-9-yl) methyl ((S)-1-(((S)-1-((4-(hydroxymethyl) phenyl) amino)-1-oxo-5-ureidopentan-2-yl) amino)-3-methyl-1-oxobutan-2-yl) carbamate (4).

To a stirred solution of (S)-2-((S)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)-5-ureidopentanoic acid (12 g, 24.2 mmol) in a mixture of DCM and

- MeOH (2:1) (150 mL) was added 4-Aminobenzyl alcohol (3.57 g, 29.04 mmol) and EEDQ (11.9 g, 48.4 mmol). The reaction mixture was stirred at room temperature for overnight. Reaction progress was monitored by TLC. TLC system: 10% MeOH in DCM (RF: 0.4, UV visible). The solvent was removed and to the residue was added diethyl ether (500 mL) and sonicated for 5 - 10 mins. The solid was filtered, washed with additional ether and dried to afford (9H-fluoren-9-yl) methyl ((S)-1-(((S)-1-((4-(hydroxymethyl) phenyl) amino)-1-oxo-5-ureidopentan-2-yl) amino)-3-methyl-1-oxobutan-2-yl) carbamate (11 g, 75.86%) as a white solid. ¹H NMR (300 MHz, DMSO-d₆) δ ppm 9.92 - 10.01 (m, 1 H) 8.09 (br d, J=7.70 Hz, 1 H) 7.89 (d, J=7.34 Hz, 2 H) 7.74 (br t, J=6.79 Hz, 2 H) 7.54 (br d, J=8.07 Hz, 2 H) 7.41 (br t, J=7.70 Hz, 3 H) 7.28 - 7.36 (m, 2 H) 7.23 (br d, J=8.44 Hz, 2 H) 5.96 (br t, J=5.32 Hz, 1 H) 5.40 (s, 2 H) 5.09 (t, J=5.69 Hz, 1 H) 4.36 - 4.48 (m, 3 H) 4.18 - 4.33 (m, 3 H) 3.93 (br dd, J=8.62, 7.15 Hz, 1 H) 2.87 - 3.09 (m, 3 H) 1.91 - 2.08 (m, 1 H) 1.55 - 1.78 (m, 2 H) 1.29 - 1.51 (m, 2 H) 0.87 (br t, J=7.34 Hz, 6 H); LCMS: 85.44%, RT: 1.80 min, M+H: 602.36.
- Step 4: (9H-fluoren-9-yl) methyl ((S)-3-methyl-1-(((S)-1-((4-(((4-nitrophenoxy) carbonyl) oxy) methyl) phenyl) amino)-1-oxo-5-ureidopentan-2-yl) amino)-1-oxobutan-2-yl) carbamate (5):
- To a stirred solution of (9H-fluoren-9-yl)methyl ((S)-1-(((S)-1-((4-(hydroxymethyl)phenyl)amino)-1-oxo-5-ureidopentan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate (11 g, 18.3 mmol) and Bis(4-nitrophenyl)carbonate (11.12 g, 36.6 mmol) in DMF (60 mL) at 23 °C was added DIPEA (4.72 g, 36.6 mmol) and the mixture was stirred at room temperature for 1 hr. Reaction progress was monitored by TLC. TLC system: 10% MeOH in DCM (RF: 0.4, UV visible). After completion, diethyl ether (200 mL) was added to the reaction and stirred for 30 min. The solid was filtered, washed with additional diethyl ether and dried to afford (9H-fluoren-9-yl)methyl ((S)-3-methyl-1-(((S)-1-((4-(((4-nitrophenoxy)carbonyl)oxy)methyl)phenyl)amino)-1-oxo-5-ureidopentan-2-yl)amino)-1-oxobutan-2-yl)carbamate (10 g, 71.42%) as a white solid. ¹H NMR (300 MHz, DMSO-d₆) δ ppm 10.14 (s, 1 H) 8.26 - 8.37 (m, 2 H) 8.14 (br d, J=7.34 Hz, 1 H) 7.89 (br d, J=7.34 Hz, 2 H) 7.70 - 7.80 (m, 2 H) 7.53 - 7.68 (m, 4 H) 7.26 - 7.48 (m, 6 H) 5.93 - 6.04 (m, 1 H) 5.37 - 5.46 (m, 2 H) 5.25 (s, 2 H) 4.38 - 4.49 (m, 1 H) 4.17 - 4.35 (m, 3 H) 3.88 - 4.01 (m, 1 H) 2.91 - 3.09 (m, 2 H) 1.99 (dt, J=13.39, 6.88 Hz, 1 H) 1.56 - 1.75 (m, 2 H) 1.31 - 1.50 (m, 2 H) 0.88 (br t, J=7.52 Hz, 6 H); LCMS: 91.44%, RT: 2.16min, 767.11.

Step 5: 4-((S)-2-((S)-2-(((9H-fluoren-9-yl) methoxy) carbonyl) amino)-3-methylbutanamido)-5-ureidopentanamido) benzyl tert-butyl ethane-1, 2-diyldicarbamate (6).

To a stirred solution of (9H-fluoren-9-yl)methyl ((S)-3-methyl-1-(((S)-1-((4-((4-nitrophenoxy)carbonyl)oxy)methyl)phenyl)amino)-1-oxo-5-ureidopentan-2-yl)amino)-1-oxobutan-2-yl)carbamate (5 g, 6.5 mmol) in DMA (50 mL) at 0 °C, HOAt (0.97 g, 7.15 mmol) and 2,6-Lutidine (2.78 g, 26 mmol) were added and stirred for 5 min at 0 °C, then tert-butyl (2-aminoethyl)carbamate (1.14 g, 7.15 mmol) and DIPEA (2.51 g, 19.5 mmol) was added and allowed to stir at 23 °C for 2 h. Reaction progress was monitored by TLC. TLC system: 10% MeOH in DCM. After completion, the reaction mixture was poured into MTBE (1000 mL) and stirred for 1 h, resulting solid was filtered, washed with MTBE and dried to afford 4-((S)-2-((S)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)-5-ureidopentanamido)benzyl tert-butyl ethane-1,2-diyldicarbamate (5 g, 97%) as a pale yellow solid. ¹H NMR (300 MHz, DMSO-d₆) δ ppm 10.06 (s, 1 H) 8.12 (br d, J=7.34 Hz, 1 H) 7.89 (d, J=7.34 Hz, 2 H) 7.74 (br t, J=6.60 Hz, 2 H) 7.53 - 7.62 (m, 2 H) 7.42 (br t, J=8.07 Hz, 3 H) 7.23 - 7.36 (m, 4 H) 7.19 (br s, 1 H) 6.80 (br s, 1 H) 5.98 (br t, J=5.50 Hz, 1 H) 5.41 (s, 2 H) 4.94 (s, 2 H) 4.36 - 4.49 (m, 1 H) 4.17 - 4.34 (m, 3 H) 3.93 (br dd, J=8.80, 7.34 Hz, 1 H) 2.94 - 3.09 (m, 6 H) 2.78 (s, 1 H) 1.91 - 2.07 (m, 2 H) 1.56 - 1.76 (m, 2 H) 1.37 (s, 10 H) 1.18 (br d, J=5.14 Hz, 2 H) 0.87 (br t, J=7.34 Hz, 6 H); LCMS: 85.27%, RT: 2.01min, M+H: 788.43.

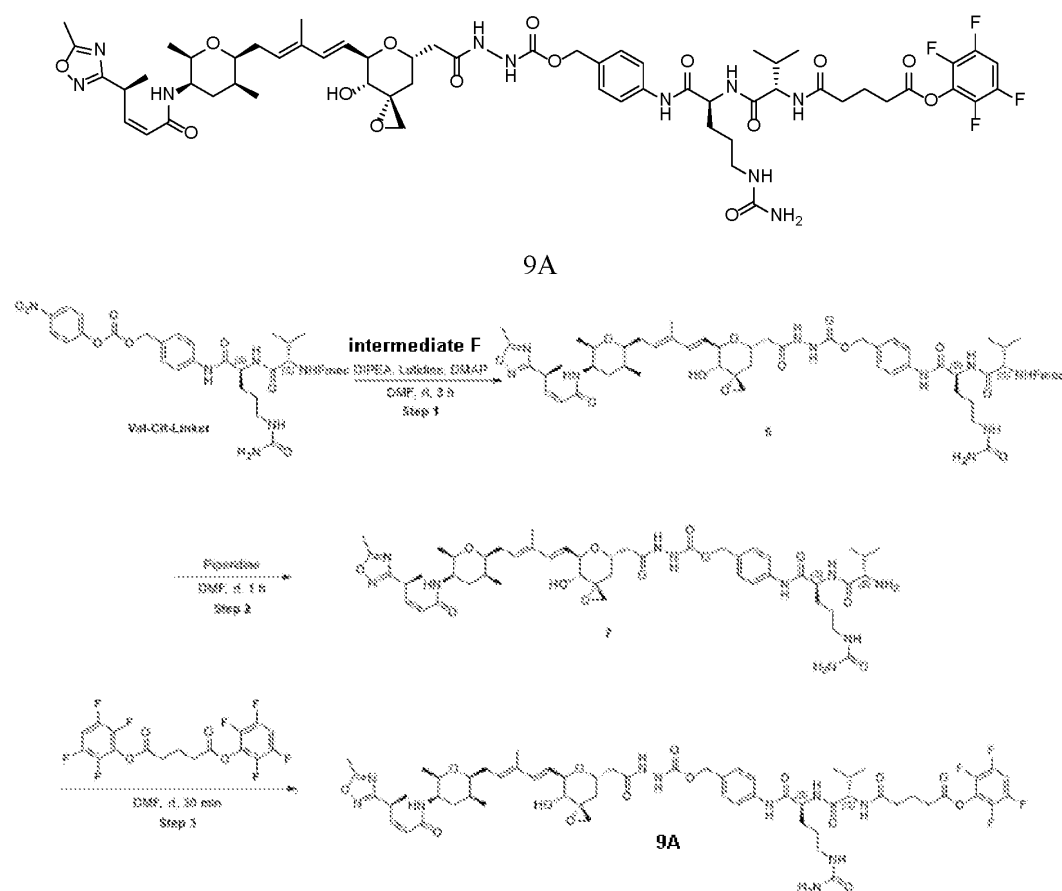
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Step 6: 4-((S)-2-((S)-2-amino-3-methylbutanamido)-5-ureidopentanamido) benzyl tert-butyl ethane-1, 2-diyldicarbamate (Compound-E).

To a stirred solution of 4-((S)-2-((S)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)-5-ureidopentanamido)benzyl tert-butyl ethane-1,2-diyldicarbamate in DMF (50 mL) at room temperature was added piperidine and the mixture was stirred at room temperature for 1 h. The reaction progress was monitored by TLC. TLC system: 10% MeOH in DCM. After 1 h, the reaction mixture was poured into diethyl ether (500 mL), stirred for 1 h, solid was filtered and dried to afford 4-((S)-2-((S)-2-amino-3-methylbutanamido)-5-ureidopentanamido)benzyl tert-butyl ethane-1,2-diyldicarbamate (2.4 g, 68.5%) as a pale yellow solid. ¹H NMR (300 MHz, DMSO-d₆) δ ppm 10.11 (s, 1 H) 8.12 (br d, J=7.34 Hz, 1 H) 7.58 (d, J=8.80 Hz, 2 H) 7.28 (br d, J=8.44 Hz, 2 H) 7.19 (br s, 1 H) 6.79 (br s, 1 H) 5.91 - 6.02 (m, 1 H) 5.40 (s, 2 H) 4.94 (s, 2 H) 4.47 (br s, 1 H) 2.90 - 3.08 (m, 8 H) 2.74 (br d, J=6.60 Hz, 1 H) 1.93 (br dd, J=11.74, 6.97 Hz, 1 H) 1.53 - 1.74 (m, 3 H) 1.37 (s, 12 H) 0.88 (d, J=6.60 Hz, 3 H) 0.78 (d, J=6.97 Hz, 3 H); LCMS: 87.71%, RT: 1.31min, M+H: 566.27.

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Example 28. Preparation of Compound 9A



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Step 1: Synthesis of 4-((S)-2-((S)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)-5-ureidopentanamido)benzyl 2-(2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((S,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetyl)hydrazine-1-carboxylate.

To a stirred solution of (9H-fluoren-9-yl)methyl ((S)-3-methyl-1-(((S)-1-(((4-((4-nitrophenoxy)carbonyl)oxy)methyl)phenyl)amino)-1-oxo-5-ureidopentan-2-yl)amino)-1-oxobutan-2-yl)carbamate (87 mg, 0.114 mmol) in DMA (5.0 mL) was added HOAt (25 mg, 0.190 mmol), Lutidine (40 mg, 0.38 mmol), (S,Z)-N-((2R,3R,5S,6S)-6-((2E,4E)-5-((3R,4R,5R,7S)-7-(2-hydrazinyl-2-oxoethyl)-4-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)-3-methylpenta-2,4-dien-1-yl)-2,5-dimethyltetrahydro-2H-pyran-3-yl)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamide (55 mg, 0.095 mmol) & DIPEA (49 mg, 0.38 mmol) at rt. The reaction mixture was stirred at rt for 3 h. The progress of the reaction was monitored by

LCMS. After 3 h, crude LCMS showed 35% of desired product m/z peak. The crude reaction mixture was directly purified by Prep. HPLC techniques and the collected fractions were lyophilized to afford 4-((S)-2-((S)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)-5-ureidopentanamido)benzyl 2-(2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((S,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetyl)hydrazine-1-carboxylate (35 mg, 33%) as a white solid. LCMS: 74.35%, RT: 2.41 min, M+H: 1201.86.

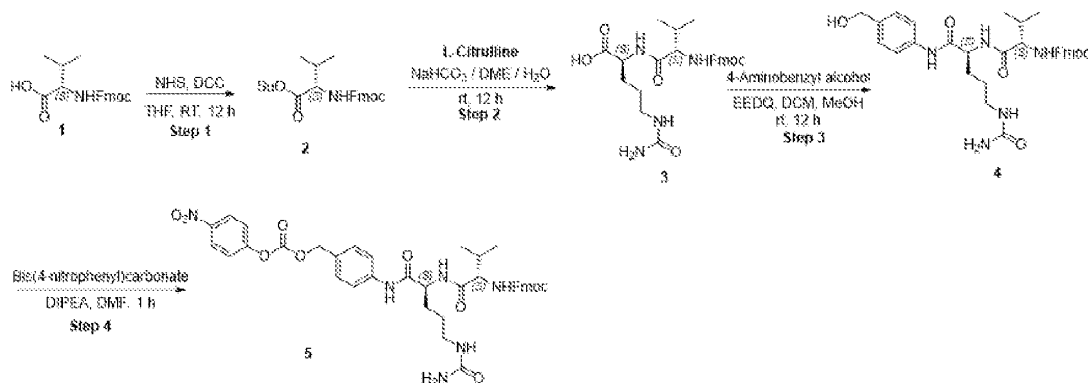
- 10 Step 2: Synthesis of 4-((S)-2-((S)-2-amino-3-methylbutanamido)-5-ureidopentanamido)benzyl 2-(2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((S,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetyl)hydrazine-1-carboxylate.
- 15 To a stirred solution of 4-((S)-2-((S)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)-5-ureidopentanamido)benzyl 2-(2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((S,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetyl)hydrazine-1-carboxylate (40 mg, 0.033 mmol) in DMF (2.0 mL) was added piperidine (14 mg, 0.165 mmol) was added and the reaction was stirred at rt for 1 h. The progress of the reaction was monitored by LCMS. Crude LCMS showed 36% of desired product mass. The reaction mixture was directly purified by Prep. HPLC techniques & the collected pure fractions were lyophilized to afford 4-((S)-2-((S)-2-amino-3-methylbutanamido)-5-ureidopentanamido)benzyl 2-(2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((S,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetyl)hydrazine-1-carboxylate (16 mg, 50%) as a white solid. LCMS: 84.64%, 1.87min, M+H: 979.64

- 30 Step 3: Synthesis of 4-((S)-2-((S)-3-methyl-2-(5-oxo-5-(2,3,5,6-tetrafluorophenoxy)pentanamido)butanamido)-5-ureidopentanamido)benzyl 2-(2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((S,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetyl)hydrazine-1-carboxylate.

To a stirred solution of 4-((S)-2-((S)-2-amino-3-methylbutanamido)-5-ureidopentanamido)benzyl 2-(2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((S,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetyl)hydrazine-1-carboxylate (15 mg, 0.015 mmol) in DMF (1.5 mL) was added bis(2,3,5,6-tetrafluorophenyl) glutarate (32 mg, 0.075 mmol) at rt. The reaction mixture was stirred at rt for 30 min. The progress of the reaction was monitored by LCMS. Crude LCMS showed 26% of desired product mass. The reaction mixture was directly purified by Prep. HPLC techniques and the pure fractions were collected at -78 °C & lyophilized to afford 4-((S)-2-((S)-3-methyl-2-(5-oxo-5-(2,3,5,6-tetrafluorophenoxy)pentanamido)butanamido)-5-ureidopentanamido)benzyl 2-(2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((S,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetyl)hydrazine-1-carboxylate (1 mg) as a white solid. LCMS: 92.48%, 1.89min, M+H: 1241.80

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Synthesis of Val-Cit-Linker:



Step 1: Synthesis of 2,5-dioxopyrrolidin-1-yl (((9H-fluoren-9-yl)methoxy)carbonyl)-L-valinate.

To a stirred solution of (((9H-fluoren-9-yl)methoxy)carbonyl)-L-valine (10 g, 29.5 mmol) and N-Hydroxysuccinamide (3.73 g, 32.45 mmol) in THF (200 mL) was added DCC (6.69 g, 32.45 mmol) in portions under ice-bath. After the addition the reaction mixture was stirred at room temperature for over night. TLC system: 50% EtOAc in Hexane (Rf: 0.5, UV visible). The reaction mixture was cooled to -5 °C, filtered and washed with cold THF (50 mL), the filtrate was concentrated in vacuum to get crude compound. The crude residue was purified by trituration with MTBE and n-pentane to afford 2,5-dioxopyrrolidin-1-yl (((9H-

fluoren-9-yl)methoxy)carbonyl)-L-valinate (12.2 g, 95.31%) (C3783-015-A2) as a white solid.

¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 7.77 (d, J=7.63 Hz, 2 H) 7.59 (br d, J=7.41 Hz, 2 H) 7.37 - 7.43 (m, 2 H) 7.29 - 7.35 (m, 2 H) 5.25 (br d, J=9.16 Hz, 1 H) 4.69 (dd, J=9.26, 4.80 Hz, 1 H) 4.45 (d, J=6.65 Hz, 2 H) 4.21 - 4.28 (m, 1 H) 3.21 (s, 1 H) 2.82 - 2.88 (m, 5 H) 2.29 - 2.40 (m, 1 H) 1.19 (s, 3 H) 1.06 (br dd, J=9.81, 6.98 Hz, 6 H)
LCMS (C3783-015-A2): 98.92% (437.27, M+H), RT: 2.61 min.

Step 2: Synthesis of (S)-2-((S)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)-5-ureidopentanoic acid.

Fmoc-Val-NHS (15 g, 34.4 mmol) in 90 mL of DME was added to a solution of L-citrulline (6.32 g, 36.12 mmol) in 50 mL of THF and NaHCO₃ (3.03 g, 36.12 mmol) in 90 mL of water. The mixture was stirred at room temperature overnight. The reaction progress was monitored by TLC. TLC system: 10% MeOH in DCM (R_f: 0.1, UV visible). Aqueous citric acid (450 mL of a 15% solution in water) was added and the mixture was extracted with 10% IPA/EtOAc (2 x 500 mL). The organic layers were washed with water (3 x 500 mL) and the solvent was evaporated under vacuum. The resulting solid was triturated with 500 mL of ethyl ether and filtered to yield the product. The resulting solid was triturated with 500 mL of diethyl ether and filtered to yield (S)-2-((S)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)-5-ureidopentanoic acid (11.5 g, 67.64%) (C3783-023-A2) as a white solid.

¹H NMR (300 MHz, DMSO-d₆) δ ppm 12.33 - 12.64 (m, 1 H) 8.15 (br d, J=7.34 Hz, 1 H) 7.89 (d, J=7.70 Hz, 2 H) 7.60 - 7.80 (m, 2 H) 7.21 - 7.50 (m, 4 H) 5.93 (br t, J=5.69 Hz, 1 H) 5.37 (s, 2 H) 4.07 - 4.41 (m, 4 H) 3.92 (br dd, J=8.80, 6.97 Hz, 1 H) 3.20 - 3.47 (m, 6 H) 2.95 (q, J=6.24 Hz, 2 H) 1.89 - 2.07 (m, 1 H) 1.48 - 1.79 (m, 2 H) 1.30 - 1.47 (m, 2 H) 1.00 - 1.14 (m, 1 H) 0.87 (dd, J=9.35, 6.79 Hz, 6 H)
LCMS (C3783-023-A2): 97.37% (497.06, M+H), RT: 1.75 min.

Step 3: Synthesis of (9H-fluoren-9-yl)methyl ((S)-1-(((S)-1-((4-(hydroxymethyl)phenyl)amino)-1-oxo-5-ureidopentan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate.

To a stirred solution of (S)-2-((S)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)-5-ureidopentanoic acid (12 g, 24.2 mmol) in a mixture of DCM and MeOH (2:1) (150 mL) was added 4-Aminobenzyl alcohol (3.57 g, 29.04 mmol) and EEDQ

(11.9 g, 48.4 mmol). The reaction mixture was stirred at room temperature for overnight. Reaction progress was monitored by TLC. TLC system: 10% MeOH in DCM (Rf: 0.4, UV visible). The solvent was removed and to the residue was added diethyl ether (500 mL) and sonicated for 5 - 10 mins. The solid was filtered, washed with additional ether and dried to afford (9H-fluoren-9-yl)methyl ((S)-1-(((S)-1-((4-(hydroxymethyl)phenyl)amino)-1-oxo-5-ureidopentan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate (11 g, 75.86%) as a white solid.

¹H NMR (300 MHz, DMSO-d₆) δ ppm 9.92 - 10.01 (m, 1 H) 8.09 (br d, J=7.70 Hz, 1 H) 7.89 (d, J=7.34 Hz, 2 H) 7.74 (br t, J=6.79 Hz, 2 H) 7.54 (br d, J=8.07 Hz, 2 H) 7.41 (br t, J=7.70 Hz, 3 H) 7.28 - 7.36 (m, 2 H) 7.23 (br d, J=8.44 Hz, 2 H) 5.96 (br t, J=5.32 Hz, 1 H) 5.40 (s, 2 H) 5.09 (t, J=5.69 Hz, 1 H) 4.36 - 4.48 (m, 3 H) 4.18 - 4.33 (m, 3 H) 3.93 (br dd, J=8.62, 7.15 Hz, 1 H) 2.87 - 3.09 (m, 3 H) 1.91 - 2.08 (m, 1 H) 1.55 - 1.78 (m, 2 H) 1.29 - 1.51 (m, 2 H) 0.87 (br t, J=7.34 Hz, 6 H)

LCMS: 85.44%, RT: 1.80 min, M+H: 602.36

15

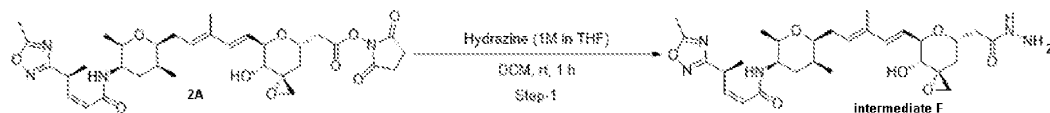
Step 4: Synthesis of (9H-fluoren-9-yl)methyl ((S)-3-methyl-1-(((S)-1-((4-((4-nitrophenoxy)carbonyl)oxy)methyl)phenyl)amino)-1-oxo-5-ureidopentan-2-yl)amino)-1-oxobutan-2-yl)carbamate.

To a stirred solution of (9H-fluoren-9-yl)methyl ((S)-1-(((S)-1-((4-(hydroxymethyl)phenyl)amino)-1-oxo-5-ureidopentan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate (11 g, 18.3 mmol) and Bis(4-nitrophenoxy)carbonate (11.12 g, 36.6 mmol) in DMF (60 mL) at 23 °C was added DIPEA (4.72 g, 36.6 mmol) and the mixture was stirred at room temperature for 1 hr. Reaction progress was monitored by TLC. TLC system: 10% MeOH in DCM (Rf: 0.4, UV visible). After completion, diethyl ether (200 mL) was added to the reaction and stirred for 30 min. The solid was filtered, washed with additional diethyl ether and dried to afford (9H-fluoren-9-yl)methyl ((S)-3-methyl-1-(((S)-1-((4-((4-nitrophenoxy)carbonyl)oxy)methyl)phenyl)amino)-1-oxo-5-ureidopentan-2-yl)amino)-1-oxobutan-2-yl)carbamate (10 g, 71.42%) as a white solid.

¹H NMR (300 MHz, DMSO-d₆) δ ppm 10.14 (s, 1 H) 8.26 - 8.37 (m, 2 H) 8.14 (br d, J=7.34 Hz, 1 H) 7.89 (br d, J=7.34 Hz, 2 H) 7.70 - 7.80 (m, 2 H) 7.53 - 7.68 (m, 4 H) 7.26 - 7.48 (m, 6 H) 5.93 - 6.04 (m, 1 H) 5.37 - 5.46 (m, 2 H) 5.25 (s, 2 H) 4.38 - 4.49 (m, 1 H) 4.17 - 4.35 (m, 3 H) 3.88 - 4.01 (m, 1 H) 2.91 - 3.09 (m, 2 H) 1.99 (dt, J=13.39, 6.88 Hz, 1 H) 1.56 - 1.75 (m, 2 H) 1.31 - 1.50 (m, 2 H) 0.88 (br t, J=7.52 Hz, 6 H)

LCMS (SR-12580-7-A2): 91.44%, RT: 2.16min, 767.11.

Preparation of intermediate F.



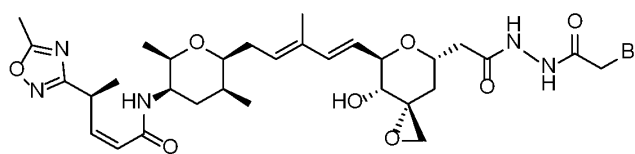
5

Synthesis of (S,Z)-N-((2R,3R,5S,6S)-6-((2E,4E)-5-((3R,4R,5R,7S)-7-(2-hydrazineyl-2-oxoethyl)-4-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)-3-methylpenta-2,4-dien-1-yl)-2,5-dimethyltetrahydro-2H-pyran-3-yl)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamide.

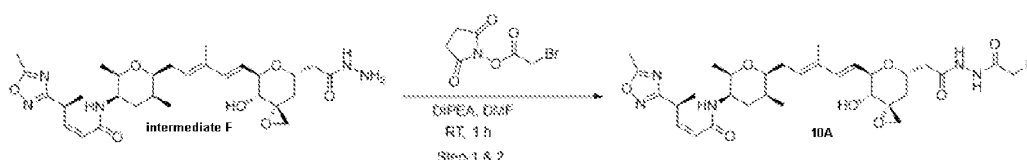
To a stirred solution of 2,5-dioxopyrrolidin-1-yl 2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((S,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetate (20 mg, 0.03 mmol) in DCM (2 mL) was added Hydrazine (1M in THF) (70 μ L, 0.3 mmol) stirred for 30 min at RT, reaction progress was monitored by LCMS. Crude LCMS showed 68% of desired product mass. The reaction mixture was evaporated under reduced pressure to get crude compound (20 mg), crude compound purified by Prep. HPLC. The pure fractions were collected at -78 $^{\circ}$ C and lyophilized to afford (S,Z)-N-((2R,3R,5S,6S)-6-((2E,4E)-5-((3R,4R,5R,7S)-7-(2-hydrazineyl-2-oxoethyl)-4-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)-3-methylpenta-2,4-dien-1-yl)-2,5-dimethyltetrahydro-2H-pyran-3-yl)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamide (9.4 mg, 53.7 %) as a white solid.

^1H NMR (400 MHz, CHLOROFORM-d) δ ppm 7.29 (br s, 1 H), 6.36 (d, J=15.80 Hz, 1 H), 6.14 - 6.18 (m, 1 H), 6.05 (br d, J=9.05 Hz, 1 H), 5.81 (dd, J=11.34, 0.76 Hz, 1 H), 5.59 - 5.65 (m, 1 H), 5.52 (br t, J=6.98 Hz, 1 H), 5.09 - 5.14 (m, 1 H), 4.44 - 4.48 (m, 1 H), 4.22 (t, J=6.43 Hz, 1 H), 3.89 - 3.99 (m, 3 H), 3.66 - 3.70 (m, 1 H), 3.51 - 3.55 (m, 2 H), 3.00 (d, J=4.47 Hz, 1 H), 2.68 - 2.73 (m, 2 H), 2.60 (d, J=6.10 Hz, 1 H), 2.56 (s, 3 H), 2.34 - 2.41 (m, 1 H), 2.19 - 2.26 (m, 1 H), 2.02 - 2.04 (m, 1 H), 1.91 - 1.95 (m, 3 H), 1.77 - 1.82 (m, 2 H), 1.76 (s, 3 H), 1.47 (d, J=6.98 Hz, 3 H), 1.16 (d, J=6.32 Hz, 3 H), 0.98 (d, J=7.30 Hz, 3 H). LCMS: 90.9% purity, M+H: 574.5, RT: 2.05.

30 Example 29. Preparation of compound 10A.



10A



- 5 Synthesis of (S,Z)-N-((2R,3R,5S,6S)-6-((2E,4E)-5-((3R,4R,5R,7S)-7-(2-(2-(2-bromoacetyl)hydrazineyl)-2-oxoethyl)-4-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)-3-methylpenta-2,4-dien-1-yl)-2,5-dimethyltetrahydro-2H-pyran-3-yl)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamide.

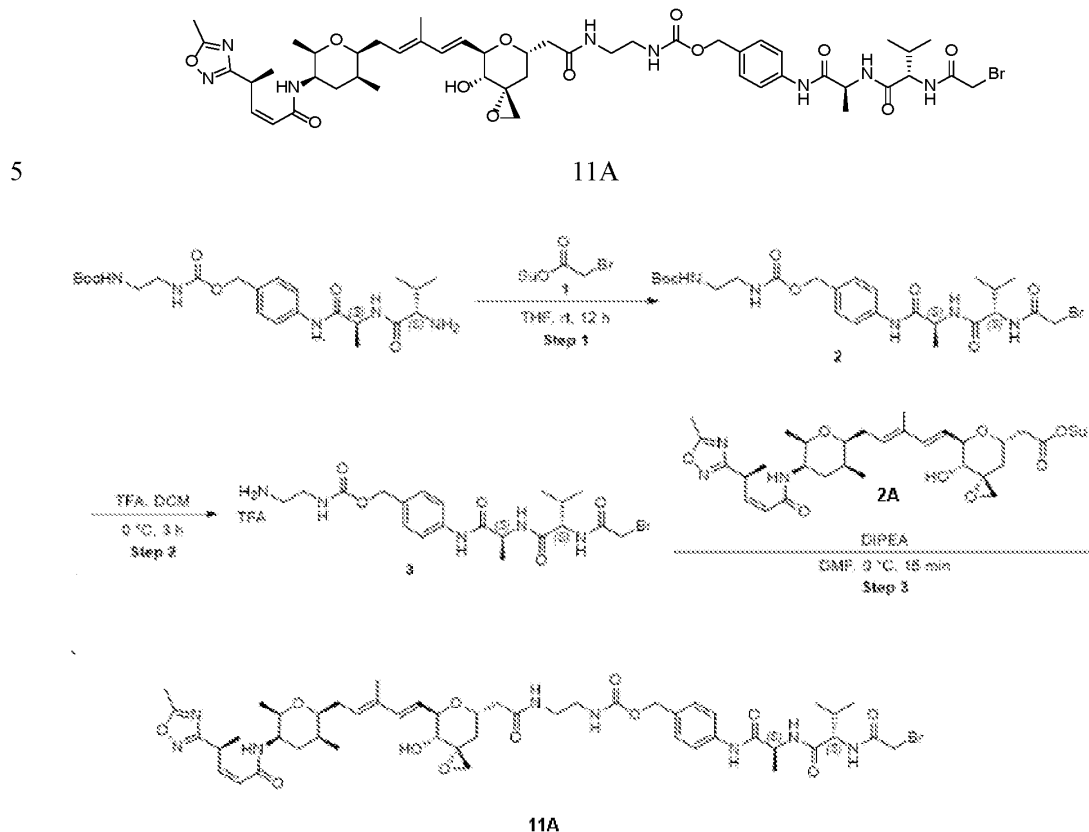
To a stirred solution of 2,5-dioxopyrrolidin-1-yl 2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((S,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetate (14 mg, 0.02 mmol) in DMF (1.5 mL) was added 2,5-dioxopyrrolidin-1-yl 2-bromoacetate (7.1 mg, 0.03 mmol) stirred for 1h at RT, when LCMS indicated completion of the reaction, reaction mass quenched with 0.1% Formic acid solution (10 uL), reaction mass directly purified through prep HPLC, collected fractions at -78° C and lyophilized to afford (S,Z)-N-((2R,3R,5S,6S)-6-((2E,4E)-5-((3R,4R,5R,7S)-7-(2-(2-(2-bromoacetyl)hydrazineyl)-2-oxoethyl)-4-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)-3-methylpenta-2,4-dien-1-yl)-2,5-dimethyltetrahydro-2H-pyran-3-yl)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamide as off white solid.

20

¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 8.71 (br s, 1 H), 8.55 (br s, 1 H), 6.40 (br d, J=15.65 Hz, 1 H), 6.15 (dd, J=11.25, 9.78 Hz, 1 H), 6.04 (br d, J=8.80 Hz, 1 H), 5.81 (d, J=11.25 Hz, 1 H), 5.64 (dd, J=15.65, 6.36 Hz, 1 H), 5.54 (br t, J=6.85 Hz, 1 H), 5.06 - 5.14 (m, 1 H), 4.42 - 4.49 (m, 1 H), 4.25 - 4.31 (m, 1 H), 3.87 - 4.01 (m, 3 H), 3.67 (td, J=6.36, 4.40 Hz, 1 H), 3.49 - 3.57 (m, 2 H), 3.02 (d, J=4.40 Hz, 1 H), 2.78 - 2.87 (m, 2 H), 2.70 - 2.77 (m, 1 H), 2.56 (s, 3 H), 2.34 - 2.42 (m, 1 H), 2.16 - 2.26 (m, 2 H), 2.11 (br dd, J=14.43, 5.14 Hz, 1 H), 1.91 - 2.03 (m, 3 H), 1.76 (s, 4 H), 1.47 (d, J=7.34 Hz, 3 H), 1.17 (d, J=6.36 Hz, 3 H), 0.98 (d, J=7.34 Hz, 3 H)

LCMS: 98.18% purity, M+H: 694.37, RT: 3.58

Example 30. Preparation of compound 11A.



Step 1: Synthesis of 4-((S)-2-((S)-2-(2-bromoacetamido)-3-methylbutanamido)propanamido)benzyl tert-butyl ethane-1,2-diyl dicarbamate.

To a stirred solution of 4-((S)-2-((S)-2-amino-3-methylbutanamido)propanamido)benzyl tert-butyl ethane-1,2-diyl dicarbamate (500 mg, 1.04 mmol) in THF (15 mL) at room temperature was added 2,5-dioxopyrrolidin-1-yl 2-bromoacetate (369 mg, 1.56 mmol) and the reaction mixture was stirred at room temperature for 12 h. The progress of the reaction was monitored by TLC. TLC mobile phase: 10% MeOH in DCM (Rf: 0.5, UV active). The reaction mixture was concentrated under reduced pressure to get crude compound. The crude compound was triturated with diethyl ether (50 mL) and dried to afford 4-((S)-2-((S)-2-(2-bromoacetamido)-3-methylbutanamido)propanamido)benzyl tert-butyl ethane-1,2-diyl dicarbamate (400 mg, 63%) as an off-white solid.

LCMS (BT-17195-82-A1): 96.61%, RT: 2.18min, M+H: 600.30.

¹H NMR (400 MHz, DMSO-d₆) δ ppm 9.92 - 10.01 (m, 1 H), 8.28 - 8.37 (m, 2 H), 7.57 (d, J=8.50 Hz, 2 H), 7.28 (d, J=8.50 Hz, 2 H), 7.11 - 7.22 (m, 1 H), 6.75 - 6.86 (m, 1 H), 4.88 - 4.99 (m, 2 H), 4.33 - 4.44 (m, 1 H), 4.24 (dd, J=8.72, 6.76 Hz, 1 H), 3.89 - 4.02 (m, 2 H), 3.38 (q, J=7.01 Hz, 1 H), 2.92 - 3.04 (m, 4 H), 1.93 - 2.04 (m, 1 H), 1.37 (s, 9 H), 1.31 (d, J=7.08 Hz, 3 H), 1.09 (t, J=7.03 Hz, 1 H), 0.86 (dd, J=17.77, 6.76 Hz, 6 H)

Step 2: Synthesis of 4-((S)-2-((S)-2-(2-bromoacetamido)-3-methylbutanamido)propanamido)benzyl (2-aminoethyl)carbamate.

To a stirred solution of 4-((S)-2-((S)-2-(2-bromoacetamido)-3-methylbutanamido)propanamido)benzyl tert-butyl ethane-1,2-diyl dicarbamate (350 mg, 0.58 mmol) in DCM (10 mL) was added trifluoroacetic acid (991 mg, 8.6 mmol) at 0°C and stirred for 3 h at rt. The progress of the reaction was monitored by TLC. TLC mobile phase: 10% MeOH in DCM (R_f: 0.5, UV active). The reaction mixture was concentrated under reduced pressure to get crude compound (300 mg). The crude compound was purified by trituration with diethyl ether and dried to afford 4-((S)-2-((S)-2-(2-bromoacetamido)-3-methylbutanamido)propanamido)benzyl (2-aminoethyl)carbamate (200 mg, TFA Salt) (BT-17195-87-A1) as a pale yellow solid.

LCMS (BT-17195-87-A1): 84.48%, RT: 1.64min, M+H: 500.26.

¹H NMR (400 MHz, DMSO-d₆) δ ppm 9.98 (s, 1 H), 8.32 - 8.32 (m, 1 H), 8.29 - 8.37 (m, 1 H), 7.73 (br d, J=0.87 Hz, 3 H), 7.58 (d, J=8.61 Hz, 2 H), 7.26 - 7.38 (m, 3 H), 4.97 (s, 2 H), 4.34 - 4.44 (m, 1 H), 4.23 (dd, J=8.72, 6.76 Hz, 1 H), 3.90 - 4.04 (m, 2 H), 3.19 - 3.27 (m, 2 H), 2.86 (br d, J=2.94 Hz, 2 H), 1.93 - 2.04 (m, 2 H), 1.31 (d, J=7.08 Hz, 3 H), 1.17 (t, J=7.08 Hz, 1 H), 0.87 (dd, J=17.22, 6.76 Hz, 6 H)

Step 3: Synthesis of 44-((S)-2-((S)-2-(2-bromoacetamido)-3-methylbutanamido)propanamido)benzyl (2-(2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((S,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl) pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro [2.5]octan-5-yl)acetamido)ethyl)carbamate.

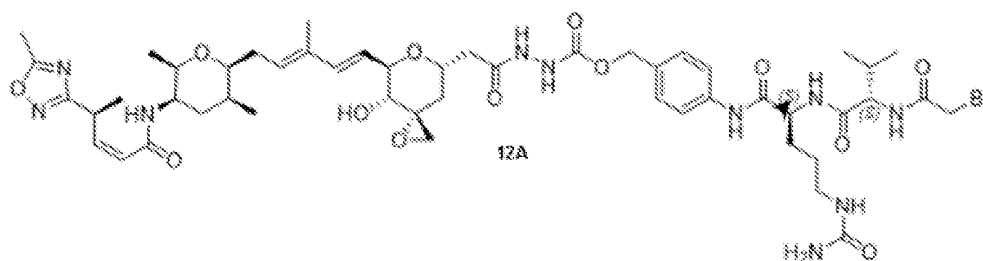
To a stirred solution of 2,5-dioxopyrrolidin-1-yl 2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((S,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetate (90 mg, 0.137 mmol) & 4-((S)-2-((S)-2-(2-bromoacetamido)-3-methylbutanamido)propanamido)benzyl (2-aminoethyl)carbamate (102

mg, 0.205 mmol) in DMF (2.0 mL) was added DIPEA (0.026 g, 0.205 mmol) at 0 °C. After 15 min at 0 °C, the reaction mixture was quenched with 0.1M formic acid. Crude LCMS showed 20% of desired product mass. The reaction mixture was directly submitted for Prep. HPLC purification. The pure fractions were collected at -78 °C and lyophilized to afford 44-
 5 ((S)-2-((S)-2-(2-bromoacetamido)-3-methylbutanamido)propanamido)benzyl (2-(2-((3R,5S,7R,8R)-7-((1E,3E)-5-((2S,3S,5R,6R)-3,6-dimethyl-5-((S,Z)-4-(5-methyl-1,2,4-oxadiazol-3-yl)pent-2-enamido)tetrahydro-2H-pyran-2-yl)-3-methylpenta-1,3-dien-1-yl)-8-hydroxy-1,6-dioxaspiro[2.5]octan-5-yl)acetamido)ethyl)carbamate as a white solid.

LCMS: 94.49%, RT: 2.47 min, M+H: 1041.58.

10 ¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 8.37 (s, 1 H), 7.53 - 7.65 (m, 2 H), 7.31 (br d, J=7.96 Hz, 3 H), 7.04 - 7.13 (m, 1 H), 6.51 - 6.65 (m, 2 H), 6.25 - 6.39 (m, 1 H), 6.09 - 6.20 (m, 1 H), 6.00 - 6.08 (m, 1 H), 5.76 - 5.86 (m, 1 H), 5.43 - 5.67 (m, 2 H), 5.12 - 5.28 (m, 2 H), 5.01 - 5.09 (m, 2 H), 4.56 - 4.70 (m, 1 H), 4.19 - 4.43 (m, 4 H), 3.82 - 3.98 (m, 3 H), 3.54 - 3.64 (m, 2 H), 3.40 - 3.51 (m, 4 H), 3.20 - 3.36 (m, 4 H), 2.89 - 2.95 (m, 1 H), 2.46 -
 15 2.75 (m, 9 H), 2.31 - 2.42 (m, 2 H), 2.13 - 2.26 (m, 2 H), 1.87 - 1.98 (m, 1 H), 1.79 - 1.85 (m, 2 H), 1.69 - 1.77 (m, 6 H), 1.46 (d, J=6.98 Hz, 8 H), 1.23 - 1.32 (m, 2 H), 1.10 - 1.18 (m, 4 H), 0.89 - 1.03 (m, 11 H)

Example 31. Preparation of compound 12A.

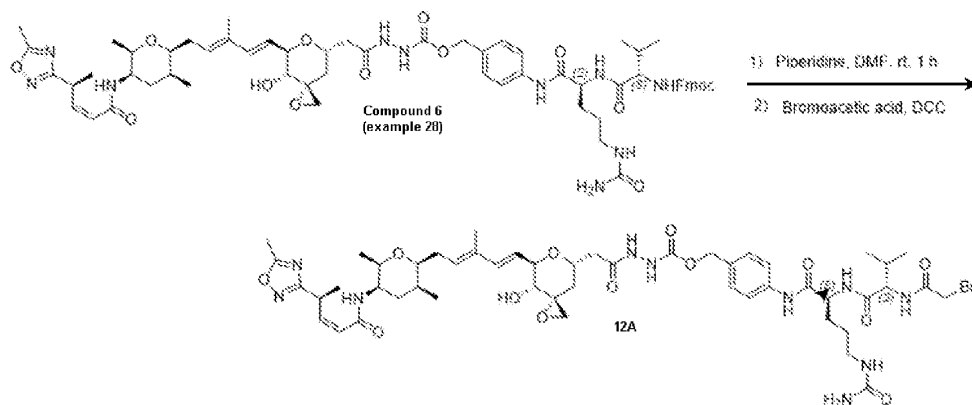


20

12A

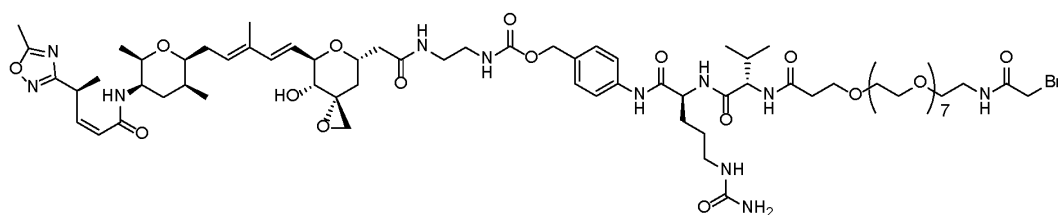
Compound 12A was prepared according to the following synthetic scheme using compounds and intermediates described herein, and using synthetic procedures similar to

the ones provided herein.

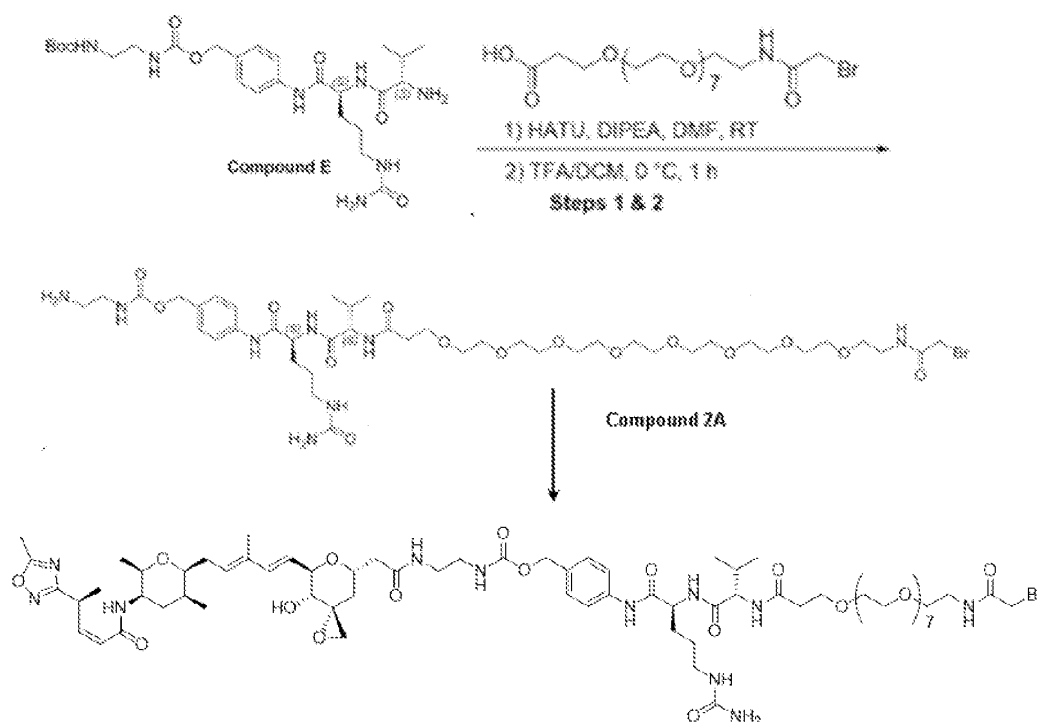


Example 32. Preparation of compound 13A.

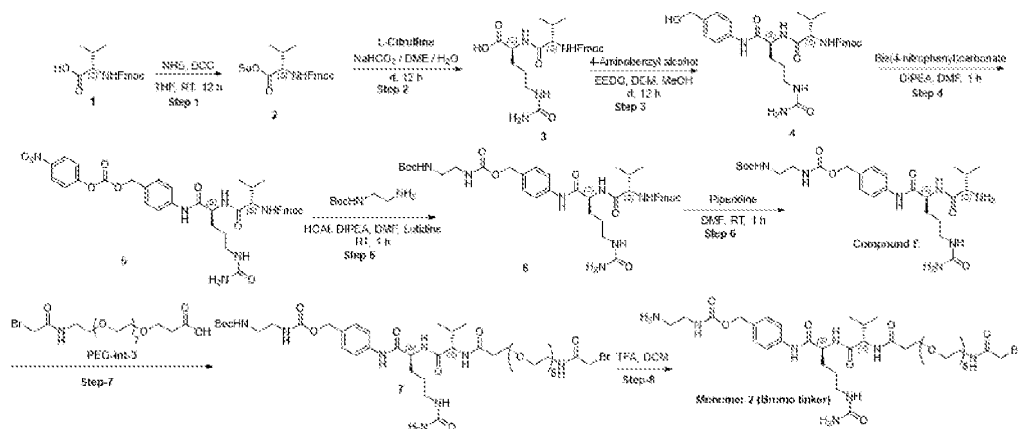
Compound 13A was prepared according to the following synthetic scheme using
5 compounds and intermediates described herein, and using synthetic procedures similar to the
ones provided herein.



13A



Preparation of intermediate.



5

Step 1: Synthesis of 2, 5-dioxopyrrolidin-1-yl (((9H-fluoren-9-yl)methoxy)carbonyl)-L-valinate (1).

To a stirred solution of (((9H-fluoren-9-yl)methoxy)carbonyl)-L-valine (10 g, 29.5 mmol) and N-Hydroxysuccinamide (3.73 g, 32.45 mmol) in THF (200 mL) was added DCC (6.69 g, 32.45 mmol) in portions under ice-bath. After the addition the reaction mixture was

stirred at room temperature for overnight. TLC system: 50% EtOAc in Hexane (RF: 0.5, UV visible). The reaction mixture was cooled to -5 °C, filtered and washed with cold THF (50 mL), the filtrate was concentrated in vacuum to get crude compound. The crude residue was purified by trituration with MTBE and n-pentane to afford 2, 5-dioxopyrrolidin-1-yl (((9H-fluoren-9-yl)methoxy)carbonyl)-L-valinate (12.2 g, 95.31%) (C3783-015-A2) as a white solid. ¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 7.77 (d, J=7.63 Hz, 2 H) 7.59 (br d, J=7.41 Hz, 2 H) 7.37 - 7.43 (m, 2 H) 7.29 - 7.35 (m, 2 H) 5.25 (br d, J=9.16 Hz, 1 H) 4.69 (dd, J=9.26, 4.80 Hz, 1 H) 4.45 (d, J=6.65 Hz, 2 H) 4.21 - 4.28 (m, 1 H) 3.21 (s, 1 H) 2.82 - 2.88 (m, 5 H) 2.29 - 2.40 (m, 1 H) 1.19 (s, 3 H) 1.06 (br dd, J=9.81, 6.98 Hz, 6 H); LCMS (C3783-015-A2): 98.92% (437.27, M+H), RT: 2.61 min.

Step 2: Synthesis of (S)-2-((S)-2-((((9H-fluoren-9-yl) methoxy)carbonyl)amino)-3-methylbutanamido)-5-ureidopentanoic acid (3).

Fmoc-Val-NHS (15 g, 34.4 mmol) in 90 mL of DME was added to a solution of L-citrulline (6.32 g, 36.12 mmol) in 50 mL of THF and NaHCO₃ (3.03 g, 36.12 mmol) in 90 mL of water. The mixture was stirred at room temperature overnight. The reaction progress was monitored by TLC. TLC system: 10% MeOH in DCM (RF: 0.1, UV visible). Aqueous citric acid (450 mL of a 15% solution in water) was added and the mixture was extracted with 10% IPA/EtOAc (2 x 500 mL). The organic layers were washed with water (3 x 500 mL) and the solvent was evaporated under vacuum. The resulting solid was triturated with 500 mL of ethyl ether and filtered to yield the product. The resulting solid was triturated with 500 mL of diethyl ether and filtered to yield (S)-2-((S)-2-((((9H-fluoren-9-yl) methoxy) carbonyl) amino)-3-methylbutanamido)-5-ureidopentanoic acid (11.5 g, 67.64%) (C3783-023-A2) as a white solid. ¹H NMR (300 MHz, DMSO-d₆) δ ppm 12.33 - 12.64 (m, 1 H) 8.15 (br d, J=7.34 Hz, 1 H) 7.89 (d, J=7.70 Hz, 2 H) 7.60 - 7.80 (m, 2 H) 7.21 - 7.50 (m, 4 H) 5.93 (br t, J=5.69 Hz, 1 H) 5.37 (s, 2 H) 4.07 - 4.41 (m, 4 H) 3.92 (br dd, J=8.80, 6.97 Hz, 1 H) 3.20 - 3.47 (m, 6 H) 2.95 (q, J=6.24 Hz, 2 H) 1.89 - 2.07 (m, 1 H) 1.48 - 1.79 (m, 2 H) 1.30 - 1.47 (m, 2 H) 1.00 - 1.14 (m, 1 H) 0.87 (dd, J=9.35, 6.79 Hz, 6 H); LCMS (C3783-023-A2): 97.37% (497.06, M+H), RT: 1.75 min.

Step 3: Synthesis of (9H-fluoren-9-yl) methyl ((S)-1-(((S)-1-((4-(hydroxymethyl) phenyl) amino)-1-oxo-5-ureidopentan-2-yl) amino)-3-methyl-1-oxobutan-2-yl) carbamate (4):

To a stirred solution of (S)-2-((S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)-5-ureidopentanoic acid (12 g, 24.2 mmol) in a mixture of DCM and

MeOH (2:1) (150 mL) was added 4-Aminobenzyl alcohol (3.57 g, 29.04 mmol) and EEDQ (11.9 g, 48.4 mmol). The reaction mixture was stirred at room temperature for overnight. Reaction progress was monitored by TLC. TLC system: 10% MeOH in DCM (RF: 0.4, UV visible). The solvent was removed and to the residue was added diethyl ether (500 mL) and sonicated for 5 - 10 mins. The solid was filtered, washed with additional ether and dried to afford (9H-fluoren-9-yl) methyl ((S)-1-(((S)-1-((4-(hydroxymethyl) phenyl) amino)-1-oxo-5-ureidopentan-2-yl) amino)-3-methyl-1-oxobutan-2-yl) carbamate (11 g, 75.86%) as a white solid. ¹H NMR (300 MHz, DMSO-d₆) δ ppm 9.92 - 10.01 (m, 1 H) 8.09 (br d, J=7.70 Hz, 1 H) 7.89 (d, J=7.34 Hz, 2 H) 7.74 (br t, J=6.79 Hz, 2 H) 7.54 (br d, J=8.07 Hz, 2 H) 7.41 (br t, J=7.70 Hz, 3 H) 7.28 - 7.36 (m, 2 H) 7.23 (br d, J=8.44 Hz, 2 H) 5.96 (br t, J=5.32 Hz, 1 H) 5.40 (s, 2 H) 5.09 (t, J=5.69 Hz, 1 H) 4.36 - 4.48 (m, 3 H) 4.18 - 4.33 (m, 3 H) 3.93 (br dd, J=8.62, 7.15 Hz, 1 H) 2.87 - 3.09 (m, 3 H) 1.91 - 2.08 (m, 1 H) 1.55 - 1.78 (m, 2 H) 1.29 - 1.51 (m, 2 H) 0.87 (br t, J=7.34 Hz, 6 H); LCMS: 85.44%, RT: 1.80 min, M+H: 602.36.

Step 4: Synthesis of (9H-fluoren-9-yl) methyl ((S)-3-methyl-1-(((S)-1-((4-((4-nitrophenoxy) carbonyl) oxy) methyl) phenyl) amino)-1-oxo-5-ureidopentan-2-yl) amino)-1-oxobutan-2-yl) carbamate (5).

To a stirred solution of (9H-fluoren-9-yl)methyl ((S)-1-(((S)-1-((4-(hydroxymethyl)phenyl)amino)-1-oxo-5-ureidopentan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate (11 g, 18.3 mmol) and Bis(4-nitrophenyl)carbonate (11.12 g, 36.6 mmol) in DMF (60 mL) at 23 °C was added DIPEA (4.72 g, 36.6 mmol) and the mixture was stirred at room temperature for 1 hr. Reaction progress was monitored by TLC. TLC system: 10% MeOH in DCM (RF: 0.4, UV visible). After completion, diethyl ether (200 mL) was added to the reaction and stirred for 30 min. The solid was filtered, washed with additional diethyl ether and dried to afford (9H-fluoren-9-yl)methyl ((S)-3-methyl-1-(((S)-1-((4-((4-nitrophenoxy)carbonyl)oxy)methyl)phenyl)amino)-1-oxo-5-ureidopentan-2-yl)amino)-1-oxobutan-2-yl)carbamate (10 g, 71.42%) as a white solid. ¹H NMR (300 MHz, DMSO-d₆) δ ppm 10.14 (s, 1 H) 8.26 - 8.37 (m, 2 H) 8.14 (br d, J=7.34 Hz, 1 H) 7.89 (br d, J=7.34 Hz, 2 H) 7.70 - 7.80 (m, 2 H) 7.53 - 7.68 (m, 4 H) 7.26 - 7.48 (m, 6 H) 5.93 - 6.04 (m, 1 H) 5.37 - 5.46 (m, 2 H) 5.25 (s, 2 H) 4.38 - 4.49 (m, 1 H) 4.17 - 4.35 (m, 3 H) 3.88 - 4.01 (m, 1 H) 2.91 - 3.09 (m, 2 H) 1.99 (dt, J=13.39, 6.88 Hz, 1 H) 1.56 - 1.75 (m, 2 H) 1.31 - 1.50 (m, 2 H) 0.88 (br t, J=7.52 Hz, 6 H); LCMS: 91.44%, RT: 2.16min, 767.11.

Step 5: Synthesis of 4-((S)-2-((S)-2-(((9H-fluoren-9-yl) methoxy) carbonyl) amino)-3-methylbutanamido)-5-ureidopentanamido) benzyl tert-butyl ethane-1, 2-diyldicarbamate (6).

To a stirred solution of (9H-fluoren-9-yl)methyl ((S)-3-methyl-1-(((S)-1-((4-(((4-nitrophenoxy)carbonyl)oxy)methyl)phenyl)amino)-1-oxo-5-ureidopentan-2-yl)amino)-1-oxobutan-2-yl)carbamate (5 g, 6.5 mmol) in DMA (50 mL) at 0 °C, HOAt (0.97 g, 7.15 mmol) and 2,6-Lutidine (2.78 g, 26 mmol) were added and stirred for 5 min at 0 °C, then tert-butyl (2-aminoethyl)carbamate (1.14 g, 7.15 mmol) and DIPEA (2.51 g, 19.5 mmol) was added and allowed to stir at 23 °C for 2 h. Reaction progress was monitored by TLC. TLC system: 10% MeOH in DCM. After completion, the reaction mixture was poured into MTBE (1000 mL) and stirred for 1 h, resulting solid was filtered, washed with MTBE and dried to afford 4-((S)-2-((S)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)-5-ureidopentanamido)benzyl tert-butyl ethane-1,2-diyldicarbamate (5 g, 97%) as a pale yellow solid. ¹H NMR (300 MHz, DMSO-d₆) δ ppm 10.06 (s, 1 H) 8.12 (br d, J=7.34 Hz, 1 H) 7.89 (d, J=7.34 Hz, 2 H) 7.74 (br t, J=6.60 Hz, 2 H) 7.53 - 7.62 (m, 2 H) 7.42 (br t, J=8.07 Hz, 3 H) 7.23 - 7.36 (m, 4 H) 7.19 (br s, 1 H) 6.80 (br s, 1 H) 5.98 (br t, J=5.50 Hz, 1 H) 5.41 (s, 2 H) 4.94 (s, 2 H) 4.36 - 4.49 (m, 1 H) 4.17 - 4.34 (m, 3 H) 3.93 (br dd, J=8.80, 7.34 Hz, 1 H) 2.94 - 3.09 (m, 6 H) 2.78 (s, 1 H) 1.91 - 2.07 (m, 2 H) 1.56 - 1.76 (m, 2 H) 1.37 (s, 10 H) 1.18 (br d, J=5.14 Hz, 2 H) 0.87 (br t, J=7.34 Hz, 6 H); LCMS: 85.27%, RT: 2.01min, M+H: 788.43.

20

Step 6: Synthesis of 4-((S)-2-((S)-2-amino-3-methylbutanamido)-5-ureidopentanamido) benzyl tert-butyl ethane-1, 2-diyldicarbamate (Compound-E).

To a stirred solution of 4-((S)-2-((S)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)-5-ureidopentanamido)benzyl tert-butyl ethane-1,2-diyldicarbamate in DMF (50 mL) at room temperature was added piperidine and the mixture was stirred at room temperature for 1 h. The reaction progress was monitored by TLC. TLC system: 10% MeOH in DCM. After 1 h, the reaction mixture was poured into diethyl ether (500 mL), stirred for 1 h, solid was filtered and dried to afford 4-((S)-2-((S)-2-amino-3-methylbutanamido)-5-ureidopentanamido)benzyl tert-butyl ethane-1,2-diyldicarbamate (2.4 g, 68.5%) as a pale yellow solid. ¹H NMR (300 MHz, DMSO-d₆) δ ppm 10.11 (s, 1 H) 8.12 (br d, J=7.34 Hz, 1 H) 7.58 (d, J=8.80 Hz, 2 H) 7.28 (br d, J=8.44 Hz, 2 H) 7.19 (br s, 1 H) 6.79 (br s, 1 H) 5.91 - 6.02 (m, 1 H) 5.40 (s, 2 H) 4.94 (s, 2 H) 4.47 (br s, 1 H) 2.90 - 3.08 (m, 8 H) 2.74 (br d, J=6.60 Hz, 1 H) 1.93 (br dd, J=11.74, 6.97 Hz, 1 H) 1.53 - 1.74 (m, 3 H) 1.37 (s, 12 H) 0.88 (d, J=6.60 Hz, 3 H) 0.78 (d, J=6.97 Hz, 3 H); LCMS: 87.71%, RT: 1.31min, M+H: 566.27.

30

Step 7: Synthesis of tert-butyl (4-((32S,35S)-1-iodo-32-isopropyl-2,30,33-trioxo-35-(3-ureidopropyl)-6,9,12,15,18,21,24,27-octaoxa-3,31,34-triazahexatriacontan-36-amido)benzyl)ethane-1,2-diylldicarbamate (7).

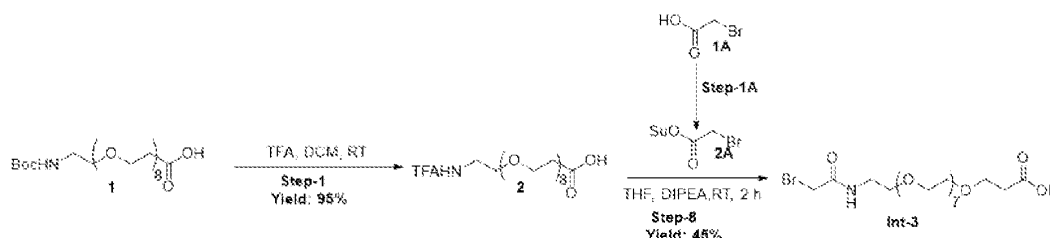
- 5 To a stirred solution of 4-((S)-2-((S)-2-amino-3-methylbutanamido)-5-ureidopentanamido)benzyl tert-butyl ethane-1,2-diylldicarbamate(1 g, 1.8 m mol), 1-bromo-2-oxo-6,9,12,15,18,21,24,27-octaoxa-3-azatriacontan-30-oic acid(3.54 g, 6.3 m mol) in THF(30 ml), DCC(0.74 g, 3.6 m mol) and DIPEA(0.696 g, 5.4 m mol) were added at RT. The reaction mixture was stirred at RT for 3 h. Diethyl ether (75 ml) was added to the reaction
- 10 mixture. The obtained solid was filtered and washed Diethyl ether (40 ml). The solid was dried under high vacuum to get product (2 g, LCMS showed 79.7% of desired product, Yield 79%). TLC showed COMPLEX. TLC: Mobile phase: 10% MeOH in DCM. The compound as a white colour solid. ¹H NMR (400 MHz, DMSO-d₆) δ ppm 9.98 (s, 1 H) 8.22 - 8.39 (m, 1 H) 8.11 (br d, J=7.30 Hz, 1 H) 7.87 (br d, J=8.50 Hz, 1 H) 7.59 (br d, J=8.17 Hz, 2 H) 7.27 (br d, J=8.17 Hz, 2 H) 7.18 (br s, 1 H) 6.79 (br s, 1 H) 5.99 (br s, 1 H) 5.58 (br d, J=7.74 Hz, 1 H) 5.41 (br s, 2 H) 4.94 (br s, 2 H) 4.34 - 4.43 (m, 1 H) 4.23 (br t, J=7.57 Hz, 1 H) 3.86 (s, 1 H) 3.57 - 3.64 (m, 3 H) 3.39 - 3.55 (m, 33 H) 3.11 - 3.27 (m, 4 H) 2.99 (br dd, J=11.28, 5.83 Hz, 7 H) 2.32 - 2.42 (m, 1 H) 1.97 (dq, J=13.00, 6.35 Hz, 1 H) 1.47 - 1.80 (m, 8 H) 1.32 - 1.44 (m, 12 H) 1.25 (br d, J=5.78 Hz, 9 H) 0.94 - 1.15 (m, 4 H) 0.85 (br dd, J=12.70, 6.70
- 15 Hz, 7 H); LCMS: 79.73% (1109.3, M+H), RT: 2.83 min.
- 20

Step 8 Synthesis of 4-((32S,35S)-1-iodo-32-isopropyl-2,30,33-trioxo-35-(3-ureidopropyl)-6,9,12,15,18,21,24,27-octaoxa-3,31,34-triazahexatriacontan-36-amido)benzyl(2-(2,2,2-trifluoroacetamido)ethyl)carbamate (Monomer-2-Bromo linker).

- 25 To a solution 4-((32S,35S)-1-bromo-32-isopropyl-2,30,33-trioxo-35-(3-ureidopropyl)-6,9,12,15,18,21,24,27-octaoxa-3,31,34-triazahexatriacontan-36-amido)benzyl tert-butyl ethane-1,2-diylldicarbamate(2 g, 1.8 m mole) in DCM(43 ml) was added TFA(3.078 g, 27 m mole) at 0 °C. The reaction mixture was stirred at RT for 4 h. The reaction progress was monitored by LCMS & TLC. LCMS showed SM was consumed. TLC showed complex.
- 30 TLC: Mobile phase: 10% MeOH in DCM. The reaction solvent was concentrated under reduced pressure to get crude. The crude was added water (5 ml) and obtained solid was filtered. The solid (1 g) was submitted for analysis LCMS & 1HNMR showed no desired product. The water was lyophilized to get 2 g, (LCMS showed 60) of desired product). The 2 g, LCMS showed 60%, was purified by Prep.HPLC. The obtained fractions was lyophilized

to get pure product as a TFA salt (600 mg, LCMS 95%, Yield 30%) as an off white gummy.
 Prep.HPLC CONDITION: Mobile phase A: - 0.1%TFA (aq), Mobile phase B: - Acetonitrile,
 Column: - xselectPhenyl hexyle (150*25) mm, 5u, Flow: - 16ml/min, Method :-(t/%B): 0/20,
 2/20, 10/60, Solubility: - ACN + THF + Water Temperature: - Ambient. ¹H NMR (400 MHz,
 5 DMSO-d₆) δ ppm 9.95 - 10.02 (m, 1 H) 8.22 - 8.37 (m, 1 H) 8.11 (d, J=7.52 Hz, 1 H) 7.87 (d,
 J=8.72 Hz, 1 H) 7.72 (br s, 2 H) 7.60 (d, J=8.50 Hz, 2 H) 7.25 - 7.39 (m, 3 H) 5.09 - 6.22 (m,
 18 H) 4.31 - 4.43 (m, 1 H) 4.22 (dd, J=8.45, 6.81 Hz, 1 H) 3.86 (s, 2 H) 3.56 - 3.66 (m, 2 H)
 3.39 - 3.55 (m, 32 H) 3.17 - 3.27 (m, 4 H) 2.90 - 3.08 (m, 2 H) 2.81 - 2.90 (m, 2 H) 2.29 -
 2.46 (m, 2 H) 1.89 - 2.02 (m, 1 H) 1.52 - 1.77 (m, 2 H) 1.30 - 1.48 (m, 2 H) 0.85 (dd,
 10 J=12.37, 6.81 Hz, 6 H); LCMS: 95.3% (1009.2, M+H), RT: 3.29 min.

Preparation of



Step 1A: Synthesis of 2, 5-dioxopyrrolidin-1-yl 2-bromoacetate (2A).

15 A 250 mL round bottom flask was charged with 2-bromoacetic acid (4 g, 29 mmol),
 N-hydroxysuccinimide (3.67 g, 31.9 mmol) and DCM (40 ml). The solution stirred in an ice
 bath as N-N dicyclohexylcarbodiimide (6.58 g, 31.9 mmol) was added. The reaction stirred in
 the ice bath for one hour and an additional hour at room temperature. TLC: Mobile phase -
 10% MeOH in DCM, R_f- 0.15. The reaction mixture was filtered through a sintered glass
 20 funnel and concentrated in vacuo to give a crude white solid. The solid was dissolved in
 warm DCM (40 mL) and recrystallized with hexane (33 mL). The solid was collected by
 Filtration, washed with hexane (20 ml) and dried in vacuo to get 2, 5-dioxopyrrolidin-1-yl 2-
 bromoacetate (6 g, 1HNMR, Yield 88%) as a white colour solid. ¹H NMR (400 MHz,
 CHLOROFORM-d) δ ppm 4.10 (s, 2 H) 2.82 - 2.95 (m, 4 H)

25

Step 1: Synthesis of 1, 1, 1-trifluoro-2-oxo-6, 9, 12, 15, 18, 21, 24, 27-octaoxa-3-
 azatriacontan-30-oic acid (2).

To a solution of 2, 2-dimethyl-4-oxo-3, 8, 11, 14, 17, 20, 23, 26, 29-nona-5-
 azadotriacontan-32-oic acid (6 g, 11.1 m moles) in DCM (400 mL) was added TFA (40 mL)

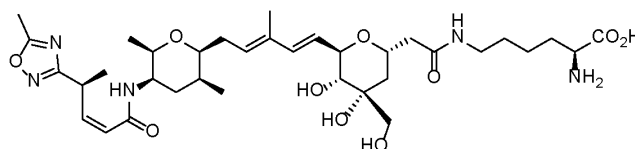
at 0°C. The reaction mixture was stirred at 0°C for 1 h when TLC indicated completion of the reaction. Mobile phase: 10% MeOH in DCM, RF: 0.15. Reaction mixture was concentrated under reduced pressure to get 1, 1, 1-trifluoro-2-oxo-6, 9, 12, 15, 18, 21, 24, 27-octaoxa-3-azatriacontan-30-oic acid as a yellow gum. (5.5 g, Yield: 92%). ¹H NMR (400 MHz, DMSO-d₆) δ ppm 7.55 - 7.91 (m, 4 H) 3.55 - 3.65 (m, 8 H) 3.44 - 3.55 (m, 24 H) 2.91 - 3.05 (m, 2 H) 2.44 (t, J=6.38 Hz, 2 H).

Step 2: Synthesis of 1-bromo-2-oxo-6, 9, 12, 15, 18, 21, 24, 27-octaoxa-3-azatriacontan-30-oic acid (Int-3).

To a solution of 1,1,1-trifluoro-2-oxo-6,9,12,15,18,21,24,27-octaoxa-3-azatriacontan-30-oic acid (2.4 g, 4.5 m mol) in THF (48 ml) was added DIPEA (2.9 g, 22.5 m mol) at RT. The reaction mixture was stirred at RT for 5 min. 2, 5-dioxopyrrolidin-1-yl 2-bromoacetate (1.38 g, 5.85 m mole) was added to the reaction mixture at RT. The reaction mixture was stirred at RT for 2 h. The reaction progress was monitored by TLC. TLC showed complex. The reaction progress was monitored by LCMS (LCMS showed 2% of SM). The reaction mixture was stirred for another 30 min. The reaction mixture was concentrated under reduced pressure to get crude (7 g, LCMS showed 41% of desired product). The crude (7 g) was dissolved in DCM (300 ml) and washed with water (30 ml). The DCM layer was dried over sodium sulphate and concentrated under reduced pressure to get (250 mg, LCMS showed 7.9% of desired product). The H₂O layer was lyophilized to get 5 g, LCMS showed 38.6%, of the desired product. The product (5 g) was dissolved in EtOAc (500 ml) and washed with 10 ml of water. The ethyl acetate layer was dried over sodium sulphate and concentrated under reduced pressure to get product which was taken forward in subsequent reaction (3.5 g, Yield 62%) as off white gum. LCMS: 62.42% (562.1, M+H), RT: 2.471 min.

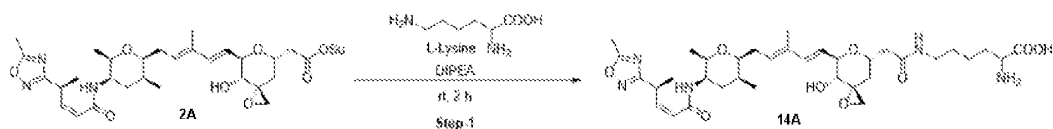
25

Example 33. Preparation of compound 14A

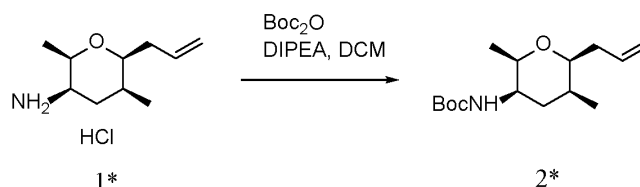


Compound 14A was prepared according to the following synthetic scheme using compound 2A of example 21.

LCMS: 88.10% purity, M+H: 688.44, RT: 1.96.

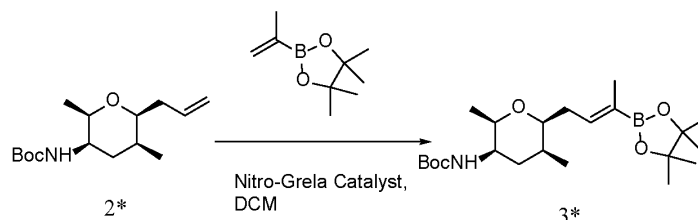


Example 34. Preparation of compound 2*.



- 5 To compound 1* (50 g, 24.17 mol) and dry DCM at 0°C was added DIPEA (4 equiv.) and Boc₂O (2 equiv.) in sequence and stirred at room temperature for 16 h. The reaction mixture was concentrated at 40°C to get crude (160 g) product. The crude product was purified by combiflash chromatography (using 330 g, silica loader 230-400 silica, for slurry 60-120 200 g silica, 5% EA in hexanes as eluent (1375 mL of EtOAc and 11.25 L of
- 10 Hexanes), to provide compound 2* as pale yellow liquid (Yield: 60.59 g (93.2%); GC Purity – 95.14%)

Example 35. Preparation of compound 3*.



- 15 Compound 3* was prepared as described by the following steps.
- 1 Compound 2* (20 g, 1.0 eq) was added to an inerted 3 L reaction vessel.
 2. Dichloromethane (2 L, 100 mL/g) was added to the reaction vessel and degassed with a stream of subsurface nitrogen for at least 20 min.
 3. Nitro-Grela catalyst (10 g, 0.50 g/g) was added to the inerted reaction vessel and degassed with a stream of subsurface nitrogen for at least 10 min.
 4. Isopropenylboronic acid pinacol ester (140 mL, 7.08 mL/g) was added to the reaction vessel.
 5. The reaction mixture was heated to reflux.
- 20

6. The reaction was held for at least 3 hr at 60 °C.

7. The reaction mixture was cooled to 15 to 25 °C.

8. The crude reaction mixture was added to silica gel (500 g, 25 g/g compound 2*) and concentrate the contents of the rotovap flask until all solvent is removed.

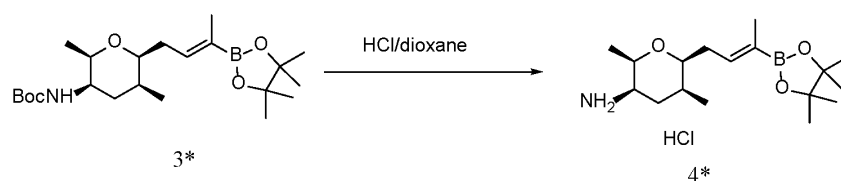
5 9. The crude product was purified by silica gel chromatography.

10. The product containing fractions were concentrated to dryness to give compound 2* (11.6 g, 23% yield) as a yellow, sticky liquid (low melting solid).

Figure 24A shows the ^1H NMR spectrum of isolated compound 3* while Figure 24B shows the ^{13}C NMR spectrum of isolated compound 3*.

10 In one embodiment the catalyst is a metathesis catalyst (e.g., a ruthenium metathesis catalyst). In one embodiment the solvent is a hydrocarbon solvent, ether (cyclic and acyclic ether) solvent, halogenated solvent, or other polar aprotic solvent or water or a mixture thereof.

15 Example 36. Preparation of Compound 4*.



Compound 4* was prepared as described by the following steps.

1. Compound 3 (6.6 g, 1.0 eq) was added to an inerted 100 mL reaction vessel.

2. 1,4-Dioxane (33.0 mL, 5.0 mL/g) was added to the reaction vessel and cooled to 0-5 °C.

3 4N HCl/dioxane (13.2 mL, 2.0 mL/g) was added to the reaction vessel and stirred. The reaction mixture was allowed to heat to 15-25 °C.

4. The reaction mixture was held for at least 3 hr at 15 to 25 °C.

5. The reaction mixture was concentrated to dryness.

25 7. MTBE (33.0 mL, 5.0 mL/g) was added to the reaction vessel.

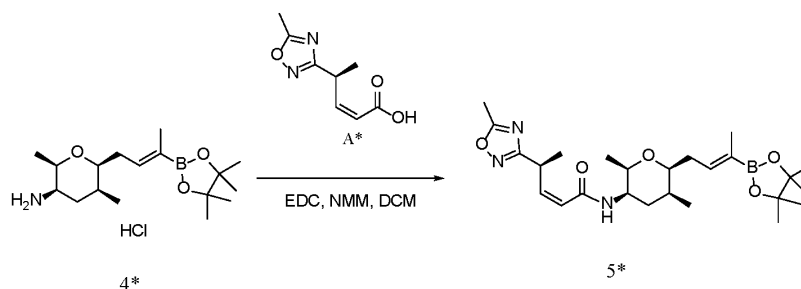
8. The reaction mixture was concentrated to dryness.

9. The solids were dried at 40 °C under vacuum to give compound 4 (5.1 g, 88% yield) as an off-white solid.

Figure 25A shows the ^1H NMR spectrum of isolated compound 4 while Figure 25B
30 shows the ^{13}C NMR spectrum of isolated compound 4*.

In one embodiment the acid is a mineral acid. In one embodiment the solvent is a hydrocarbon solvent, ether (cyclic and acyclic ether) solvent, halogenated solvent, or other polar aprotic solvent or water or a mixture thereof.

5 Example 37. Preparation of Compound 5*.



Compound 5* was prepared as described by the following steps.

1. Intermediate A* (0.78 g, 1.05 eq) as added to an inerted 50 mL glass vessel.
2. Dichloromethane (14 mL, 10 mL/g) was added to the vessel.
- 10 3. Compound 4 (1.4 g, 1.0 equiv.) was added to the vessel and cooled to -25 to -15 °C.
4. Triethylamine (1.14 mL, 0.81 mL/g) was added to the vessel.
5. n-Propylphosphonic anhydride (4.83 mL, 3.45 mL/g) was added to the vessel over 10 min.
- 15 6. The reaction mixture was held at least 2 hr at -25 to -15 °C.
7. Saturated ammonium chloride (28 mL, 20 mL/g) was added to a second glass vessel.
8. The reaction mixture was transferred into the second glass vessel and the stirred mixture was allowed to warm to 15-25 °C.
- 20 9. Stirring was stopped and the layers were allowed to separate. The bottom organic layer was removed and transferred to a third glass vessel.
10. Dichloromethane (28 mL, 20 mL/g) was added to the second glass vessel and stirred for at least 5 min.
11. Stirring was stopped and the layers were allowed to separate. The bottom organic
- 25 layer was removed and transferred to the third glass vessel.
12. Dichloromethane (28 mL, 20 mL/g) was added to the second glass vessel and stirred for at least 5 min.

13. Stirring was stopped and the layers were allowed to separate. The bottom organic layer was removed and transferred to the third glass vessel.

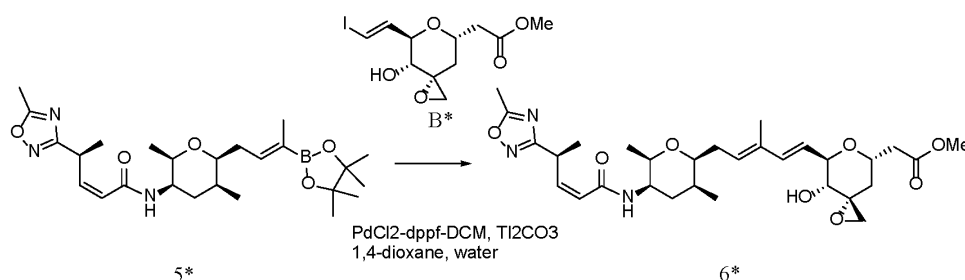
14. Anhydrous sodium sulfate (10 g, 7.1 g/g) was added to the crude reaction mixture in the third glass vessel. The mixture was filtered and the filtrate was concentrated.

5 15. The contents were filtered and the filtrate was concentrated to dryness to give compound 5 (0.95 g, 50% yield - corrected) as a brown solid.

In one embodiment the coupling agent is an anhydride (e.g., alkyl phosphonic anhydride) or a carbodiimide. In one embodiment the solvent is a hydrocarbon solvent, ether (cyclic and acyclic ether) solvent, halogenated solvent, or other polar aprotic solvent or water
10 or a mixture thereof. In one embodiment the base is an amine base.

Figure 26A shows the ^1H NMR spectrum of isolated compound 5* while Figure 26B shows the ^{13}C NMR spectrum of isolated compound 5*.

15 Example 38. Preparation of Compound 6*.



Compound 6* was prepared as described by the following steps.

1. Compound 5* (2.0 g, 1.0 eq) and intermediate B (1.8 g, 0.98 eq) to added to an inerted 100 mL glass vessel.
- 20 2. 1,4-Dioxane (16 mL, 8 mL/g) and water (4 mL, 2 mL/g) were added to the glass vessel and degassed with a stream of subsurface nitrogen for at least 10 min.
3. $\text{Pd(dppf)Cl}_2\text{-DCM}$ (0.35 g, 0.17 g/g) was added to the glass vessel.
4. Thallium carbonate (5.9 g, 3.0 g/g) to the 100 mL was added to the glass vessel and degassed with a stream of subsurface nitrogen for at least 10 min.
- 25 5. The reaction components were held at 15-25 °C with stirring for at least 4 hr.
6. EtOAc (20 mL, 10 mL/g) was added to the glass vessel and the reaction components were stirred at 15-25 °C for at least 5 min.
7. The reaction mixture was filtered into a clean vessel.

8. Water (20 mL, 10 mL/g) was added to the glass vessel and the reaction components were stirred at 15-25 °C for at least 10 min.

9. Stirring was stopped the layers were allowed to separate for at least 10 min.

10. The bottom layer was removed and discarded.

5 11. Saturated NaCl (20 mL, 10 mL/g) was added to the glass vessel and stirred at 15-25 °C for at least 10 min.

12. Stirring was stopped and the layers were allowed to separate for at least 10 min.

13. The bottom layer was removed and discarded.

10 14. Anhydrous sodium sulfate (5 g, 2.5 g/g) was added to the glass vessel and the reaction components were stirred at 15-25 °C for at least 10 minutes.

15. The contents of the vessel were filtered and the filtrate was transferred into a clean roto-evaporator flask.

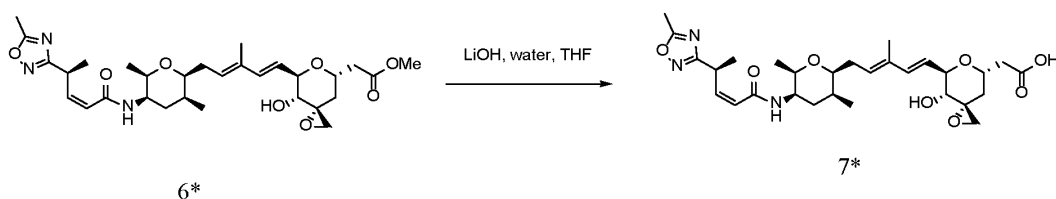
16. The contents of the roto-evaporator flask were concentrated until all solvent was removed give compound 6 (3.0 g, 86% yield) as a brown viscous liquid.

15 Figure 27A shows the ¹H NMR spectrum of crude compound 6* while Figure 27B shows the ¹³C NMR spectrum of crude compound 6*.

In one embodiment the catalyst is a metal coupling catalyst (e.g., a palladium catalyst). In one embodiment the solvent is a hydrocarbon solvent, ether (cyclic and acyclic ether) solvent, halogenated solvent, or other polar aprotic solvent or water or a mixture thereof.

20

Example 39. Preparation of Compound 7*



Compound 7* was prepared as described by the following steps.

25 1. Compound 6* (2.4 g, 1.0 eq) and THF (72 mL, 30 mL/g) were added to an inerted 500 mL glass vessel.

2. A solution of lithium hydroxide (150 mg, 0.062 g/g) and water (72 mL, 30 mL/g) were added to the vessel and stirred at 15-25 °C for 2 hr.

3. MTBE (144 mL, 60 mL/g) was added to the vessel and the reaction components were stirred at 15-25 °C for at least 10 min.

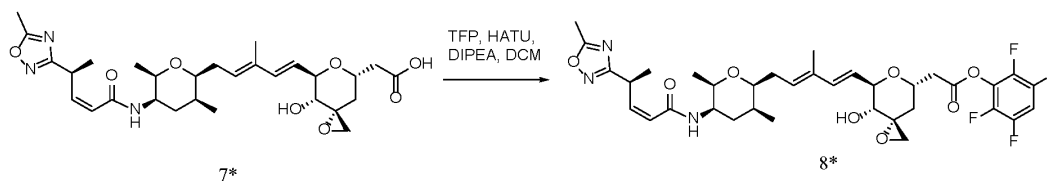
30

4. Stirring was stopped and the layers allowed to separate for at least 10 min.
5. The layers were separated and the bottom, aqueous layer back was transferred into the glass vessel.
6. 85% aq. NaH_2PO_4 (30 mL, 12.5 mL/g, pH 7) was added to the vessel.
- 5 7. EtOAc (144 mL, 60 mL/g) was added to the vessel and stirred at 15-25 °C for at least 10 minutes.
8. Stirring was stopped and the layers were allowed to separate for at least 10 minutes.
9. The layers were separated and the organic layer was transferred back into the glass
- 10 vessel.
10. Anhydrous sodium sulfate (1 g, 0.4 g/g) was added to the vessel and the reaction components were stirred at 15-25 °C for at least 10 min.
11. The contents of the glass vessel were filtered and the filtrate was transferred into a clean roto-evaporator flask.
- 15 12. The contents of the roto-evaporator flask were concentrated until all solvent was removed give compound 7 (1.2 g, 50% yield) as a brown solid.

Figure 28 shows the ^1H NMR spectrum of crude compound 7*.

- In one embodiment the base is a hydroxide base. In one embodiment the solvent is a hydrocarbon solvent, ether (cyclic and acyclic ether) solvent, halogenated solvent, or other
- 20 polar aprotic solvent or water or a mixture thereof.

Example 40. Preparation of Compound 8*



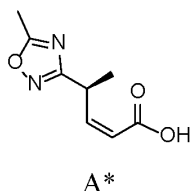
- Compound 8* was prepared as described by the following steps.
- 25 1. Compound 7* (1.2 g, 1.0 eq) and dichloromethane (60 mL, 50 mL/g) were added to an inerted 250 mL glass vessel with agitation.
 2. HATU (0.90 g, 0.75 g/g) was added to the vessel.
 3. DIPEA (0.7 mL, 0.58 mL/g) was added to the vessel.
 4. Tetrafluorophenol (0.36 g, 0.30 g/g) was added to the glass vessel and the reaction
 - 30 components were stirred at 15-25 °C for at least 1 hour.

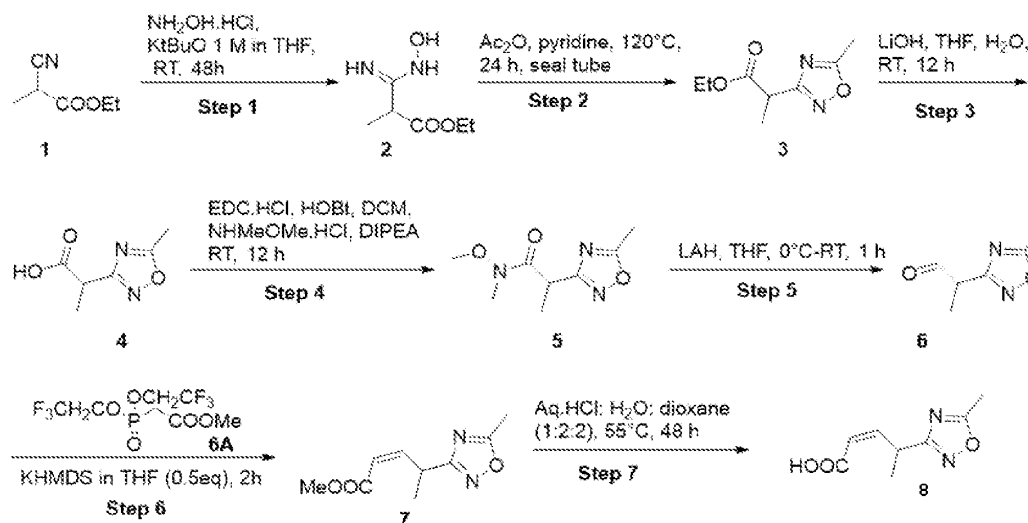
5. Water (50 mL, 41.7 mL/g) was added to the vessel
6. Dichloromethane (50 mL, 41.7 mL/g) was added to the vessel and the reaction components were stirred at 15-25 °C for at least 10 min.
7. Stirring was stopped and the layers were allowed to separate for at least 10 minutes.
8. The layers were separated and the bottom organic layer was transferred into a clean flask.
9. Anhydrous sodium sulfate (5 g, 4.2 g/g) was added to the organic layer and the mixture was stirred at 15-25 °C for at least 5 min.
10. The contents of the flask were filtered and the filtrate was transferred into a clean roto-evaporator flask.
11. The contents of the roto-evaporator flask were concentrated until all solvent was removed.
13. The crude product was purified by preparative HPLC
14. All the product containing fractions were combined and EtOAc (750 mL) and 10% aq. NaHCO₃ (750 mL) were added to the combined fractions. The biphasic mixture was agitated, the layers were allowed to separate.
15. The layers were separated and the organic layers was dried with sodium sulfate (5 g, 4.2 g/g)
16. The mixture was filtered and the organic layer was concentrated to dryness to give compound 8 (0.4 g, 45% yield) as an off-white solid.

Figure 29A shows the ¹H NMR spectrum of compound 8 while Figure 29B shows the ¹³C NMR spectrum of compound 8*.

In one embodiment the base is an amine base. In one embodiment the coupling agent is a uronium coupling agent. In one embodiment the solvent is a hydrocarbon solvent, ether (cyclic and acyclic ether) solvent, halogenated solvent, or other polar aprotic solvent or water or a mixture thereof.

Example 41. Preparation of compound A*.





Step 1: Synthesis of ethyl 3-(hydroxyl amino)-3-imino-2-methylpropanoate (2).

To a stirred solution of ethyl 2-cyanopropanoate (20 g, 15.7 mmol) in THF (500 mL) was added a solution of KOtBu (314 mL, 31.4 mmol, 1 M in THF) at 0°C . After 10 min hydroxyl amine hydrochloride (27 g, 39.3 mmol) was added & the reaction mixture was stirred at room temperature for 48 h. The progress of the reaction was monitored by TLC. TLC indicated SM got consumed. TLC system (50% EtOAc in Hexane, Rf: 0.1, KMnO_4 active). The reaction mass evaporated under reduced pressure, residue was purified by silica column using 40% ethyl acetate in hexane as an eluent. The pure fractions were collected and evaporated under reduced pressure to get ethyl 3-(hydroxyl amino)-3-imino-2-methylpropanoate (5.5 g, 21.8%) as colourless oil.

^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ ppm 1.17 (t, $J = 7.15$ Hz, 3 H), 1.24 (d, $J = 7.15$ Hz, 3 H), 3.10 - 3.20 (m, 1 H), 3.92 - 4.23 (q, 2 H), 5.31 - 5.45 (br, 2 H).

LCMS: 43.4% (161.13, $\text{M}+\text{H}$), RT = 0.37 min. and 49.48% (161.13, $\text{M}+\text{H}$), RT = 0.41 min

Step 2: Synthesis of ethyl 2-(5-methyl-1,2,4-oxadiazol-3-yl) propanoate (3)

To a stirred solution of ethyl 3-(hydroxyl amino)-3-imino-2-methylpropanoate (5.5 g, 34.3 mmol) in pyridine (50 mL) was added acetic anhydride (9.7 mL, 10.3 mmol) in seal tube at room temperature. The reaction mixture was heated at 120°C for 24 h, progress of the reaction was monitored by TLC. TLC indicated SM got consumed. TLC system (50% EtOAc in Hexane, Rf: 0.5, KMnO_4 active).

The reaction mass was evaporated under reduced pressure, residue was purified by silica column using 20% ethyl acetate in hexane as an eluent. The pure fractions were collected

and evaporated under reduced pressure to get ethyl 2-(5-methyl-1, 2, 4-oxadiazol-3-yl) propanoate (4.5 g, 71.4%) as yellow coloured oil.

¹H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 1.26 (t, *J* = 7.09 Hz, 3 H), 1.58 - 1.62 (d, 3 H), 2.61 (s, 3 H), 3.89 - 4.00 (q, 1 H), 4.15 - 4.26 (q, 2 H).

5 LCMS: 86.56% (185.22, M+H), RT = 1.48 min.

Step 3: Synthesis of 2-(5-Methyl-1, 2, 4-oxadiazol-3-yl) propanoic acid (4).

To a stirred solution of ethyl 2-(5-methyl-1, 2, 4-oxadiazol-3-yl) propanoate (4.5 g, 24.4 mmol) in MeOH:THF:H₂O (45 ml, 4:4:1) was added LiOH.H₂O (4 g, 9.7 mmol) at 0°C and
10 stirred the reaction mixture at room temperature for 12 h. The progress of the reaction was monitored by TLC. TLC indicated SM got consumed. TLC system (50% EtOAc in Hexane, R_f: 0.1, KMnO₄ active).

The reaction mass evaporated under reduced pressure, pH of residue was adjusted ~1 using 6N HCl (~10 ml) then extracted with ethyl acetate (25 ml x 2). The combined organic
15 layer was dried using Na₂SO₄ and concentrated under reduced pressure to get 2-(5-methyl-1, 2, 4-oxadiazol-3-yl) propanoic acid (3.5 g, 92%) as colourless oil.

¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 1.41 (d, *J* = 7.23 Hz, 3 H), 2.57 (s, 3 H), 3.88 - 3.97 (q, 1 H), 12.65 - 12.82 (br s, 1 H).

LCMS: 94.38% (157.1, M+H), RT = 1.05 min.

20

Step 4: Synthesis of N-methoxy-N-methyl-2-(5-methyl-1, 2, 4-oxadiazol-3-yl) propanamide (5).

To a stirred solution of 2-(5-methyl-1, 2, 4-oxadiazol-3-yl) propanoic acid (3.5 g, 22.4 mmol) in DCM (100 ml), EDC.HCl (6.4 g, 33.6 mmol), HOBt (4.5 g, 33.6 mmol) DIPEA
25 (11.7 ml, 67.2 mmol) were added at 0°C. After 10 min, N, O-dimethyl hydroxyl amine hydrochloride (3.2 g, 33.6 mmol) was added at 0°C. The reaction mixture was stirred at room temperature for 12 h. The progress of the reaction was monitored by TLC. TLC indicated SM got consumed. TLC system (50% EtOAc in Hexane, R_f: 0.2, KMnO₄ active).

The reaction mass was diluted with DCM (40 mL) and washed with saturated solution
30 of sodium bicarbonate (30 ml). The organic layer was separated and dried over sodium sulphate. The organic layer was evaporated under reduced pressure and obtained 5 g of crude N-methoxy-N-methyl-2-(5-methyl-1, 2, 4-oxadiazol-3-yl) propanamide. The crude compound was purified by silica column using 40% ethyl acetate in hexane as an eluent. The pure fractions

were collected and evaporated under reduced pressure to get N-methoxy-N-methyl-2-(5-methyl-1, 2, 4-oxadiazol-3-yl) propanamide (2.2 g, 50%) as colourless oil.

¹H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 1.57 (d, *J* = 7.03 Hz, 3 H), 2.60 (s, 3 H), 3.26 (s, 3 H), 3.72 (s, 3 H), 4.34 - 4.42 (q, 1 H).

5 LCMS: 96.65% (200.16, M+H), RT = 1.21 min.

Step 5: 2 Synthesis of -(5-methyl-1, 2, 4-oxadiazol-3-yl) propanal (6).

To a stirred solution of LAH (380 mg, 10 mmol) in THF (10 ml) was added N-methoxy-N-methyl-2-(5-methyl-1, 2, 4-oxadiazol-3-yl) propanamide (1 g, 50 mmol) in THF (20 ml)
10 slowly at 0°C. The reaction mixture was stirred at room temperature for 1 h. The progress of the reaction was monitored by TLC. TLC indicated SM got consumed. TLC system (40% EtOAc in Hexane, R_f: 0.3, KMnO₄ active and 2, 4-DNP slightly active).

The reaction mixture was quenched using 1 N HCl (~3 ml) at 0°C and stirred for 10 min and added ethyl acetate (20 ml). The reaction mixture was filtered through celite bed. The
15 filtrate was dried using sodium sulphate and minimized volume up to 4 mL (~800 mg, crude) of 2-(5-methyl-1, 2, 4-oxadiazol-3-yl) propanal. As such crude 2-(5-methyl-1, 2, 4-oxadiazol-3-yl) propanal used for Step 6.

ESMS: 40.4% (141.1, M+H), RT = 2.24 min. (Crude)

20 Step 6: Synthesis of methyl (Z)-4-(5-methyl-1, 2, 4-oxadiazol-3-yl) pent-2-enoate (7).

To a stirred solution of 18-crown-6-ether (7.5 g, 28.5 m mol) in dry THF (30 ml) was added methyl 2-(bis(2,2,2-trifluoroethoxy) phosphoryl) acetate (1.3 ml, 6.2 m mol) at 0°C followed by added 1 M KHMDS in THF (6.2 ml, 6.2 mmol) at -78°C. The reaction mixture maintained at -78°C for 30 min then added 2-(5-methyl-1, 2, 4-oxadiazol-3-yl) propanal (~800
25 mg) in dry THF (10 ml) at -78°C and allow the reaction mixture at room temperature gradually & stirred at same temperature for 2 h. The progress of the reaction was monitored by TLC. TLC indicated SM got consumed. TLC system (30% EtOAc in Hexane, R_f: 0.5, KMnO₄ active).

The reaction mixture was quenched using H₂O (30 ml) and extracted in ethyl acetate
30 (50 ml x 2). The combined organic layer was dried over sodium sulphate and concentrated under reduced pressure to get 600 mg of crude methyl (Z)-4-(5-methyl-1, 2, 4-oxadiazol-3-yl) pent-2-enoate. The crude compound was purified by silica column using 10% ethyl acetate in hexane as an eluent. The pure fractions were collected and evaporated under reduced pressure

to get methyl (Z)-4-(5-methyl-1, 2, 4-oxadiazol-3-yl) pent-2-enoate (420 mg, 59% pure by LCMS, 37%) as yellow coloured oil.

¹H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 1.44 - 1.48 (d, 3 H), 2.57 (s, 3 H) 3.74 (s, 3 H), 5.01 - 5.10 (q, 1 H), 5.92 - 5.89 (d, *J* = 10 Hz, 1 H), 6.35 - 6.46 (dd, *J* = 10 Hz, 1 H).

5 LCMS: 59% (197.21, M+H), RT = 1.57 min.

Z geometric isomer confirm by JJ coupling constant value of olefin bond: 10 Hz

Step 7: Synthesis of (Z)-4-(5-methyl-1, 2, 4-oxadiazol-3-yl) pent-2-enoic acid (8).

Methyl (Z)-4-(5-methyl-1, 2, 4-oxadiazol-3-yl) pent-2-enoate (550 mg, 28 mmol, crude
10 80% pure by LCMS) was added in dioxane (10 ml) & 2 N HCl (5 ml). The reaction mixture was heated at 55 °C for 48 h. The progress of the reaction was monitored by TLC. TLC indicated SM got consumed. TLC system (30% EtOAc in Hexane, R_f: 0.1, KMnO₄ active). The reaction mixture was concentrated under reduced pressure and purified using Prep HPLC to get pure (Z)-4-(5-methyl-1, 2, 4-oxadiazol-3-yl) pent-2-enoic acid (138 mg, 28.6%) as
15 colourless oil.

¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 1.33 (d, *J* = 7.02 Hz, 3 H), 2.55 (s, 3 H), 4.86 - 5.08 (q, 1 H), 5.84 (dd, *J* = 11.40 Hz, 1 H), 6.24 (m, *J* = 10.52 Hz, 1 H), 11.64 - 12.96 (br s, 1 H).

LCMS: 98.59% (183.21, M+H), RT = 1.4 min.

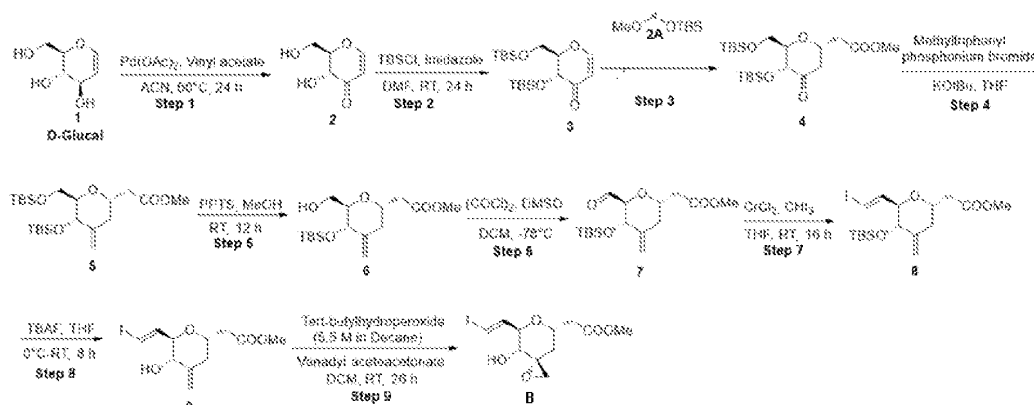
20 Step 8: Synthesis of

The racemic mixture of methyl (Z)-4-(5-methyl-1, 2, 4-oxadiazol-3-yl) pent-2-enoic acid (8) from step 7 was subjected to chiral chromatography to provide the desired enantiomer (intermediate A*).

25 Example 42. Preparation of compound B*.



B*



Step 1: Synthesis of (2R, 3R)-3-hydroxy-2-(hydroxymethyl)-2H-pyran-4(3H)-one (2).

A mixture of compound 1 (137.5 g, 0.941 mole), Pd (OAc)₂ (5.9 g, 0.026 mole) in ACN (550 ml) was added vinyl acetate (250 g, 2.907 mole) at RT. The reaction mixture was stirred at 60 °C for 24 h. The reaction mixture was filtered through a celite and washed with EtOAc (100 ml). The solvent was concentrated under reduced pressure to get residue. The residue was recrystallization from a mixture of acetone (68 ml), EtOAc (68 ml). Afford a (2R, 3R)-3-hydroxy-2-(hydroxymethyl)-2H-pyran-4(3H)-one (75 g, 55%) as an off white solid. TLC system (EtOAc, RF=0.39, UV active). ¹H NMR (400 MHz, DMSO-d₆) δ = 7.60 (d, J = 5.9 Hz, 1H), 5.61 (d, J = 4.9 Hz, 1H), 5.30 (d, J = 5.4 Hz, 1H), 5.00 (t, J = 5.6 Hz, 1H), 4.16 - 4.06 (m, 2H), 3.82 - 3.76 (m, 1H), 3.73 - 3.66 (m, 1H).

Step 2: Synthesis of (2R, 3R)-3-(tert-butyldimethylsilyloxy)-2-((tert-butyldimethylsilyloxy)methyl)-2H-pyran-4(3H)-one (3).

To a solution of compound (2R, 3R)-3-hydroxy-2-(hydroxymethyl)-2H-pyran-4(3H)-one (100 g, 0.69 mole) in DMF (1900 ml), were added TBS-Cl (277 g, 2.65 mole), Imidazole (140.9 g, 2.07 mole) at RT. The reaction mixture was stirred at RT for 24 h. The progress of the reaction was monitored by TLC. TLC indicated SM was consumed. TLC system (5% EtOAc in Pet Ether, RF=0.5, UV active). After completion of reaction, the reaction mixture was diluted with diethyl ether (2500 ml) and washed with water (1000 ml), brine (1000 ml). The diethyl ether layer was dried over anhydrous sodium sulphate. Then filtered, concentrated, crude formed. Crude purified by column chromatography (100-200 silica gel/eluent 2% EtOAc in Pet Ether) to give (2R, 3R)-3-(tert-butyldimethylsilyloxy)-2-((tert-butyldimethylsilyloxy)methyl)-2H-pyran-4(3H)-one (200 g, 77.5%) as an off white solid. ¹H NMR (400 MHz, CHLOROFORM-d) δ = 7.30 - 7.25 (m, 1H), 5.30 (d, J = 5.8 Hz, 1H), 4.45

- 4.40 (m, 1H), 4.18 (td, J = 2.6, 12.2 Hz, 1H), 3.99 (d, J = 2.8 Hz, 2H), 0.91 (d, J = 2.5 Hz, 18H), 0.23 (s, 3H), 0.11 - 0.05 (m, 6H); LCMS: 99.48% (373.37, M+H), RT: 3.47 min.

Step 3: Synthesis of methyl 2-((2S, 5R, 6R)-5-(tert-butyldimethylsilyloxy)-6-((tert-butyldimethylsilyloxy) methyl)-4-oxotetrahydro-2H-pyran-2-yl) acetate (4).

To a stirred solution of (2R,3R)-3-(tert-butyldimethylsilyloxy)-2-((tert-butyldimethylsilyloxy)methyl)-2H-pyran-4(3H)-one (55 g, 0.147 mole) in DCM(2000 ml) was added tert-butyl(1-methoxyvinyl)dimethylsilane (55 g, 0.292 mole) at RT. The reaction mixture was stirred at RT for 2 h. After BF₃.Et₂O (63.25 g, 3.03 mole) was added to the reaction mixture at -78 °C. The reaction mixture was stirred at -78 °C for 1 h. The progress of the reaction was monitored by TLC. TLC indicated SM was consumed. TLC system (15% EtOAc in Pet Ether, RF=0.5, PMA active). The reaction mixture was quenched with saturated NH₄Cl (500 ml) and extracted with DCM (2x250 ML). The DCM layer was washed with saturated NaHCO₃ (500 ml), brine (500 ml). The DCM layer was dried over anhydrous sodium sulphate. Then mixture was filtered, concentrated, and crude formed. The crude material was purified by column chromatography (100-200 silica gel/eluent 3% EtOAc in Pet Ether) to give methyl 2-((2S, 5R, 6R)-5-(tert-butyldimethylsilyloxy)-6-((tert-butyldimethylsilyloxy) methyl)-4-oxotetrahydro-2H-pyran-2-yl) acetate (25 g, 37.9%) as a transparent gum. ¹H NMR (400 MHz, CHLOROFORM-d) δ = 4.92 - 4.72 (m, 1H), 4.33 (d, J = 8.8 Hz, 1H), 3.91 - 3.78 (m, 2H), 3.74 - 3.58 (m, 4H), 2.83 - 2.60 (m, 2H), 2.52 - 2.38 (m, 2H), 0.99 - 0.79 (m, 18H), 0.15 (s, 3H), 0.11 - 0.01 (m, 9H)

Step 4: Synthesis of methyl 2-((2S, 5S, 6R)-5-(tert-butyldimethylsilyloxy)-6-((tert-butyldimethylsilyloxy) methyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (5).

To a suspension of methyltriphenylphosphoniumbromide (12 g, 0.033 mole) in THF (180 ml) at 0 °C, was added a THF (80 ml) solution of KOtBu (3.33 g, 0.029 mole). After 1 h, a THF (60 ml) solution of methyl 2-((2S, 5R, 6R)-5-(tert-butyldimethylsilyloxy)-6-((tert-butyldimethylsilyloxy) methyl)-4-oxotetrahydro-2H-pyran-2-yl) acetate (10 g, 0.022 mole) was added drop wise. Then reaction mixture allowed to 25 °C and stirred for 1 hr. The progress of the reaction was monitored by TLC. TLC indicated SM was consumed. TLC system (5% EtOAc in Pet Ether, RF=0.3, PMA active). The reaction mixture was quenched with saturated NH₄Cl (100 ml) and extracted with EtOAc (2x150 ml). The EtOAc layer was dried over anhydrous sodium sulphate. Then filtered, concentrated, crude formed. Crude purified by column chromatography (100-200 silica gel/eluent 5% EtOAc in Pet Ether) to

give methyl 2-((2S, 5S, 6R)-5-(tert-butyldimethylsilyloxy)-6-((tert-butyldimethylsilyloxy) methyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (4 g, 40.4%) as a yellow colour liquid. ¹H NMR (400 MHz, CHLOROFORM-d) δ = 5.07 (s, 1H), 4.89 - 4.86 (m, 1H), 4.30 - 4.23 (m, 1H), 4.02 (d, J = 6.5 Hz, 1H), 3.75 - 3.66 (m, 5H), 3.47 (td, J = 4.7, 6.4 Hz, 1H),
5 2.65 (dd, J = 7.3, 15.0 Hz, 1H), 2.48 (dd, J = 6.8, 15.0 Hz, 1H), 2.42 - 2.28 (m, 2H), 0.95 - 0.84 (m, 18H), 0.12 - 0.01 (m, 12H)

Step 5: Synthesis of methyl 2-((2S, 5S, 6R)-5-(tert-butyldimethylsilyloxy)-6-(hydroxymethyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (6).

10 To a stirred solution of compound methyl 2-((2S, 5S, 6R)-5-(tert-butyldimethylsilyloxy)-6-((tert-butyldimethylsilyloxy) methyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (75.1 g, 0.168 mole) in MeOH (751 ml) was added PPTS (59.4 g, 0.236 mole) at RT. The reaction mixture was stirred at RT for 16 h. The progress of the reaction was monitored by TLC. TLC indicated SM was consumed. TLC system (25% EtOAc in Pet
15 Ether, RF=0.3, PMA active). The reaction mixture was quenched with saturated NaHCO₃ (1270 ml) and extracted with EtOAc (2x 1500 ml). The EtOAc layer was dried over anhydrous sodium sulphate. Then filtered, concentrated, crude formed. Crude purified by column chromatography (100-200 silica gel/eluent 25% EtOAc in Pet Ether) to methyl 2-
20 ((2S, 5S, 6R)-5-(tert-butyldimethylsilyloxy)-6-(hydroxymethyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (39 g, 69.8%) as a yellow color gum. ¹H NMR (400 MHz, CHLOROFORM-d) δ = 5.11 (s, 1H), 4.88 (s, 1H), 4.42 - 4.35 (m, 1H), 3.93 (d, J = 7.3 Hz, 1H), 3.71 - 3.64 (m, 5H), 3.56 - 3.50 (m, 1H), 2.73 (dd, J = 8.8, 15.4 Hz, 1H), 2.51 - 2.42 (m, 2H), 2.31 (dd, J = 3.8, 13.4 Hz, 1H), 2.15 (t, J = 6.4 Hz, 1H), 0.94 - 0.91 (m, 9H), 0.10 - 0.08 (m, 3H), 0.05 - 0.03 (m, 3H). LCMS: 80.17% (331.39, M+H), RT: 2.78 min

25

Step 6: Synthesis of methyl 2-((2S, 5S, 6S)-5-(tert-butyldimethylsilyloxy)-6-formyl-4-methylenetetrahydro-2H-pyran-2-yl) acetate (7).

To a stirred solution of oxalyl chloride (17.2 g, 0.136 mole) in DCM (214 ml) at -78 °C was slowly added DMSO (21 g, 0.27 mole). The reaction mixture was stirred at -78 °C to
30 -60 °C for 20 min. Then a solution of compound to methyl 2-((2S, 5S, 6R)-5-(tert-butyldimethylsilyloxy)-6-(hydroxymethyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (30 g, 0.09 mole) in DCM (414 ml) was added drop wise. The reaction mixture was slowly warmed to -45 °C over 30 min. After TEA (52.8 g, 0.522 mole) was added at -45 °C and the reaction mixture was warmed to 0 °C over 10 min. The reaction progress was monitored by

TLC. TLC showed SM was consumed. TLC system (20% EtOAc in Pet Ether, RF=0.35, PMA active). The reaction mixture was quenched with saturated NH₄Cl (500 ml) and extracted with DCM (2x500 ML). The DCM layer was washed with brine (1000 ml). The DCM layer was dried over anhydrous sodium sulphate. Then filtered, concentrated, crude formed. Crude purified by column chromatography (100-200 silica gel/eluent 20% EtOAc in Pet Ether) to give methyl 2-((2S,5S,6S)-5-(tert-butyldimethylsilyloxy)-6-formyl-4-methylenetetrahydro-2H-pyran-2-yl)acetate (22 g, 73.8%) as a yellow color gum. ¹H NMR (400 MHz, CHLOROFORM-d) δ = 9.76 - 9.73 (m, 1H), 5.05 - 5.00 (m, 1H), 4.93 - 4.87 (m, 1H), 4.36 (d, J = 4.3 Hz, 1H), 4.29 - 4.20 (m, 1H), 4.12 - 4.08 (m, 1H), 3.73 - 3.67 (m, 4H), 2.78 - 2.69 (m, 1H), 2.55 - 2.40 (m, 1H), 2.27 - 2.20 (m, 2H), 0.96 - 0.87 (m, 9H), 0.12 - 0.02 (m, 6H); LCMS: 97.53% (329.34, M+H), RT: 2.60 min

Step 7: Synthesis of methyl 2-((2S, 5S, 6R)-5-((tert-butyldimethylsilyl) oxy)-6-((E)-2-iodovinyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (8).

To a stirred solution of methyl 2-((2S, 5S, 6S)-5-((tert-butyldimethylsilyl) oxy)-6-formyl-4-methylenetetrahydro-2H-pyran-2-yl) acetate (2 g, 6.088 m moles) in THF (140 mL), stirred at 26°C, CrCl₂ (4.489 g, 36.531 m moles) and CHI₃ (7.191 g, 18.265 m moles) were added and stirred at the same temperature for 16 h. TLC indicated completion of the reaction (TLC system: 20% of EtOAc in pet ether, Rf: 0.7, PMA active). Reaction mixture was diluted with ice-cold water (50 mL) and the product was extracted with EtOAc (2x50 mL). Combined organic layer was dried over Na₂SO₄ and concentrated to get the crude product (2.8 g) which was purified by normal phase chromatography using silica (100-200 mesh) and 4% of EtOAc in pet ether as an eluent. Collected fractions were evaporated under reduced pressure at 28°C to get methyl 2-((2S, 5S, 6R)-5-((tert-butyldimethylsilyl) oxy)-6-((E)-2-iodovinyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate as a pale yellow liquid (800 mg, Yield 28%). ¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 6.49 - 6.62 (m, 1 H) 6.31 - 6.44 (m, 1 H) 5.09 - 5.22 (m, 1 H) 4.83 - 4.96 (m, 1 H) 4.35 - 4.45 (m, 1 H) 3.84 - 3.92 (m, 1 H) 3.75 - 3.82 (m, 1 H) 3.65 - 3.72 (m, 3 H) 2.63 - 2.72 (m, 1 H) 2.42 - 2.55 (m, 2 H) 2.25 - 2.36 (m, 1 H) 0.87 - 0.96 (m, 9 H) -0.01 - 0.02 (m, 6 H); LCMS: 85.58% (453, M+H & 475 M+23 (Na adduct), RT: 2.8 min.

Step 8: Synthesis of methyl 2-((2S, 5S, 6R)-5-hydroxy-6-((E)-2-iodovinyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (9).

To a solution of methyl 2-((2S, 5S, 6R)-5-((tert-butyldimethylsilyl) oxy)-6-((E)-2-iodovinyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (800 mg, 1.768 m moles) in THF (40 mL), TBAF (1M in THF, 2.122 mL, 2.122 m moles) was added at 0°C then slowly allowed to reach to RT over 2 h when TLC indicated completion of the reaction (TLC system: 30% EtOAc in pet ether, Rf: 0.2, PMA active). Reaction mixture was quenched by adding NH₄Cl (Aq) (20 mL) and the product was extracted with EtOAc (2x20mL). Combined organic layer was dried over Na₂SO₄ and concentrated to get the crude product (2 g) which was purified by normal phase chromatography using silica (100-200 mesh) and 20% of the EtOAc in pet ether as an eluent. Collected fractions were evaporated to get methyl 2-((2S, 5S, 6R)-5-hydroxy-6-((E)-2-iodovinyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate as a pale yellow liquid (450 mg, Yield 76%). ¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 6.67 (br d, J=4.80 Hz, 2 H) 5.11 - 5.15 (m, 1 H) 4.95 - 5.00 (m, 1 H) 4.26 - 4.34 (m, 1 H) 4.08 - 4.16 (m, 1 H) 3.88 - 3.95 (m, 1 H) 3.66 - 3.73 (m, 3 H) 2.60 - 2.70 (m, 1 H) 2.44 - 2.51 (m, 1 H) 2.35 - 2.42 (m, 2 H) 1.96 - 2.00 (m, 1 H); LCMS: 89% (339.04, M+H), RT: 1.69 min

15

Step 9: Synthesis of methyl 2-((3R, 5S, 7R, 8R)-8-hydroxy-7-((E)-2-iodovinyl)-1, 6-dioxaspiro [2.5] octan-5-yl) acetate (intermediate C).

To a solution of methyl 2-((2S, 5S, 6R)-5-hydroxy-6-((E)-2-iodovinyl)-4-methylenetetrahydro-2H-pyran-2-yl) acetate (450 mg, 1.33 m moles) in DCM (27 mL), stirred at 0°C, V(acac)₂ (35.2 mg, 0.133 m moles) followed by ^{tert}BuOOH (5-6 M in decane, 0.53 mL, 2.66 m moles) was added and allowed to stir at RT for 4 h when TLC indicated completion of the reaction (TLC system: 50% EtOAc in pet ether, Rf: 0.3, PMA active). Reaction mixture was filtered through celite, washed with DCM (20 mL). The filtrate was concentrated to get the crude product (500 mg) which was purified by normal phase chromatography using silica (100-200 mesh) and 25% of EtOAc in pet ether as an eluent. Collected fractions were evaporated to get methyl 2-((3R, 5S, 7R, 8R)-8-hydroxy-7-((E)-2-iodovinyl)-1, 6-dioxaspiro [2.5] octan-5-yl) acetate as a pale yellow gum (220 mg, Yield 46%). ¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 6.70 (dd, J=14.60, 4.90 Hz, 1 H) 6.49 - 6.57 (m, 1 H) 4.55 (s, 1 H) 4.08 - 4.13 (m, 1 H) 3.47 - 3.52 (m, 1 H) 2.97 - 3.01 (m, 1 H) 2.88 - 2.96 (m, 1 H) 2.59 - 2.69 (m, 2 H) 2.18 (dd, J=14.17, 5.34 Hz, 1 H) 1.79 - 1.86 (m, 1 H) 1.68 - 1.76 (m, 1 H).

30

Example 43. MDR Studies.

Methods:

Isogenic MDR1-low MES-SA and MDR1-high MES-SA/ MX2 uterine sarcoma cell lines were purchased from ATCC. MES-SA/ MX2 cell line was obtained on Mitoxantrone-selection of parental MES-SA cells until resistance to the type II topoisomerase I inhibitor was obtained, and resistance was correlated with MDR1 overexpression. Elacridar and
5 Tariquidar are third-generation inhibitors highly specific for MDR1 inhibition while Valspodar is a second-generation p-Glycoprotein inhibitor that also interacts with CYP 3A4. MES-SA cells were grown in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS) and MES-SA/ MX2 were grown in McCoy's 5A medium supplemented with 45% Waymouth's MB 752/1(Sigma-Aldrich) and 10% FBS. The isogenic lines were grown
10 in the presence of 0.013 nanomolar to 5 micromolar concentrations of ADC payloads SN38, DXd, MMAE and the Thailanstatin ADC metabolites- a membrane-permeable ThA13 d2 methyl ester representative of the lysine conjugations and the 301-metabolite released by the cysteine conjugates. Cytotoxicity was measured after 5 days using an ATP-cell titer glo luciferase read-out. Each data point represents an average of triplicate wells for each cell line-
15 payload combination. Untreated wells were used to control for total cell numbers (100% live) for each cell line.

Conclusion:

Active species of ADC payloads are substrates for various p-Glycoproteins such as MDR1. There is a 282-fold increase in IC₅₀ required by MMAE to kill MES-SA/ MX2 cells
20 (MDR1-high) over parental MES-SA (MDR1-low) cells (Fig 30C, closed squares vs circles). This is attributed to MDR1-specific activity as this increase is blocked by MDR-1 inhibitors Elacridar, Tariquidar and Valspodar. MMAE's potency returns to baseline MES-SA control IC₅₀s in MES-SA/ MX2 cells treated with MDR1 inhibitors even in presence of MDR1 overexpression (Fig C, closed squares vs triangles), suggesting that the loss of potency was
25 driven by MDR1 transporter overexpression.

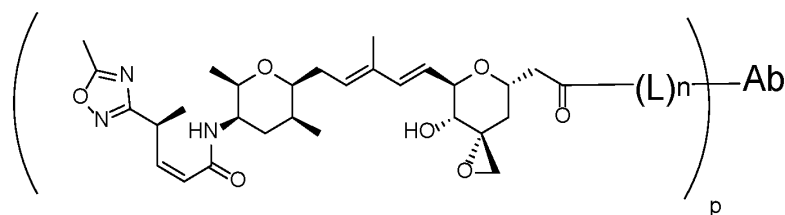
Similarly, increase in ADC-payload IC₅₀s required to kill MES-SA/ MX2 cells were seen in the presence of SN38 (5.2-fold, Fig 30D), DXd (9.67-fold, Fig 30E), ThA13 d2 Methyl ester (0.75-fold, Fig 30A) and ThA13 d2 301 (Fig 30B, 8.48-fold) upon comparison
30 of MES-SA/MX2 vs their corresponding MES-SA curves (closed squares vs circles) that returned to baseline when treated with multiple MDR1 inhibitors. The results suggest that Thailanstatin ADCs release payloads that are poor substrates for multi-drug resistance transporter MDR1, especially the species released by lysine conjugates.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, the descriptions and

examples should not be construed as limiting the scope of the invention. All patents, patent applications, and references cited throughout the specification are expressly incorporated by reference.

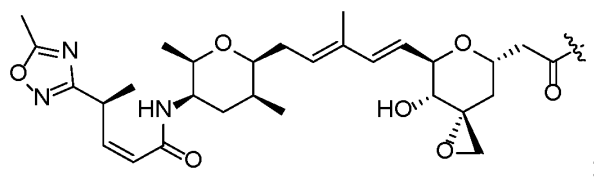
WHAT IS CLAIMED IS:

1. An antibody-drug conjugate compound comprising an antibody covalently attached to a drug moiety directly (i.e., without a linker) or through a linker of Formula I:



I

or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein the drug moiety is:



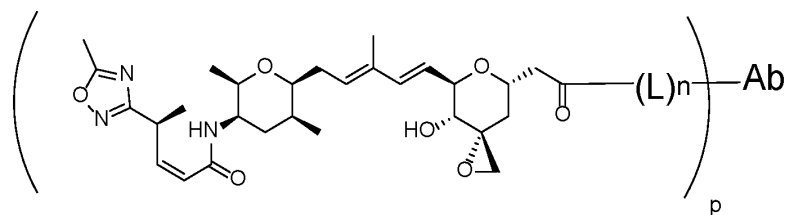
L is a linker;

n is 0 or 1;

p is 1 to 20; and

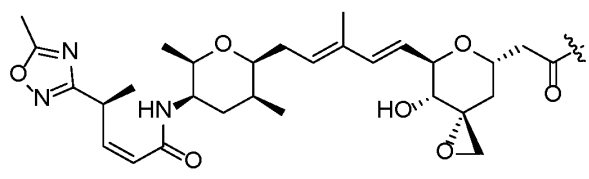
Ab is an antibody.

2. The antibody-drug conjugate compound of claim 1 comprising an antibody covalently attached to a drug moiety directly (i.e., without a linker) or through a linker of Formula I:



I

or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein the drug moiety is:



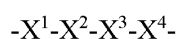
L is a linker;

n is 0 or 1;

p is 1 to 20; and

Ab is an antibody that binds to Trop2.

3. The antibody-drug conjugate compound of claim 1 or claim 2, wherein n is 0 (i.e., the linker (L) is absent).
4. The antibody-drug conjugate compound of claim 1 or claim 2, wherein the antibody is attached to the drug moiety by a sidechain nitrogen of a lysine residue of the antibody.
5. The antibody-drug conjugate compound of claim 1, wherein n is 1.
6. The antibody-drug conjugate of any one of claims 1, 2 or 5, wherein the linker L has the formula



wherein,

X^1 is a bond, $-N(R^a)-$, $-N(R^a)-N(R^a)-$, $-O-$, $-N-O-$, $-S-$, $-C(=O)-$, $-C(=O)N(R^a)$, $C(=S)N(R^a)-$, $-C(=O)O-$, $-C(=O)S-$, $-N(R^a)SO_2-$, $-OC(=O)N(R^a)-$, $-(R^a)HC(=O)N(R^a)-$, or $-N(R^a)C(=S)N(R^a)-$, wherein R^a is H or (C_1-C_6) alkyl;

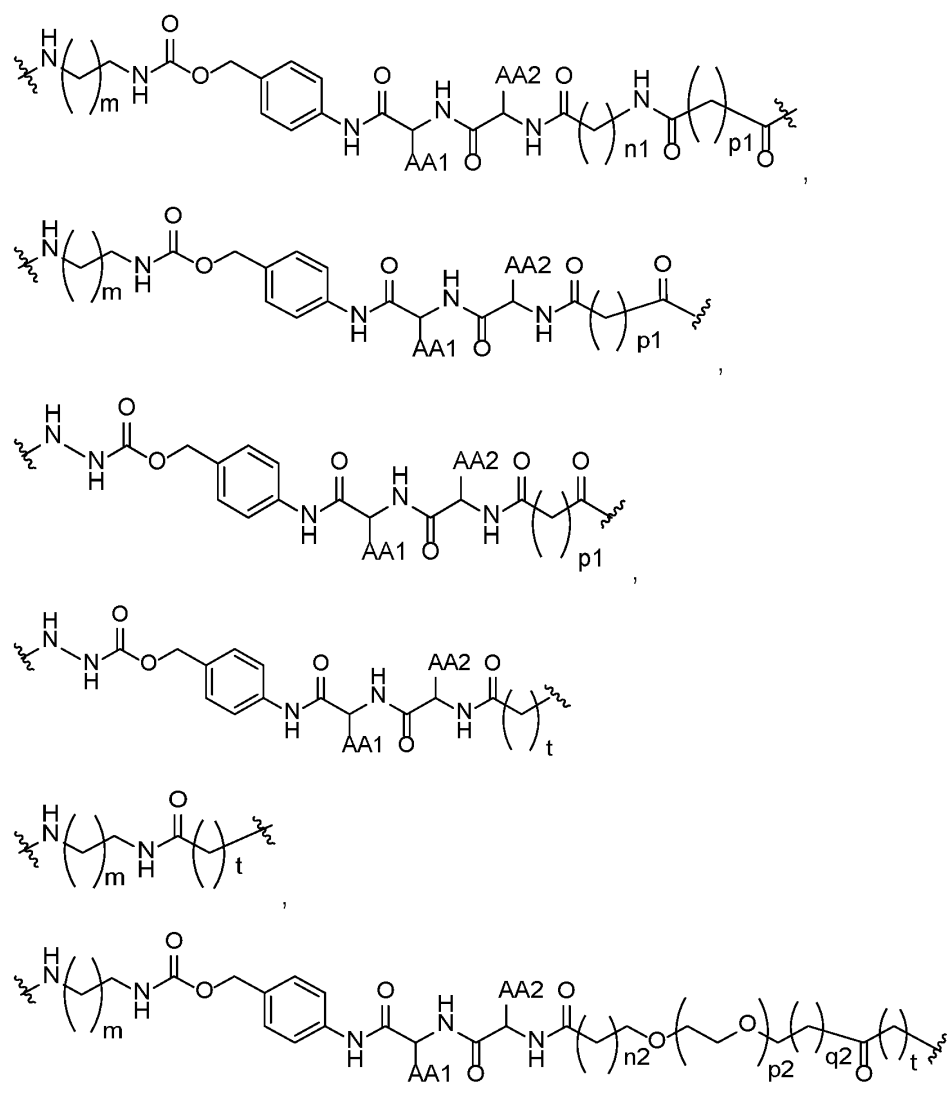
X^2 is a bond or a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain having from 1 to 30 carbon atoms, wherein one or more of the carbon atoms is optionally replaced by $(-O-)$, $(-S-)$, $-N(R^b)-$, a divalent 6-10 membered aryl or a divalent 5-20 membered heteroaryl wherein R^b is H or (C_1-C_6) alkyl, wherein the hydrocarbon chain is optionally substituted on carbon with one or more substituents selected from the group consisting of halo, hydroxy, mercapto, oxo(=O), and thioxo(=S), and wherein the divalent 6-10 membered aryl or a divalent 5-20 membered heteroaryl is optionally substituted with one more halo or (C_1-C_6) alkyl;

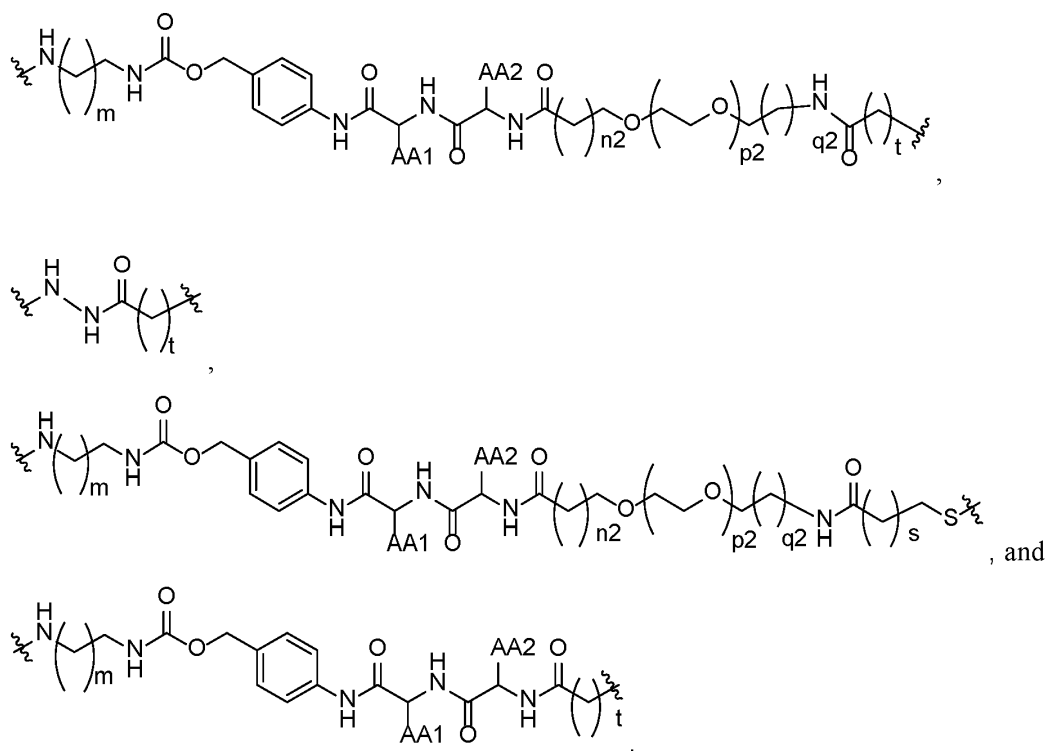
X^3 is a bond or a peptide (e.g., a peptide comprising 1, 2, 3, 4, 5, or 6 amino acid residues); and

X⁴ is a bond or a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain having from 1 to 60 carbon atoms, wherein one or more of the carbon atoms is optionally replaced by (-O-), (-S-), or -N(R^c)- wherein R^c is H or (C₁-C₆)alkyl, wherein the hydrocarbon chain is optionally substituted on carbon with one or more substituents selected from the group consisting of halo, hydroxy, mercapto, oxo(=O), and thioxo(=S);

wherein at least one of X¹, X², or X³ is not a bond.

7. The antibody-drug conjugate compound of claim 1 or claim 2, wherein n is 0 (i.e., L is absent) or n is 1 and L is a linker selected from the group consisting of:





wherein:

each m is independently 1, 2, 3, or 4;

each n1 is independently 1, 2, 3, 4, 5, or 6;

each p1 is independently 1, 2, 3, 4, 5, or 6;

each t is independently 1, 2, 3, or 4;

each n2 is independently 1, 2, 3, 4, 5, or 6;

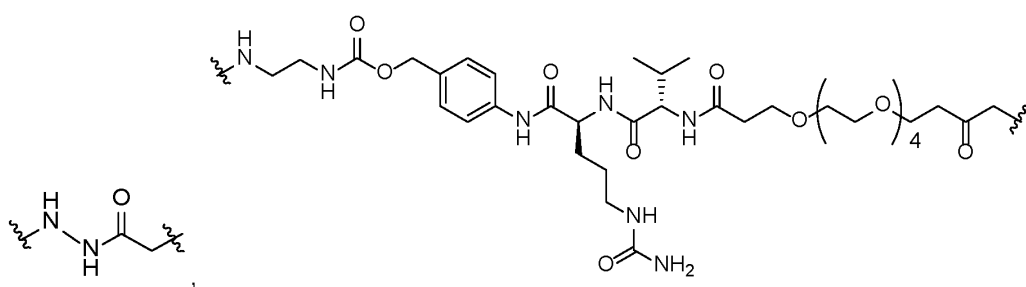
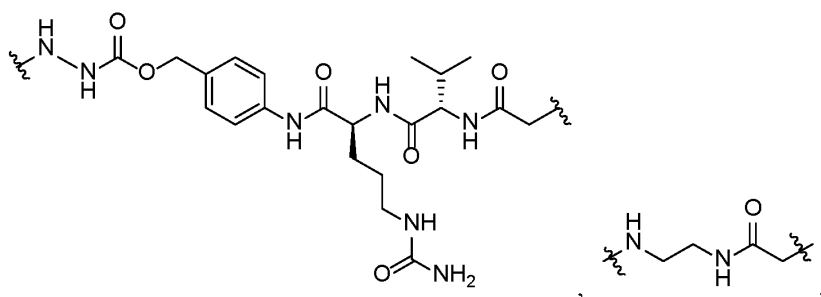
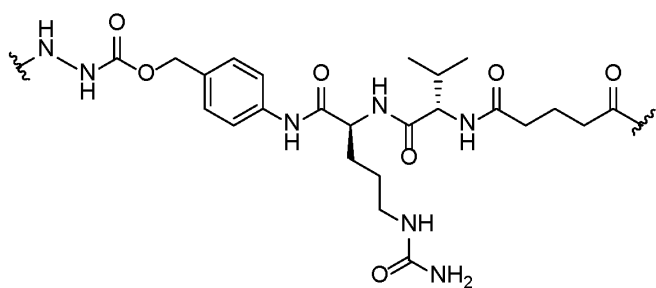
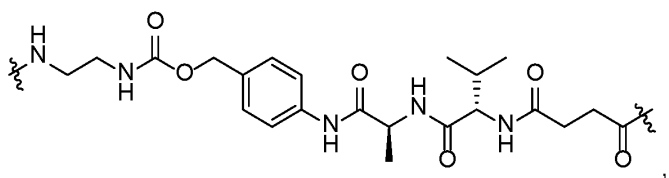
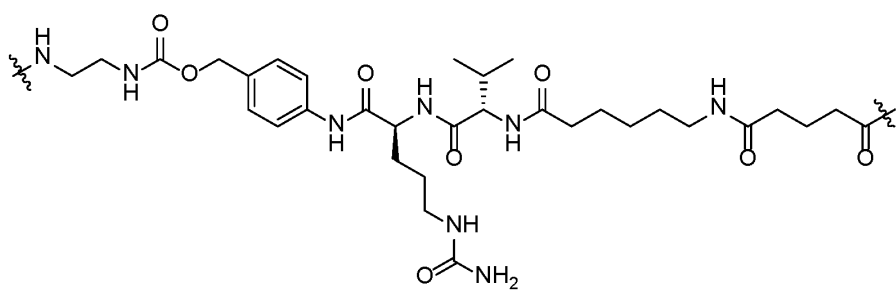
each p2 is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;

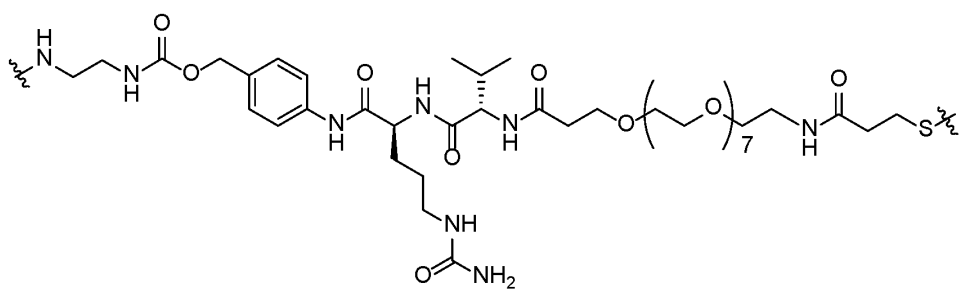
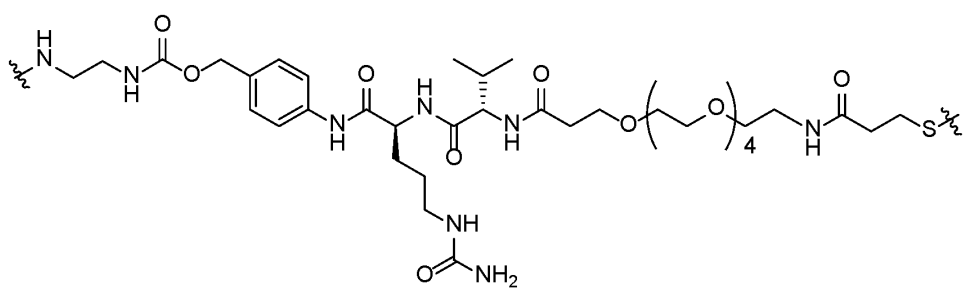
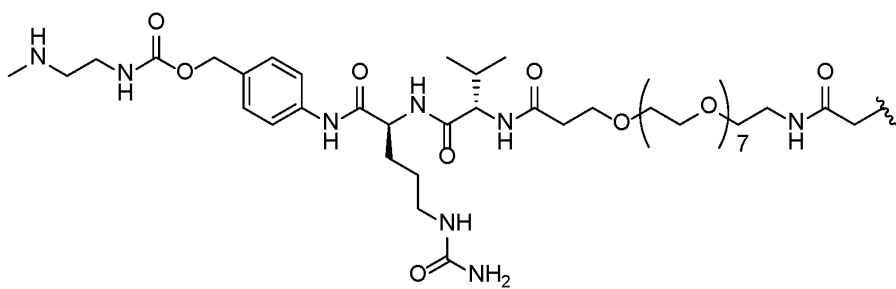
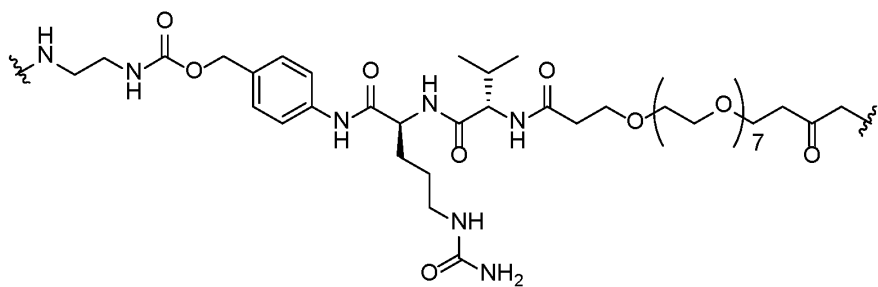
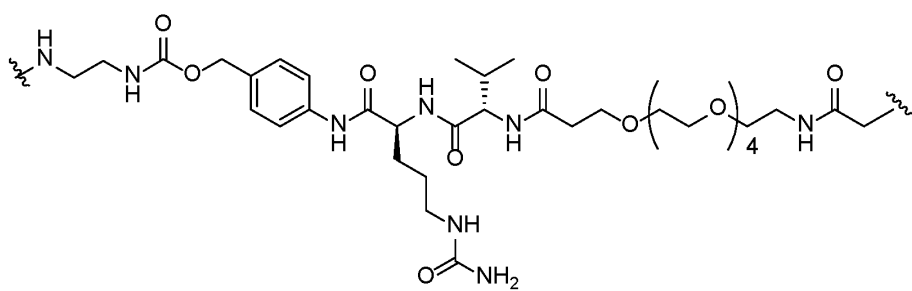
each q2 is independently 1, 2, 3, 4, 5, or 6;

each s is independently 1, 2, 3, or 4; and

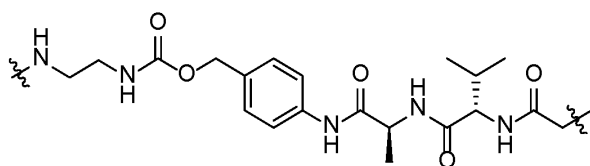
each AA1 and AA2 are independently a sidechain of an amino acid.

8. The antibody-drug conjugate compound of claim 1, wherein n is 0 (i.e., L is absent) or n is 1 and L is a linker selected from the group consisting of:





and



9. The antibody-drug conjugate compound of any one of claims 1-8, wherein the antibody is an anti-Trop2 antibody or fragment thereof and comprises an immunoglobulin heavy chain variable region polypeptide and an immunoglobulin light chain variable region polypeptide, wherein:

the immunoglobulin heavy chain variable region polypeptide comprises a complementarity determining region 1 (HCDR1) comprising an amino acid sequence that is at least 75% identical to NYGMN (SEQ ID NO: 14), a complementarity determining region 2 (HCDR2) comprising an amino acid sequence that is at least 75% identical to WINTKTGEPTYAEEFKG (SEQ ID NO: 16) or WINTKTGEPTYAQEFTG (SEQ ID NO: 21), and a complementarity determining region 3 (HCDR3) comprising an amino acid sequence that is at least 75% identical to GGYGSSYWYFDV (SEQ ID NO: 18); and/or

the immunoglobulin light chain variable region polypeptide comprises a complementarity determining region 1 (LCDR1) comprising an amino acid sequence that is at least 75% identical to KASQDVSIABA (SEQ ID NO: 27), a complementarity determining region 2 (LCDR2) comprising an amino acid sequence that is at least 75% identical to SASYRYT (SEQ ID NO: 29), and a complementarity determining region 3 (LCDR3) comprising an amino acid sequence that is at least 75% identical to QQHYITPLT (SEQ ID NO: 31).

10. The antibody-drug conjugate compound of any one of claims 1-8, wherein the antibody is an anti-Trop2 antibody or fragment thereof and comprises an immunoglobulin heavy chain variable region polypeptide and an immunoglobulin light chain variable region polypeptide, wherein:

the immunoglobulin heavy chain variable region polypeptide comprises a complementarity determining region 1 (HCDR1) comprising an amino acid sequence that is at least 85% identical to NYGMN (SEQ ID NO: 14), a complementarity determining region 2 (HCDR2) comprising an amino acid sequence that is at least 85% identical to WINTKTGEPTYAEEFKG (SEQ ID NO: 16) or WINTKTGEPTYAQEFTG (SEQ ID NO: 21), and a complementarity determining region 3 (HCDR3) comprising an amino acid sequence that is at least 85% identical to GGYGSSYWYFDV (SEQ ID NO: 18); and/or

the immunoglobulin light chain variable region polypeptide comprises a complementarity determining region 1 (LCDR1) comprising an amino acid sequence that is at least 85% identical to KASQDVSIABA (SEQ ID NO: 27), a complementarity determining region 2 (LCDR2) comprising an amino acid sequence that is at least 85% identical to SASYRYT (SEQ ID NO: 29), and a complementarity determining region 3 (LCDR3) comprising an amino acid sequence that is at least 85% identical to QQHYITPLT (SEQ ID NO: 31).

11. The antibody-drug conjugate compound of any one of claims 1-8, wherein the antibody is an anti-Trop2 antibody or fragment thereof and comprises an immunoglobulin heavy chain variable region polypeptide and an immunoglobulin light chain variable region polypeptide, wherein:

the immunoglobulin heavy chain variable region polypeptide comprises a complementarity determining region 1 (HCDR1) comprising NYGMN (SEQ ID NO: 14), a complementarity determining region 2 (HCDR2) comprising WINTKTGEPTYAEEFKG (SEQ ID NO: 16) or WINTKTGEPTYAQEFTG (SEQ ID NO: 21), and a complementarity determining region 3 (HCDR3) comprising GYGSSYWYFDV (SEQ ID NO: 18); and/or

the immunoglobulin light chain variable region polypeptide comprises a complementarity determining region 1 (LCDR1) comprising KASQDVSIABA (SEQ ID NO: 27), a complementarity determining region 2 (LCDR2) comprising SASYRYT (SEQ ID NO: 29), and a complementarity determining region 3 (LCDR3) comprising QQHYITPLT (SEQ ID NO: 31).

12. The antibody-drug conjugate compound of any one of claims 1-8, wherein the antibody is an anti-Trop2 antibody or fragment thereof and comprises an immunoglobulin heavy chain variable region polypeptide and an immunoglobulin light chain variable region polypeptide, wherein:

the immunoglobulin heavy chain variable region polypeptide comprises a complementarity determining region 1 (HCDR1) comprising SEQ ID NO: 14, a complementarity determining region 2 (HCDR2) comprising SEQ ID NO: 16, and a complementarity determining region 3 (HCDR3) comprising SEQ ID NO: 18.

13. The antibody-drug conjugate compound of any one of claims 1-8, wherein the antibody is an anti-Trop2 antibody or fragment thereof and comprises an immunoglobulin

heavy chain variable region polypeptide and an immunoglobulin light chain variable region polypeptide, wherein:

the immunoglobulin heavy chain variable region polypeptide comprises a complementarity determining region 1 (HCDR1) comprising SEQ ID NO: 14, a complementarity determining region 2 (HCDR2) comprising SEQ ID NO: 21, and a complementarity determining region 3 (HCDR3) comprising SEQ ID NO: 18.

14. The antibody-drug conjugate compound of any one of claims 1-8, the antibody is an anti-Trop2 antibody or fragment thereof and comprises an immunoglobulin heavy chain variable region polypeptide and an immunoglobulin light chain variable region polypeptide, wherein:

the immunoglobulin light chain variable region polypeptide comprises a complementarity determining region 1 (LCDR1) comprising SEQ ID NO: 27, a complementarity determining region 2 (LCDR2) comprising SEQ ID NO: 29, and a complementarity determining region 3 (LCDR3) comprising SEQ ID NO: 31.

15. The antibody-drug conjugate compound of any one of claims 1-8, wherein the antibody is an anti-Trop2 antibody or fragment thereof and comprises an immunoglobulin heavy chain variable region polypeptide comprising an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 1-4, and an immunoglobulin light chain variable region polypeptide comprising an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 5-6.

16. The antibody-drug conjugate compound of any one of claims 1-8, wherein the antibody is an anti-Trop2 antibody or fragment thereof and comprises an immunoglobulin heavy chain variable region polypeptide comprising an amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 1-4, and an immunoglobulin light chain variable region polypeptide comprising an amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 5-6.

17. The antibody-drug conjugate compound of any one of claims 1-8, wherein the antibody is an anti-Trop2 antibody or fragment thereof and comprises an immunoglobulin heavy chain variable region polypeptide comprising an amino acid sequence that is at least 97% identical to any one of SEQ ID NOs: 1-4, and an immunoglobulin light chain variable

region polypeptide comprising an amino acid sequence that is at least 97% identical to any one of SEQ ID NOs: 5-6.

18. The antibody-drug conjugate compound of any one of claims 1-8, wherein the antibody is an anti-Trop2 antibody and comprises an immunoglobulin heavy chain variable region polypeptide comprising an amino acid sequence that is at least 99% identical to any one of SEQ ID NOs: 1-4, and an immunoglobulin light chain variable region polypeptide comprising an amino acid sequence that is at least 99% identical to any one of SEQ ID NOs: 5-6.

19. The antibody-drug conjugate compound of any one of claims 1-8, wherein the antibody is an anti-Trop2 antibody or fragment thereof and comprises an immunoglobulin heavy chain variable region polypeptide comprising an amino acid sequence of any one of SEQ ID NOs: 1-4, and an immunoglobulin light chain variable region polypeptide comprising an amino acid sequence of any one of SEQ ID NOs: 5-6.

20. The antibody-drug conjugate compound of any one of claims 1-8, wherein the antibody is an anti-Trop2 antibody or fragment thereof and comprises an immunoglobulin heavy chain variable region polypeptide comprising an amino acid sequence of any one of SEQ ID NOs: 2 or 4, and an immunoglobulin light chain variable region polypeptide comprising an amino acid sequence of any one of SEQ ID NOs: 5-6.

21. The antibody-drug conjugate compound of any one of claims 1-8, wherein the antibody is an anti-Trop2 antibody and comprises an immunoglobulin heavy chain polypeptide comprising a human IgG1, IgG2, IgG3, or IgG4 Fc domain.

22. The antibody-drug conjugate compound of any one of claims 1-8, wherein the antibody is an anti-Trop2 antibody and comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 7, 9, 10, 11, or 34-37, and an immunoglobulin light chain polypeptide comprising an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 8 or 12.

23. The antibody-drug conjugate compound of any one of claims 1-8, wherein the antibody is an anti-Trop2 antibody and comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 7, 9, 10, 11, or 34-37, and an immunoglobulin light chain polypeptide comprising an amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 8 or 12.
24. The antibody-drug conjugate compound of any one of claims 1-8, wherein the antibody is an anti-Trop2 antibody and comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence that is at least 97% identical to any one of SEQ ID NOs: 7, 9, 10, 11, or 34-37, and an immunoglobulin light chain polypeptide comprising an amino acid sequence that is at least 97% identical to any one of SEQ ID NOs: 8 or 12.
25. The antibody-drug conjugate compound of any one of claims 1-8, wherein the antibody is an anti-Trop2 antibody and comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence that is at least 99% identical to any one of SEQ ID NOs: 7, 9, 10, 11, or 34-37, and an immunoglobulin light chain polypeptide comprising an amino acid sequence that is at least 99% identical to any one of SEQ ID NOs: 8 or 12.
26. The antibody-drug conjugate compound of any one of claims 1-8, wherein the antibody is an anti-Trop2 antibody and comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence of any one of SEQ ID NOs: 7, 9, 10, 11, or 34-37.
27. The antibody-drug conjugate compound of any one of claims 1-8, wherein the antibody is an anti-Trop2 antibody and comprises an immunoglobulin light chain polypeptide comprising an amino acid sequence of any one of SEQ ID NOs: 8 or 12.
28. The antibody-drug conjugate compound of any one of claims 1-8, wherein the antibody is an anti-Trop2 antibody and comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence that is any one of SEQ ID NOs: 7, 10, 35, or

37, and an immunoglobulin light chain polypeptide comprising an amino acid sequence that is any one of SEQ ID NOs: 8 or 12.

29. The antibody-drug conjugate compound of any one of claims 1-8, wherein the antibody is an anti-Trop2 antibody and comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence that is any one of SEQ ID NOs: 7, or 37, and an immunoglobulin light chain polypeptide comprising an amino acid sequence of SEQ ID NO: 8.

30. An antibody or fragment thereof comprising an immunoglobulin heavy chain variable region polypeptide and an immunoglobulin light chain variable region polypeptide, wherein:

the immunoglobulin heavy chain variable region polypeptide comprises a complementarity determining region 1 (HCDR1) comprising an amino acid sequence that is at least 90% identical to NYGMN (SEQ ID NO: 14), a complementarity determining region 2 (HCDR2) comprising an amino acid sequence that is at least 90% identical to WINTKTGEPTYAQEFTG (SEQ ID NO: 21), and a complementarity determining region 3 (HCDR3) comprising an amino acid sequence that is at least 90% identical to GGYGSSYWYFDV (SEQ ID NO: 18); and/or

the immunoglobulin light chain variable region polypeptide comprises a complementarity determining region 1 (LCDR1) comprising an amino acid sequence that is at least 90% identical to KASQDVSIABA (SEQ ID NO: 27), a complementarity determining region 2 (LCDR2) comprising an amino acid sequence that is at least 90% identical to SASYRYT (SEQ ID NO: 29), and a complementarity determining region 3 (LCDR3) comprising an amino acid sequence that is at least 90% identical to QQHYITPLT (SEQ ID NO: 31).

31. The antibody or fragment thereof of claim 30, wherein:

the immunoglobulin heavy chain variable region polypeptide comprises a complementarity determining region 1 (HCDR1) comprising NYGMN (SEQ ID NO: 14), a complementarity determining region 2 (HCDR2) comprising WINTKTGEPTYAQEFTG (SEQ ID NO: 21), and a complementarity determining region 3 (HCDR3) comprising GGYGSSYWYFDV (SEQ ID NO: 18); and/or

the immunoglobulin light chain variable region polypeptide comprises a complementarity determining region 1 (LCDR1) comprising KASQDVSIABA (SEQ ID NO:

27), a complementarity determining region 2 (LCDR2) comprising SASYRYT (SEQ ID NO: 29), and a complementarity determining region 3 (LCDR3) comprising QQHYITPLT (SEQ ID NO: 31).

32. The antibody or fragment thereof of any one of claims 30-31, wherein:
the immunoglobulin heavy chain variable region polypeptide comprising an amino acid sequence that is at least 95% identical to SEQ ID NO:4, and
the immunoglobulin light chain variable region polypeptide comprising an amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 5-6; or
the immunoglobulin heavy chain variable region polypeptide comprising an amino acid sequence that is at least 95% identical to SEQ ID NO:4, and
the immunoglobulin light chain variable region polypeptide comprising an amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 6.

33. The antibody or fragment thereof of any one of claims 30-31, wherein:
the immunoglobulin heavy chain variable region polypeptide comprising an amino acid sequence that is at least 97% identical to SEQ ID NO:4, and
the immunoglobulin light chain variable region polypeptide comprising an amino acid sequence that is at least 97% identical to any one of SEQ ID NOs: 5-6 or
the immunoglobulin heavy chain variable region polypeptide comprising an amino acid sequence that is at least 97% identical to SEQ ID NO:4, and
the immunoglobulin light chain variable region polypeptide comprising an amino acid sequence that is at least 97% identical to any one of SEQ ID NOs: 6.

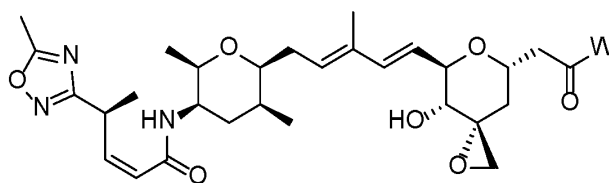
34. The antibody or fragment thereof of any one of claims 30-31, wherein:
the immunoglobulin heavy chain variable region polypeptide comprising an amino acid sequence that is at least 99% identical to SEQ ID NO:4, and
the immunoglobulin light chain variable region polypeptide comprising an amino acid sequence that is at least 99% identical to any one of SEQ ID NOs: 5-6; or
the immunoglobulin heavy chain variable region polypeptide comprising an amino acid sequence that is at least 99% identical to SEQ ID NO:4, and
the immunoglobulin light chain variable region polypeptide comprising an amino acid sequence that is at least 99% identical to any one of SEQ ID NOs: 6.

35. The antibody or fragment thereof of any one of claims 30-31, wherein:
the immunoglobulin heavy chain variable region polypeptide comprising SEQ ID NO:4, and
the immunoglobulin light chain variable region polypeptide comprising SEQ ID NO: 5 or 6;
or
the antibody or fragment thereof of any one of claims 30-31, wherein:
the immunoglobulin heavy chain variable region polypeptide comprising SEQ ID NO:4, and
the immunoglobulin light chain variable region polypeptide comprising SEQ ID NO: 6.
36. The antibody of any one of claims 30-31, wherein the antibody comprises an immunoglobulin heavy chain polypeptide comprising a human IgG1, IgG2, IgG3, or IgG4 Fc domain.
37. The antibody of any one of claims 30-31, wherein the antibody comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 7, or 37, and an immunoglobulin light chain polypeptide comprising an amino acid sequence that is at least 95% identical to any one of SEQ ID NOs: 8 or 12.
38. The antibody of any one of claims 30-31, wherein the antibody comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence that is at least 97% identical to any one of SEQ ID NOs: 7, or 37, and an immunoglobulin light chain polypeptide comprising an amino acid sequence that is at least 97% identical to any one of SEQ ID NOs: 8 or 12.
39. The antibody of any one of claims 30-31, wherein the antibody comprises an immunoglobulin heavy chain polypeptide comprising an amino acid sequence that is at least 99% identical to any one of SEQ ID NOs: 7, or 37, and an immunoglobulin light chain polypeptide comprising an amino acid sequence that is at least 99% identical to any one of SEQ ID NOs: 8 or 12.

40. The antibody of any one of claims 30-31, wherein the antibody comprises an immunoglobulin heavy chain polypeptide comprising any one of SEQ ID NOs: 7, or 37, and an immunoglobulin light chain polypeptide comprising any one of SEQ ID NOs: 8 or 12.

41. The antibody of any one of claims 30-31, wherein the antibody comprises an immunoglobulin heavy chain polypeptide comprising any one of SEQ ID NOs: 7, or 37, and an immunoglobulin light chain polypeptide comprising SEQ ID NO: 8.

42. A compound of formula II



II

or a salt thereof,

wherein

W is a leaving group or -L-X;

L is a linker; and

X is a reactive group.

43. The compound of claim 42, wherein W is a leaving group.

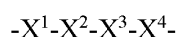
44. The compound of claim 42 or 43, wherein the leaving group is halo, -O(C₁-C₆)alkyl, -Oaryl, -Oheteroaryl or -Oheterocyclyl, wherein the -O(C₁-C₆)alkyl, -Oaryl, Oheteroaryl or Oheterocyclyl are each optionally substituted with one or more substituents independently selected from the group consisting of oxo, halo, (C₁-C₆)alkyl and -O(C₁-C₆)alkyl.

45. The compound of claim 42, wherein W together with the carbonyl group to which it is attached forms an activated ester.

46. The compound of claim 42, wherein W is -Ophenyl optionally substituted with one or more halo or W is 2,5, dioxopyrrolidinyl.

47. The compound of claim 42, wherein W is -Ophenyl optionally substituted with one or more halo.
48. The compound of claim 42, wherein W is -Ophenyl optionally substituted with one or more fluoro.
49. The compound of claim 42, wherein W is a 2,3,5,6,-tetrafluorophenoxy.
50. The compound of claim 42, wherein W is 2,5, dioxopyrrolidininyloxy.
51. The compound of claim 42, wherein W is L-X.

52. The compound of claim 42 or claim 51, wherein L has the formula



wherein,

X¹ is a bond, -N(R^a)-, -N(R^a)-N(R^a)-, -O-, -N-O-, -S-, -C(=O)-, -C(=O)N(R^a), C(=S)N(R^a)-, -C(=O)O-, -C(=O)S-, -N(R^a)SO₂-, -OC(=O)N(R^a)-, -(R^a)HC(=O)N(R^a)-, or -N(R^a)C(=S)N(R^a)-, wherein R^a is H or (C₁-C₆)alkyl;

X² is a bond or a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain having from 1 to 30 carbon atoms, wherein one or more of the carbon atoms is optionally replaced by (-O-), (-S-), -N(R^b)-, a divalent 6-10 membered aryl or a divalent 5-20 membered heteroaryl wherein R^b is H or (C₁-C₆)alkyl, wherein the hydrocarbon chain is optionally substituted on carbon with one or more substituents selected from the group consisting of halo, hydroxy, mercapto, oxo(=O), and thioxo(=S), and wherein the divalent 6-10 membered aryl or a divalent 5-20 membered heteroaryl is optionally substituted with one more halo or (C₁-C₆)alkyl;

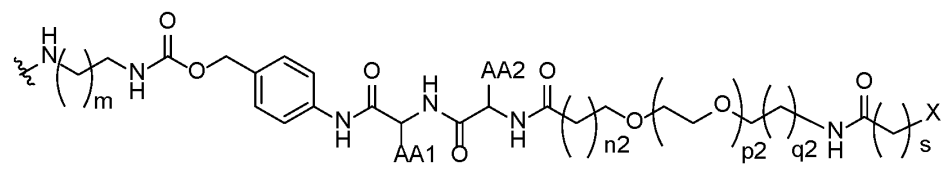
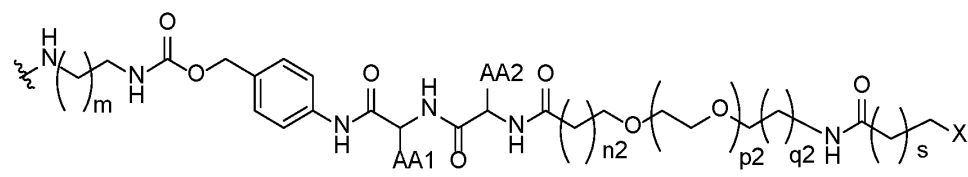
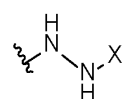
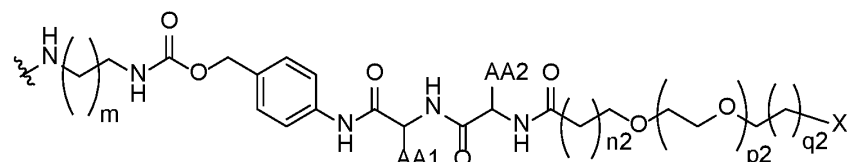
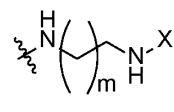
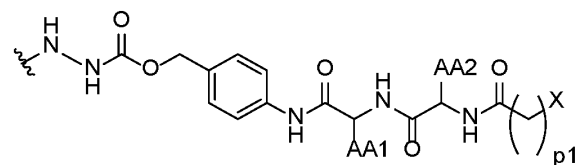
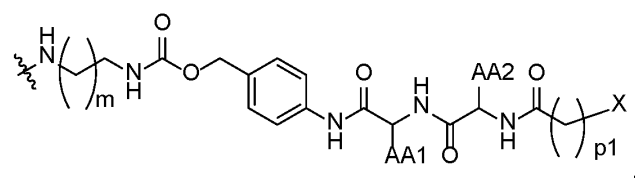
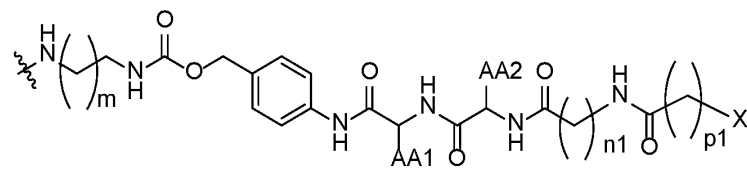
X³ is a bond or a peptide (e.g., a peptide comprising 1, 2, 3, 4, 5, or 6 amino acid residues); and

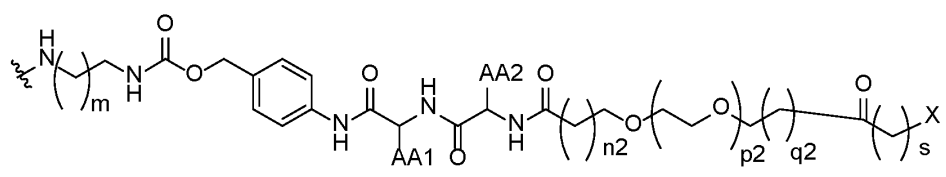
X⁴ is a bond or a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain having from 1 to 60 carbon atoms, wherein one or more of the carbon atoms is optionally replaced by (-O-), (-S-), or -N(R^c)- wherein R^c is H or (C₁-C₆)alkyl, wherein the hydrocarbon chain is optionally substituted on carbon with one or more substituents selected from the group consisting of halo, hydroxy, mercapto, oxo(=O), and

thioxo(=S);

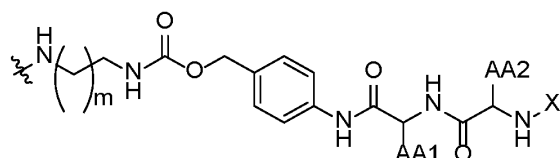
wherein at least one of X^1 , X^2 , or X^3 is not a bond.

53. The compound of any one of claims 42, 51, or 52 wherein W is selected from the group consisting of .





and



wherein:

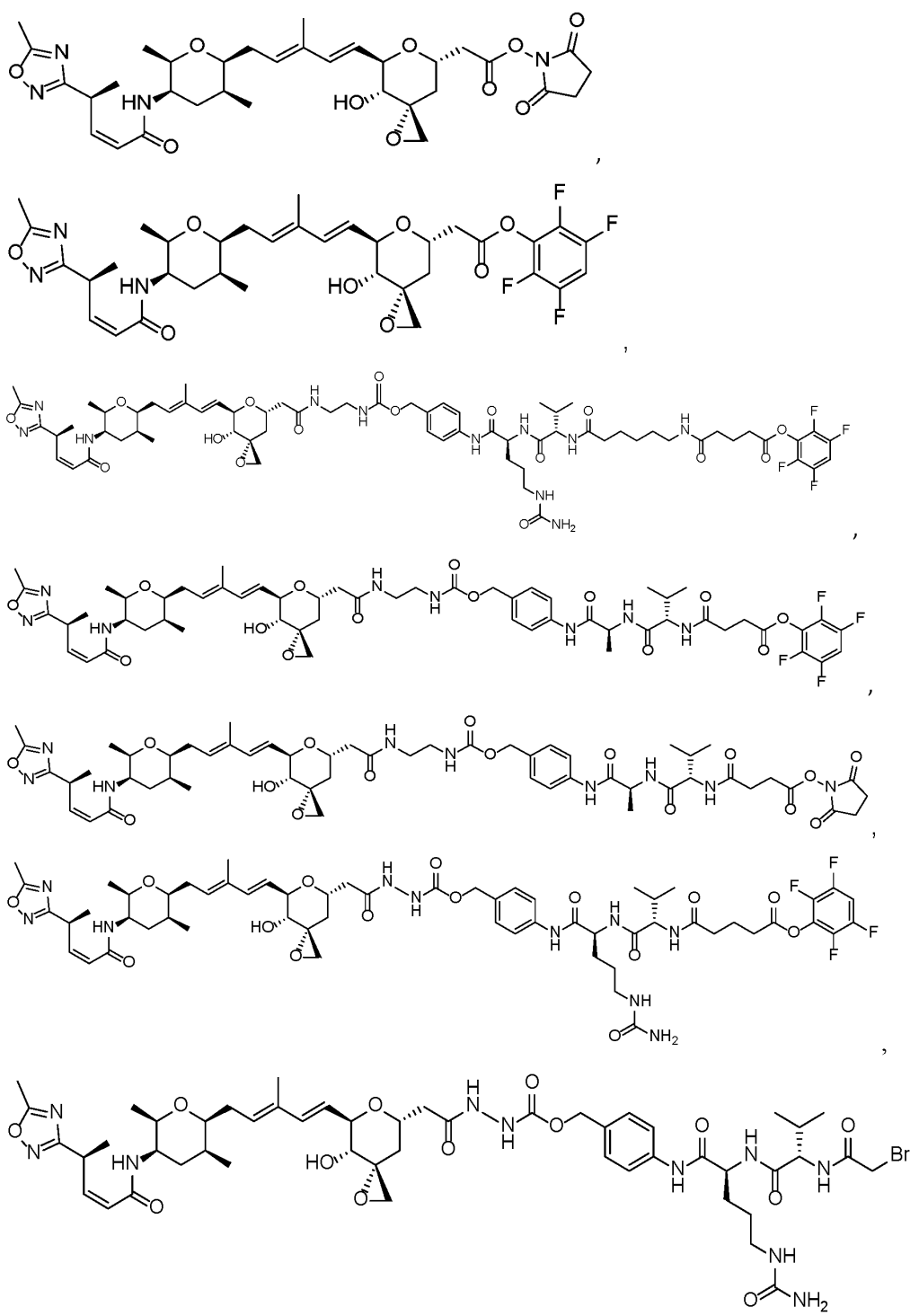
- each m is independently 1, 2, 3, or 4;
- each n1 is independently 1, 2, 3, 4, 5, or 6;
- each p1 is independently 1, 2, 3, 4, 5, or 6;
- each t is independently 1, 2, 3, or 4;
- each n2 is independently 1, 2, 3, 4, 5, or 6;
- each p2 is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;
- each q2 is independently 1, 2, 3, 4, 5, or 6;
- each s is independently 1, 2, 3, or 4; and
- each AA1 and AA2 are independently a sidechain of an amino acid.

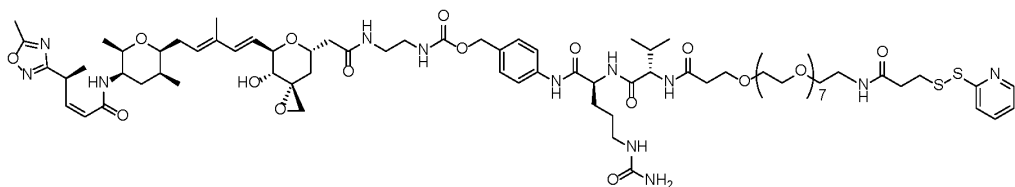
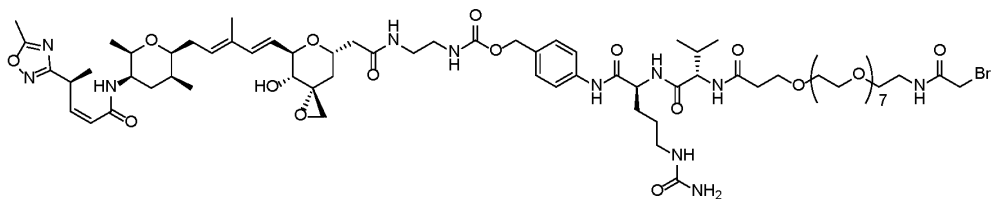
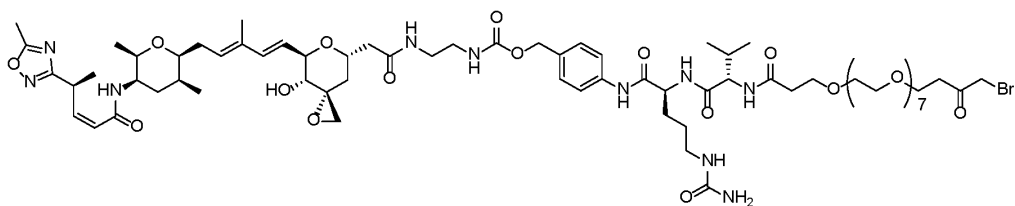
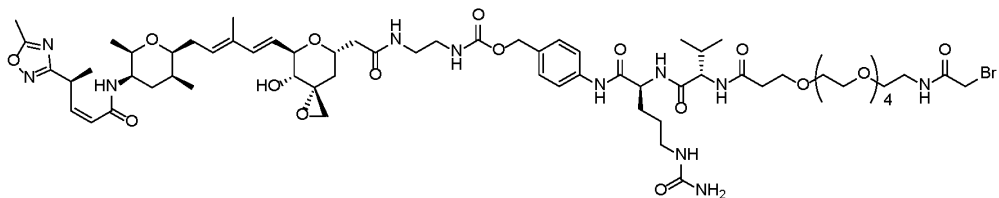
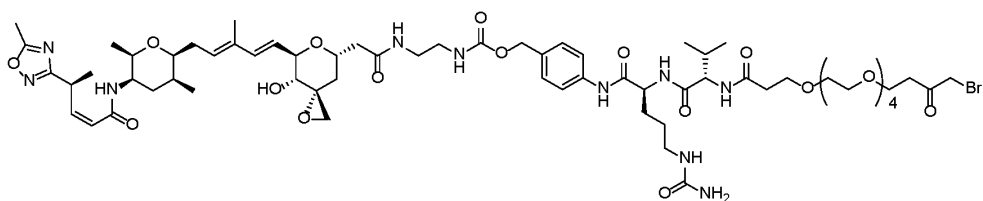
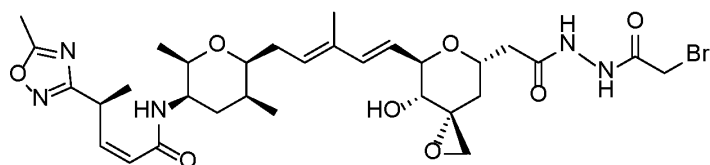
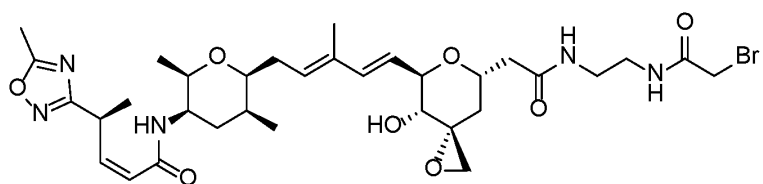
54. The compound of any one of claims 42, 51, or 52, wherein each X is independently selected from an ester, activated ester, haloacetamide, halo, and disulfanylheteroaryl.

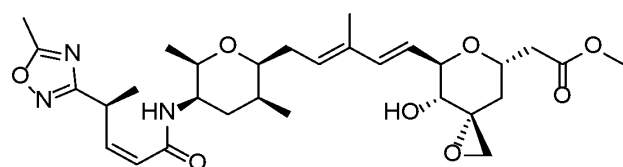
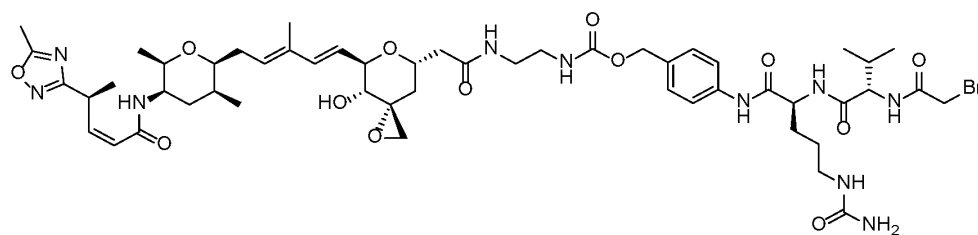
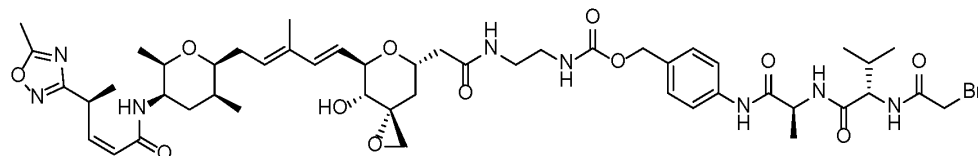
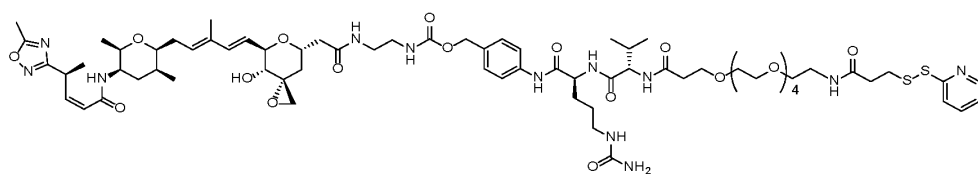
55. The compound of any one of claims 42, 51, or 52, wherein each X is selected from 2,3,5,6,-tetrafluorophenoxycarbonyl, 2,5-dioxypyrollidinylloxycarbonyl, disulfanylpyridinyl, and bromoacetyl.

56. The compound of any one of claims 42, 51, or 52, wherein each X is selected from 2,3,5,6,-tetrafluorophenoxycarbonyl and bromoacetyl.

57. The compound of claim 42 selected from the group consisting of

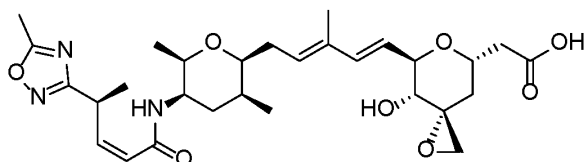
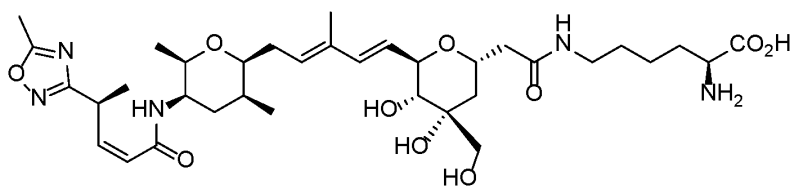
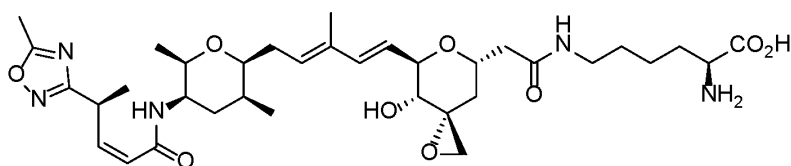


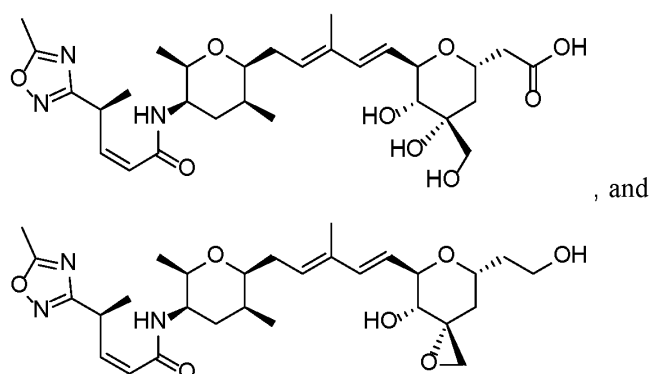




or a salt thereof.

58. A compound of selected from the group consisting of





or a salt thereof (e.g., a pharmaceutically acceptable salt thereof).

59. The antibody-drug conjugate compound according to any one of claims 1-29, wherein p is 1, 2, 3, 4, 5, 6, 7, or 8.

60. The antibody-drug conjugate compound according any one of claims 1-29, comprising a mixture of the antibody-drug conjugate compounds, wherein the average drug loading per antibody in the mixture of antibody-drug conjugate compounds is about 1 to about 5.

61. A pharmaceutical composition comprising the antibody-drug conjugate compound according to any one of claims 1-29, 59, or 60 and a pharmaceutically acceptable diluent, carrier or excipient.

62. A method for treating cancer in a patient in need thereof, comprising administering a therapeutically effective amount of the antibody drug conjugate compound of any one of claims 1-29, 59, or 60, or the pharmaceutical composition of claim 61 to the patient in need thereof.

63. The method of claim 62, wherein the cancer is selected from lung cancer, non-small cell lung cancer, pancreatic cancer, esophageal cancer, gastric cancer, bladder cancer, ovarian cancer, breast cancer, uterine cancer and head and neck cancer.

64. The method of claim 62, wherein the cancer is selected from lung cancer (e.g., non-small cell lung cancer, lung adenocarcinoma, and lung large cell carcinoma), pancreatic

cancer, esophageal cancer, gastric cancer, bladder cancer, ovarian cancer, breast cancer, and uterine cancer.

65. The method of any one of claims 62-64, wherein the patient has Trop2 positive tumor cells.

66. The method of any one of claims 62-64, wherein the patient has tumor cells comprising one or more mutation that is a loss-of-function mutation in a tumor suppressor gene, and/or a gain-of-function mutation in an oncogenic driver gene and/or amplification of an oncogenic driver gene.

67. The method of any one of claims 62-66, wherein the patient has tumor cells that comprises one or more mutation in a gene selected from the group consisting of KRAS, BRAF, EGFR, ERBB2, ERBB3, APC, CNGA2, ARID1A, SLC34A2, ROS1, FGFR3, TACC3, BAIAP2L1, PIK3CA, PIK3R1, BRCA2, PTEN, STK11, CDKN2A, SMAD4, TP53, UGT2B17, SMARCA2, and SMARCA4.

68. The method of claim 66 or claim 67, wherein the one or more mutation comprises a point mutation.

69. The method of claim 67, wherein the one or more mutation comprises a mutation selected from the group consisting of KRas G12V, BRAF G466V, EGFR L861Q, PIK3CA E545K, APC R1450*, PIK3R1 R386fs, PIK3R1 R639ter, TP53 V218, ARID1A L649fs/R693ter and SMARCA4 G1162C.

70. The method of claim 66 or claim 67, wherein the one or more mutation comprises a gain-of-function mutation.

71. The method of claim 66 or claim 67, wherein the patient has tumor cell that comprises one or more mutation in a gene selected from the group consisting of KRAS, BRAF, EGFR, SLC34A2, ROS1, FGFR3, TACC3, BAIAP2L1, PIK3CA, PIK3R1, and BRCA2.

72. The method of claim 66 or claim 67, wherein the one or more mutation comprises a loss-of-function mutation.

73. The method of claim 66 or claim 67, wherein the subject has tumor cell that comprises one or more mutation in a gene selected from the group consisting of PTEN, STK11, CDKN2A, SMAD4, and TP53.
74. The method of claim 66 or claim 67, wherein the one or more mutation comprises fusion mutation of two genes.
75. The method of claim 74, wherein the one or more mutation comprise fusion mutation selected from the group consisting of SLC34A2-ROS1, FGFR3-TACC3, and FGFR3-BAIAP2L1.
76. The method of claim 66 or claim 67, wherein the one or more mutation comprises a BRCA-deficient mutation, FGFR3 gain-of-function mutation, TP53 loss-of-function mutation, or SMARCA4 loss-of-function mutation.
77. The method of claim 66 or claim 67, wherein one or more copy number variations (CNV) comprises a partial or total loss of PTEN, STK11, CDKN2A, CNGA2, SMAD4, UGT2B17; or gain/ amplification of ERBB2, ERBB3, PIK3CA.
78. The method of any one of claims 62-77, wherein the patient has HPV positive tumor cells.
79. The method of any one of claims 62-77, wherein the patient has HPV39 positive tumor cells.
80. The method of any one of claims 62-79, wherein the patient is unresponsive to standard-of-care (SOC) therapy.
81. The method of any one of claims 62-80, wherein the patient's tumor is resistant to MEK or BET inhibitor therapy (e.g., in Lung adenocarcinoma), anti-EGFR therapy (e.g., in NSCLC), or multi-kinase inhibitor therapies (e.g., such as Sorafenib or AZ628 inhibitors in Gastric cancer).

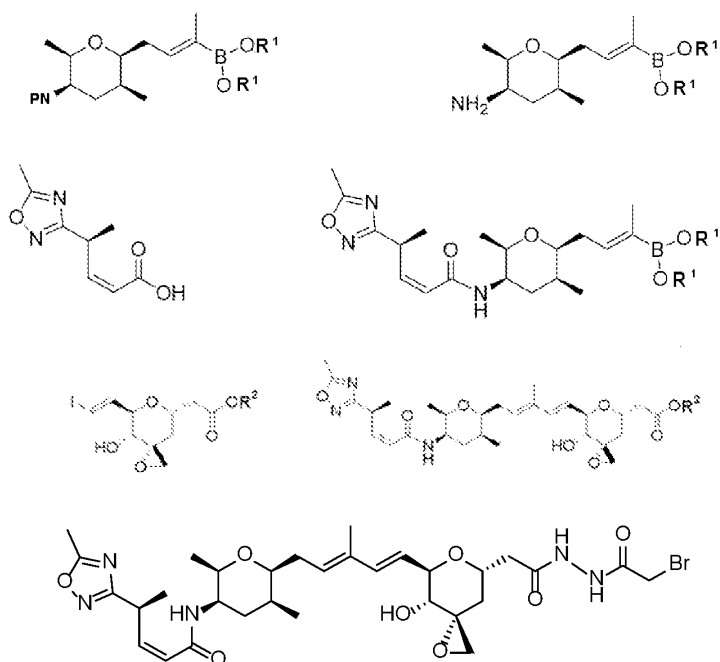
82. An article of manufacture comprising a pharmaceutical composition of claim 61, a container, and a package insert or label indicating that the pharmaceutical composition can be used to treat cancer.

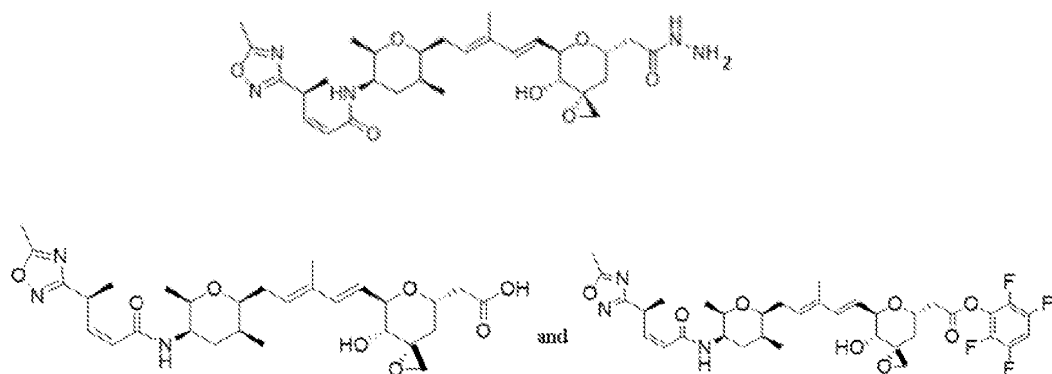
83. Use of an antibody-drug conjugate compound according to any one of claims 1-29, 59, or 60, or a pharmaceutically acceptable salt thereof, in the manufacture of a medicament for the treatment of cancer in a mammal.

84. An antibody-drug conjugate compound according to any one of claims 1-29, 59, or 60, or a pharmaceutically acceptable salt thereof, for use in the therapeutic treatment of cancer.

85. Use of an antibody-drug conjugate compound according to any one of claims 1-29, 59, or 60, or a pharmaceutically acceptable salt thereof, for medical therapy.

86. A compound selected from the group consisting of:





wherein:

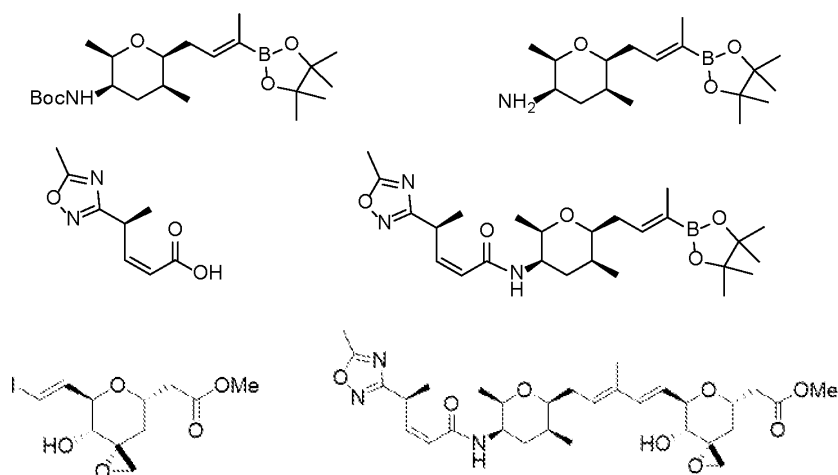
P is an amine nitrogen protecting group;

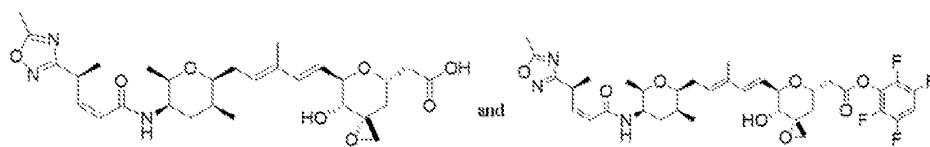
each R^1 is independently a (C₁-C₆)alkyl, or two R^1 groups together with the atoms to which they are attached form a heterocyclyl optionally substituted with one or more (C₁-C₆)alkyl; and

R^2 is a (C₁-C₆)alkyl or phenyl, wherein the phenyl is optionally substituted with one or more halo or nitro;

or a salt thereof.

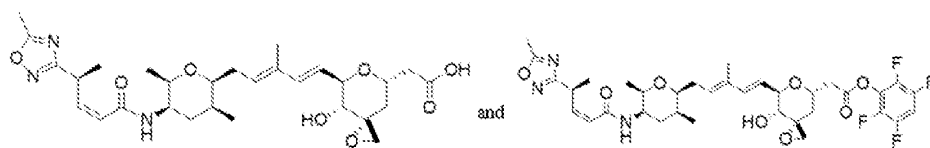
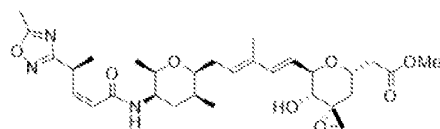
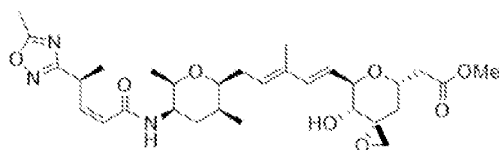
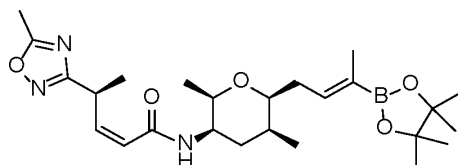
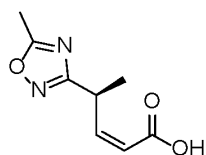
87. The compound of claim 86 selected from the group consisting of:





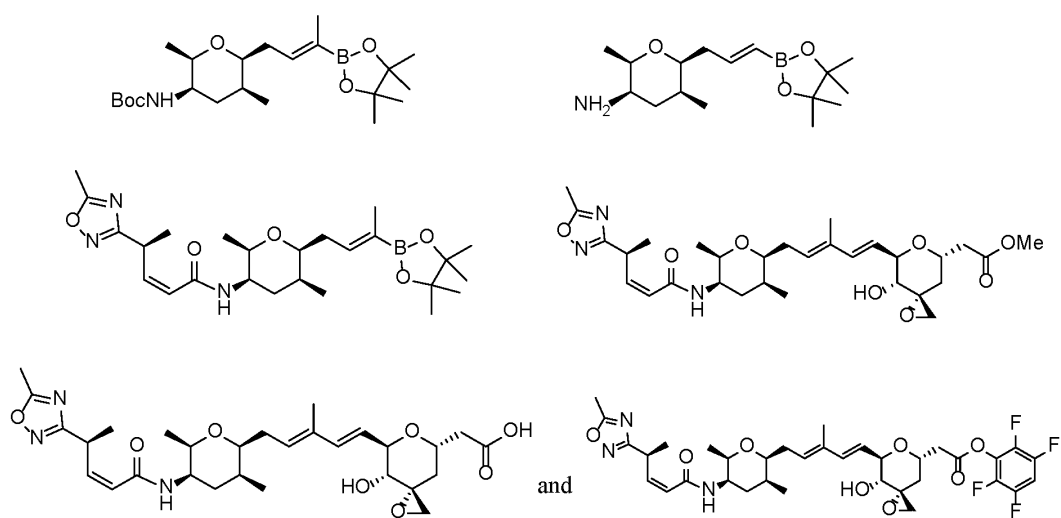
or a salt thereof.

88. The compound of claim 86 selected from the group consisting of:



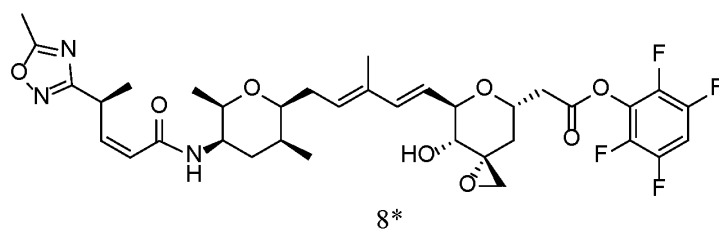
or a salt thereof.

89. The compound of claim 86 selected from the group consisting of:

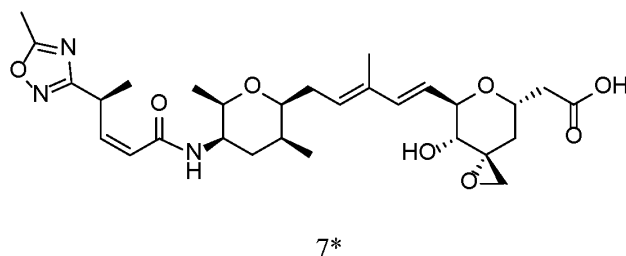


or a salt thereof.

90. A method for preparing a compound of formula 8*:

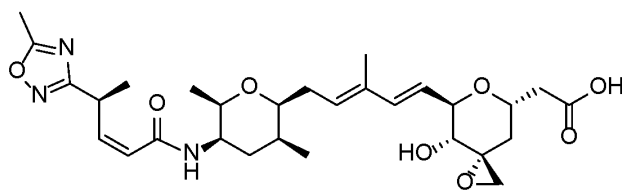


or a salt thereof, comprising converting a corresponding compound of formula 7*:



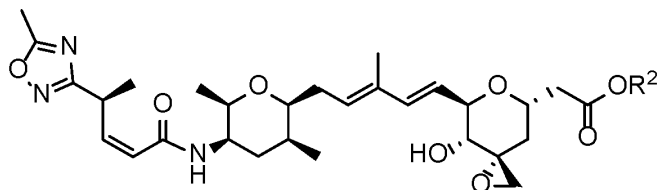
or a salt thereof, to the compound of formula 8* or a salt thereof.

91. A method for preparing a compound of formula 7*:



7*

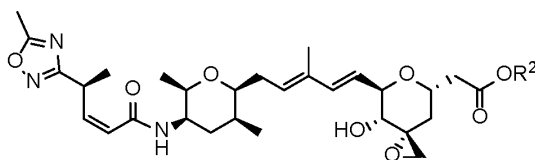
or a salt thereof, comprising converting a corresponding compound of formula 6a*:



6a*

or a salt thereof, to the compound of formula 7* or a salt thereof,
wherein R² is a (C₁-C₆)alkyl.

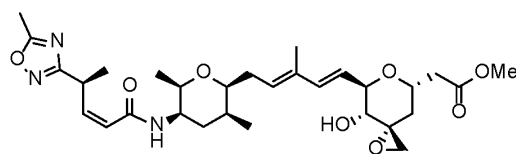
92. The method of claim 90, wherein the compound of formula 7* or a salt thereof is prepared, comprising converting a corresponding compound of formula 6a*:



6a*

or a salt thereof, to the compound of formula 7* or a salt thereof,
wherein R² is a (C₁-C₆)alkyl.

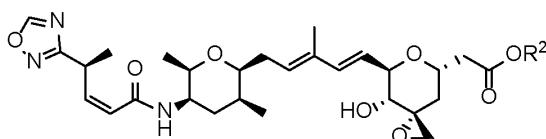
93. The method of claim 91 or claim 92, wherein the compound of formula 6a* is a compound of formula 6*:



6*

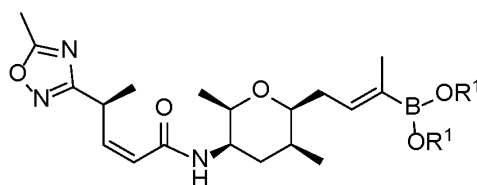
or a salt thereof.

94. A method for preparing a compound of formula 6a*:



6a*

or a salt thereof, comprising converting a corresponding compound of formula 5a*:



5a*

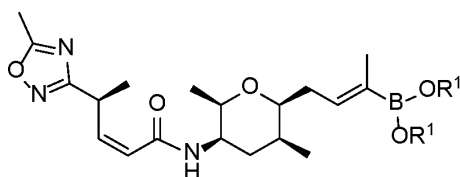
or a salt thereof, to the compound of formula 6a* or a salt thereof,

wherein:

each R¹ is independently a (C₁-C₆)alkyl, or two R¹ groups together with the atoms to which they are attached form a heterocyclyl optionally substituted with one or more (C₁-C₆)alkyl; and

R² is a (C₁-C₆)alkyl.

95. The method of claim 91 or claim 92, wherein the compound of formula 6a* or a salt thereof is prepared, comprising converting a corresponding compound of formula 5a*:

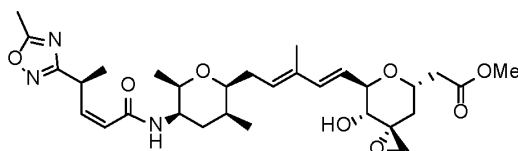


5a*

or a salt thereof, to the compound of formula 6 or a salt thereof,

wherein each R¹ is independently a (C₁-C₆)alkyl, or two R¹ groups together with the atoms to which they are attached form a heterocyclyl optionally substituted with one or more (C₁-C₆)alkyl.

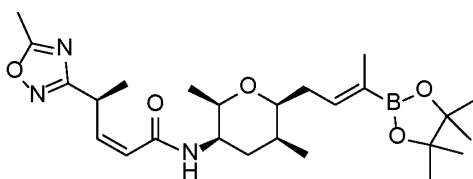
96. The method of claim 94 or claim 95, wherein the compound of formula 6a* is a compound of formula 6*:



6*

or a salt thereof.

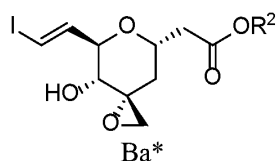
97. The method of claim 94 or claim 95, wherein the compound of formula 5a* is a compound of formula 5*:



5*

or a salt thereof.

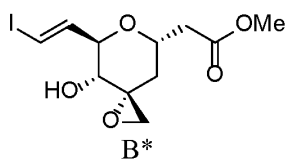
98. The method of claim 94 or claim 95, wherein the compound of formula 6a* is prepared, comprising reacting the compound of formula 5a* or a salt thereof, with a compound of formula Ba*:



or a salt thereof, to the compound of formula 6a*,

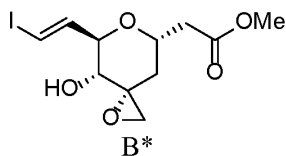
wherein R² is a (C₁-C₆)alkyl.

99. The method of claim 98, wherein the compound of formula Ba* is a compound of formula B*:



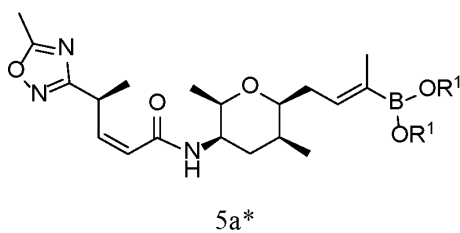
or a salt thereof.

100. The method of claim 96, wherein the compound of formula 6* is prepared comprising reacting the compound of formula 5a* or a salt thereof, with a compound of formula B*:

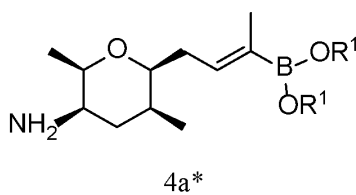


or a salt thereof, to provide the compound of formula 6*.

101. A method for preparing a compound of formula 5a*:



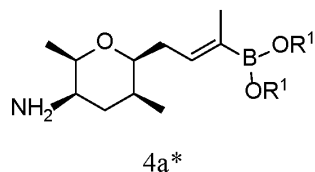
or a salt thereof, comprising converting a corresponding compound of formula 4a*:



or a salt thereof, to the compound of formula 5a* or a salt thereof,

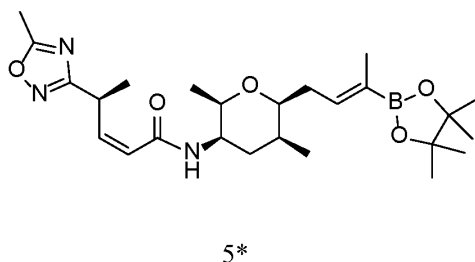
wherein each R¹ is independently a (C₁-C₆)alkyl, or two R¹ groups together with the atoms to which they are attached form a heterocyclyl optionally substituted with one or more (C₁-C₆)alkyl.

102. The method of claim 94 or claim 95, wherein the compound of formula 5a* or a salt thereof is prepared, comprising converting a corresponding compound of formula 4a*:

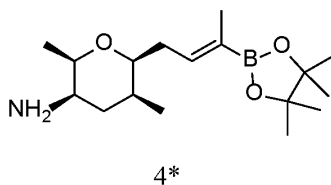


or a salt thereof, to the compound of formula 5a* or a salt thereof.

103. The method of claim 101 or claim 102, wherein the compound of formula 5a* is a compound of formula 5*:

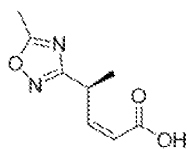


or a salt thereof, and the compound of formula 4a* is a compound of formula 4*:



or a salt thereof.

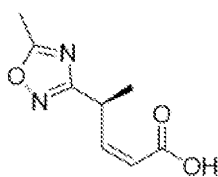
104. The method of claim 101 or claim 102, wherein the compound of formula 5a* is prepared comprising reacting the compound of formula 4a* or salt thereof with a compound of formula



A*

or salt thereof, to provide the compound of formula 5a* or a salt thereof.

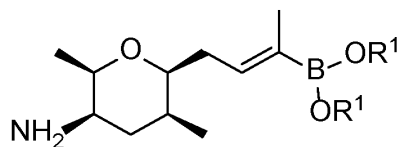
105. The method of claim 103, comprising reacting the compound of formula 4* or salt thereof, with a compound of formula A*:



A*

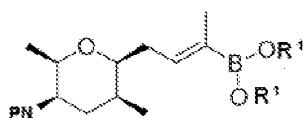
or salt thereof, to provide the compound of formula 5* or a salt thereof.

106. A method for preparing a compound of formula 4a*:



4a*

or a salt thereof, comprising converting a corresponding compound of formula 3a*:



3a*

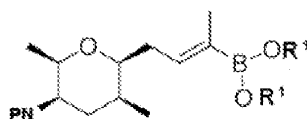
or a salt thereof, to the compound of formula 4a* or a salt thereof,

wherein:

P is an amine nitrogen protecting group; and

each R¹ is independently a (C₁-C₆)alkyl, or two R¹ groups together with the atoms to which they are attached form a heterocyclyl optionally substituted with one or more (C₁-C₆)alkyl.

107. The method of claim 101 or claim 102, wherein the compound of formula 4a* or a salt thereof is prepared, comprising converting a corresponding compound of formula 3a*:



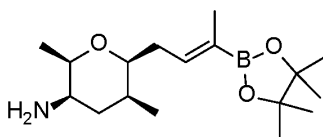
3a*

or a salt thereof, to the compound of formula 4a* or a salt thereof,

wherein P is an amine nitrogen protecting group; and

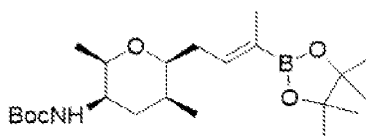
each R¹ is independently a (C₁-C₆)alkyl, or two R¹ groups together with the atoms to which they are attached form a heterocyclyl optionally substituted with one or more (C₁-C₆)alkyl.

108. The method of claim 106 or claim 107, wherein the compound of formula 4a* or a salt thereof is a compound of formula 4*:



4*

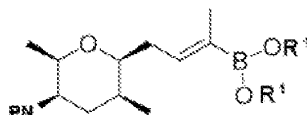
or a salt thereof, and the compound of formula 3a* or a salt thereof is a compound of formula 3*:



3*

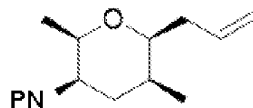
or a salt thereof.

109. A method for preparing a compound of formula 3a*:



3a*

or a salt thereof, comprising converting a corresponding compound of formula 2a*:



2a*

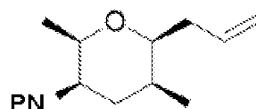
or a salt thereof, to the compound of formula 3a* or a salt thereof,

wherein:

P is an amine nitrogen protecting group; and

each R¹ is independently a (C₁-C₆)alkyl or two R¹ groups together with the atoms to which they are attached form a heterocyclyl optionally substituted with one or more (C₁-C₆)alkyl.

110. The method of claim 21 or claim 22, wherein the compound of formula 3a* or a salt thereof is prepared, comprising converting a corresponding compound of formula 2a*:

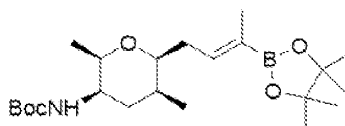


2a*

or a salt thereof, to the compound of formula 3a* or a salt thereof,

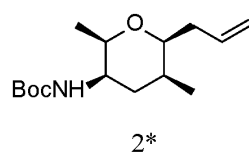
wherein P is an amine nitrogen protecting group.

111. The method of claim 109 or claim 110, wherein the compound of formula 3a* or a salt thereof, is a compound of formula 3*:



3*

or a salt thereof, and the compound of formula 2a* is a compound of formula 2*:



or a salt thereof.